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Review Paper

Application of mathematical modelling in the meat chain

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ABSTRACT

The objective of this study was to give an overview on the application of mathematical modelling in the meat sector. The literature review employed VOSViewer as a bibliometric tool and showed three directions of research. The first is associated with models related to meat consumption, mainly in terms of consumer patterns, exposure assessment and potential health risks. The second is linked with modeling various intrinsic and extrinsic meat quality cues. The third explores modeling environmental impacts in the meat chain.

The main conclusion of this study is that mathematical modeling is an emerging scientific dimension but with limited application by the main meat chain actors. There is a need to develop user-friendly guidelines for application of this type of modeling in the meat sector.

1. Introduction

Mathematical modelling is a tool that enables understanding system/process characteristics and predicts its outcomes (*Sandeep & Irudayaraj*, 2001). The main approach when setting a mathematical model is to articulate an idea behind the model, to analyze potential theories associated with the phenomenon and to define the mathematical language (*Bender*, 1978). However, modelling in food science is a challenge due to three reasons: (i) (mis)understanding of the phenomena in focus, (ii) mathematical problems when setting the experiment and (iii) uncertainties related to food data (*Trystram*, 2012).

Mathematical modeling throughout the food chain aims to model and/or simulate different phenomena from the micro-scale to the entire food chain, with the potential to upscale certain phenomena across the chain (*Djekic et al.*, 2019). In order to successfully develop a model, it is crucial to understand the phenomenon through both theory by exploring existing knowledge and practice by performing different measurements (*Trystram*, 2012). However beside building a model by formulating the problem and outlining it mathematically, it is important to test the model and verify its robustness (*Bender*, 1978). This can be obtained by checking various what-if scenarios. Depending on the model, it may focus on optimizing a product/process or enhancing predictive simulation and improving control (*Djekic et al.*, 2019). In general models can be categorized as product-based, process-based or product-process based (*Fito et al.*, 2007).

A cross-European survey on the application of mathematical models through the food chain outlined that the main predictors for the use of models are the country where companies operate (developed opposed to less developed) and the size of the food company (large opposed to small and medium-sized companies), while the food sector is less important (*Djekic et al.*, 2019). These results reveal the lack

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of resources and knowledge as an issue of importance, since different competencies are needed for developing and using various models. These competences comprise food science, applied mathematics, advanced statistics, food engineering etc. (*Trystram*, 2012). Unfortunately, despite the scientific development of different models, their application is still scarce. This is even pronounced when validation of the models needs to be applied in the food companies (*Djekic & Smigic*, 2024). Some authors suggest the development of user-friendly guidelines (*Allais et al*., 2007), especially for small food companies.

When it comes to the meat sector, there are different modelling approaches from product-based models, considering meat as an orthotropic material with independent mechanical/thermal characteristics across three perpendicular directions (*Djekic et al*., 2022), consumer based models by applying Monte Carlo simulation associated with the exposure assessment of a certain population to meat product additives (*Petrović et al*., 2022), to different lifecycle assessment models throughout the meat chain (*Djekic & Tomasevic*, 2016).

Since meat science is a promising research field with many interesting phenomena where mathematical tools may be applied, the main objective of this paper was to give an overview of different mathematical models associated with the meat sector and to identify scientific breakthroughs in future studies.

2. Materials and methods

To conduct a literature review associating mathematical modelling and meat science, a text mining concept was applied using VOSViewer tool. This bibliometric analysis enabled the understanding of the current research flows. The detailed search string was as follows: ("mathema*" OR "model" OR "predictive" OR "simulation") AND ("meat" OR "meat science" OR "meat chain")). This bibliometric string was applied in the scientific database Web of Science. The cut-off criterion was the inclusion of keywords more than seven times.

3. Results

The search revealed 15,584 results. To narrow the results, the following criteria applied: (i) only research and review papers, (ii) papers from 2015 to date; (iii) papers only in English language. The outcome was 9,718 results. This search showed that these publications were written by 37,181 authors, with 8,004 affiliations from 150 countries. The top five countries/regions for the selected period were China with 2,340 publications (24.1% of total publications), USA with 1,526 (15.7%), Brazil with 563 (5.8%), Spain with 536 (5.5%) and Italy with 511 (5.3%). Out of 9,718 publications, 82 were highly cited papers with one paper reaching over 3,700 citations. These 82 papers were further processed.

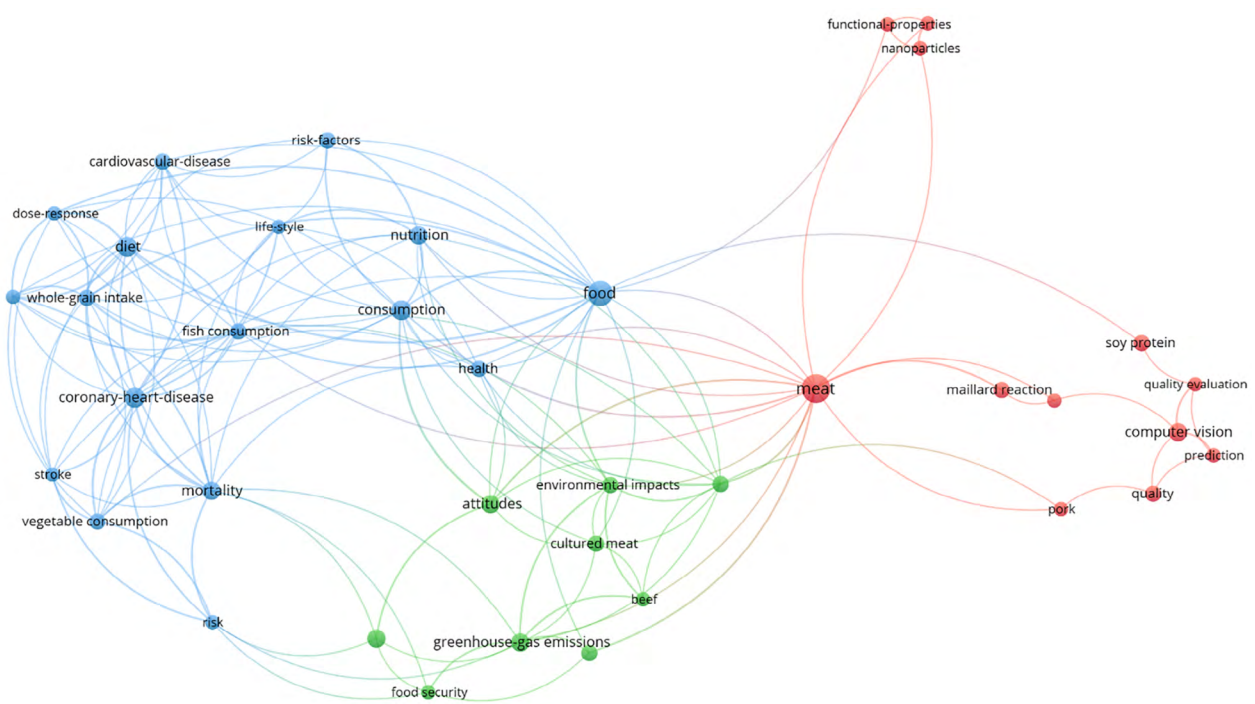

Figure 1. Network visualization of inter-linkage between meat and mathematical modelling

Figure 1 depicts the network visualization of titles, abstracts and keywords of the highly cited manuscripts that were published in the last 10 years, based on the selected strings of keywords as three clusters (outlined in different colors).

The blue cluster (consumption) is associated with models relating to meat consumption and potential health risks, such as cardiovascular or coronary heart issues. The red cluster (quality) is mainly related to different models with the aim of explaining different physico-chemical and intrinsic quality cues of meat and meat products. The green (environmental) cluster is related to modelling environmental impacts and the carbon footprint of meat throughout the meat chain.

4. Discussion

Modelling related to meat consumption has two main directions: (a) exposure assessment modelling and (b) health risk modelling. An exposure assessment is a qualitative and/or quantitative estimation of the probability that a certain population is exposed to a biological, chemical, or physical agent through food consumption (*Udovicki & Djekic*, 2024). To calculate the exposure of a certain a target compound, such as additives in meat products, it is essential to obtain data on meat consumption habits, to know the main demographic characteristics of the population (age, sex, weight and body mass index) and to perform chemical analysis of the presence of additive concentrations in meat products (*Petrović et al.*, 2022). The modelling approach to any exposure assessment is either deterministic or probabilistic (*Udovicki & Djekic*, 2024). A further step in exposure assessment is to perform a health risk assessment by combining exposure diet levels and food contamination levels to predict death and mortality *(Udovicki et al.*, 2019).

Quality-related modelling is focused on meat products, meat technology processes or both and is the most diverse area of research throughout the multiverse of intrinsic and extrinsic meat quality cues (*Rajic et al.*, 2022). An interesting quality-based modelling approach was proposed where consumer sensory scores were used as a basis for eating quality prediction scores further associated with individual quality grades for different cuts as outlined in the Meat Standards Australia (*Pannier et al.*, 2025). Other examples are the application of mathematical models predicting pig carcass composition by using total lean meat percentage and carcass weight (*Tomovic et al*., 2019), or the application of the structural equation model on investigating relationships between carcass and meat quality traits in cattle (*Bresolin et al*., 2022)**.**

Novel food technologies are also being modelled, such as the application of a sarcoplasmic model to investigate the effects of high-pressure processing on beef color (*Denzer et al*., 2023). The same technology modelled whether high-pressure processing increased the *in vitro* protein digestibility (*Lee et al*., 2023). Color was subject to analyses by applying computer vision systems (*Tomasevic et al*., 2019, 2021). Mass transfer is a subject of different studies, such as the case of wet salting of caiman meat with the application of mathematical models enabling the prediction of mass transfer kinetics until equilibrium conditions (*Sanches et al*., 2023).

When it comes to food safety, machine learning was used as a tool for microbial predictive modelling to anticipate the growth of *Escherichia coli* in raw ground beef depending on storage temperatures (*Al et al*., 2024). Other examples are the use of interaction models in predicting growth of *Staphylococcus aureus* in beef (*Cheng et al.*, 2023) or predictive modeling of the growth of *Clostridium perfringens* during the cooling process of cooked pork (*Juneja et al.*, 2021).

Food oral processing and digestion are also subject to modelling. Food breakdown during mastication, eating pattern and bite size are important quality cues associated with meat (*Đekić*, 2023). Modelling food oral processing has three milestones: (i) *ex ante*, associated with testing meat properties prior to mastication, (ii) ongoing, analyzing oral processing parameters, and (iii) *ex post,* studying swallowing and digestibility (*Djekic et al*., 2022). *In vitro* gastrointestinal models can further study if freezing-then-aging treatment of beef has any effect on the protein digestibility and release of potentially bioactive compounds (*Lee et al*., 2024).

Environmental modelling is mainly performed using life-cycle assessment approach as outlined in ISO 14040 (*ISO*, 2006). Depending on the boundaries, the life cycle in the meat chain can span from "the cradle to the gate" covering livestock farm, slaughterhouse, meat processing plant, retail and households (*Djekic & Tomasevic*, 2016), or part of the life-cycle such as "cradle-to-gate" covering meat production and processing, "gate-to-gate" analyzing only the meat processing phase, "gate-to-grave" analyzing environmental impacts associated with the

consumers and food waste or different other combinations such as "cradle-to-market" or "cradle-to-use" (*Djekic et al*., 2018). Apart from in modelling, the environmental impacts, such as global warming potential, acidification potential, eutrophication potential or ozone layer depletion, are the most often used environmental potentials in the meat chain (*Djekic & Tomasevic*, 2019).

5. Conclusion

The main conclusion of this overview is that mathematical modelling in meat science is a scientific perspective with various innovations and novelties being developed, but its application by meat producers and other actors in the meat chain is at a very low level. Future research should focus on developing user-friendly models applicable in the meat chain continuum.

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Review Paper

Empty barns, overcrowded clinics: restoring balance in veterinary education priorities

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ABSTRACT

The veterinary profession in Southeast Europe faces a structural imbalance: while companion-animal medicine dominates employment opportunities and student preferences, food-animal health, public health, and One Health functions are increasingly under-resourced. This study examines veterinary education establishments (VEEs) from Slovenia, Croatia, Bosnia and Herzegovina, Serbia, and North Macedonia to assess how international standards (WOAH Day-1 Competencies; EAEVE/ESEVT) and market forces shape veterinary education. Although VEEs already achieved or tend to accomplish and maintain compliance with accreditation requirements and update curricula to meet EU standards, implementation often reflects student demand and clinical caseloads rather than regional epidemiological or food security needs. Our findings highlight the need for VEEs to rebalance curricula by embedding WOAH's competencies alongside EAEVE's standards, strengthening extramural learning in food-animal and public-health contexts, and developing joint regional strategies such as shared electives, micro-credentials, and mobility programs. Without such proactive measures, veterinary education risks reinforcing market-driven specialization at the expense of essential societal roles. These lessons extend beyond Southeast Europe, offering a framework for aligning veterinary education globally with both professional demand and public-good imperatives.

1. Introduction

Throughout its history, the veterinary profession has continually adapted to meet society's needs. Today, however, it faces a profound structural shift in which an increasing concentration of resources and expertise in companion animal sector threatens to erode its capacity to serve broader public-good functions. The Federation of Veterinarians in Europe (FVE) survey from 2023 shows that the most common employment sector is clinical practice (63%), predominantly small animal clinical practice (70%), followed by public service (14%), education and research (6%) and industry (4%). In

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terms of caseload, companion animals account for 58% of all cases treated, compared to 10% for cattle, 7% for equines, and 3% for pigs. This market profile reflects rising demand for companion-animal care but poses concerns about sustaining food-animal, public-health, and One Health capacities of profession.

The workforce trend is closely intertwined with profession's educational basis. The standards and accreditation systems that shape veterinary education, as the frameworks used to define graduate competencies, strongly influence how future veterinarians are prepared to meet—or fail to meet—societal needs beyond the dominant companion-animal sector. The Southeast European region offers a distinctive case for studying veterinary education perspectives. Emerging from a centrally planned economic model with publicly owned veterinary stations (clinics) established for each municipality, was aimed to secure all range of professional services on self-sustainable basis. More than 35 years into the transition to market economies, the region now spans EU members (Slovenia and Croatia) and EU candidate states (Bosnia and Herzegovina, Serbia and North Macedonia). Despite varied EU integration levels, countries remain interconnected through significant cultural, societal and economic aspects including intra-regional trade in live animals, meat, dairy, and feed. These trade flows sustain rural economies and regional food security but also create shared vulnerabilities to transboundary animal diseases and joint public-health responsibilities.

It is commonly accepted that the EAEVE-aligned curricula and competencies across borders can strengthen disease prevention and treatment preparedness, facilitate EU compliance, and enhance market competitiveness. However, such harmonization, in response to current employment opportunities, can reduce flexibility for local adaptation, potentially marginalizing region-specific needs, and fostering an over-standardized approach that may not adequately reflect diverse epidemiological, cultural, or economic contexts. While Southeast Europe's conditions are unique, the lessons from this collaborative evaluation—particularly in balancing market demand with public-good functions—can inform other small economies with high agricultural trade and similar veterinary workforce challenges that are by rule dependent on single Veterinary educational establishment (VEE). To address identified challenges, this study aims to analyze current accreditation and quality assurance standards and impact on how VEEs shape veterinary education trends and priorities, survey educational statistics from five regional veterinary faculties to document institutional facts and realities in enrolment, curriculum content, postgraduate offerings, and graduate orientation toward professional sectors and recommend future development and strategic pathways for VEEs highlighting how regional collaboration and innovations can strengthen their respond to professional demand, keep One Health competencies, and reinforce proactive educational design.

2. Materials and methods

2.1 Analysis of relevant accreditation and quality assurance standards in veterinary education

The first step consisted of a comparative analysis of two major veterinary education quality frameworks:

- WOAH Day 1 Competencies for Graduating Veterinarians (2012)
- EAEVE)/ESEVT Standards and Indicators (2023)

The analysis examined competency domains, Quality assurance and governance and sectoral balance.

2.2 Survey of educational data in participating VEEs

A structured survey instrument was developed and administered to five VEEs, two from EU member states (Ljubljana and Zagreb – both EAEVE accredited) and three from EU candidate countries (Sarajevo, Belgrade, and Skopje).

The survey comprised five sections covering:

- Student demographics and enrolment trends (past five academic years)
- Postgraduate and specialization programs, including emerging areas
- Curriculum structure, reforms, and educational priorities
- Employment opportunities trends and systemic issues (interest in public-good sectors, graduate employment)
- Institutional perspectives on external drivers of curriculum direction, partnerships, and mobility programs

Questionnaires were distributed electronically, with responses validated through follow-up communication with designated institutional contacts. The survey captured both quantitative data (enrolment, graduation numbers, gender ratios, elective choice trends) and qualitative descriptions (curriculum reform narratives, employer engagement practices, mobility participation).

2.3. Analysis of population and livestock data

Cross-analysis of population and animal data were used to identify gaps between veterinary capacity and production realities. The Livestock Unit (LSU) is used as a standardized measure of animal biomass, using coefficients that convert species counts to a common reference (1 LSU = an adult dairy cow's annual feed requirement). Livestock units (LSU) were calculated using conversion coefficients established in the EU Farm Structure Survey framework (Commission Regulation (EC) No 1200/2009). For comparability, we applied the following simplified coefficients: cattle = 1.0, pigs = 0.3, sheep = 0.1, goats = 0.1, poultry = 0.007, and use formula: LSU total = (Cattle × 1.0) + (Pigs × 0.3) + (Sheep × 0.1) + (Goats × 0.1) + (Poultry × 0.007) Non companion animals veterinarian's ratio for further analysis is approximated at 30 % of registered veterinary workforce based on FVE survey (2023).

2.4. Formulation of recommendations for future collaboration and study programs improvements.

Insights from the standards analysis, survey results and literature review were used to develop evidence-based recommendations.

3. Results

3.1. Competency Frameworks: WOAH vs EAEVE approaches and educational impacts

Veterinary graduate competencies are foundational to curriculum design, accreditation standards, and workforce alignment. Two dominant frameworks—those of the World Organization for Animal Health (*WOAH*, 2012) and the European Association of Establishments for Veterinary Education (*EAEVE*, 2023) present distinctive yet complementary visions for the profession's entry-level expectations. Understanding their orientation, structure, and impact is critical for rebalancing veterinary education.

Table 1. Comparative summary of structural distinctions of WOAH/EAEVE standards

Dimension	WOAH (2012)	EAEVE/ESEVT (2023)
Orientation	Global minimum set of competencies for WOAH members, regulatory support	European professional mobility and clinical readiness
Competency format	Broad domains (e.g., zoonoses, epidemiology)	Specific clinical and public health competencies grouped under 5 core categories
Primary focus	National veterinary services, disease surveillance, One Health	Balanced training for all major animal species, integrated public health modules
Public health emphasis	Strong (food safety, transboundary animal diseases, veterinary public health (VPH)	Moderate to strong (integrated in core curriculum)
Clinical skills detail	Limited	Extensive (clinical reasoning, procedures, species-specific care)
Communication & ethics	Implicit	Explicit, including client interaction and ethical conduct
Legal/regulatory training	Emphasis on legislation for public service roles	Included in understanding business, ethics, and professional responsibilities
Use in accreditation	Informal benchmarking	Mandatory in ESEVT accreditation

3.2. Student enrolment and gender distribution

Graduation numbers varied widely by institution size, from ~30 graduates per year in smallest VEE to over 120 in largest one. Across all VEEs female graduates predominated, typically constituting 60–85% of the total.

3.3. Postgraduate and specialist programs

All VEEs offer postgraduate education, though the number and scope vary:

- Belgrade and Zagreb run extensive specialist modules covering clinical, public health, food safety, and epidemiology.
- Sarajevo's offer centers on "Veterinary Medicine and Public Health." with wide variety of tracking
- Skopje delivers a specialist program in *Food Safety and Quality* and doctoral tracks in veterinary medicine and food safety.
- Ljubljana provides interdisciplinary doctoral program in biomedicine and environmental protection.

No institutions dedicate programs for emerging fields such as digital veterinary medicine, veterinary business management, aquatic health disease management though some integrate relevant topics (e.g., One Health, economics and management) into core curricula.

Table 2. Data on student enrolment and gender distribution are presented for each VEEs

VEE	Enrolment and gender distribution
University of Belgrade	The recorded a steady enrolment around 1,340–1,370 students annually, with a growing female majority (from 53% to 65%).
University of Zagreb	The maintained ~900–1,000 students across Croatian and English programs, with a female share consistently above 75%.
University of Sarajevo	The enrolled ~300–380 students annually, with females comprising ~65%–70% of the cohort.
University of Skopje	The showed smaller cohorts (120–134 students) but a similarly high female proportion (~67%).
University of Ljubljana	The averaged ~430 students per year, with an ~84% female majority.

3.4. Curriculum structure and reforms

All VEEs reported curriculum updates within the last five years, generally aimed at alignment with EAEVE and EU requirements. External Drivers of curriculum direction are:

- EAEVE and EU Directive (2005/36/EC) compliance;
- National accreditation standards;
- Employer feedback through councils or structured surveys;
- Labor market demands and professional chamber requirements for practical skills and public-good sector readiness.

3.5. Interest in public sector

All VEEs observed declining student interest in food-animal medicine, public health, and public sector careers:

- Belgrade recorded a drop in public health elective enrolments from 147 (2019/20) to 59 (2024/25).
- Zagreb noted that most final-year students select the companion-animal ("Pets") track.
- Ljubljana's internal survey found decreasing interest in most veterinary areas between Year 1 and Year 6, except for dogs/cats, pharmacy, and research.

Table 2. Comparative overview of VEEs curriculum structure

Institution	Program type	Duration/ ECTS	Notable inclusions	Curriculum updates
Ljubljana (SLO)	Integrated I and II cycle study	6 years/360 ECTS	EAEVE-accredited; Broad species coverage; electives in public health, communication skills, entrepreneurship, aquaculture health	Increased early clinical exposure, optimized rotations, added courses on exotic animal medicine, environmental safety, antimicrobial resistance (AMR), and sustainable protein sources.
Zagreb (CRO)	Integrated I and II cycle study	6 years/360 ECTS	EAEVE-accredited; structured electives in food safety, public health, and companion-animal medicine tracking in final year	Conducted comprehensive learning outcomes mapping, increasing visibility of Day One Competencies and harmonized with the Croatian Qualifications Framework.
Sarajevo (BiH)	Integrated I and II cycle study	5 years/300 ECTS	Bologna-model; core veterinary training; public health and food safety emphasis in later years; postgraduate program in Veterinary Medicine & Public Health	Modernized content to meet EU Directive 2005/36/ EC, adding up to 30% new content.
Belgrade (SRB)	Integrated I and II cycle study	6 years/360 ECTS	Strong food-animal base with growing companion-animal focus	Reduced overall teaching hours, shortened program from 12 to 11 semesters, introduced a One Health course, and expanded clinical rotations in the final semester.
Skopje (MKD)	Integrated veterinary medicine program	5,5 years/ 330 ECTS	Covers all major species; includes food safety, public health, and One Health elements;	Extended program to six years (360 ECTS), introduced one-semester courses for balanced workload, and added new electives.

3.6. Graduate employment trends

Employment outcomes are generally favorable, though tracking systems differ:

- Some VEEs rely on national agencies or professional chambers for employment data (Belgrade, Zagreb, Skopje, Ljubljana).
- Sarajevo does not systematically track graduate employment.

3.7. Partnerships and mobility

All faculties maintain partnerships with private practices, farms, food industry facilities, and public health institutes, providing clinical and field training opportunities. Participation in EU mobility programs (Erasmus+, CEEPUS) is continuously growing, supporting both student and staff exchanges.

3.8. Population statistic

Table 3. Livestock statistics and livestock unit calculation (LSU) per country

Country (Year)	Cattle	Pigs	Poultry	Sheep	Goats	LSU total*
Slovenia (2024)	454,000	232,000	6,500,000	116,800	25,100	**583,290**
Croatia (2022)	422,000	945,000	10,918,000	643,000	82,000	**854,426**
Bosnia & Herzegovina (2024)	383,000	447,000	17,547,000	1,099,000	62,000	**756,029**
Serbia (2024, prelim.)	698,605	2,349,176	14,773,571	1,759,424	~180,000*	**1,700,715**
North Macedonia (2023)	148,693	193,412	1,562,000**	587,073	85,528	**284,911**

Sources: Slovenia (2024): Statistical Office of the Republic of Slovenia (SURS); Croatia (2022): Croatian Bureau of Statistics (DZS). Bosnia & Herzegovina (2024): Agency for Statistics of BiH (BHAS). Serbia (2024, prelim.): Republic Statistical Office of Serbia (RZS, preliminary); goat numbers estimated from a 2021 research article (*Frontiers in Veterinary Science*). North Macedonia (2023): State Statistical Office of North Macedonia (SSO).

Notes: *Serbia goat number approximate; North Macedonia poultry estimated from 2022 yearbook with +11.8% growth in 2023.

Table 4. Veterinarian Workforce Distribution (ratio estimates based on FVE, 2023)

Country	Population (2025 est.)	FVE Ratio (vets / 1,000 pop.)	Estimated Total vets	Companion-animal vets (70%)	Food/ Public vets (30%)
Slovenia	2,130,850	0.44	≈ **938**	≈ **657**	≈ **281**
Croatia	3,964,392	0.28	≈ **1,110**	≈ **777**	≈ **333**
Bosnia & Herzegovina	3,137,079	0.20	≈ **627**	≈ **439**	≈ **188**
Serbia	6,689,039	0.39	≈ **2,609**	≈ **1,826**	≈ **783**
North Macedonia	1,812,654	0.13	≈ **236**	≈ **165**	≈ **71**

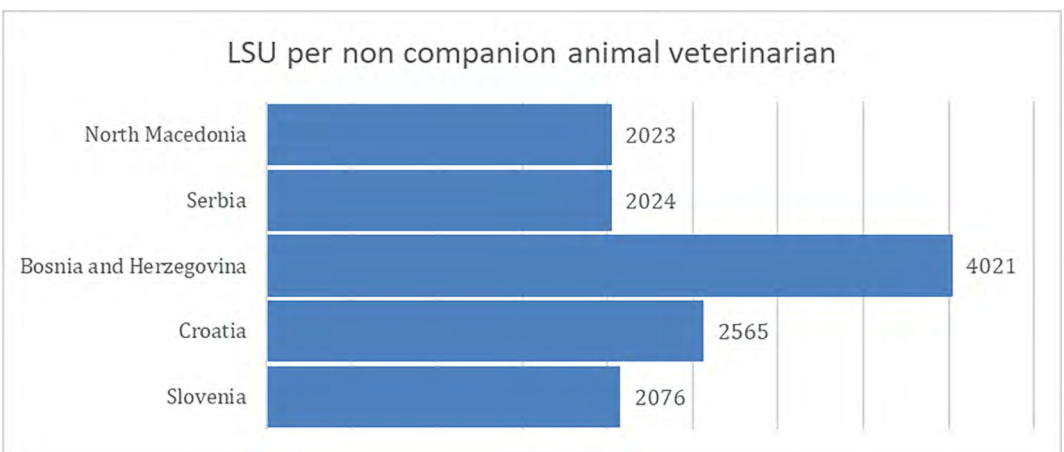

Figure 1. Estimation of total LSU per currently practicing non companion animal veterinarian for each country

4. Discussion

As a public good, veterinary profession, including its educational base, has a responsibility to balance with societal needs, ensuring that specialization thrives without compromising critical capacities. While a current market-driven focus on companion-animal services has demonstrated direct correlation with development of deeper specialization and technical excellence in veterinary education (*Drzemalla et al.*, 2025), it risks narrowing the profession's scope—leaving essential roles in food-animal medicine, food security and safety, disease events surveillance, and One Health underresourced, and reshaping educational institutions in ways that may weaken their ability to serve society with a balanced workforce (*Leighton*, 2004, *Enticott et al.*, 2011, *Fejzic and Šerić-Haračić*, 2017). Today, VEEs face divergent driving forces: to follow the trending companion-animal market pull or to steer curricula and policy toward broader societal resilience. This challenge is complicated by two parallel phenomena: the corporatization of companion-animal practice, which reshapes clinical priorities and early-career pathways, and persistent workforce vulnerabilities such as high attrition, rural recruitment gaps, and mental health pressures (*Diana et al.*, 2025; *Zoetis*, 2025). VEEs, particularly public ones, bear a special responsibility in sustaining and shaping the veterinary profession to meet future needs and requirements. Their role extends beyond cooperation in developing educational models (*Arnold and Warman*, 2025), rather a new proactive stance is demanded - one that anticipates societal and professional challenges and strategically prepares graduates to respond. Veterinary education cannot stand in isolation. It is intrinsically tied to the profession and, now more than ever, requires systematic and proactive alignment between academic foundations and the evolving imperatives of veterinary profession.

The first objective of our study was to examine how international frameworks—principally WOAH's Day One competencies and the EAEVE/ESEVT standards—shape veterinary education, and the results (Table 1.) highlight both their complementary strengths and the structural gaps that emerge in practice. WOAH's approach emphasize the veterinarian's role in safeguarding public health, ensuring national veterinary service quality, and supporting emergent issue such as biosecurity, emergency responses and antimicrobial resistance (AMR). The WOAH framework is globally oriented and explicitly emphasizes that veterinarians must be equipped not only in clinical care but also in public-goods areas such as food security and safety, AMR, and One Health. Complementing this global perspective, EAEVE's competency structure, as embedded in the ESEVT SOP 2023 and Annex 2, aligns with EU directives (2005/36/EC, 2013/55/EU) and the European Higher Education Area's (EHA) quality assurance standards (ESG 2015). The EAEVE model's focus on clinical competence has profoundly shaped European veterinary curricula. VEEs are required to provide extensive hands-on training, through teaching hospitals and extramural placements. This shift has professionalized veterinary training, enhanced graduate autonomy, and facilitated EU workforce mobility. While it is true that implementation often becomes companion-animal heavy (due to student preferences, hospital caseloads, or VEEs resources), this is not a feature of EAEVE policy itself, but rather a consequence of how VEEs allocate teaching time to meet compliance. The framework itself does not deprioritize public health. Rather, the practical weight in curricula may drift toward clinical companion-animal training due to student demand and clinical practice economics but this is an implementation challenge, not a design flaw in ESEVT. However, in practice, the predominance of clinical training has sometimes meant that public-good roles receive less attention. Graduates as per various studies feel underprepared for careers in food safety, rural practice, and government service—domains central to WOAH's competency vision (*Enticott et al*., 2011, *Sander and Miller*, 2021). This competency mismatch raises concerns about resilience during public health emergencies, where veterinary services play key roles (FVE, 2023). These findings suggest that while VEEs meet set of accreditation criteria, they may lag in internalizing and operationalizing holistic competencies that support public-health resilience.

Curriculum rebalancing and implementation must avoid choosing between models. Instead, integrated frameworks can leverage EAEVE's clinical depth and WOAH's public health breadth. Curriculum designers should map each course outcome to both EAEVE and WOAH standards, ensure exposure to public health and food-animal practice and collaborate with public-sector agencies for training placements. Our findings demonstrate concrete evidence of the regional pattern:

- Curriculum orientation, if aimed to follow market trends and requirements, will be heavily weighted toward companion-animal practice,

with limited structural emphasis on food-animal medicine and public health.

- Postgraduate offerings lack systematic focus on emerging fields such as digital veterinary medicine, aquatic animal health, public advocacy, economics, communication and population-level medicine (One health).
- Graduate career aspirations follow similar trends: rising student interest in private clinic employment, declining inclination toward public-good roles.

These observations mirror literature (*National Research Council*, 2013; *Cary et al.*, 2017; *AVA*, 2025) highlighting the profession's urban tilt and the consequent neglect of food production and population health training — a concerning trend given the region's heavy reliance on agri-food trade and epidemiological risk interconnectivity.

Given that Southeast Europe represents a region with post-socialist educational legacies, varying levels of EU integration (two EU member states and three candidate countries), and dense agri-food trade networks, studied VEEs operate within a unique socio economics and political context. Traditional regional interconnectedness—the trade of livestock, dairy, and meat production—carries both economic consequences and epidemiological vulnerabilities. Mixed human migrations and transboundary animal diseases have repeatedly posed the risk to spread across regional borders (*Angeloni et al*., 2023), underscoring the urgency of veterinary capacity for surveillance, epidemiological response, and certification. Thus, there is both a market imperative—to sustain significant value in intra-regional and EU-bound trade—and a public health imperative—to mitigate disease risks through education and preparedness. VEEs that embed these dual priorities into curricula enhance regional resilience and alignment with EU market requirements. Our study strongly supports a proactive stance by VEEs: not merely following market signals but standing guard to veterinary professional identity to include public-good service and orientation to emerging fields. Formulating regional collaborative strategies—joint curriculum mapping, shared micro credentials in One Health, food safety, epidemiology, and economics—is essential.

Across all VEEs surveyed, total enrolment over the past five academic years has remained relatively stable, with some institutions reporting slight declines in recent intakes. Female students consistently outnumber male students, representing between 60% and 85% of total enrolments depending on the institution. Veterinary medicine, previously a male-dominated profession in region of our study, has experienced a significant increase in the number of women studying at VEEs and practicing in all fields of the profession. Female predominance in veterinary medicine continues to increase globally. In the United States, women now account for 81% of 2024 DVM graduates and about 74% of the active workforce (*AVMA*, 2024). In the United Kingdom, the most recent RCVS survey reported that 61% of respondents were female, mirroring registration data and indicating a growing trend (*RCVS*, 2024). At the European level, the FVE survey showed that around 65% of veterinarians are women, with younger cohorts displaying even higher proportions (*FVE*, 2023). This demographic momentum suggests that feminization will continue as older, more male cohorts retire and younger, predominantly female cohorts enter the profession. Despite this clear trend, there is no updated, cross-national causal analysis that explains *why* feminization has occurred or what the future consequences might be for workforce balance, leadership structures, and professional valuation. The most robust causal explanation remains the U.S.-focused study by *Lincoln* (2010), which attributes the feminization largely to supply-side dynamics and the withdrawal of male applicants, rather than wage or tuition effects. Since then, most available studies have been descriptive workforce counts or qualitative discussions on leadership and representation underscoring the need for more systematic, longitudinal, and comparative research (*Vezeau et al*., 2025).

Additional dimension in assessing the balance between veterinary education outcomes and societal needs is the relationship between the number of veterinarians available and the livestock biomass measured in LSU. Based on the assumption, drawn from the *FVE* (2023) survey, that only 30% of veterinarians are engaged in livestock, equine, and public health practice, we calculated ratio on number of non-companion animal veterinarians/LSU. So, in France, each non-companion veterinarian is responsible for about 3,000 livestock units (LSU). This very high ratio reflects the scale of the national herd relative to available veterinary capacity. In Spain, the figure is lower but still substantial, at around 1,800 LSU per non-companion vet, largely driven by the country's intensive swine and poultry sectors. Germany shows a lighter burden, with

approximately 1,300 LSU per non-companion vet, thanks to its comparatively large veterinary workforce. Italy has the lowest ratio among the four, with about 1,000 LSU per non-companion vet**,** suggesting relatively more veterinary capacity per LSU.

Our study sample shows that while veterinary workforce size broadly aligns with veterinarian to population ratios reported by the FVE survey (ranging from 0.13 to 0.44 veterinarians per 1,000 inhabitants), the distribution of veterinarians relative to LSU reveals structural imbalances. For example, Serbia, with an estimated 1.7 million LSU, maintains approximately 2,609 veterinarians, of whom fewer than one-third are engaged in food-animal or public-sector roles. This equates to roughly 2,170 LSU per non-companion animal veterinarian. In Slovenia, with a smaller livestock base of ~583,000 LSU, and about 281 non-companion animal veterinarians, study shoved lower burden of 2,075 LSU per veterinarian in production and public roles**.** Bosnia and Herzegovina presents a particularly sharp contrast: despite a total of 756,029 LSU, only about 188 veterinarians are estimated to work in food-animal or public-good domains. This represents nearly 4,000 LSU per veterinarian**,** indicating a significant service gap. No internationally recognized benchmark exists for an optimal LSU-to-veterinarian ratio. However, our analysis suggest that veterinary coverage for production animals, surveillance, and rural practice is under severe strain, with direct implications for biosecurity, zoonotic disease monitoring, and trade certification. These findings reinforce the argument that current educational and professional pathways trend to channel graduates into companion-animal practice, might keep public-good and production roles systematically under-resourced in long term.

Finally, we identified the several priorities for VEEs to address challenges and issue discussed in this study:

- Broaden professional impact by reinforcing the veterinary profession's capacity to address societal needs beyond companion-animal care.
- Safeguard and expand public-good competencies in food-animal health, veterinary public health, food safety, and One Health.
- Drive curriculum reform to ensure a balanced distribution of learning outcomes across market-oriented and public-good domains.
- Foster regional collaboration in curriculum design, resource pooling, and harmonization of competencies to strengthen workforce resilience.

6. Conclusions

The findings of this study confirm a regional structural imbalance in veterinary profession. While training in food-animal medicine remains a core component of existing curricula, the challenge lies in sustaining student interest and ensuring that these pathways remain attractive compared to companion-animal careers. Addressing this requires more than preserving curriculum hours; it demands measures that enhance visibility, engagement, and perceived value of food-animal, public health, and One Health roles. The study demonstrates that VEEs in Southeast Europe operate at the intersection of post-socialist legacies, EU integration, and agri-food trade dependencies. Current accreditation schemes, while ensuring baseline quality and harmonization, risk reinforcing this imbalance. If VEEs align curricula too closely with accreditation indicators and market demand, they may inadvertently follow the companion-animal trend, narrowing their societal contribution instead of broadening it. This context highlights the urgent need for a systematic and strategic approach that recognizes education and the profession as inseparable. VEEs must proactively rebalance training by embedding WOAH's public-good competencies alongside EAEVE's standards, ensuring graduates are prepared not only for private practice but also for roles in regulatory services, food safety, and One Health. Demographic shifts, including feminization and attrition pressures, further underline the importance of forward-looking workforce strategies. Regional collaboration—through joint curriculum innovations, shared training infrastructures, and incentive schemes for non-companion-animal careers—offers a practical path forward. While regionally grounded, these lessons resonate more widely: veterinary education should not merely mirror the market or accreditation frameworks but actively shape the profession's capacity to serve as both a responsive service and a public good central to food security, trade, and societal resilience.

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Review Paper

Advances and functional perspectives on next-generation edible films and coatings for meat products

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ABSTRACT

Edible films and coatings (EFCs) are emerging as sustainable alternatives to conventional meat packaging. Composed of plant- and animal-based biopolymers, they act as barriers to oxygen and moisture while providing active protection when combined with natural antioxidants and antimicrobials. Advances in single-layer, multilayer, and Layer-by-Layer (LbL) systems have improved their mechanical strength, barrier performance, and controlled release capabilities. The integration of plant extracts, essential oils, and polyphenols enhances oxidative stability, inhibits microbial growth, and maintains meat quality. Hybrid structures combining plant and animal materials further extend shelf life under refrigeration. With their bioactive and innovative designs, EFCs offer effective solutions for meat preservation and environmental sustainability.

1. Introduction

The demand for sustainable, clean-label, and high-quality meat products has accelerated research into edible films and coatings (EFCs) as eco-friendly packaging solutions. Conventional petroleum-based packaging, while offering good barrier and mechanical properties, contributes to environmental pollution and micro plastic accumulation due to its non-biodegradability (*Gheorghita et al*., 2020; *Bremenkamp,* 2025). Biodegradable films and coatings derived from renewable resources, on the other hand, provide both protective barriers and bioactivity, especially when formulated with natural antioxidants and antimicrobials (*Vilca et al*., 2023; *Stoleru et al*., 2024). Studies have shown that these systems can effectively reduce oxidative rancidity and microbial spoilage, thereby extending the shelf life and quality of meat products while aligning with sustainability goals (*Umaraw et al*., 2020; *Sayadi et al*., 2022; *Smaoui et al*., 2022; *Fatima et al*., 2024).

Although the terms "film" and "coating" are sometimes used interchangeably, they differ in structure and application. Edible films are preformed thin layers, typically manufactured by casting or extrusion, and can be applied as standalone sheets around meat products to improve oxygen and moisture barrier performance (*Suhag et al*., 2020). In contrast, edible coatings are applied directly to the surface of meat by dipping, spraying, or brushing, creating a continuous and integrated layer that adheres tightly to the product matrix (*Ansrade et al*., 2012; *Umaraw et al*., 2020). Coatings are particularly advantageous for incorporating bioactive

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compounds, such as clove oil, argan oil, lemongrass oil, apple peel, post-biotics, or polyphenolic plant extracts, enabling active protection against oxidation and microbial growth (*Shin et al.*, 2017; *Stoleru et al.*, 2021; *Abbasi et al*., 2023; *Jafari et al*., 2025). The functionality of EFCs depends on both raw material composition and structural design. Plant-based biopolymers (e.g., starch, pectin, cellulose, zein) provide excellent film-forming ability and gas barrier properties, whereas animal-derived polymers (e.g., gelatin, whey protein isolate (WPI), casein) exhibit superior oxygen barrier and mechanical performance (*Azeredo et al*., 2022). Hybrid systems, such as WPI combined with carnauba wax or gelatin-zein composites, combine these advantages and significantly enhance oxidative stability (*Kandasamy et al*., 2021; *Pires et al*., 2024). Structurally, EFCs are classified into single-layer, multilayer, and Layer-by-Layer (LbL) systems (*Xiao et al*., 2016). While single-layer films are simple but often require additives to improve barrier properties, multilayer structures—typically composed of protein and lipid layers—provide superior control over moisture and oxygen transmission (*Wang et al*., 2022; *Grzebieniarz et al*., 2023; *Dargahi et al*., 2024; *Kiattijiranon et al*., 2024). Advanced LbL coatings, created through sequential deposition of oppositely charged polymers, allow precise thickness control and controlled release of bioactive compounds (*Siddiqui et al*., 2023; *Jurić et al.*, 2023). EFCs play a crucial role in maintaining the quality attributes of meat by extending shelf life, preserving color stability, reducing lipid and protein oxidation, and enhancing sensory characteristics (*Sánchez-Ortega et al*., 2014; *Sharma & Jambhulkar et al*., 2014). Antioxidant-enriched films have been shown to reduce thiobarbituric acid reactive substances (TBARS) by 30–50%, while coatings with essential oils or polyphenolic compounds inhibit microbial spoilage and improve consumer acceptability (*Ansarian et al*., 2021; *Xue et al*., 2021; *Bai et al.*, 2022; *Smaoui et al*., 2022). This review aims to provide a comprehensive overview of plant- and animal-based biopolymers, structural approaches, such as single-layer, multilayer, and LbL systems, and the integration of functional additives, while discussing current advancements and future perspectives for next-generation EFCs that align with sustainability and clean-label requirements.

2. Structural types of edible films and coatings

The structure of EFCs strongly affects barrier capacity, mechanical strength, flexibility, and bioactive release (*Moeini et al*., 2022). Polymer arrangement, lipid/protein layers, and additive distribution influence food protection (*Gómez-Estaca et al*., 2023). Accordingly, EFCs can be tailored to reduce oxygen permeability, delay spoilage, and improve sensory quality of meat products (*Alias et al*., 2022; *Karnwal et al*., 2025). EFCs are classified as single-layer, multilayer, or layer-by-layer (LbL) systems, each with distinct benefits. Single-layer films, based on polysaccharides, proteins, or lipids, are simple and economical, but often show weak water vapor resistance (*Jamroz et al*., 2019; *Lopes et al*., 2020; *Agarwal et al*., 2021; *Bashkar et al*., 2023; *Grzebieniarz et al*., 2023). Incorporating active compounds, like essential oils or antioxidants, improves their performance, as shown with starch films containing oregano (*Shen et al*., 2022) or chitosan with rosemary oil (*Farokhzad et al*., 2023). Multilayer films, combining complementary layers, such as protein for oxygen barrier and lipid for moisture resistance, achieve superior protective effects (*Alias et al*., 2022; *Jiang et al*., 2022). They significantly reduce water vapor transmission and enhance oxidative stability in meat (*Anukiruthika et al*., 2020; *Xie*, 2023), and are widely used for fresh cuts, sausages, and ready-to-eat products. LbL coatings, based on sequential deposition of oppositely charged polymers (e.g., chitosan/pectin), create nanostructured films with tunable thickness and controlled release (*Yang et al*., 2019; *Andrade et al*., 2021; *Hernandes-Garcia et al*., 2022; *Cai et al*., 2023). When enriched with essential oils or phenolic extracts, they inhibit microbes and delay oxidation (*Vital et al*., 2016; *Langroodi et al*., 2021; *He & Wang*, 2022; *Antonino et al*., 2023). Nano emulsions and nano cellulose further enhance stability, transparency, and barrier capacity, enabling extended preservation of meat and seafood (*Kim et al*., 2018; *Zhang et al*., 2024; *Khorami et al*., 2024).

3. Integrated biopolymer systems and functional additives for meat preservation

EFCs are primarily developed from plant-based and animal-based biopolymers, which serve as the main structural matrices and determine their functional performance. Plant-derived materials are widely used in EFCs due to their renewability, biodegradability, and excellent film-forming abilities. Polysaccharides, such as starch, pectin, cellulose derivatives (e.g., carboxymethyl cellulose, hydroxypropyl methylcellulose), and alginate, are known for their strong oxygen barrier properties (*Díaz-Montes & Castro-Muñoz*, 2021). Due to their hydrophilic nature, these matrices are often combined with lipid components, like carnauba and candelilla wax or essential oils, to enhance moisture resistance (*Devi et al*., 2024). Plant proteins, including soy protein isolate, zein, pea protein, and pumpkin seed protein, contribute mechanical strength, flexibility, and transparency, making them ideal for edible film formation (*Khwaldia et al*., 2024). In addition to structural roles, plant-based matrices are frequently functionalized with natural bioactive compounds to improve oxidative and microbial stability. Natural antioxidants, such as grape seed extract, green tea catechins, rosemary, and pomegranate peel extract, are incorporated to scavenge free radicals and inhibit lipid oxidation (*Xie et al*., 2023). For example, starch-based films enriched with oregano essential oils have significantly delayed oxidative rancidity and color deterioration in fish fillets (*Martins et al.*, 2021). Similarly, natural antimicrobials like thyme, oregano, cinnamon, and clove essential oils—rich in phenolic compounds such as carvacrol and thymol—have been shown to effectively reduce spoilage and pathogenic microorganisms (*Antonino et al*., 2024). Also, starch-based films with oregano oil, for instance, have achieved a 50% reduction in total coliform counts on beef during storage (*Cestari et al*., 2021).

Animal-derived biopolymers, including gelatin, whey protein isolate (WPI), casein, collagen, and egg albumin, are valued for their superior mechanical properties and oxygen barrier performance (*Benbettaïeb et al*., 2016; *Rahman et al*., 2023). These polymers can form strong, transparent films, and when combined with lipid components such as beeswax or milk fat derivatives, they provide enhanced water vapor resistance (*Castro-Rosas et al*., 2016; *Devi et al.*, 2022). Animal-based films are also highly compatible with bioactive additives. Enrichment with natural antioxidants, like rosemary, elderberry, guava leaf, shallot, and green tea, can significantly reduce lipid oxidation and maintain the desirable red color of meat during storage (*Hamann et al*., 2022; *Lackner et al*., 2024*; Chang et al*., 2023; *Velaverde et al*., 2024; *Yeddes et al.,* 2025). Hybrid films that combine plant and animal polymers (e.g., gelatin-zein composites or WPI-carnauba wax layers) have demonstrated synergistic effects, improving both barrier properties and active functionalities (*Fitriani et al*., 2023). For example, gelatin-chitosan-based edible nano emulsion coating incorporating rosemary extract and ε-poly-L-lysine (ε-PL) has demonstrated superior antimicrobial and antioxidant efficacy for ready-to-eat carbonado chicken. The nano emulsion significantly reduced total viable bacterial counts, TBARS values, and pH variation over 16 days of refrigerated storage, thereby extending shelf life by at least 6 days compared to uncoated samples and coarse emulsions *(Zhao et al*., 2024). Advanced Layer-by-Layer (LbL) coatings, formed through the sequential electrostatic deposition of oppositely charged biopolymers, such as chitosan and alginate, have demonstrated enhanced preservation effects when loaded with natural bioactive agents. Recent studies have shown that bilayer systems incorporating fucoidan from *Sargassum angustifolium*, grapefruit seed extract, or combinations of tannic acid and cinnamaldehyde nano emulsions not only extend the shelf life of fish and shrimp products significantly (up to 16 days) but also provide strong antimicrobial action, lipid oxidation inhibition, and even real-time freshness monitoring through pH-sensitive layers (*Kim et al*., 2018; *Khorami et al*., 2024; *Wu et al*., 2024). EFCs not only delay lipid and protein oxidation but also inhibit spoilage and pathogenic microorganisms, thereby extending shelf life and preserving desirable sensory attributes (Table 1). Another important function of EFCs is moisture retention and texture preservation, as they minimize water loss and prevent toughening during cold storage (*Umaraw et al*., 2020; *Karnwal et al.*, 2025). Hybrid biopolymer films (e.g., gelatin-zein or WPI-carnauba wax composites) ensure juicier and more tender meat over time (*Arcan & Yemenicioğlu*, 2013*; Zhang et al*., 2018; *Cheng et al*., 2022; *Hu et al*., 2023). Sensory evaluations further confirm that natural bioactives such as rosemary extract or green tea polyphenols enhance flavor stability and consumer acceptability (*Sharma et al*., 2021).

Table 1. Summary of Edible Films and Coatings for Meat Preservation

Film/Coating Type	Active Compounds	Main Application	Key Results	References
Alginate coatings	Essential oils	Fresh meat	Inhibited microbial spoilage; reduced weight loss	*Gheorghita et al*., 2020
Edible alginate films with pineapple peel extract	Phenolic antioxidants	Beef	Antioxidant protection; oxidative stability maintained	*Lourenço et al*., 2020
Chitosan film with oregano & thyme essential oil	Oregano & thyme essential oil	Beef	Antimicrobial effect; improved oxidative stability	*Gaba et al*., 2022
Antimicrobial/ antioxidant coatings	Natural extracts	Chicken meat	Improved shelf life & safety	*Moura-Alves et al*., 2023
Polysaccharide-based coatings	Essential oils, bioactive nanosystems	Meat & fish	Antimicrobial and antioxidant effects	*Hashemi et al*., 2023
Fish gelatin-pectin coatings	Lemongrass essential oil	Chicken meat	Antimicrobial and sensory improvement	*Carraro et al*., 2023
Gelatin/carboxymethyl chitosan/guar gum coatings	Shallot waste extracts	Beef	Reduced microbial load; safety improvement	*Nguyen et al*., 2023
Ethyl cellulose/gelatin-carboxymethyl chitosan bilayer film	Euryale ferox polyphenols	Cooked meat	Antioxidant and antimicrobial effect	*Cai et al*., 2023
Composite coatings	Nanocellulose + chitosan	Meat	Barrier enhancement; antimicrobial and antioxidant activity	*Costa et al*., 2021
pH-sensitive bilayer film (smart packaging)	Tannic acid–cinnamaldehyde nanoemulsion + alizarin	Shrimp	UV protection, antimicrobial effect, pH monitoring, freshness indicator	*Wu et al*., 2024
Graphene oxide multilayer films	Graphene oxide + chitosan + poly(l-lactic acid-poly butylene *itaconate* copolymers	Chilled meat	High barrier properties, low O_2 permeability, delayed spoilage	*Zhang et al*., 2024
Curcumin-loaded chitosan/egg yolk film	Curcumin	Pork	Freshness preservation; antioxidant activity	*Jiang et al*., 2024
Olive leaf extract coatings	Olive leaf phenolics	Cooked meat	Antioxidant and antimicrobial protection	*Şen & Güleç*, 2024
Acorn phenolic-rich coatings	Acorn extract	Chicken patties	Improved oxidative stability, sensory quality	*Vallejo-Torres et al*., 2024
Chitosan nanocomposites	Biopreservatives, nanoparticles	Meat	Strong antimicrobial and antioxidant properties	*Heras et al*., 2024
κ-Carrageenan-based coatings	Resveratrol, quercetin	Goat patties	Antioxidant effect, shelf-life extension	*Şengül & Şen*, 2025
Alginate-based coatings + irradiation	Myrtle, rosemary extracts	Beef	Strong antioxidant and antimicrobial protection	*Smeti et al*., 2025
Sodium alginate coatings + preservatives	Potassium sorbate	Pork	Antimicrobial effect, shelf life extension	*Jiang et al*., 2025
Nanocellulose-based coatings	Forsythia essential oil	Fresh meat	Antimicrobial and barrier improvement	*Cheng et al*., 2024

4. Conclusion

Edible films and coatings represent a promising pathway toward reducing reliance on conventional plastic packaging in the meat industry, offering protective functionality while supporting clean-label and environmental objectives. By leveraging the complementary properties of plant-derived and animal-derived biopolymers, these systems can be tailored to improve moisture and gas barrier capacity, mechanical integrity, and controlled release of active agents. Advances in multilayer and LbL structures have expanded the design possibilities, enabling better integration of bioactive components for oxidative and microbial stability. Nevertheless, their broader implementation depends on overcoming economic, technological, and regulatory barriers, as well as ensuring consumer trust. Future developments are expected to focus on intelligent and responsive packaging concepts, integration of by-product valorization strategies, and scalable production technologies, strengthening the role of EFCs in sustainable meat preservation.

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Review Paper

From gene to table – DNA barcoding the backbone of next-generation food integrity and safety

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ABSTRACT

The authenticity and safety of food are critical issues in global supply chains, where mislabelling, adulteration and contamination continue to challenge food integrity, consumer-trust and safety, and public health. DNA barcoding, a molecular identification method based on short genetic sequences, is a powerful tool for species verification in a wide range of food products, including meat, fish, plant-based foods, and processed foods. This study explores the application of DNA barcoding with an emphasis on its role against food fraud, consumer protection and public health. Advances in barcoding technologies, coupled with accessible databases enable identification of species even in complex or degraded samples. Case examples illustrate its successful implementation in regulatory, industrial, and research settings. The integration of DNA barcoding into traceability systems supports a more transparent, accountable, and trustworthy food supply industry. As food systems evolve, DNA-based verification is poised to become a core component of next-generation food integrity strategies.

1. Introduction

Food quality and safety are critical issues in the increasing global food trade characterized by complex supply chains. Although consumers increasingly demand sustainable, transparent, and healthy food products — and despite stricter regulations and technological progress — food fraud remains a persistent issue. It poses risks to public health, causes economic damage, compromises religious dietary requirements, and may even contribute to the endangerment of protected species (*Li et al*., 2020; *Filonzi et al*., 2023). Food fraud involves deliberate mislabelling, species substitution, and exposure to toxins, pathogens and allergens (*Ortea et al.,* 2012) threatening consumer health and undermine consumer trust (*Dawan and Ahn*, 2022). Mislabelling rates across various food sectors including seafood, meat, botanicals, spices, and probiotics exceed on average by > 20% (*Gorini et al.,* 2023) to a maximum of >70% for certain food categories (*Stamatis et al.,* 2015). The most common type of food fraud is the replacement of a component with a similar cheaper one, followed by undeclared ingredients (*Stamatis et al.,* 2015).

Since the 1980s worldwide research strategies have been aiming to develop and improve

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technologies to control authenticity and safety of foods (*Katerinopoulou et al*., 2020). Amongst the analytical tests used for food authentication DNA-based techniques are most effective. DNA barcoding, a molecular technique that uses short, standardized genetic markers to identify species (*Hebert et al.,* 2003), has become a cornerstone in addressing these challenges (*Kumar et al.,* 2009). Originally developed for taxonomic and biodiversity research, DNA barcoding has been adapted to food science to authenticate raw and processed food products, even when morphological characteristics are degraded or lost (*Dawn and Ahn*, 2022). The technique has evolved further with the integration of next-generation sequencing (NGS), enabling DNA metabarcoding—a high-throughput approach capable of detecting multiple species in complex mixtures (*Dobrovolny et al*., 2022). This has proven especially valuable in uncovering adulteration in meat products and composite foods. For example, a 2024 study applying metabarcoding to EU meat samples revealed numerous undeclared animal and plant species, providing evidence of hidden substitutions and labelling violations (*Mottola et al*, 2024). However, the field is not without its limitations. A 2024 systematic review of metabarcoding in animal-origin food authentication highlighted persistent issues such as inconsistent primer sets, platform-dependent biases, and the need for standardized quality control procedures before the method can be widely adopted for regulatory use (*Giusti et al*., 2024). In the seafood industry, DNA barcoding continues to reveal significant mislabelling — for instance, a 2021 Italian border-inspection study found 22.5 % mislabelling in imported seafood, with cephalopods mislabelled at 43.8 % and fish at 14.0 % — sometimes involving endangered or protected species (*Filonzi et al*., 2023; *Guardone et al*., 2021). The economic impact of food fraud remains uncertain, with global estimates ranging between $ 10 billion (*Robson et al*., 2020) to at least $ 65 billion (*Agetu*, 2020). In 2017 alone, authorities in Europe seized approximately 9,800 tonnes of non-authentic food (*Kendall et al*., 2019). These findings illustrate the global scale of the problem and the importance of molecular tools in enforcing food law and protecting endangered species.

This review provides a comprehensive overview of DNA barcoding and its technological advancements, with a focus on its role in ensuring food authenticity and traceability “from gene to table” (*Liu K et al*, 2022). Current applications across food categories are reviewed, regulatory perspectives are addressed, and emerging trends including portable sequencing and the integration with blockchain technology are examined (*Singh and Sharma*, 2023).

2. Principles and technological advancements in DNA barcoding

The selection of DNA barcoding markers depends on the target organism. For animals, the cytochrome c oxidase I (COI) remains the standard. In plants, mitochondrial genes evolve too slow, so plastid genes like *rbcL* and *matK* are used instead (*CBOL Plant Working Group*, 2009). For fungi, the internal transcribed spacer (ITS) region offers high discriminatory power (*Schoch et al.*, 2012).

The barcoding workflow typically begins with sample collection from food matrices (Figure 1). DNA is then extracted using commercial kits or CTAB-based protocols designed to overcome food-specific inhibitors like fats or tannins. Target regions are amplified via PCR, often using nested or multiplex strategies in complex mixtures (*Handy et al.*, 2011). Sequencing is performed through either traditional Sanger methods or whole-genome sequencing. Traditional Sanger sequencing, while reliable, is limited by low throughput and an inability to resolve multiple species in complex mixtures. These constraints have been overcome by the introduction of next-generation sequencing (NGS) technologies. This enables DNA metabarcoding—simultaneous identification of multiple species within a single mixed sample—making the technique particularly valuable for testing multi-ingredient and processed foods like sausages, herbal blends, and convenience meals.

In cases where DNA is degraded—such as in canned or heavily processed foods—shorter “mini-barcodes” (less than 200 base pairs) improve amplification success (*Stahl et al*., 2023). Mini-barcoding achieved identification rates above 90% across a range of difficult food matrices (*Stahl et al.,* 2023). Nevertheless, the shorter length of mini-barcodes may limit their ability to discriminate between closely related species, and issues such as misidentified specimens, sample confusion, or contamination can further compromise accuracy. However, advances in reference databases and bioinformatic methods have improved reliability, and combining multiple loci or integrating mini-barcodes with full-length markers allows robust species identification (*Galimberti et al.,* 2013; *Cheng et al*., 2023).

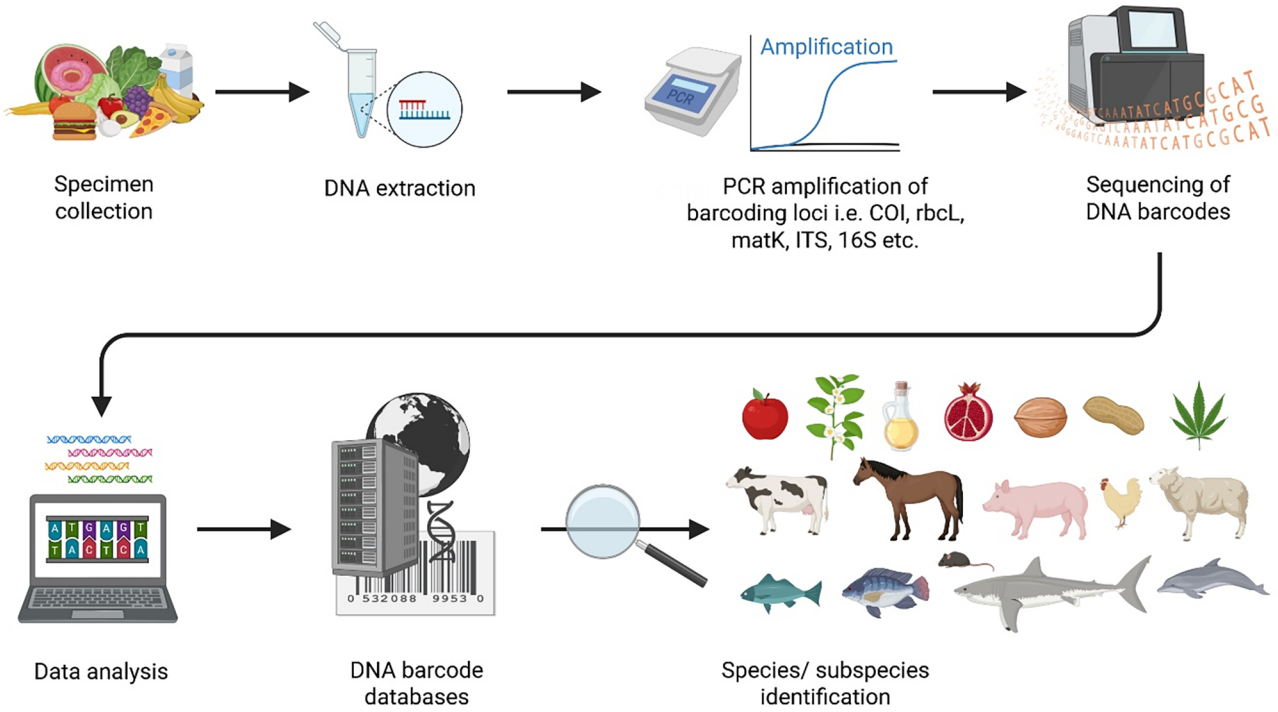

Figure 1. The principles of the DNA- barcoding technique (Created in BioRender. Ruppitsch W. (2025) https://BioRender.com/bwtr3ev»)

The effectiveness of these tools depends heavily on the quality of the reference databases used. Customized regional or institutional databases are increasingly being incorporated into barcoding workflows to complement public repositories and improve identification accuracy. Such tailored datasets, for example those developed for mammalian and poultry species authentication, allow laboratories to use relevant barcode sequences adapted to their validation needs (*Dobrovolny et al*., 2022). Two of the most common global databases are the Barcode of Life Data System (BOLD), which is curated specifically for barcoding and linked to voucher specimens (*Ratnasingham and Hebert,* 2007), and GenBank, which offers broader coverage but is less curated and more prone to misidentifications (*Nilsson et al.,* 2006). To enhance consistency and data interoperability, initiatives such as the FAIR data principles (Findable, Accessible, Interoperable, Reusable) have been introduced. These guidelines aim to improve database standardization and facilitate the integration of barcoding outputs into regulatory and traceability systems (*Wilkinson et al*., 2016). Hybrid strategies—such as cross-validating GenBank hits with BOLD entries or building in-house curated databases—are becoming increasingly common for ensuring accuracy in critical applications like regulatory inspections and certification.

Collectively, these technological advancements have pushed DNA barcoding from a niche laboratory method into a mainstream, scalable, and field-deployable tool with broad applications in food integrity and safety.

3. Applications in food integrity and safety

One of the most pressing concerns in global food markets is species mislabelling and substitution, which remains prevalent in seafood, meat, and plant-based products. For instance, studies in Thailand have shown that over 24% of seafood products were mislabelled, including cases where endangered species like *Thunnus maccoyii* (Southern bluefin tuna) were substituted for more common varieties (*Senathipath et al.,* 2024). DNA-based methods, including metabarcoding and DNA barcoding, have revealed the presence of undeclared animal species in a substantial proportion of meat products, highlighting widespread mislabelling and the utility of these molecular tools for verifying species composition and ensuring food authenticity (*Tantuan and Viljoen*, 2021; *Hellberg et al.,* 2017). In the plant sector, DNA markers such as *rbcL*, *matK*, and ITS2 have uncovered extensive adulteration in herbal supplements and traditional medicines (*Harris et al.,* 2024; *Chen et al.,* 2022). High-value oils, such

as olive oil from protected regions, can also benefit from DNA-based traceability tools, which help authenticate geographic origin and detect adulteration with lower-quality oils (*Agrimonti et al.,* 2011). DNA barcoding is also instrumental in detecting adulterants and contaminants that pose health risks to consumers. Even trace amounts of undeclared allergens like peanuts, soy, or crustaceans can trigger serious allergic reactions. DNA-based analyses have proven effective in identifying such allergens in processed foods and sauces, as well as banned substances like bushmeat or endangered species in imported products (*Staats et al*., 2016). Using metabarcoding techniques, numerous instances of undeclared animal DNA have been detected in products labelled as vegan or allergen-free, which can lead to product recalls and stricter regulatory oversight (*Giusti et al.,* 2023; *BeVeg*, 2023). The method also enables detection of toxic plant species that may be accidentally or fraudulently incorporated into teas, supplements, or traditional remedies—situations where visual inspection would be inadequate *(Sucher and Carles*, 2008).

Beyond authenticity and allergen control, DNA barcoding plays a growing role in verifying compliance with religious and ethical food certifications. Halal certification, for example, requires the exclusion of any porcine material; barcoding is routinely employed to test for pork DNA in meat, gelatine, and additives (*Shabani et al.*, 2015). Kosher regulations similarly restrict specific ingredients and combinations, and DNA-based tests can detect the presence of shellfish or non-kosher fish in processed foods (*Andronache et al.*, 2025). Even vegan-labelled products are increasingly scrutinized using metabarcoding to ensure they are free of trace animal DNA, especially in shared production facilities (*Srivathsan et al.,* 2021).

DNA barcoding is expanding into the realm of microbial food safety (Sabater et al.,2021). Although originally developed for multicellular organisms, adaptations using 16S rRNA and ITS rRNA sequencing now allow for the identification of bacterial, fungal, and parasitic contaminants. This approach has been applied to detect key pathogens such as *Salmonella*, *Listeria*, and *E. coli* in meat and dairy products, as well as toxigenic fungi like *Aspergillus* spp. and *Fusarium* spp. in cereals and spices (*Muñoz-Martinez et al.,* 2025; *Gonzalez-Escalona et al.,* 2017), and protozoan contaminants like Cryptosporidium spp. and Giardia spp. in water and fresh produce. DNA metabarcoding has been applied in Sardinian sheep cheese processing environments, identifying bacterial strains that could contribute to product contamination and spoilage, highlighting the utility of metabarcoding for monitoring microbial risks in cheese production (*Giagnoni et al*., 2025). Applications of DNA barcoding in food integrity and safety are shown in Table 1.

4. Regulatory and industrial perspective

Food fraud within the European Union is regulated under Regulation (EC) No. 178/2002 of the European Parliament. Despite significant efforts toward its prevention, access to reliable and publicly available information remains limited (*Brooks et al.,* 2021). European mechanisms such as the Rapid Alert System for Food and Feed (RASFF), established in 1979, and the EU Food Fraud Network,

Table 1. Applications of DNA barcoding in food integrity and safety

Application area	Description
Species mislabelling	Identification of incorrectly labelled or substituted species in meat, fish, etc.
Detection of allergens and contaminants	Detection of trace allergens (e.g., peanut, soy) and harmful substances.
Religious and ethical standards	Verification of the absence of prohibited ingredients in Halal, Kosher, and vegan products.
Microbial contamination	Identification of bacteria, fungi, and parasites relevant to food safety.
Authentication of plant-based products	Analysis of teas, supplements, and herbal blends for undeclared or toxic species.
Verification of declared composition	Detection of undeclared or illegal ingredients in complex processed foods.

launched in 2013, have improved data sharing and cross-border collaboration in addressing food fraud (*Brooks et al*., 2021). However, the complexity of international supply chains, combined with the highly complex nature of many food products, increases the probability that fraud remains undetected (*Manning & Monaghan*, 2019).

One contributing factor is that the Hazard Analysis and Critical Control Points (HACCP) system, while effective for food safety, was not designed for the detection of fraudulent practices (*Kowalska,* 2018). To address this gap, complementary frameworks such as Threat Analysis and Critical Control Points (TACCP) and Vulnerability Assessment and Critical Control Points (VACCP) are increasingly being adopted (*Brooks et al.,* 2021). The effectiveness of these systems, however, depends heavily on the application of advanced analytical techniques capable of verifying food authenticity and preventing adulterated products from entering the supply chain (*Manning & Soon*, 2016; *Fox et al.,* 2018).

Building on this foundation, DNA-based techniques such as DNA barcoding and metabarcoding are increasingly regarded as valuable tools to strengthen global food authenticity and traceability systems. In the EU, the European Food Safety Authority (EFSA) has acknowledged their scientific potential, particularly regarding identity verification of novel foods including plants, animals, fungi, and algae, but has not yet formally endorsed their widespread use (*Veveris,* 2024). Metabarcoding, for instance, shows particular promise in detecting undeclared species or complex plant compositions, though its regulatory application remains under development by EFSA and EU Member States (*Mottola et al*., 2024; *Giusti et al*, 2024).

In the United States, the Food and Drug Adminsitration (FDA) has integrated DNA-based methods into its seafood inspections and guidance materials to support regulatory compliance and ensure seafood authenticity (*Handy et al.,* 2011). These methods use standardized DNA sequences, such as those in the Regulatory Fish Encyclopedia, to detect mislabelled or substituted species throughout the supply chain, enhancing traceability, preventing fraud, and supporting consumer protection (*Haile et al.,* 2008). DNA barcoding represents a valuable tool that helps the FDA ensure the safety and integrity of the U.S. food supply (*Jones et al*., 2013).

Countries like Canada, Australia, and China also apply DNA barcoding in customs, Halal/Kosher certification, and herbal verification (*Fathima et al.,* 2024; *Aghayeva et al.,* 2021). Seafood processors use DNA barcoding to confirm species at each supply chain step, helping detect mislabelling and thus improve traceability (*Pardo et al*., 2020). The technology is also being embedded in blockchain platforms for meat and herbal supplements, linking product records to genetic proof (*Gorini et al*., 2023).

Globally, the Codex Alimentarius Commission has begun incorporating molecular tools into its food authenticity standards, encouraging harmonization. While Codex recognizes the importance of DNA-based methods for detection and identification of species and specific proteins in foods, their use is not yet mandatory, and implementation depends on national authorities (*Codex*, 2011). So far, FDA and the Joint Research Centre (JRC) of the European Commission have released detailed protocols, and Codex is evaluating molecular methods for inclusion in international guidelines, however standardized and validated protocols are still needed to ensure harmonized application across countries (*JRC*, 2024; *Codex*, 2023).

5. Challenges and limitations

Despite its advantages, DNA barcoding faces several limitations that hinder its universal application in food authenticity and safety. A major technical barrier is DNA degradation in processed foods. Thermal, chemical, or mechanical treatments often fragment DNA, making standard barcode regions like COI, *rbcL* or ITS difficult to amplify (*Meusnier et al*., 2008). Mini-barcodes (<200 bp) have been developed to overcome this, though they may lack full taxonomic resolution (*Shokralla et al*., 2015). Another critical issue is the incompleteness and inconsistency of reference databases (*Kartzinel et al*., 2025). Many food-relevant species are underrepresented, especially among plants, fungi, and minor seafood taxa. Misannotations in public databases such as GenBank can result in misidentifications, prompting researchers to cross-reference with BOLD or use curated in-house databases (*Ratnasingham and Hebert*, 2007).

A lack of global harmonization means results accepted in one region may not be valid in another (*Geary et al*., 2019). Data sovereignty is also a concern, particularly where genetic resources from indigenous regions are used without consent, raising compliance issues with the Nagoya Protocol (*Prathapan and Rajan*, 2020). Misidentification due to contaminated or incomplete data can lead to costly

recalls and reputational harm. Costs and infrastructure constraints remain a major hurdle in low- and middle-income countries. Although technologies have improved accessibility, expenses for reagents, limited lab capacity, and lack of trained staff are persistent challenges (*Yuan et al.,* 2025). Additionally, global databases are biased toward species from developed regions, limiting their relevance for foods originating from Africa, Asia, and Latin America. Greater investment in local databases, training, and technology transfer is essential for equitable global adoption (*Yuan et al*.,2025).

6. Future perspectives

DNA barcoding is evolving beyond species identification into a central tool for transparency, adaptability, and sustainability. Its convergence with blockchain, artificial intelligence (AI), and ecosystem monitoring opens new opportunities for traceability and risk management. Blockchain integration offers secure, tamper-proof traceability by linking DNA-verified species data to immutable digital records. Pilot projects in high-value sectors like seafood and organic meat have already connected QR codes on packaging to DNA-based authenticity logs (*Gröppel-Klein et al.,* 2023; *FAO*, 2022). This model could become standard for certified products like Halal or sustainably sourced goods. AI and machine learning are transforming how barcoding data is processed. These tools automate species classification, detect anomalies, and integrate genetic, geographic, and trade data to predict fraud and supply chain vulnerabilities (*Lokan et al.*, 2024). Barcoding also supports climate-resilient food systems by enabling rapid identification of shifting species and empowering small producers to validate products for competitive markets and it contributes to reduce food waste through improved sorting, labelling, and quality control (*Gorini et al.,* 2023). In this broader role, DNA barcoding is poised to become essential infrastructure for building ethical, transparent, and sustainable food systems worldwide.

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Original Scientific Paper

Fatty acid composition of Visočka pečenica, a traditional dry-cured meat product from Bosnia and Herzegovina

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ABSTRACT

Visočka pečenica is a traditional dry-cured beef meat product from Bosnia and Herzegovina. It is the first meat product in Bosnia and Herzegovina to be protected by a geographical indication, and it is particularly appreciated for its aroma, taste and smoky properties. In this study, the fatty acid composition of 40 Visočka pečenica made in traditional (artisanal) and industrial production systems was examined. The fatty acid composition was determined by gas chromatography. Of the saturated fatty acids, the most abundant were palmitic and stearic acids (respectively, constituting 23.46% and 15.29% of the total fatty acids - traditional; 23.41% and 16.16% - industrial production). From the group of monounsaturated fatty acids, the most abundant was oleic acid, accounting for 45.61% of the total fatty acids in products from traditional production and 42.48% in industrial products. The most abundant polyunsaturated fatty acid was linoleic acid, which made up 7.61% of the total fatty acids (traditional production) and 7.41% (industrial production). PCA analysis of fatty acid composition showed that twenty fatty acids were characteristic of Visočka pečenica from industrial production and four fatty acids of products from traditional production.

1. Introduction

Cured meat products are highly valued products in the diet of the population both in Bosnia and Herzegovina (BiH) and in the world, and are good sources of high biological value protein. Those products with specific names and from specific geographic regions of a country are more regarded as traditional (*Radovanović et al*., 2003). The properties and quality of these products are greatly influenced by, among other things, the general characteristics of the climate, and especially by the specific climatic conditions of the geographical area in which they are produced (*Radovanović et al*., 2005).Visočka pečenica is a traditional, dry-cured beef meat product from BiH that has been produced in the municipality of Visoko for many years. It is produced from the highest quality parts of beef carcasses, dry salted only with table salt and cold smoked and dried (*Begić et al*., 2023).

Although cured meat products are a source of high-quality protein, negative aspects of this type of product are also known as a result of the high content of animal fat. Lipids in meat are found in muscle tissue and in the associated adipose tissue. Adi-

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pose tissue contains triglycerides and muscle tissue, in addition to triglycerides in the membranes of muscle fibres, which also contains phospholipids (*Barbir et al*., 2014). Research has shown that the fatty acid composition of meat consists of an average of 40% saturated (SFA), 40% monounsaturated (MUFA) and about 2-25% polyunsaturated (PUFA) fatty acids, with oleic acid being the most common in all types of meat and meat products (*Woods and Fearon*, 2009).

The fatty acid composition of cured meat products is influenced by various factors, primarily the type, feeding and breeding of animals, as well as the applied technological processes and parameters during production (*Siciliano et al*., 2013). There has been increased interest in recent years in manipulating the fatty acid composition of meat and meat products. This is because meat is seen to be a major source of fat in the diet and especially of saturated fatty acids, which have been implicated in diseases associated with modern lifestyles, especially in developed countries (*Wood et al*., 2004). A relatively high cholesterol content and a low ratio of polyunsaturated to saturated fatty acids are risk factors for some disorders, such as coronary heart disease (*Cordain et al*., 2005). Saturated fatty acids are well known to raise total and low-density lipoprotein (LDL) cholesterol (*Valsta et al*., 2005). Saturated fatty acids are correlated with increased risk of cardiovascular disease while a high intake of monounsaturated and n-3 polyunsaturated fatty acids has an inverse effect (*Fernández et al*., 2007). Given the importance of fatty acid composition on the nutritional acceptability of meat and meat products, the aim of this study was to examine the fatty acid composition of Visočka pečenica produced traditionally (artisanally) and industrially. Fatty acid composition tests on Visočka pečenica from these two production systems have not been conducted so far.

2. Materials and methods

2.1. Production and sampling of Visočka pečenica

Experimental production of Visočka pečenica was performed in traditional and industrial conditions. Altogether, 40 Visočka pečenica were sampled twice for research needs, halfway through and at the end of the smoking processes. Accordingly, 80 samples were analyzed. Visočka pečenica were prepared from different carcass cuts (round-*Musculus gluteobiceps*, *Musculus gluteus medius*, *Musculus semitendinosus* and; back musculature-*Musculus longissimus dorsi* (MLD)). During the smoking phase, the products were spread over two levels. The first level was, in relation to the hearth in the smokehouse, at a height of 2 m. The second height studied was 4 m above the hearth. Looking at the phases, the experimental design meant that during smoking, five samples of both anatomical regions (round and back) were placed on each level of the smokehouse. The experimental design, from the choice of raw material to the technological processing to the method of sampling, was identical for both production systems. However, on comparing the traditional and industrial production systems, differences found were related to the amount of added salt, the length of smoking time, and the parameters of the smoking process (temperature and relative humidity inside the smokehouse, outside air temperature and the temperature of the stokehole).

2.2. Methods

Fatty acid methyl esters were prepared using the standard method (*BAS ISO*, 2018), which involves dissolving glycerides in isooctane and trans-esterification using a methanolic solution of potassium hydroxide; the fatty acid composition was determined using the gas chromatography method. The fat content of the Visočka pečenica was determined using the reference method (*BAS ISO,* 2007), which involves Soxhlet fat extraction with organic solvents. The fat obtained through extraction was further used to determine the composition of fatty acids. Approximately 60 mg of extracted fat was weighed into a glass tube with a glass stopper and 4 mL of isooctane was added. After the fat was dissolved, 200 µL of methanolic potassium hydroxide solution was added. After adding the methanolic potassium hydroxide solution, vigorous shaking was performed twice for 30 s, after which the mixture was left to react at room temperature. After the mixture became clear, a glycerol layer was separated at the bottom of the tube, and 1 g of sodium hydrogen sulphate monohydrate was added to neutralize the mixture. Vigorous shaking was once again performed twice for 30 s. When the crystals had settled, 500 µL of the resulting sample solution was transferred to an injection vial and 1 mL of isooctane was added, after which the vial was closed and shaken. For the determination of fatty acid composition, a Shimadzu QP2010 gas chromatograph with

mass spectrophotometer was used with a Supelco SP - 2560 capillary column (100 m × 0.25 mm × 0.20 μm). The chromatographic determination conditions were as follows: injector and detector temperatures 220°C, column temperature 170°C, injected volume 1 μL. Helium with a flow rate of 0.9 mL/min was used as the carrier gas. Fatty acids methyl esters were identified by comparison with retention times of 37 fatty acids methyl esters of the standard mixture (SupelcoTM 37 Component FAME Mix) analyzed under the same conditions. The fatty acid content was expressed as % of the total identified fatty acids. For the correlation and presentation of the results multivariate data analysis was used - analysis of the basic components or PCA analysis. Statistical analyses were performed using Past software 3.15 (*Hammer et al*., 2001).

3. Results and discussion

The fatty acid compositions of the Visočka pečenica from traditional production are shown in Table 1. The analysis determined that, among the three fatty acid types, Visočka pečenica from traditional production were the richest in monounsaturated fatty acids due to the large amount of oleic acid (C18:1 c n-9), which was also the most abundant fatty acid (it accounted for 45.61% of the total fatty acids). The next-largest share was made up of saturated fatty acids. The most abundant saturated fatty acids were palmitic (C16:0) with 23.46% of total fatty acids and stearic fatty acid (C18:0) with 15.29%. The smallest share was made up of polyunsaturated fatty acids, and the most abundant among them was linoleic fatty acid (C18:2 c n-6) that constituted 7.61% of the total fatty acids. Comparing the composition of fatty acids halfway through and at the end of the smoking process, no major differences in their amounts were found. With the finished smoking process (compared to half-smoked product), there was slightly less of the palmitic, oleic and linoleic fatty acids and more stearic fatty acid. The Visočka pečenica prepared from round had a higher content of stearic fatty acid compared to the MLD products. In contrast, the MLD Visočka pečenica had higher contents of palmitic, oleic and linoleic fatty acids compared to the round Visočka pečenica.

The fatty acid composition of the Visočka pečenica from the industrial production system are shown in Table 2. The largest proportion of the three

Table 1. Fatty acid composition of Visočka pečenica from traditional production (% of total identified fatty acids)

Fatty acid (%)	TRHH	TMHH	TRLH	TMLH	TRHE	TMHE	TRLE	TMLE
C8:0	0.11	0.08	0.10	0.09	0.11	0.06	0.09	0.09
C10:0	0.12	0.06	0.10	0.08	0.14	0.05	0.08	0.10
C12:0	0.09	0.06	0.08	0.05	0.08	0.03	0.06	0.08
C14:0	1.34	1.21	1.38	1.09	1.35	1.16	1.11	1.09
C15:0	0.01	0.00	0.00	0.00	0.02	0.00	0.00	0.01
C16:0	22.98	23.73	23.40	23.87	22.36	23.24	24.47	23.63
C17:0	0.24	0.22	0.20	0.16	0.36	0.17	0.11	0.25
C18:0	16.56	14.90	15.20	14.22	16.95	15.44	14.39	14.69
C20:0	0.24	0.22	0.21	0.16	0.35	0.17	0.17	0.30
C14:1	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.01
C16:1	3.25	2.76	3.01	2.22	3.61	2.55	2.77	2.98
C17:1	0.48	0.44	0.50	0.43	0.72	0.35	0.50	0.50
C18:1 t n-9	0.14	0.07	0.10	0.05	0.14	0.05	0.11	0.08
C18:1 c n-9	44.05	45.81	45.50	47.54	43.44	46.45	46.84	45.26
C20:1	0.77	0.39	0.60	0.43	0.77	0.35	0.55	0.45
C22:1 n-9	0.29	0.22	0.25	0.22	0.29	0.17	0.22	0.30
C18:2 c n-6	7.47	7.95	7.50	7.70	7.12	8.13	6.65	8.34
C18:2 t n-6	0.01	0.01	0.01	0.00	0.02	0.00	0.01	0.01
C18:3 n-6	0.14	0.14	0.11	0.11	0.23	0.07	0.11	0.15
C20:2 n-6	0.29	0.24	0.30	0.22	0.34	0.23	0.33	0.25
C20:3 n-6	0.19	0.17	0.20	0.16	0.28	0.12	0.22	0.20
C20:4 n-6	0.34	0.34	0.35	0.33	0.36	0.29	0.33	0.34
C18:3 n-3	0.44	0.50	0.45	0.44	0.50	0.46	0.44	0.45
C20:3 n-3	0.44	0.48	0.45	0.43	0.45	0.46	0.44	0.44

*T-traditional production; M-MLD, R-round samples; H-higher, L-lower smoking heights; H-halfway through, E-end of the smoking process.

fatty acid groups in these Visočka pečenica was monounsaturated fatty acids, due to the high proportion of oleic fatty acid (C18:1 c n-9), which was the most abundant fatty acid compared to the others (it constituted 42.48% of the total fatty acids). The next-largest proportion was made up of the saturated fatty acids, of which the most abundant were palmitic (C16:0) with 23.41% and stearic fatty acid (C18:0) with 16.16% of total fatty acids. The smallest proportion was made up of the polyunsaturated fatty acids, of which linoleic fatty acid (C18:2 c n-6) was present in the largest amount, accounting for 7.41% of total fatty acids. In comparing the fatty acid composition of Visočka pečenica from industrial production halfway through and at the end of the smoking process, the longer smoking time resulted in some small changes. Namely, with longer smoking, the contents of stearic and linoleic acids were lower, while the contents of palmitic and oleic acids were numerically higher. On comparing the different cuts, the Visočka pečenica prepared from round were richer in stearic and oleic acids, while those prepared from MLD were richer in palmitic and linoleic acids.

In comparing the two production systems, it can be concluded that the largest proportion of fatty acids in the Visočka pečenica from both production types was monounsaturated fatty acids, with a higher proportion in traditionally produced than in industrially produced products. Oleic fatty acid was the most abundant fatty acid in Visočka pečenica from both production systems, in a higher quantity in those from traditional production (45.61%) compared to those from industrial production (42.48%). *Subašić* (2024) and *Saičić et al.* (2010) indicated that oleic acid was the most abundant of the fatty acids in beef prosciutto, with higher levels (59.18% and 49.92% reported, respectively) compared to those listed in our current study. After monounsaturated fatty acids, the largest proportion of fatty acids in Visočka pečenica from both types of production was saturated fatty acid group, with a higher proportion in industrial than in traditional Visočka pečenica. The most abundant saturated fatty acids in the Visočka pečenica were palmitic (C16:0) (23.46% - traditional and 23.41% - industrial) and stearic (C18:0) (15.29% - traditional and 16.16% - industrial) acids. In accordance with the above, *Saičić et al*. (2010) and *Subašić* (2024) also recorded palmitic and stearic acid as the most abundant of the saturated fatty acid group in beef prosciutto. Among the three fatty acid types, the low-

Table 2. Fatty acid composition of Visočka pečenica from industrial production (% of total identified fatty acids)

Fatty acid (%)	IRHH	IMHH	IRLH	IMLH	IRHE	IMHE	IRLE	IMLE
C8:0	0.16	0.14	0.13	0.14	0.13	0.13	0.12	0.12
C10:0	0.17	0.17	0.16	0.15	0.14	0.16	0.14	0.13
C12:0	0.13	0.11	0.10	0.09	0.09	0.12	0.09	0.09
C14:0	1.63	1.55	1.58	1.59	1.57	1.59	1.55	1.19
C15:0	0.07	0.04	0.04	0.04	0.05	0.04	0.04	0.02
C16:0	23.28	23.78	22.82	23.43	24.06	23.03	23.27	23.60
C17:0	0.52	0.39	0.39	0.42	0.28	0.49	0.37	0.35
C18:0	15.87	16.77	16.15	16.13	16.75	15.49	16.15	15.94
C20:0	0.34	0.35	0.31	0.26	0.19	0.31	0.27	0.22
C14:1	0.06	0.05	0.04	0.04	0.04	0.04	0.05	0.04
C16:1	4.07	3.11	3.51	3.66	3.05	3.98	3.10	3.63
C17:1	0.83	0.71	0.75	0.82	0.46	0.71	0.50	0.51
C18:1 t n-9	0.25	0.22	0.22	0.23	0.19	0.22	0.18	0.18
C18:1 c n-9	41.53	41.35	42.92	42.19	42.11	42.67	43.80	43.28
C20:1	0.80	0.84	0.79	0.79	0.74	0.71	0.73	0.75
C22:1 n-9	0.29	0.31	0.29	0.26	0.28	0.27	0.27	0.25
C18:2 c n-6	7.47	7.55	7.29	7.37	7.40	7.61	7.12	7.48
C18:2 t n-6	0.01	0.02	0.03	0.02	0.01	0.01	0.01	0.01
C18:3 n-6	0.13	0.17	0.13	0.09	0.14	0.12	0.09	0.09
C20:2 n-6	0.42	0.44	0.39	0.41	0.42	0.40	0.37	0.40
C20:3 n-6	0.38	0.43	0.37	0.34	0.42	0.35	0.32	0.35
C20:4 n-6	0.49	0.45	0.48	0.45	0.46	0.44	0.46	0.40
C18:3 n-3	0.55	0.57	0.58	0.53	0.56	0.53	0.50	0.53
C20:3 n-3	0.55	0.48	0.53	0.55	0.46	0.58	0.50	0.44

* I-industrial production; M-MLD, R-round samples; H-higher, L-lower smoking heights; H-halfway through, E-end of the smoking process.

est proportion in the Visočka pečenica was the polyunsaturated fatty acids, with a higher proportion in industrial than in traditional products. The most abundant of the polyunsaturated fatty acid group was linoleic acid (both production types), with a higher proportion in traditional (7.61%) than in industrial (7.41%) products. In relation to the above results, *Subašić* (2024) reported higher amounts of linoleic acid (11.30%), while *Saičić et al*. (2010) reported lower amounts (3.13%) in beef prosciutto. *Tomić et al*. (2009) conducted a study of the fatty acid composition of Uzice beef prshuta produced from *M. semitendinosus* and *M. longissimus lumborum et thoracis* using two different salting processes. The study determined the palmitic, stearic and oleic acids were the highest of all the fatty acids in all tested samples. Considering the salting processes used, there were no significant differences in the fatty acid composition of the tested samples (*Tomić et al*., 2009). In the fatty acid composition of the dried beef product, *cecina*, *Fernández et al*. (2015) and *Molinero et al*. (2008) found the most abundant fatty acids were oleic, palmitic, stearic and linoleic, which is in line with the results of our study. These same fatty acids were also the most abundant in pastirma, the Turkish dry meat product (*Çakıcı et al*., 2015; *Aksu and Kaya* 2002; *Aksu et al*., 2017), in Kazakh dry-cured beef (*Sha et al*., 2016), and in Dalmatian and Istrian prosciutto (*Marušić Radovčić et al*., 2018; *Marušić et al*., 2013). Approximate results of the fatty acid composition were given by *Pleadin et al.* (2017) in their study of prosciutto originating from households in BiH. The most abundant saturated fatty acids were palmitic acid, accounting for 23.73% and stearic acid, accounting for 13.81% of total fatty acids. The most abundant of the monounsaturated fatty acid group was oleic acid, accounting for 45.40%, and from the polyunsaturated fatty acid group, linoleic acid making up 10.65% of total fatty acids (*Pleadin et al*., 2017). *Pleadin et al*. (2016) examined the fatty acid composition of traditional cured meat products from Croatia and Slovenia, including Dalmatian and Karst prosciutto. The authors found the composition of fatty acids was in the order of monounsaturated > saturated > polyunsaturated acids, and oleic, palmitic and stearic fatty acids as the most abundant in the products. *Pleadin et al*. (2015) examined seasonal variations in the composition of fatty acids in Istrian and Dalmatian prosciutto, with the highest proportion of total fatty acids being oleic acid, followed by palmitic, stearic and linoleic acids. The authors stated that the season of production can significantly affect the composition of fatty acids in the cured meat products (*Pleadin et al*. 2015).

Figure 1 shows the principal component analysis (PCA) of the Visočka pečenica from traditional and industrial production according to the composition of fatty acids. It can be seen that the Visočka

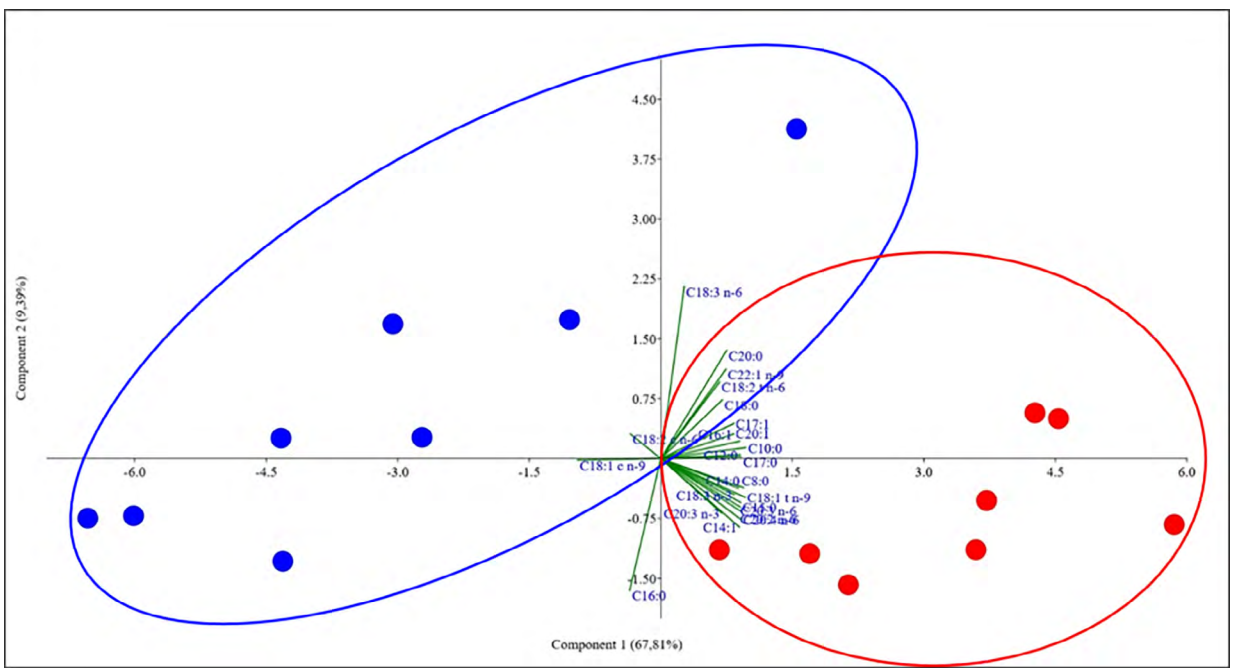

Figure 1. Principal component analysis of Visočka pečenica from traditional and industrial production according to the composition of fatty acids
(*T-traditional, I-industrial production; M-MLD, R-round samples; H-higher, L-lower smoking heights; H-halfway through, E-end of the smoking process)

pečenica from industrial production were grouped in relation to 20 fatty acids out of a total of 24 identified (red ellipsoid). The reason for this grouping was due to the larger amount of those 20 fatty acids in the industrial products than in the traditional products. Visočka pečenica from traditional production were more dispersed and positioned in three quadrants (blue ellipsoid; Figure 1).

The Visočka pečenica made from round, smoked at the higher height and sampled at the end of the smoking process from traditional production (TRHE) were completely separated from all other products and were characterized by a high content of γ-linolenic acid (C18:3 n-6). A similar group of products, only smoked only at the lower 2 m height, was positioned completely opposite on the graph in Figure 1, characterized by the highest content of palmitic fatty acid (C16:0). Oleic fatty acid (C18:1 c n-9) was characteristic of Visočka pečenica from traditional production (TMLH, TRLE and TMHE) which had the highest contents of this fatty acid. Linoleic fatty acid (C18:2 c n-6) was positioned in relation to traditionally produced MLD Visočka pečenica smoked at the lower height of 2 m and sampled at the end of the smoking process, which had the highest content of this fatty acid. Fatty acids C20:0, C22:1 n-9, C18:2 t n-6 and C18:0 were characteristic of Visočka pečenica from both production systems (TRHE, IRLH and IMHH). Other fatty acids were characteristic of and found in the highest amounts in industrial Visočka pečenica, as can be seen in Figure 1.

4. Conclusion

This study presents the first results of the fatty acid composition of Visočka pečenica. The largest proportion of the fatty acid composition of products from both production systems (traditional and industrial) was the monounsaturated fatty acids, of which oleic acid the most abundant acid in relation to all identified fatty acids. Both the monounsaturated fatty acids and oleic acid content were higher in Visočka pečenica from traditional than from industrial production. The content of saturated fatty acids was lower than that of monounsaturated fatty acids in both production systems. Palmitic and stearic fatty acids were the most abundant saturated fatty acids, with a higher proportion in Visočka pečenica from industrial than traditional production. The content of polyunsaturated fatty acids was the lowest of the three fatty acid types in the Visočka pečenica, with a higher proportion in industrial than in traditional products. The most abundant polyunsaturated fatty acid was linoleic fatty acid. With a longer (finished) smoking process, in Visočka pečenica from both production systems, there was a slight decrease in the content of polyunsaturated fatty acids. In industrial Visočka pečenica with the longer smoking process (compared with half-smoking), the content of saturated fatty acids was lower, while the content of monounsaturated fatty acids was higher. The opposite trend was observed in traditionally produced Visočka pečenica. In both production systems, Visočka pečenica made from round were richer in saturated fatty acids, but the MLD Visočka pečenica contained more polyunsaturated fatty acids. To date, many studies have been conducted on the modification of the fatty acid composition of meat and meat products in order to improve their nutritional value. The results of this study can serve as a basis for future research aimed at improving the nutritional value of the fatty acid composition of traditional Visočka pečenica.

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Review Paper

Mechanisms, exposure, and reduction strategies of polycyclic aromatic hydrocarbons in charcoal-grilled meat

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ABSTRACT

Meat cooked on a charcoal grill produces polycyclic aromatic hydrocarbons (PAHs) through pyrolysis, incomplete combustion, and fat dripping onto heat sources. High meat consumption and the use of charcoal in traditional Serbian grilling techniques encourage the formation of PAHs, especially carcinogenic substances like benzo[a]pyrene. PAH levels are greatly influenced by variables like fuel type, grilling method, temperature, and fat content. Indirect grilling, pre-treatments, and marinades high in antioxidants all successfully lower PAH contamination. Dietary PAHs have been linked by epidemiological data to an increased risk of cancer, and grilling environments present additional health risks due to occupational exposure. Preventive measures must be put in place to reduce exposure to PAHs and the health risks they pose. This review emphasizes the pressing need for additional research and public health initiatives to lower PAH exposure while maintaining culinary customs.

1. Introduction

According to 2022 data, the average annual meat consumption worldwide was about 44.14 kg per person. In the European Union, it reached around 78.83 kg. Serbia's consumption, at approximately 79.37 kg per person, is close to the EU average (*OurWorldInData et al*., 2019). Despite economic challenges, Serbia maintains high meat consumption. This shows the strong influence of cultural and traditional habits on dietary choices, often regardless of economic status. Meat has been a staple in the Serbian diet for centuries. Grilling is seen as a cultural symbol and social activity, especially with dishes like "pljeskavica" and "ćevapčići" (*Baltic et al.*, 2018; *Brankov et al*., 2017; *Stevanović et al*., 2016; *Tomašević et al*., 2023; *Trovic et al*., 2021). Grilling in Serbia usually involves cooking quickly at high temperatures using charcoal or wood. This can create polycyclic aromatic hydrocarbons (PAHs), which are chemical compounds that may cause cancer (*Du et al*., 2025; *Kartalović et al*., 2022). PAHs result from the incomplete burning of organic material and are especially common in smoked and grilled meat products (*Kim et al*., 2021; *Ma et al*., 2024). Epidemiological studies suggest a connection between PAH exposure and higher risks of several cancers, including colorectal and

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lung cancer (*Cross et al*., 2007; *Farvid et al*., 2021; *IARC*, 2010). Given the importance of meat in Serbian cooking and the common grilling practice, it is essential to assess consumer exposure to PAHs and the related health risks from eating grilled meat.

2. Mechanisms of PAH formation during grilling

Although the specific process involved in PAH formation is yet to be established, three mechanisms are available for PAH formation from food when undergoing grilling: (1) pyrolysis of organic materials, (2) incomplete fuel combustion and (3) dripping of fat from food onto the heat source (*Du et al*., 2025; *Duedahl-Olesen and Ionas*, 2022; *Palade et al*., 2023). Organic materials like carbohydrates, lipids, proteins and others play an imperative role in PAH formation (*Du et al.*, 2025; *Zhang et al*., 2021). This process is substrate-dependent: carbohydrates degrade through caramelization and pyrolysis, while proteins and lipids contribute via Maillard reactions and thermal breakdown (*Hu et al*., 2021). At high temperatures (>200°C), these compounds break down to yield low-molecular-weight light rings and highly reactive intermediate free radicals (*Singh et al*., 2023). The light rings and free radicals combine to yield stable polynuclear aromatics, which then condense to form PAHs via the Diels-Alder reaction (*Llamas et al*., 2017). Additionally, unsaturated intermediates formed during lipid or sugar pyrolysis can undergo ring closures and condensation facilitated by thermal energy and radical recombination (*Hu et al*., 2021). Moreover, the Maillard reaction, an intricate chemical reaction in the course of thermal treatment of meat products, has a significant function in pyrolysis of organic matter towards the generation of PAHs in grilled food (*Nie et al*., 2018a). Pyrolysis of protein in meat gives rise to free amino acids which, through the Maillard reaction, react with reducing sugars to form intermediate products, such as Amadori rearrangement products (ARPs) (*Ishak et al*., 2022). Intermediate products continue to decompose to form PAHs, such as phenanthrene (Phe), pyrene (Pyr), and benzo[a] pyrene (BaP) (Britt et al., 2004). This transformation is influenced by pH, water activity, and reaction time, which modulate the degradation pathways of Maillard intermediates (*Hu et al*., 2021). One of the key pathways for PAH formation is the incomplete combustion of fuel, which generates reactive hydrocarbon radicals and low molecular weight (LMW) PAHs (*Afe et al*., 2020; *Iko Afé et al*., 2020). During this process, several mechanisms are involved, including the hydrogen abstraction-carbon addition (HACA) mechanism (*Kislov et al*., 2013). As the temperature increases, free radicals and LMW PAHs undergo cyclization and polymerization reactions, ultimately forming more complex, high molecular weight PAHs. (*Knize et al*., 1999; *Nie et al.*, 2018b). Furthermore, PAHs can also be produced by pouring fat over a heat source. Fat drips from foods onto heat sources produce incomplete combustion of fat and the emission of light PAHs. These PAHs then migrate via smoke to the foods, where they are adsorbed (*Zhu et al*., 2022). *Chen and Chen* (2001) identified fatty acids as being able to form hydroperoxides on oxidation, which produce cyclic compounds, such as cyclohexene, by intramolecular cyclization. These ringed compounds can further stack to give benzene rings and eventually grow into PAHs by the Diels-Alder mechanism. The combined action of these three formation pathways under the grilling process result in the occurrence of high concentrations of PAHs in grilled food, which need to be considered to ensure the protection of human health.

3. Factors influencing PAH levels during meat grilling

The development of polycyclic aromatic hydrocarbons (PAHs) in grilled foods can be affected by multiple variables (*Du et al*., 2025), including the type of food being cooked (*Lee et al.*, 2016; *Sahin et al*., 2020), the nature of the fuel used (Kim et al., 2021), the time of the grilling process (*Kao et al*., 2014), as well as the temperature and intensity of heat exposure during grilling (*Onopiuk et al*., 2021; *Sumer and Oz*, 2023). The composition of fuel, and the level of direct contact between food and the flame, are instrumental in determining the levels of PAHs in grilled foods. Several studies have proven that charcoal grilling produces much higher PAH levels compared to gas grilling (*Alomirah et al*., 2011; *Anjum et al*., 2019; *Chung et al*., 2021; *Farhadian et al.*, 2010a; *Lateef and Walker*, 2015). This disparity is mostly explained by the emissions produced during charcoal combustion, which significantly contribute to PAH formation (*Chaemsai et al*., 2016; *Iko Afé et al*., 2020). Furthermore, the type of charcoal used, i.e., white, black, or extruded, affects the PAH content, with extruded charcoal generating the highest concentrations (*Kim et al*., 2021). The grilling method, whether direct or indirect, plays a crucial role

in PAH levels. *Sumer and Oz* (2023) observed that direct grilling yields considerably greater PAH concentrations than indirect grilling. Supporting this, *Lee et al.* (2016) and *Anjum et al*. (2019) demonstrated that cooking with direct charcoal fire can increase benzo[a]pyrene (BaP) levels by five times or more compared to indirect techniques. This significant increase is attributed to factors such as the proximity of food to the heat source, elevated temperatures, and smoke and tar production from fat dripping onto the heat source, all promoting PAH formation (*Hamidi et al*., 2022; *Lee et al*., 2016). Temperature, cooking time, and fat content play key roles in PAH formation during grilling. Longer cooking times generally result in higher PAH accumulation due to enhanced pyrolytic and oxidative reactions; however, temperature exerts a stronger influence on PAH synthesis (*Min et al*., 2018; *Rose et al*., 2015). Indirect grilling at moderate temperatures tends to produce lower PAH levels, whereas direct grilling over open flames or charcoal at high temperatures generates substantially more PAHs. This increase is primarily due to incomplete combustion and pyrolysis of fat drippings (*Liu et al*., 2019; *Onopiuk et al*., 2021; *Singh et al*., 2023). Elevated temperatures also facilitate the transformation of lighter PAHs into more complex and toxic compounds (*Wang et al*., 2017; *Zhang et al*., 2021). Fat content in meat greatly influences PAH levels; higher fat content promotes dripping and pyrolysis on heated surfaces, increasing PAH formation (*Babaoglu et al*., 2017; *Hamidi et al*., 2022; *Perelló et al*., 2008). Studies confirm that fattier cuts accumulate more PAHs than lean cuts under identical grilling conditions (*Dost and İdeli*, 2012; *Lee et al*., 2016). Substituting fatty meats with leaner cuts or plant-based alternatives could reduce dietary PAH exposure, although cultural habits likely would impact the acceptance of such changes (*Wang et al*., 2022).

4. Ways to reduce PAH formation in grilled meat

A significant amount of research has looked into different ways to reduce the formation of polycyclic aromatic hydrocarbons (PAHs) in grilled meats. Marination and changes to traditional grilling methods stand out as the most effective approaches (*Du et al*., 2025; *Hasyimah et al*., 2022; *Mehl et al*., 2019). Marinating meat before grilling is seen as a straightforward yet powerful way to lower PAH levels under regular cooking conditions. This effectiveness largely comes from the antioxidants in the marinade that neutralize free radicals produced during cooking. These radicals are key players in PAH formation (*Arun et al*., 2023; *Esfahani Mehr et al.*, 2019; *Kao et al.*, 2014; *Nor Hasyimah et al*., 2020). Several studies have shown the antioxidant power of various marinades, confirming their role in blocking PAHs (*Viegas et al*., 2012; *Wang et al*., 2017). However, marination can have downsides, as foods in marinades or sauces can char more easily. This outcome can ironically increase PAH levels on the food surface (*Ahmad Kamal et al*., 2018). Therefore, careful control of grilling conditions is vital to achieve a balance between lowering PAHs and avoiding excessive charring. Moreover, the detailed ways in which antioxidants interact with free radicals during cooking and their overall effect on PAH levels still need more exploration (*Zhang et al*., 2021).

Besides marination, changing grilling methods can significantly cut down PAH exposure. Pre-treatments like steaming or microwave heating before grilling reduce PAH levels by decreasing cooking time and limiting direct contact with smoke and pyrolysis products (*Farhadian et al*., 2010b). Additionally, wrapping meat in materials like aluminium foil or banana leaves can prevent PAH deposition by acting as a physical barrier. Notably, banana leaves work better than aluminium foil (*Farhadian et al*., 2010b; *Gorji et al.*, 2016). Another important factor in PAH formation is the contact of fat drippings with the heat source, which leads to pyrolysis and further PAH generation. Keeping fat drippings away from flames or hot surfaces significantly lowers PAH formation (*Duedahl-Olesen and Ionas*, 2022; *Farhadian et al*., 2010b; *Lee et al.*, 2016; *Min et al*., 2018). In summary, these findings show that using antioxidant-rich marinades, controlled grilling techniques, pre-treatment methods, and physical barriers can work together to lower PAH contamination in grilled meats. This strategy can help reduce dietary exposure and related health risks.

5. Health risks

Polycyclic aromatic hydrocarbons (PAHs) comprise over 600 compounds, with benzo[a]pyrene (BaP) being the most studied and classified by IARC as carcinogenic to humans (Group 1). Others, such as benzo[b]fluoranthene (BbF), benzo[a] anthracene (BaA), and chrysene (CHR), are classified as possibly carcinogenic to humans (Group 2B) based on limited evidence (*International Agency*

for Research on Cancer, 2010). PAHs occur in complex mixtures with potential synergistic toxicity and, unlike spoilage markers, are undetectable by smell, taste, or appearance, increasing unintentional intake (*Sampaio et al*., 2021). Due to their lipophilicity, PAHs accumulate in high-fat foods (beef, poultry, fish) and are absorbed via dietary lipids, particularly BaP, which travels systemically bound to lipoproteins, accumulating in the liver and intestinal mucosa (*Mallah et al*., 2022; *Sampaio et al*., 2021). Once inside cells, PAHs undergo bioactivation via CYP1A1 into reactive epoxides that bind DNA and proteins, initiating carcinogenesis (*Afé et al*., 2021). This mechanism is supported by findings of DNA adducts in colorectal tumours of individuals with high meat intake (*Cheng et al*., 2021). The gut microbiota also influences PAH toxicity by modulating enterohepatic recycling, hepatic enzyme expression, and local PAH biotransformation, while microbial dysbiosis could act synergistically with PAHs and other meat-derived carcinogens to promote mutagenesis and oncogene activation (*Abu-Ghazaleh et al*., 2020). Additionally, microbial metabolism can generate estrogenic PAH metabolites, adding potential endocrine-disrupting effects (*Afé et al*., 2021). An early study from a Slovenian-populated region of Hungary reported unusually high stomach cancer incidence in a population consuming home-smoked meats prepared with softwood, a practice linked to elevated PAH formation (*Fritz and Soos*, 1980).

Contemporary epidemiological studies and meta-analyses consistently demonstrate a strong association between high intake of red and processed meats, major dietary sources of PAHs, and increased cancer risk. In the NIH-AARP cohort, individuals with the highest red meat intake had a 20-60% higher risk of colorectal, liver, lung, and oesophageal cancers, while high processed meat intake was associated with 20% greater colorectal and 16% greater lung cancer risk (*Cross et al*., 2007). *Chan et al*. (2011) confirmed a dose-response relationship: each 100 g/day of red meat raised colorectal cancer risk by 17%, and 50 g/day of processed meat raised this risk by 18%, up to a plateau. Additional carcinogens in processed meats, including nitrosyl-heme and heterocyclic amines, likely act synergistically with PAHs. A meta-analysis of 148 studies (*Farvid et al*., 2021) extended these findings to multiple cancers, including breast, colon, lung, and liver. However, the E3N French cohort showed no clear link between dietary PAH4 intake and overall mortality, except for increased lung cancer mortality in current smokers, suggesting additive effects of inhaled and ingested PAHs. Beyond this, PAH exposure has been associated with poorer outcomes in breast cancer survivors and may contribute to lung cancer in never-smokers via inhalation of cooking-related emissions (*LoPiccolo et al*., 2024). Occupational exposure to PAHs, particularly in grilling environments, also presents a significant health concern. Restaurant grill workers experience markedly elevated internal PAH burdens, with *Oliveira et al.,* (2020) reporting a ninefold increase in total urinary hydroxylated PAH metabolites (OHPAHs) during workdays. Grilling over charcoal briquettes emits extremely high PAH levels, with all solid fuel scenarios exceeding the incremental lifetime cancer risk (ILCR) threshold of 10^{-4}, indicating unacceptable inhalation-related risks (*Badyda et al.,* 2022). These exposures correspond to measurable genetic damage; *Cao et al.,* (2021) found a 45% increase in PIG-A gene mutation frequency among barbecue workers, with strong correlations between urinary PAH levels and biomarkers of genotoxicity. These findings confirm that traditional grilling poses not only dietary but also inhalation-based carcinogenic risks, especially in occupational settings. Health risk assessments should consider both exposure routes and promote ventilation, exposure limits, and safer cooking practices.

6. Conclusion

Grilled meat is an important part of Serbian culture and is widely consumed. However, we still do not fully understand the risks associated with exposure to harmful PAHs. Several factors contribute to the formation of PAHs, including the grilling method, type of fuel, fat content, and temperature. This highlights the need for strategies that are tailored to local practices. Using antioxidant-rich marinades, improving grilling techniques, and educating the public can help lower PAH intake while preserving culinary traditions. There is increasing evidence that links dietary PAHs to negative health effects, but several challenges make it hard to interpret these findings. Oral intake is just one way people can be exposed to PAHs, as inhalation and skin contact also play a role. Observational studies, different influencing factors, individual genetic differences, and the presence of other carcinogens in processed meats make it difficult to pinpoint the exact impact of PAHs. Current biomonitoring tools are not able to

separate the different exposure routes. This shows the need for thorough long-term research and standardized assessment methods to better grasp how dietary PAHs affect health.

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Original Scientific Paper

Cyclopiazonic acid in meat products originating from different production practices

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ABSTRACT

The consumption of dry-fermented and dry-cured meat products is widespread, primarily due to their desirable sensory characteristics and nutritional value. However, the intake of these products may pose health risks associated with contamination by mycotoxins. One of the mycotoxins that can be present in meat products is cyclopiazonic acid (CPA). The present study aimed to compare the occurrence of CPA in the same types of meat products originating from different production practices. Traditional and industrial practices are characterized by different production conditions, including temperature, humidity, and seasonal variations, which, in traditional production, are often not strictly controlled. Therefore, comparing mycotoxin contamination in meat products with regard to production practices is important for food safety. The results revealed a notable difference in CPA prevalence, with a markedly higher occurrence in traditionally produced meat products (21.7%) compared to those from industrial production (2.9%). Among the traditional products, CPA levels ranged from 6.1 µg/kg to 335.5 µg/kg. In contrast, CPA was detected in only two industrial meat products, with levels of 2.7 µg/kg in a sausage and 17.6 µg/kg in a *Pečenica*. These findings suggest that industrially produced meat products are generally safer for consumers with respect to CPA contamination. Nevertheless, despite the low incidence observed in industrial products, ongoing surveillance is necessary, particularly given the current lack of established regulatory limits for CPA in all foodstuffs.

1. Introduction

Cyclopiazonic acid (CPA) is a mycotoxin produced by various moulds belonging to the genera *Aspergillus* and *Penicillium*. Numerous studies have confirmed that *Aspergillus flavus*, *Aspergillus tamarii*, and *Aspergillus versicolor* are capable of synthesizing this toxin as well as *Penicillium griseofulvum, Penicillium camemberti* and *Penicillium commune* (*Burdock and Flamm*, 2000; *Zadravec et al*., 2023). Historically, CPA has been associated with cases of poisoning in both humans and animals in India, where contaminated millet was identified as the source, and strains of *A. flavus* and *A. tamarii* were isolated through microbiological analysis. CPA belongs to the category of potentially dangerous mycotoxins that can cause damage to the liver, the spleen, the pancreas, the kidneys, the salivary glands, the myocardium and the skeletal muscles. Toxic effects of CPA are most pronounced in the digestive organs, the muscles, the liver and the spleen, although the symptoms vary depending on animal breed, age and sex, mycotoxin uptake, dose and duration of exposure (*Ostry et al*., 2018).

To date, CPA has been detected in a wide range of plant-based food products, including peanuts,

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corn, figs, rice, wheat and tomatoes, as well as in animal-derived products, such as cheese, milk, and ham (*Oliveira et al.*, 2006; *da Motta and Valente Soares*, 2010; *Fernández Pinto et al*., 2010*; Ostry et al*., 2018; *Peromingo et al*., 2018; *Lešić et al*., 2022; *Andrade et al*., 2025; *Lešić et al*., 2025). Over the past five years, several studies have investigated the presence of CPA in various types of dried meat products, reporting high incidence rates and levels reaching up to 550 µg/kg (*Andrade et al*., 2025). Notably, most of these studies have focused on meat products produced in households, where manufacturing conditions are often unregulated and influenced by seasonal factors (i.e., temperature and relative humidity). In contrast, data on CPA occurrence in industrially produced meat products characterized by standardized and controlled conditions remain limited. Despite industrial production being controlled, including conditions in production facilities, and not being subject to seasonal variations, it is still important to assess the potential contamination of these products for consumer protection. Although significant progress has been made in research on CPA, regulatory limits for its presence in various food products have yet to be established. Given these observations, the present study aims to give an overview of the occurrence of CPA in some types of meat products originating from different production systems and to compare the levels of this mycotoxin in meat products originating from traditional versus industrial production systems.

2. Materials and methods

2.1. Meat products

For one year, the same types of meat products were collected from family households (n = 83) and the commercial market (n = 69). Sampling from family households was conducted at 23 locations, while commercial meat products originated from 14 medium- to large-scale meat processing industries. The collected meat products were dry-fermented sausages, *Kulen*, bacon/pancetta, dry-cured hams, dry rack and *Pečenica/Ombolo/Zarebnjak*, each with an approximate weight of 500 g. The distribution of the meat products by product type and origin is detailed in Table 1 and Table 2. Following collection, samples of the meat products were homogenized using a Grindomix GM 200 laboratory mill (Retsch, Germany) and subsequently stored at -20 °C pending mycotoxin analysis.

2.2. Chemicals and standards

The analytical standard for cyclopiazonic acid (CPA; CAS No. 18172-33-3) was obtained from Sigma-Aldrich (St. Louis, MO, USA). All solvents, including acetic acid, methanol and acetonitrile, were procured from Honeywell (Charlotte, NC, USA). Zinc acetate dihydrate (CAS No. 5970-45-6), potassium hexacyanoferrate (II) trihydrate (CAS No. 14459-95-1) and ammonium acetate (CAS No. 631-61-8) were also sourced from Sigma-Aldrich. QuEChERS extraction packets (4.0 g of $MgSO_4$, 1.0 g of NaCl, 1.0 g of sodium citrate tribasic dihydrate, and 0.5 g of sodium citrate dibasic sesquihydrate) along with syringe filters equipped with PTFE membranes (4 mm diameter, 0.2 µm pore size) were supplied by Phenomenex (Torrance, CA, USA). Captiva EMR–Lipid cartridges (3 mL) used for lipid removal were obtained from Agilent Technologies (Santa Clara, CA, USA). Carrez I solution (10.6 g of potassium hexacyanoferrate (II) trihydrate per 100 mL of deionized water) and Carrez II solution (21.9 g of zinc acetate dihydrate in 32 mL of acetic acid and diluting the solution with distilled water to a final volume of 100 mL.

2.3. Sample preparation

The sample preparation was described earlier by *Vulić et al*. (2021). Briefly, 2 g of homogenized sample was weighed, upon which 5 mL of 25 % acetic acid was added and samples were shaken on a vertical vortex shaker. Afterwards, 5 mL of organic solvent (acetonitrile) was added, and samples were extracted for 30 minutes on a head-over-head shaker (Multi RS-60, Biosan, Riga, Latvia). After the extraction, Carrez solution was added to precipitate proteins and fats, followed by the addition of QuEChERS salts, and samples were centrifuged at 5,000 rpm for 15 minutes at room temperature (Universal 320R, Hettich, Kirchlengern, Germany). Clear extracts obtained after centrifugation were passed through lipid removal columns and then filtered through a PTFE filter directly into a vial.

2.4. Instrumental analysis

A HPLC (1260 series, Agilent Technologies, Santa Clara, CA, USA) consisting of a degasser, a binary pump, an autosampler and a column compartment was coupled with a triple quadrupole mass spectrometer equipped with an electrospray ionization

(ESI) source (6410 QQQ, Agilent Technologies, Santa Clara, CA, USA). The analytical C 18 HPLC (Gemini, 150 × 4.6 mm, 5 µm particle size, 110 Å) was from Phenomenex (Torrance, CA, USA). The instrumental conditions, including chromatographic conditions as well as mass spectrometer set up, were described earlier (*Vulić et al*., 2021; *Lešić et al*., 2022)

2.5. Method validation

The calibration curve spanning from 0.5 to 10 ng/mL showed good linearity with the regression coefficient of R^2=0.998, but also in the 0.5 to 20 ng/mL range (R^2=0.996). The limit of detection (LOD) calculated from the calibration curve was 0.43 ng/mL, which corresponds to a value of 2.17 µg/kg in the meat product matrix given the sample dilution factor of 5. The limit of quantification (LOQ) calculated from LOD was 1.43 ng/mL, which corresponded to 7.15 µg/kg in the meat product matrix. The recovery of the method was assessed at two concentration levels, selected based on the established limits of detection (LOD) and quantification (LOQ). The mean recovery rates were 95.5% at 3 µg/kg and 102.1% at 10 µg/kg.

3. Results and discussion

In this study, CPA was detected in meat products taken from family households with traditional production, often characterized by uncontrolled processing conditions, in all product types except dry-cured ham with an overall occurrence rate of 21.7%. The distribution of traditionally produced meat products and descriptive statistics related to CPA levels are presented in Table 1. Among the traditionally produced meat products, the highest occurrence was observed in dry-cured neck (44.4%), followed by *Kulen* (25.0%), dry-fermented sausages (24.0%), *Pečenica/Ombolo* (20.0%), and bacon/pancetta (10.5%). As previously noted, production conditions such as ripening temperature and relative humidity, along with product-specific properties such as pH and water activity, influence the growth of surface moulds both desirable and undesirable (*Asefa et al*., 2011; *Mediani et al.*, 2022). Furthermore, the production characteristics of each meat product, particularly the duration of the ripening process, also play a critical role in mould proliferation and mycotoxin synthesis. Based on the characteristics of the products, a higher CPA occurrence rate might be expected in dry-cured hams due to their extended ripening period, which can last up to six months. This extended maturation theoretically allows extended period for mould growth and subsequent mycotoxin production, compared to products with shorter production cycles. However, in this study, CPA was not detected in dry-cured hams, which may be attributable to unfavourable environmental conditions for mould development during the production period.

The mean levels of CPA in the analyzed traditionally produced meat products ranged from 6 µg/kg in *Pečenica/Ombolo/Zarebnjak* to 74.1 µg/kg in dry-fermented sausages. The highest individual level of CPA (335.5 µg/kg) was also observed within the dry-fermented sausage category. When compared with previous studies (*Vulić et al*., 2021; *Lešić et al.*, 2021; *Lešić et al*., 2022), the pattern of CPA occurrence was largely consistent across most dry-fermented and dry-cured meat products, except for dry-cured hams.

Table 1. Cyclopiazonic acid level in different types of meat products originating from traditional practice

Origin	CPA	Dry-fermented sausages	*Kulen*	Bacon/ pancetta	Dry-cured ham	Dry rack	*Pečenica/ Ombolo/ Zarebnjak*
Traditional	N	25	16	19	13	9	10
	n+	6	4	2	/	4	2
	n %	24.0	25.0	10.5	/	44.4	20.0
	Min (µg/kg)	5.5	6.5	7.2	na	6.3	6.1
	Max (µg/kg)	335.5	27.3	9.0	na	44.3	6.8.
	Mean of positives (µg/kg) (µg/kg)	74.1	17.7	8.5	na	21.2	6.4
	Median (µg/kg)	26.8	18.6	8.5	na	17.2	6.4

Legend: CPA—cyclopiazonic acid.; n – number of analyzed meat products; n+ - number of meat products above LOQ; n % - percentage of meat products above LOQ; na—not applicable

In particular, Lešić et al. (2025) reported CPA levels in dry-cured hams ranging from 4.3 to 6.2 µg/kg. Similarly, *Peromingo et al.* (2018) detected CPA levels ranging from 36.1 to 540.1 µg/kg. These substantial differences in reported levels may be attributed to variations in production technologies/parameters of dry-cured hams as well as differing environmental conditions between production regions of Spain and Croatia. Notably, CPA was not detected in dry-cured hams in the present study. When considered alongside previous findings, this absence may be ascribed to environmental conditions during the current study period that were less conducive to mould growth and mycotoxin production.

In contrast to the traditionally produced meat products, the overall occurrence rate of CPA in industrially produced meats was notably lower, at just 2.9 %. The distribution of the industrial meat product group by the category and the descriptive statistics for CPA levels are presented in Table 2.

Table 2. Cyclopiazonic acid level in different type of meat products originating from industrial production

Origin	CPA	Dry-fermented sausages	*Kulen*	Bacon/ pancetta	Dry-cured ham	Dry rack	*Pečenica/ Ombolo/ Zarebnjak*
Industry	N	21	9	11	9	9	10
	n+	1	/	/	/	/	1
	n %	4.8	/	/	/	/	10.0
	Min (µg/kg)	2.7	Na	na	na	na	17.6
	Max (µg/kg)	2.7	Na	na	na	na	17.6
	Mean of positives (µg/kg)	2.7	Na	na	na	na	17.6
	Median (µg/kg)	2.7	Na	na	na	na	17.6

Legend: CPA—cyclopiazonic acid.; n – number of analyzed meat products; n+ - number of meat products above LOQ; n % - percentage of meat products above LOQ; na—not applicable

CPA was detected in only two industrial meat products: one dry-fermented sausage with a level of 2.7 µg/kg, and one *Pečenica/Ombolo* with a level of 17.6 µg/kg. These meat products originated from two different producers, indicating that the observed contamination cannot be attributed to poor practices at a single facility.

These findings suggest that, with few exceptions, industrial production is generally characterized by good manufacturing practices with control of crucial parameters, such as temperature and relative humidity. This observation is consistent with our previous study (*Lešić et al*., 2022), which focused specifically on *Kulen* produced using both traditional and industrial practices. Notably, in both the current and previous studies, CPA was not detected in any of the *Kulen* originating from industrial production.

When comparing the incidence of CPA occurrence in the same type of meat product originating from different production practices, it can be observed that traditional practices pose a higher risk of product contamination. As mentioned earlier, the possible reasons include inadequate temperature control, humidity and seasonal variations, as addressed by *Lešić et al*. (2022). Regarding the industrial meat products, the lack of information about potential issues during the production process prevents us concluding anything about the causes of CPA occurrence. Since CPA was detected in only two products from the industrial group, statistical comparison for traditional and industrial products was not possible. Further research, including detailed information about the production process, is necessary to explain the occurrence of CPA in industrial meat products.

The European Union (EU) has established regulatory limits for mycotoxins across various food categories. In the case of meat and meat products, maximum permitted levels (MPLs) for ochratoxin A have been defined by several EU Member States. However, for other mycotoxins, including cyclopiazonic acid (CPA), specific regulatory thresholds have not yet been established. Consequently, the findings of this study cannot be directly assessed against current legislation. Nevertheless, the results provide important data that may inform future risk assessments and contribute to the development of regulatory frameworks.

4. Conclusion

Traditional production of dry-fermented and dry-cured meat products, often conducted under unregulated and seasonally variable conditions, can been associated with the occurrence of CPA, especially at the notably higher concentrations seen. In contrast, industrial production, typically characterized by standardized and controlled processing conditions, exhibited a markedly lower incidence of CPA contamination. Due to the limited number of CPA positive findings in industrial meat products, a direct statistical comparison between traditional and industrial production systems was not feasible. Despite the low occurrence of CPA in industrial products, continued monitoring remains essential, particularly in the absence of established regulatory limits for this mycotoxin.

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Review Paper

Meat-derived bioactive peptides: application potential in functional foods

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ABSTRACT

Meat proteins constitute a rich reservoir of encrypted bioactive peptides that may be released during enzymatic or microbial hydrolysis, gastrointestinal digestion, and various food processes, subsequently exerting diverse physiological functions. These peptides show antioxidant, antimicrobial, antihypertensive, and antidiabetic properties, along with additional roles such as immunomodulatory and anti-aging effects. They have demonstrated significant potential as multifunctional ingredients, not only improving human health but also enhancing shelf life as well as techno-functional and sensory attributes of foods. Their incorporation into diverse food systems can improve oxidative stability, microbial quality, and consumer acceptance, while recent advances also point to innovative applications in antimicrobial packaging. Nevertheless, the translation of these peptides from laboratory to industry faces key challenges, including production scalability, bioavailability, and regulatory constraints. Emerging approaches, such as the utilization of nano-encapsulation techniques and protease inhibitors, are under exploration to overcome these limitations. This review aims to provide an updated overview of current findings on meat-derived biopeptides, emphasizing their bioactive properties, utilization opportunities in different food formulations, and the main challenges that must be addressed for broader application.

1. Introduction

Meat is well recognized as a major source of high-quality proteins, providing essential amino acids crucial for human nutrition. Being a major source of high-quality proteins, meat simultaneously serves as a valuable reservoir of functional protein derivatives, particularly bioactive peptides (BPs). BPs can be defined as short amino acid sequences, typically fewer than 20 residues, encrypted within parent proteins and released through hydrolysis, thereby exerting specific physiological activities with health-promoting potential (*Chew et al*., 2019; *Ozturk-Kerimoglu et al.*, 2025). Conventional methods, such as enzymatic hydrolysis, microbial fermentation, and chemical hydrolysis, have long been employed to obtain BPs from food proteins. Recently, innovative physical techniques, including microwave, ultrasound, pulsed electric fields, and high hydrostatic pressure, have also gained attention for enhancing hydrolysis efficiency, reducing enzyme use, and improving peptide purity and yield (*Zaky et al*., 2022; *Hamdi et al*., 2025). The product obtained after hydrolysis, referred to as the "hydrolysate," predominantly consists of di- and tripeptides (*Eckert et al*., 2019; *Zinina*

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et al., 2022). The process of BP production generally involves raw material pretreatments, hydrolysis, protein fractionation and purification, evaluation of bioactivity, peptide identification, *in silico* evaluation for bioactivity assessment, and confirmation of activities following chemical peptide synthesis (*Ozturk-Kerimoglu et al*., 2025). The purification of the proteins in the resulting protein hydrolysates is carried out by different techniques such as ultrafiltration, reverse osmosis, ion-exchange column chromatography (IEC), and reversed-phase high-performance liquid chromatography (RP-HPLC), while the identification of the peptide sequences is then done by advanced mass spectroscopy, namely liquid chromatography-tandem mass spectrometry (LC-MS/MS), matrix-assisted laser deionization time-of-flight (MALDI-TOF), and electrospray ionization–mass spectrometry (ESI-MS) (*Hamdi et al*., 2025).

BPs derived from various food sources have been documented to exert a broad spectrum of physiological activities, such as antioxidant, antimicrobial, antithrombotic, antihypertensive, immunomodulatory, anticancer, and lipid- or blood pressure-lowering effects; moreover, they influence not only human health but also the sensory attributes and functional characteristics of food systems (*Chew et al*., 2019; *Toldrá et al*., 2020; *Du and Li*, 2022; *Singh et al*., 2022; *Karami and Akbari-Adergani*, 2019). Beyond single-activity BPs, multifunctional peptides that act on several physiological pathways at once were also documented (*Chai et al*., 2020). Among these activities, meat-derived BPs show particular promise in antioxidant, antimicrobial, antihypertensive, and antidiabetic effects. These diverse bioactivities, along with their ability to improve functional and sensory properties, have made meat-derived BPs attractive candidates for use as functional ingredients in food processing. In this context, the present review aims to summarize the health-promoting and functional potentials of meat-derived BPs and to evaluate their possible applications in various food formulations, while also discussing the current challenges and future directions in this emerging research field.

2. Major bioactive functions of meat-derived BPs

Meat and meat products are excellent sources of BPs. Numerous BPs with diverse functional effects have been identified in various meat parts/further processed meat products, as well as in meat-derived by-products. The most frequently reported bioactive properties across various meat sources include antioxidant, antimicrobial, antihypertensive, and antidiabetic activities. In particular, antioxidant peptide sequences were identified in protein hydrolysates obtained from Spanish dry-cured ham (*Li et al*., 2021), fermented pork sausage (*Kong et al*., 2023), fermented chicken meat (*Babu et al*., 2025), and traditional Yunnan dry-cured beef (*Wang et al*., 2025), as well as from different meat by-products such as porcine liver (*López-Pedrouso et al*., 2021), porcine plasma (*Zhan et al*., 2022), chicken feet (*Ozturk-Kerimoglu et al*., 2023), and bovine bone extract (*Begum et al*., 2024). Potential antimicrobial peptides were identified in different meat-derived by-products such as bovine and porcine blood proteins (*Sanchez-Reinoso et al*., 2021), chicken feathers (*Qin et al*., 2022), and traditional ham broth (*Yang et al*., 2025). Angiotensin converting enzyme (ACE) inhibitory activity, also known as antihypertensive activity, is another critical bioactivity of the peptides derived from meat sources, which was formerly detected in different meat types/products/by-products like fish and beef skeletal muscles (*Maky and Zendo*, 2021), Indonesian traditional fermented beef (*Cangkuk*) (*Mirdhayati et al*., 2024), and beef liver hydrolysates (*Gallego et al*., 2024). Furthermore, Dipeptidyl Peptidase-IV (DPP-IV) inhibitory activity, in other words, antidiabetic activity of peptides derived from various sources such as dry-cured pork loins (*Kęska and Stadnik*, 2021), sheep skin (*Wang et al*., 2021), beef liver (*Gallego et al*., 2024), and rabbit meat (*Hu et al*., 2024) was reported. Beyond these described functions, BPs obtained from meat by-products are also mentioned to exert further effects, including opioid, anti-inflammatory, anti-tyrosinase, calcium-binding, and anti-aging activities (*Ozturk-Kerimoglu et al*., 2025).

3. Meat-derived BPs as novel ingredients in food formulations

Food protein-origin BPs are regarded as valuable candidates for the design of functional food components and the advancement of nutraceutical products. The market for BPs from food proteins is experiencing remarkable growth, expanding from USD 48.6 billion in 2020 to a forecasted USD 95.7 billion in 2028 (*Du and Li*, 2022). Besides, thanks to their minimal toxicity, efficient metabolism in the human body, and biocompatible origin, BPs have

emerged as versatile compounds with broad potential not only in the food sector but also in the feed, medical, pharmaceutical, and cosmetic industries (*Akbarian et al*., 2022; *Singh et al*., 2022; *Zaky et al*., 2022). *Chew et al.* (2019) emphasized that BPs have attracted growing interest as natural alternatives to pharmaceuticals due to their health-promoting properties. Recognized as nutraceuticals, they hold promise in preventing noncommunicable diseases (NCDs) and addressing nutritional deficiencies. Their role in preventive healthcare highlights their potential as key functional ingredients in future diets.

Table 1. Applications of meat-derived protein hydrolysates/BPs in different food products

Hydrolysate/BP	Food product	Highlighted findings	Reference
Bovine α- and β-globulin hydrolysates	Bread	•The addition of 4% (w/w) globulin papain hydrolysate (GPH) did not alter the physical quality or acceptance of bread. • *In vivo* tests in spontaneously hypertensive rats showed a significant systolic blood pressure reduction. • Antihypertensive activity of GPH remained stable after baking, supporting its potential use in functional foods.	Lafarga et al. (2016)
Microencapsulated stripped weakfish hydrolysate (*Cynoscion guatucupa*)	Yogurt	• Hydrolysate addition reduced syneresis and improved yogurt stability, especially in the microencapsulated form. • Both free and encapsulated hydrolysates provided stable antioxidant and ACE-inhibitory activities after 7 days. • Microencapsulation masked fishy flavor, resulting in sensorily acceptable functional yogurts.	Lima et al. (2021)
Yellow fin tuna (*Thunnus albacares*)	Functional beverage	• A malted grain-based health beverage was successfully fortified with tuna protein hydrolysate (TPH) derived from yellowfin tuna red meat. • 2.5% TPH was identified as the most sensorially acceptable formulation. • The enriched beverage exhibited improved nutritional, functional, antioxidant, and digestibility properties, along with good storage stability at 28 °C. • The potential of utilizing tuna cannery red meat by-products for value-added, health-promoting functional beverages was underlined.	Unnikrishnan et al. (2021)
Fish collagen-derived BPs (GPLGAAGP, GRDGEP, MTGTQGEAGR)	Yogurt	• Yogurt fortified with fish collagen-derived peptides (P1, P2, P3) showed no adverse effects on composition or sensory quality. • Protein content, water-holding capacity, and viscosity increased with peptide concentration. • At 1 mg/mL, the strongest antioxidant, ACE inhibition, and DPP-IV inhibition were observed. • Peptides P1 and P2 retained bioactivity after digestion, supporting their potential in functional yogurt formulations.	Ayati et al. (2022)
Rainbow trout roe	Mutton meat	• Alcalase-produced hydrolysate showed a higher degree of hydrolysis, stronger antioxidant activity, and higher essential amino acid content compared to pepsin. • Hydrolysate addition enhanced antioxidant and antimicrobial properties of alginate–chia seed composite coating (CC). • CC plus 1.5% hydrolysate significantly delayed microbial spoilage and lipid oxidation. • The incorporation of hydrolysate into CC effectively extended meat shelf life, meeting consumer demand for clean-label products free from synthetic preservatives.	Golpaigani et al. (2023)
Thawed drip hydrolysates from pork ham	Pork patty	• Thawed drip hydrolysates reduced thawing and cooking losses and delayed color deterioration during freeze-thaw cycles. • Protein denaturation and oxidation were inhibited, as evidenced by lower carbonyls and surface hydrophobicity, higher free sulfhydryl, and α-helix content. • 1.4% hydrolysate concentration showed the strongest protective effect, comparable to the positive control (sorbitol + sucrose).	Han et al. (2025)

BPs serve as sweetening agents, color stabilizers, texturizers, anti-caking agents, emulsifiers, flavor enhancers, and acidity regulators in food processing. In addition, BPs can enhance product quality by influencing water- and oil-holding capacity, colloidal stability, viscosity, and foaming properties (*Zaky et al*., 2022). Furthermore, growing consumer concerns regarding food safety have driven the industry to explore natural substitutes for synthetic antioxidants. In this context, antioxidant proteins and peptides are considered promising alternatives, as they exhibit equal or even superior capacity to inhibit lipid oxidation (*Aguilar-Toalá et al*., 2022; *Zaky et al*., 2022).

Table 1 presents applications of meat-derived protein hydrolysates and BPs in different food products, along with their highlighted findings. As seen in the related table, the key findings demonstrate that protein hydrolysates and BPs, mostly those derived from fish, have been successfully incorporated into diverse food systems such as meat, dairy, bakery products, and beverages. These components exhibited health-promoting bioactivities, including antioxidant, antihypertensive, antimicrobial, and enzyme-inhibitory effects. Moreover, they contributed to improved oxidative stability, microbial safety, sensory acceptance, and techno-functional properties of the products. Overall, their multifunctional potential highlights an important role in preserving food quality and developing functional foods. Specifically, umami and koku-inducing peptides from meat origin are increasingly recognized as promising functional compounds for enhancing flavor perception and sensory quality in food systems (*Mora and Toldrá*, 2025).

Although BPs can be directly incorporated into formulations, one of their most notable applications is in food packaging, where they are embedded as antimicrobial agents to enhance food safety and prolong shelf life (*Alzaydi et al*., 2023). *Notably, Si et al.* (2024) reported that utilization of composite hydrogels with antimicrobial peptides was effective in inhibiting *Staphylococcus aureus* and *Escherichia coli*, and prolonged the storage time of frozen chicken breast. Taken together, these findings underscore the growing importance of meat-derived BPs as multifunctional ingredients with significant contributions to food quality, safety, and functionality across a broad range of applications.

4. From potential to practice: challenges in BP applications in food systems

Although BPs exhibit remarkable health-promoting effects and can enhance the functional quality of foods, their widespread application still faces substantial challenges, requiring more detailed investigations and advanced trials to ensure successful adaptation into industry. The large-scale production of BPs requires effective process scale-up and strong collaborations between research laboratories and industry. For successful use in food applications, BPs must also demonstrate stability against gastrointestinal digestion and proteolytic degradation, along with sufficient absorption across intestinal cells (*Chew et al*., 2019; *Hamdi et al*., 2025).

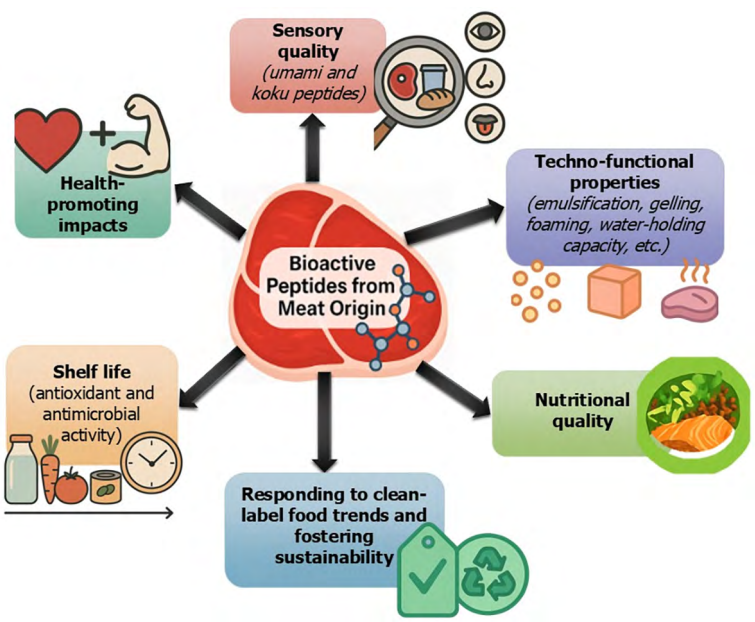

Figure 1. Potential effects of meat-derived BPs on food quality

Zaky et al. (2022) reported that despite advances in understanding peptide structure and function, their interrelationship, stability, and regulatory mechanisms remain insufficiently explored. Therefore, further pre-clinical and clinical studies are crucial to determine effective doses, bioavailability, pharmacokinetics, and the feasibility of integrating BPs into food systems. Due to certain limitations of BPs in the formulation of food and nutraceutical products, further investigations into their interactions within the food matrix, poor water solubility, hygroscopic behavior, and flavor-masking effects are also required. In this context, micro- and nano-encapsulation techniques were mentioned to enhance their compatibility and biological activity upon consumption (*Aguilar-Toalá et al*., 2022). Apart from encapsulation, the use of enzyme inhibitors such as protease blockers, as well as the application of permeation enhancers in epithelial cells, may facilitate the absorption of intact BPs (*Alzaydi et al*., 2023). Moreover, *Hamdi et al.* (2025) highlighted the considerable potential of BPs in the development of functional foods and, in particular, in anticancer research. Their ease of synthesis, structural modifiability, and low immunogenicity also make them attractive candidates for pharmaceutical applications. Nevertheless, their therapeutic use remains constrained by instability within biological systems. This limitation underscores the pressing need for comprehensive clinical studies to establish quality requirements before their full potential in healthcare and food sectors can be realized.

5. Conclusion

Meat-derived BPs are promising compounds with diverse physiological functions and wide application potential in food systems. In addition to their bioactive properties, they can enhance shelf life as well as technological and sensory properties. Their incorporation into functional food formulations is supported by scientific evidence and growing consumer demand for natural health-promoting ingredients. Yet, challenges remain in large-scale production, gastrointestinal stability, bioavailability, and regulatory approval. Advances in processing and encapsulation techniques will be key to overcoming these barriers. Overall, meat BPs hold strong promise as multifunctional ingredients, with further research needed for their full integration into food products.

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Original Scientific Paper

Fulfilling the requirements for exporting fish meat products (tuna pâté) to the United States market

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ABSTRACT

The objective of this work was to ensure all necessary prerequisites for exporting canned fish meat (tuna pâté) to the United States market are met. To accomplish this, thermocouple measurements were conducted during sterilization in the autoclave to validate the heat treatment process. Initially, a temperature distribution study was carried out during regular production, followed by a heat penetration study. In both cases, the success criteria were satisfied. The temperature distribution was monitored using nine thermocouple probes, and within one minute after the holding time (come up time), the temperature at all measured points in the autoclave medium exceeded 120°C. For the heat penetration study, eleven probes were used, eight placed in the thermal centre of the cans and three in the autoclave medium. All probes located at the thermal centre achieved F_0 values ranging from 6.88 to 8.56 minutes, confirming the safety and effectiveness of the sterilization process for tuna pâté intended for export to the United States.

1. Introduction

Despite the advancements in emerging conservation technologies which are often categorized as non-thermal processing methods (*Furukawa S., Hayakawa I.,* 2000), thermal sterilization remains the most widely used technique for food preservation (*Farid M., Abdul Gani M.A.,* 2004). Pressured vapor sterilization is economic and has a short processing time. It is non-toxic and safe for the environment (*Silindir M, et Ozer Z.A.*, 2009). In accordance with advice from the Food and Agriculture Organization of the United Nations (FAO), the extended shelf life of heat-treated meat and meat products is obtained by inhibiting or destroying micro-organisms through a heat-based process. The main steps of this preservation method involve placing the product into a container (such as a can, glass jar, or synthetic or laminated aluminium pouch) that is hermetically sealed after filling, ensuring it is impervious to external substances, and subjecting the sealed container to a controlled thermal treatment with specified temperature and duration (*FAO*, 2025).

To ensure food safety, producers are required to implement hazard analysis and critical control point (HACCP) plans. In support of this, the United States (US) Food Safety and Inspection Service (FSIS) issued a generic HACCP model as a useful

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tool (*FSIS*, 2025). The US Food and Drug Administration (FDA) has firm and strict rules and regulations which have to be met in order to import low acid canned foods (*FDA*, 2025). In order to assist food business entities, the Institute for Thermal Processing Specialists (IFTPS) issued a guideline for proper thermal processing studies (*IFTPS*, 2014). Finally, to elaborate and simplify the application to importation of canned foods to the US, the US Department of Health and Human Services issued instructions regarding the procedures to be followed in thermal processing studies in 2016 (*FDA*, 2016) and in 2017 (*FDA*, 2017).

Steam sterilization is widely employed to ensure the sterility of manufacturing equipment and products. Regulatory standards require the validation of sterilization processes, which depends on accurately estimating key microbial resistance parameters (*Faya P. et al*., 2017). It is widely acknowledged that the thermal process is a critical control point that must be closely monitored to ensure the microbiological safety of processed foods. The hazard analysis and critical control points (HACCP) system is the globally recognized approach for enhancing the safety of sterilized food products. Common challenges in implementing a HACCP system lie in identifying critical factors, determining their critical limits, and defining acceptable tolerances for those factors (*Akterian G.S. et al.*, 1999).

The industrial sterilization process relies on a specific temperature-time profile designed to ensure product shelf life and quality by inactivating target microorganisms or enzymes while minimizing the loss of nutritional components. The heating rate and the mechanism of heat transfer in canned foods—whether by conduction or convection—are influenced by various factors, including the temperature difference between the retort and the product, the surface-to-volume ratio, and the viscosity of the product (*Tattiyakul J.M., et al*, 2002).

The intensity of heat treatment significantly influences both the elimination of micro-organisms and the sensory qualities of the product. This thermal intensity can be described using physical parameters, most commonly the F-value, which quantifies the lethal effect of heat on micro-organisms. The thermal death time, measured in minutes at 121°C, is used as a reference standard for different micro-organisms (*FAO*, 2025). The minimum exposure time, denoted as F, for a decontamination process at a specific temperature is typically estimated using an empirical model that incorporates the decimal reduction time D and the temperature resistance coefficient z as parameters. These parameters are generally assumed to be independent of temperature. However, a microbiological analysis reveals that both D and z are actually temperature-dependent, suggesting that conventional models are only accurate within a limited temperature range (*Vuković*, 2012). The temperature dependence of F derived from this approach aligns closely with existing experimental data. Additionally, safety margins to ensure sterility can be easily incorporated into the process (*Doornmalen and Kopinga, 2009*). Calculating heat penetration parameters and microbial inactivation efficiency is essential for evaluating the uniformity of heat transfer in the canning industry (*Smout et al*., 2000).

To accurately measure the heat parameters during thermal processing, thermocouples are used. These are temperature indicator and measurement devices composed of two dissimilar metals which are joined together to form two junctions. When one junction is kept at an elevated temperature as compared to the other, a small thermoelectric voltage or electromotive force is generated which is proportional to the difference in temperature between the two junctions (*IFTPS*, 2014).

Aluminium is frequently used for smaller and easy-to-open cans as deep-drawn two-piece cans (the body and the bottom end are formed out of one piece and only the top end is seamed on after the filling operation). The advantages of aluminium cans compared to tin cans are their better deep-drawing capability, low weight, resistance to corrosion, good thermal conductivity and easy recyclability.

The objective of this work was to ensure all necessary prerequisites, for exporting canned fish meat (tuna pâté) to the US market are met. To accomplish this, thermocouple measurements were recorded during thermal processing of the tuna cans in the autoclave to validate the process. Specifically, a temperature distribution study was carried out during regular production, followed by a heat penetration study.

2. Materials and methods

All export requirements for the canned fish meat (tuna pâté 60 g, packed in aluminium foil with a thermally glued lid) to the US market, regarding sterilization procedure, are stated in IFTPS guidelines (*IFTPS*, 2014).

Thermal processing of tuna pâté 60 g packed in aluminium foil took place according to the following thermal formula:

$$To = 20' + \frac{20'}{120°C/2.5bar} + 15'$$

At the start of thermal processing, the first 20 minutes was the active heating phase in which temperature increased to the defined level of 120°C in the autoclave medium, then 20 minutes was the effective sterilization time at 120° C with a medium pressure of 2.5 bar in the autoclave medium. The terminating phase was 15 minutes of cooling time prior to opening the autoclave door.

To conduct a temperature distribution study (TDS), the autoclave medium temperature was 121°C, since an acceptable temperature distribution is a requirement for process establishment. A heat penetration study (HPS) then was performed under a defined thermal formula. The stages and duration of the sterilization process were the same for the TDS and the HPS.

Thermocouple measurements, required to meet the export requirements for canned fish meat for the US market, were used during the TDS in the autoclave medium and during the HPS in the thermal centre of the cans and in the autoclave medium. The TDS and HPS were carried out in accordance with the IFTPS Guidelines, which contain success criteria for the two studies (*IFTPS*, 2014). Measurements were made with an Ellab thermocouple device (E-Val Pro, serial number 411982, validated software - US FDA, 21 CFR part 11, GMP, ver. 4.6.1.0, Picture 2), hooked to thermocouples with compensating cables. The Ellab Valsuit software, Val Suite Pro ver, was used to prepare the technical report. 5.2.015.

Both the TDS and HPS were performed in a Steriflow autoclave (Roanne, France), which operated on a cascading water principle. It is a horizontal overpressure autoclave that accommodates four carts. Considering the waiting time of the canned filling before sterilization, every sterilization cycle was carried out with three carts filled with product to the top, while one cart, always positioned in the same spot in the autoclave medium, remained empty. In this way, the three-cart work practice ensured the safety of the canned product before sterilization by monitoring the residence time of the canned filling. The internal procedures of the food business entity, based on the conducted microbiological and sensory testing, defined a maximum waiting time of the filling, before sterilization, of up to 2 h. During that defined time, the filler machine in the production department can completely fill the three full carts. The position of the carts in the autoclave is shown in Figures 1 and 2. Altogether, there were 8697 cans in the autoclave during both the TDS and the HPS. Tuna pâté cans were placed in one layer on the trays, always placed and lined up in an identical manner (Figure 3). Altogether, 116 individual cans fitted on one layer on a tray, with 25 layers in the cart, making 2899 cans on one cart. The dimensions of the tuna pâté 60 g cans were L x W x H – 97 × 60 × 25.4 mm. Identical autoclave conditions were met for the separate HPS.

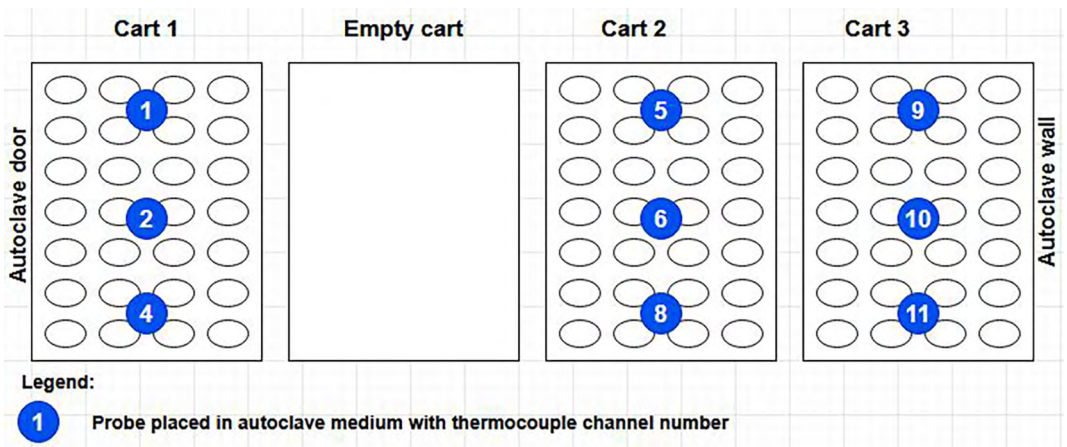

Figure 1. Plan of thermocouple probe placement in the autoclave medium (blue colour) for the temperature distribution study, seen from the side

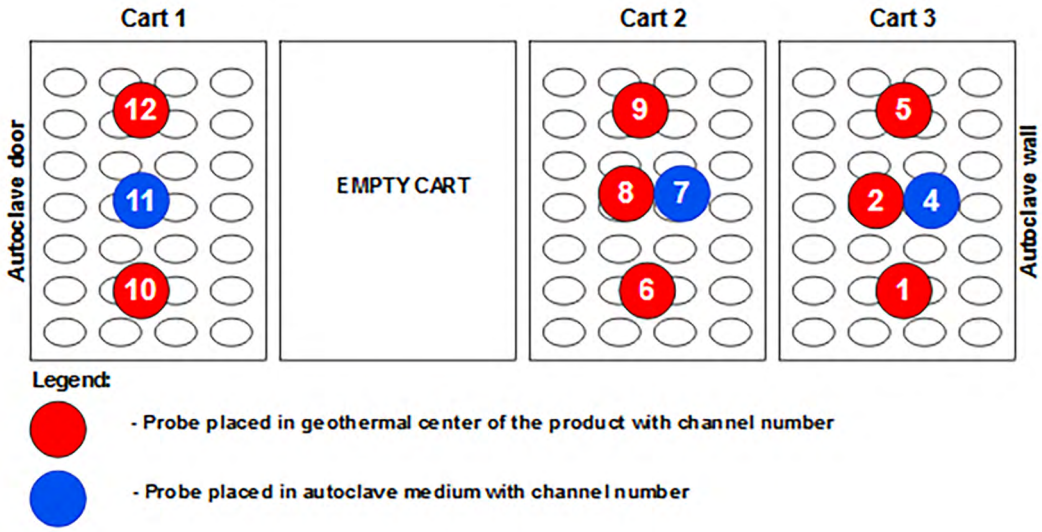

Figure 2. Schematic diagram of thermocouple probe placement in the thermal centre of the product (represented by red circles) and in the autoclave medium (blue circles), seen from the side

Figure 3. Tuna pâté cans of 60 g positioned in a cart and ready for thermal processing

For the TDS, nine thermocouple probes were used (three thermocouple probes in each of the three carts (Channels: 1, 2, 4, 5, 6, 8, 9, 10 and 11). Probes were placed outside the containers in autoclave medium (Figure 4), in accordance with IFTPS Guidelines (Chapter 4, *IFTPS*, 2014), and in the locations shown schematically in Figure 1. Temperatures were recorded at one minute intervals.

The HPS, conducted after the TDS, utilised eight thermocouple probes placed in the thermal centre of the products (Channels 1, 2, 5, 6, 7, 9, 10 and 12), in the locations depicted schematically in Figure 2. Each probe was inserted in the thermal centre of a can with an airtight seal, as depicted in the photograph, Figure 5. These eight probe locations in the autoclave were the same as in the TDS,

Figure 4. Thermocouple probes placed outside the tuna pâté cans in the carts

but in the HPS, three other probes were placed outside the cans (Channels 4, 7 and 11) to monitor the autoclave medium (Figure 2).

Figure 5. Thermocouple probe placed in the thermal centre of a tuna pâté can

3. Results

The TDS was performed during the regular production process. The temperatures recorded in the autoclave medium by the thermocouple probes throughout the thermal processing are shown in Figure 6. The first condition for the successful implementation of the TDS was to reach a temperature of at least 120°C at all control positions (thermocouple locations) within 30 seconds of reaching the effective sterilization temperature. One minute after starting the hold time, at all control positions, the temperature was above 120°C. Temperatures in the autoclave at the end of the come up time and after one minute of hold time are presented in Table 1.

Table 1. Autoclave medium temperature recorded by the probes at the end of come up time and after one minute of hold time during the temperature distribution study

Thermocouple channel	Temperature (°C)
Channel 1	120.65
Channel 2	120.69
Channel 4	121.23
Channel 5	120.32
Channel 6	120.68
Channel 8	120.53
Channel 9	120.54
Channel 10	120.38
Channel 11	120.10

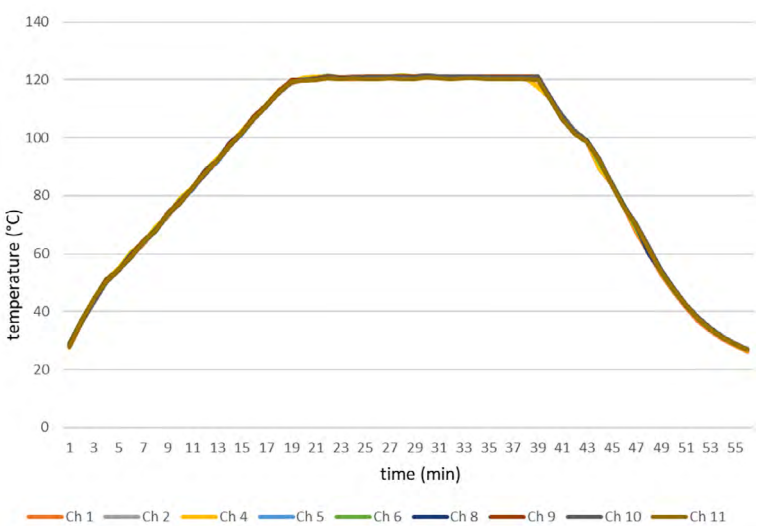

Figure 6. Temperature of the autoclave medium as recorded by the thermocouple probes at different locations in the autoclave during the thermal processing

The HPS was performed during a regular production process, and temperature results are shown in Figure 7. The temperature in the thermal centre of the product increased uniformly and consistently in every part of the autoclave medium.

At the end of the sterilization process (Table 2), acceptable F_0 values were recorded, which ranged from 6.88 (Channel 8) to 8.56 minutes (Channel 10). A graphical presentation of these F_0 values is shown in Figure 8.

Table 2. F_0 values at the end of the heat penetration study, measured in the thermal centre of the tuna pâté cans

Thermocouple channel	F_0 value (minutes)
Channel 1	7.97
Channel 2	8.18
Channel 5	7.58
Channel 6	7.78
Channel 8	6.88
Channel 9	8.05
Channel 10	8.56
Channel 12	7.29

Temperatures recorded during the HPS at the end of come up time by the probes that were placed in autoclave medium are presented in Table 3.

Table 3. Autoclave medium temperature at the end of come up time and one minute of hold time during the heat penetration study

Thermocouple channel	Temperature (°C)
Channel 4	120.78
Channel 7	120.70
Channel 11	120.28

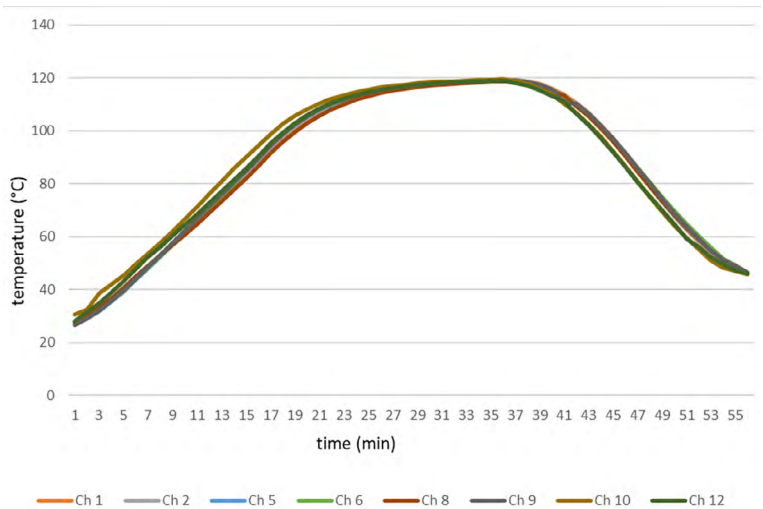

Figure 7. Temperature recorded by the different thermocouple channels in the thermal centres of tuna pâté cans during the thermal processing

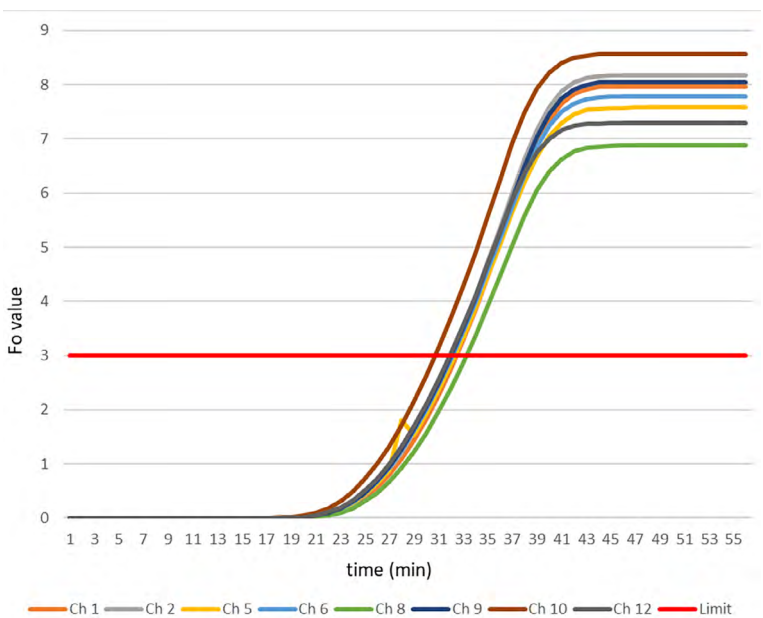

Figure 8. Achieved F_0 values during thermal processing of the tuna pâté cans

4. Discussion

Canning aims for so-called commercial sterility, meaning the food is free from microorganisms that can cause spoilage or illness under normal storage conditions (*Kinzhal M.*, 2018, *Kenneth Y.*, 2022). Validation of the sterilization process from the aspect of fulfilment all necessary conditions for export to the US market was realized through two successive studies using thermocouple probes: TDS and HPS. In both studies, the success criteria were met.

TDS is used to establish the ability of a retort process to uniformly mix and distribute the heat transfer medium, especially when the heat transfer into product may be limiting. The heating time of the autoclave medium was 20 minutes, and under the defined come up time, after 1 minute of sterilization hold time, thermocouple temperatures were above the minimum process temperature. The slowest heating point was measured by Channel 11 (120.1°C), but this was above the required temperature of 120°C, while all other thermocouple temperatures were higher at that moment. All thermocouple measurements were above the minimum process temperature after one minute of holding time. Both the uniformity and stability of temperatures in the autoclave medium were confirmed, without any fall below expected values. The position of the thermocouples was such that they were evenly distributed in all three product carts, at different levels. The success criteria for the TDS were met. Also, the three probes placed in the autoclave medium during the HPS (Table 3) revealed that the required temperatures were achieved for successful temperature distribution at the end of come up time. Product temperature increased uniformly and consistently regardless of thermocouple position, testifying to a uniform thermal process in all parts of the autoclave.

The HPS was performed to establish a consistent thermal process that would deliver commercially sterile products and to assist in evaluating process deviations (*IFTPS*, 2014). The same thermocouples were used during sterilization of the next production batch, in the same autoclave under the same production conditions. F_0 values were measured in thermal centres of the cans, in order to ensure safety and desired and expected product characteristics. Based on these microbiological considerations and including a sufficient safety margin, sterilized canned products should be produced with F_0 values of 4.0-5.5 minutes, while used autoclave medium temperatures can vary between 117 and 130 °C, which depends on the heat sensitivity of the individual products (*Desrosier et Singh*, 2025; *FAO*, 2025). In accordance with the FAO principles of meat caning and sterilization, a shelf life of up to four years at storage temperatures of 25°C or below can be achieved. The thermal death time for spores of *Clostridium botulinum* at 121°C is 2.45 minutes or in other words, an F_0-value of 2.45 is needed to inactivate all spores in the product at 121°C (*FAO*, 2025). It has been shown that F_0 values of 4 in heat-preserved products will guarantee commercial sterility (*FAO*, 2025). Measurements in our study yielded F_0 values from 6.88 (Channel 8) to 8.56 minutes (Channel 10), which is in accordance with the specified parameters for assessing HPS success, as shown in Figure 3. Comparing the F_0 values we obtained with those of other relevant pâté type products (F_0 ranged from 12.07 to 15.93 minutes and from 9.71 to 13.17 minutes), we observed a more optimal procedure that will still ensure product safety and likely will not reduce the food's biological and nutritional value (*Raseta et al.*, 2019). One condition for the successful implementation of the HPS is the achievement of a F_0 value of at least 3 in the thermal centre of the product, in accordance with the conditions that must be achieved by a can of meat intended for continental storage and transport conditions.

Overly intensive thermal processing should be avoided, since flavour changes in thermally treated meat products are caused by Maillard reactions, thermal degradation of lipids, and Maillard-lipid interactions (*Xie Q. et al., 2022*). The number and position of cans in the carts at each level was identical. The construction and shape of the cans (kidney-shaped) aluminium foil with a thermally glued lid favoured the intensity of heat transfer and the generation of suitable F_0 values in the thermal centre of the can. In similar work in Iran, it was found that cone-shaped containers exhibited the fastest heat transfer during sterilization. Container geometry significantly influences the shape and location of the slowest heating zone, as well as the final temperature, and F_0 value (*Ranjbar*, 2019).

The only thing that is constant in the entire work process is the operation of the sump water pump. During the entire process, the water circulates at the same intensity. During the one cycle of sterilization process, energy consumption was monitored

and consumption of dry saturated steam at a pressure of 6 bar was 0.4 tons, while 4.6 m³ of cold water was used.

The results obtained in both the TDS and HPS confirmed the required temperature distribution in all regions of the autoclave medium, which will ensure the safety of the tuna pâté during storage. Through these successful studies, the food business entity has increased the control level over the sterilization process as a mandatory critical control point in the production of tuna pâté intended for export to the US. Also the studies provide necessary data for further optimization of the sterilization process, from the aspect of increasing the nutritional and biological value of the product.

5. Conclusion

The success criteria for the both studies were met. First, the temperature distribution in the autoclave medium was uniform, and at the end of the come up period, all the thermocouples showed the temperature exceeded 120°C (TDS). After that, the heat penetration into the thermal centre of the product (HPS) was measured, and F_0 values were in the range of 6.88-8.56 minutes. These F_0 values are suitable to ensure the safety of the product, and all the necessary conditions were met for the export to the US market of the tuna pâté in 60 g cans, packed with a thermally glued lid.

Our findings indicate that for commercial sterilization processes, commonly used models significantly underestimate the necessary minimum exposure times at temperatures below 120°C. This has important implications for process optimization.

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Original Scientific Paper

Meat industry as our best chance for controlling transmission of a tiny but deadly tapeworm

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ABSTRACT

Echinoccocosis is a zoonotic disease which can have severe, even lethal consequences for the host, yet multiple effective means of controlling transmission and thus preventing infection, are available. *Echinococcus granulosus* and *E. multilocularis* are the two tapeworm species which are clinically most relevant in Europe and both are endemic in the Balkans. The life cycle of the tapeworm includes intermediate and dead-end hosts, ungulates and humans, as well as definitive hosts, the Canidae and Felidae. Disease caused by *E. multilocularis*, alveolar echinococcosis (AE) is more severe and also difficult to diagnose, especially in animals. Echinococcosis in livestock leads to morbidity and mortality, thereby facilitating economic losses at various levels. Reporting is mandatory, yet under-reporting is a common occurrence as is the failure to identify relevant geographical transmission foci in a timely manner, thus contributing to continuous tapeworm transmission and spread. As echinococcosis is a foodborne disease in animals and humans, the food industry, and particularly the meat industry, are key stakeholders in raising awareness, lobbying government authorities for control measures and improved diagnostics.

1. Introduction

Tapeworms of the genus *Echinoccocus* are endemic in the Balkan region, with *Echinococcus granulosus,* the 'dog tapeworm', presumed to be most frequent. *E. granulosus* is the causative agent of cystic echinococcosis (CE), or hydatid disease, which occurs in domestic and wild ungulates and humans. Echinococcosis is a zoonotic foodborne disease of high significance to public health in Europe (*Bouwknegt et al*., 2018). Aside from *E. granulosus*, which is fairly well known to veterinarians and physicians, the species with the greatest clinical impact currently in Europe is *E. multilocularis,* the 'fox tapeworm', which was detected for the first time in Serbia over a decade ago in an imported beaver (*Ćirović et al*., 2012). Alveolar echinococcosis (AE), the disease caused by *E. multilocularis*, manifests as wide-spread, infiltrative growth which results in tissue lesions and destruction. The first confirmed human case of AE in Serbia was described in 2023, and another in 2024, thus AE may appear to be an

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emerging disease (*Lalošević et al*., 2023, *Milošević et al*., 2024). However, echinococcosis has an incubation period of up to 15 years and is difficult to diagnose, which leads to an underestimated case burden and prevalence (*Casulli*, 2020; *Jorgensen et al*., 2008). Treatment is costly and complex; thus transmission control and prevention strategies are critical. To achieve an optimal outcome, the One Health approach must be implemented. In that respect, the meat industry has a vested interest and possesses the necessary economical leverage to push policy makers to enforce existing regulations on prevention, demand diligent reporting, sponsor sophisticated diagnostics and implement active surveillance. This review will highlight the importance of detecting echinococcosis, particularly AE, in food animals, as well as expose key problems in implementing effective transmission control measures proposed by the current legislation.

2. *Echinococcus* tapeworms: Life cycle and transmission

The Canidae and Felidae families are definitive hosts for *Echinococcus* spp., whereas wild and domestic ungulates are intermediate hosts and humans are dead-end hosts. In the intestines of the definitive host, the tapeworm grows into its adult stage and undergoes sexual reproduction to form thousands of infectious eggs, which are shed via feces (*Eckert et al*., 2001). Transmission of eggs occurs via the environment, mediated by soil and water, which facilitates their dispersal in the foodweb. Wild and free-range animals are commonly infected while grazing and possibly drinking from surface water sources contaminated with tapeworm eggs. The eggs reach humans and domestic animals also in trace residues of soil or when directly deposited on plants (vegetables and berries) via spray or foliar irrigation using fecally contaminated water (*Robertson et al*., 2016). A recent European multicenter study demonstrated the presence of *E. multilocularis* and *E. granulosus* eggs on 1.2 % and 1.3 % of tested vegetables and berries, respectively (*Umhang et al*., 2025). Once the eggs are ingested, the tapeworm embryo is released in the intestines. In intermediate and dead-end hosts, the tapeworm cannot develop into its adult form; instead, it penetrates the lamina propria and is transported passively to an internal organ, where it encysts to form the metacestode. Within the cyst, tapeworm larvae reproduce asexually, thereby gradually increasing the size of the cyst. As each larva is a juvenile tapeworm, once a definitive host species consumes an infected intermediate host, the transmission cycle continues (*WHO Team for Control of Neglected Tropical Diseases (NTD)*, 2001).

3. Echinococcosis

Disease begins with the establishment of the metacestode in an internal organ and progresses with cyst growth. The rate of growth is variable and estimates range from 1-50 mm annually in humans with CE (*Brunetti et al*., 2010). The cyst size is only limited by the available space and therefore depends on the localization, but eventually it leads to organ compression and obstruction of physiological processes, which can manifest as various symptoms in humans and animals (*Casulli*, 2020; *WOAH,* 2025). These are usually difficult to interpret and associate with echinococcosis specifically. In animals, CE is almost exclusively diagnosed at slaughter, by visual observation of cysts after evisceration. Unlike CE, the metacestode of *E. multilocularis* infiltrates organ tissue, causes lesions and over time, re-organizes the tissue, much like malignant tumors (*Lundstrom-Stadelmann et al*., 2025). In fact, like cancer cells, the *E. multilocularis* metacestode also has a tendency to metastasize to other organs. Since it is much more aggressive, the outcome for the infected host is uncertain. Due to the unusual disease presentation, even experienced teams in countries with high AE burdens manage to confirm AE in just over half of the human cases (*Grüner et al*., 2017). It is, therefore, likely that AE in animals is hardly ever correctly diagnosed.

3.1 Echinococcosis in food animals

Despite the fact that cysts are not infectious to humans or intermediate hosts, they are a source of infection for definitive hosts. The sanitary codex of the World Organisation for Animal Health (*WOAH*, 2025) mandates the reporting of animal cases of echinoccocosis, a practice which is also legislated by the relevant *Veterinary Law of Serbia* (2019), i.e., *Official Gazette* 91/2005, 2005; *Official Gazette* 30/2010, 2010; *Official Gazette* 93/2012, 2012; *Official Gazette* 17/2019, 2019. While the relevant law and rulebooks outline sound and effective means of discovery of cases and geographical transmission foci, the feasibility of their implementation is questionable (*Serbia*, 2006; *Serbia*, 2024, respectively *Official Gazette* 49/2006, 2006 and *Official Gazette 21/2024,* 2024). A major issue is that abscesses, lesions and other morphological changes in internal

organs are relatively common in food animals, but rarely due to parasitic infections (*Amachawadi and Nagaraja*, 2016; *Valkova et al*., 2023). Thus, a simple visual inspection is often not sufficient to identify echinococcosis (*Amachawadi and Nagaraja*, 2016; *Valkova et al*., 2023). The young age of the majority of slaughter animals (6 months to 3 years) processed at commercial facilities is a confounding factor, as it increases the odds that *E. granulosus* cysts, or lesions due to *E. multilocularis* infection, are not obvious and could be missed by visual inspection. As veterinarians trained in meat inspection are available at commercial facilities, it is certain that any organs with clearly distinguishable morphological changes would be condemned. Thus overall, cases of CE, with which they are more familiar, have a better chance of being detected as opposed to cases of AE, which is of concern. The level of echinococcosis awareness and training in visual inspection of farmers and private individuals who slaugher animals for their own consumption or commercial sale is likely to be inadequate. Thus, they may directly contribute to transmission by failing to inactivate and properly dispose infected offal. Illegal dumping of animal remains and livestock carcasses is a recognized problem in Serbia, while it is impossible to evaluate the extent of the practice of feeding raw offal to dogs.

3.2 Echinococcosis prevention

The most effective transmission control action for echinococcosis is deworming of the definitive hosts, as this stops the formation of eggs. Regular deworming of pets is mandated in Serbia and must be recorded in pet passports (*Serbia*, 2024). It is of note that any entries into the pet passport must be validated by a licensed veterinarian and/or clinic, as it is an official travel document. For travel however, proof of deworming is required only for entry into Malta, Finland and Ireland (*NetherlandsWorldwide*, 2025). In Serbia, antihelminthics with and without tapeworm efficacy in the form of tablets or pills are sold without prescription and can be administered by owners themselves. In practice, it is therefore unclear how regular deworming using drugs effective against tapeworms is to be enforced and monitored in Serbia. It stands to reason that owner would need to have their pets dewormed strictly by veterinarians, which is not mandated. Most importantly, deworming of owned animals only has a symbolic effect on transmission, as the number of stray and/or feral dogs and cats is probably similar to the number of owned pets. Additionally, *E. multilocularis* likely established itself in the domestic environment after a spillover from the sylvatic. Thus, without a more proactive and holistic approach to deworming, which includes all categories of definitive hosts, the number of cases is likely to rise.

Another control approach is vaccination of livestock to prevent infection and subsequent transmission to definitive hosts. An effective vaccine based on EG95 (a tapeworm secretory antigen) was developed in the late 1990's for sheep and since then has been used on livestock in several countries, but not in Serbia (*Lightowlers et al*., 1996, 1999; *Gauci et al*., 2022). The feasibility of implementation in Serbia

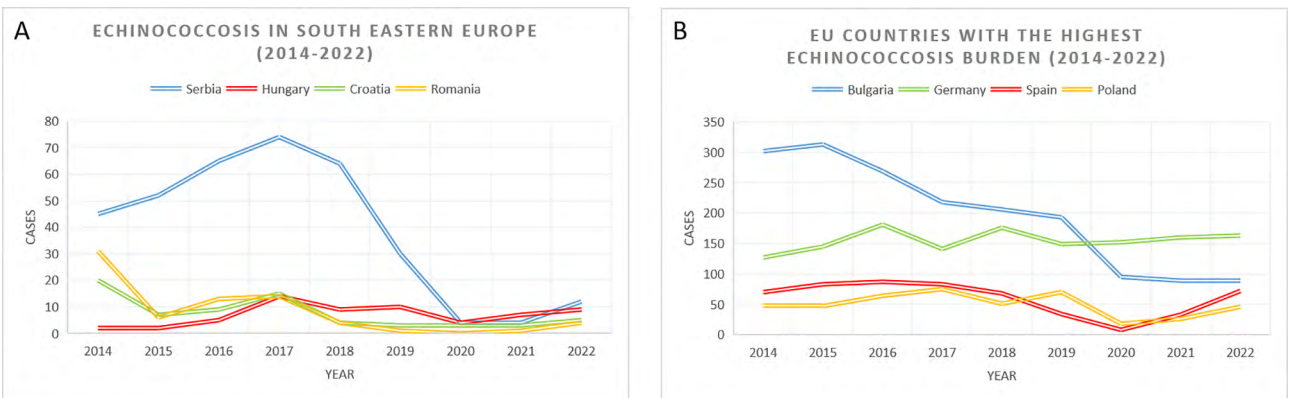

Figure 1. Officially reported human echinococcosis cases in South Eastern Europe (A) and EU countries with the highest echinococcosis burden (B) from 2014 to 2022. The blue line in A and B represents the country with the highest case number. A: Blue (Serbia), red (Hungary), green (Croatia) yellow (Romania). B: Blue (Bulgaria), green (Germany), red (Spain), yellow (Poland). The data from Serbia were extracted from annual epidemiological reports of contagious diseases from the Institute of Public Health of Serbia (IOPHOS), while the data for EU countries were retrieved from the European Centre for Disease Prevention and Control's (ECDC's) annual epidemiological reports collected from the European Surveillance System (TESSy).

must be carefully evaluated, especially as the farming industry heavily involves small farms and backyard farming (*Uzelac et al*., 2023). Based on the case numbers, the current transmission control and prevention measures in Serbia (Figure 1A) and in the EU (Figure 1B) are lacking, while both *E. granulosus* and *E. multilocularis* are abundant in the environment (Figure 2).

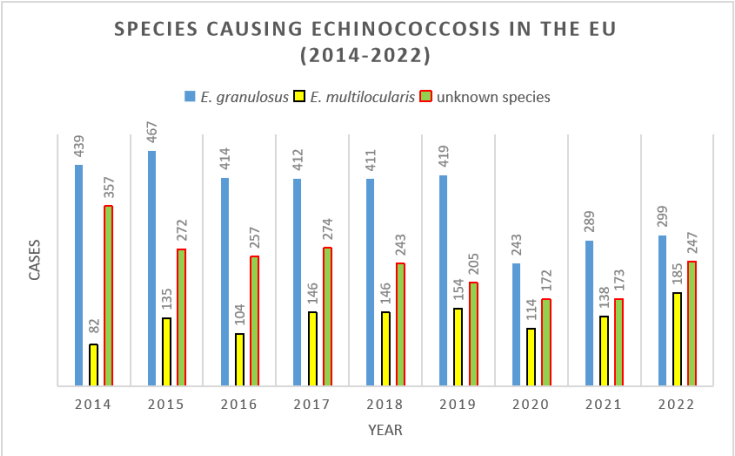

Figure 2. Tapeworm species identification in human samples in the EU (2014-2022). Blue bars are infections due to E. granulosus, yellow are E. multilocularis and green are those designated as unknown species. The data were retrieved from the European Centre for Disease Prevention and Control's (ECDC's) annual epidemiological reports and reflect data from EU countries which submitted results to the European Surveillance System (TESSy) (n = 27).

4. Discussion

Under-reporting of animal echinococcosis cases and failure to identify transmission foci directly facilitate the meat industry incurring economic losses associated with animal morbidity and death. Moreover, failure to prioritize transmission control and prevention in animals by the relevant government authorities and stakeholders will most certainly have negative consequences on public health in Serbia. Due to the high transmission capacity of a single infected definitive host, entire herds can be at risk. Organically reared and free-range animals are especially vulnerable to parasitic infections (*Kijlstra et al*., 2004). The vested interest of the meat industry is thus clear. The leverage mechanisms are its financial assets and its importance to the economy, and with that, the meat industry is ideally positioned to raise awareness on echinococcosis, as well as advocate for control and prevention. Reforms to improve detection of echinococcosis on the slaughter line can be as simple as prioritization of extensively reared animals with respect to requesting confirmatory diagnostics from government authorities and lobbying for mandatory tapeworm speciation. In order to avoid sourcing infected livestock, identification of transmission foci is key. The meat industry can, therefore, push government authorities into action and demand the establishment of a tracking system, which would provide notification on the current geographical status of transmission. These measures are, however, a stop-gap solution, vulnerable and at risk of being ineffective due to the dependency on relevant government authorities for financing, record keeping and information sharing with the meat industry. A real impact on the reduction of echinococcosis cases will only be achieved through complex reforms requiring a proactive approach by the industry though strong collaboration with the government authorities, sponsoring and investing in laboratories and testing facilities. These need to be properly equipped for high throughput, sophisticated detection and speciation with software assisted geographical surveillance of cases. With alarmingly high prevalence of *E. multilocularis* infection in wild canids in the Vojvodina region, implementing any reforms at the national level is an urgent matter (*Miljević et al.*, 2019, 2021).

5. Conclusions

Echinococcosis is a zoonotic disease with serious impact on public health, the farming industry and the economy. Slow cyst growth, a characteristic of CE, and complexity of disease presentation, a characteristic of AE, complicate diagnosis and lead to under-reporting of human and animal cases. Effective transmission control is difficult to implement and costly on a large scale. Thus, targeted and tailored measures, with respect to geography and transmission dynamics, are a better strategy. The key factors for echinococcosis control in Serbia are improved diagnostics on the slaughter line and transmission monitoring. The meat industry has a vested interest to support livestock health and production and has the leverage and financial means to push for and/or implement the necessary actions. As a major stakeholder, the meat industry may be our best chance to establish echinococcosis control and prevention in Serbia.

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Original Scientific Paper

A holistic approach model to food safety surveillance: detecting *Toxoplasma gondii* in meat

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ABSTRACT

Prevalences of infections caused by food- and waterborne parasites, some of them causing a significant disease burden, are on the increase. In the absence of systematic surveillance programmes for many of these parasites, particularly in extensive or free-range husbandry settings, research efforts are under way to design monitoring procedures and improve food safety, beyond the current standard of practice or of food safety legislation. An example is given of the commencement of these efforts, by presenting the experimental work package in a research project which entails testing of meat (heart tissue) of backyard poultry and game birds for the presence of *Toxoplasma gondii* gDNA. The high molecular prevalence of infection detected in some bird species, particularly in backyard chickens raised in rural areas, points to the need for comprehensive and timely surveillance programmes to be applied to all extensively farmed, or game animals and birds used for human consumption. Surveillance should include testing of animal feed as well as the environment (water and soil). However, until risk-based surveillance programmes are operational, meat safety will principally depend on adherence to prevention practices, such as pre-market product processing, as well as on educating consumers in hygienic handling and proper meat preparation practices.

1. Introduction

The prevalence of infections caused by food- and waterborne parasites (FWP) in humans and animals in Europe is increasing (*Bouwknegt et al.*, 2018), prompting needs for a holistic approach to their surveillance. There is a need to quickly identify reservoirs of infection, recognise emerging threats, and warn public health authorities in good time. Currently, there are no active surveillance programs for most of the FWP, and the parasitoses they cause are not notifiable for the most part.

In controlled husbandry conditions of intensive farming, parasitic diseases are easily surveyed, prevented, or treated. For instance, surveillance programmes for *Toxoplasma gondii* infection for pig farms that supply around 50% of all slaughter pigs in the Netherlands have been in place since 2012 (*Swanenburg et al.*, 2019). In addition to on-farm risk factor management (such as neutering all

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cats and covering feed storage), control of *T. gondii* infection includes a large-scale serological monitoring programme endorsed by a private food producing company. This risk-based active surveillance combines farm audits and serological testing to identify high, medium and low risk farms. A random sample of slaughter pigs is serologically monitored for *T. gondii*-specific antibodies at each delivery to abattoirs, depending on the farm status (fewer animals are sampled from low-risk farms, as opposed to those from high risk, or from a new supplier's farm; also, in the case of continuously negative serological results on repeated testing, the number sampled is decreased). This method has proven effective for *T. gondii* surveillance, as well as being affordable both in cost and time. However, this is still an unregulated programme, as opposed to that for *Trichinella* surveillance, Europe-wide. Provisions of a 2022 Serbian bylaw, harmonized with the European Union regulation from 2015, allow for the implementation of a risk-based approach in the testing for *Trichinella* infection in Serbia. Namely, pig farms could be designated '*Trichinella*-free', whereupon only 10% of animals from those farms would be subject to mandatory post-mortem *Trichinella* inspection; the enactment of this bylaw is nonetheless slow to begin (*Betić et al*., 2025 – *this Special Issue*).

In Serbia, *T. gondii* infection in food animals has been monitored to the greatest extent in pigs through individual research efforts since 2002. Seroprevalence in intensively farmed market-weight pigs over the years has been in the range of 8.3-17% (*Klun et al*., 2006; *Kuruca et al*., 2017; *Betić et al*., 2022), while a much higher seroprevalence of 30-41% has been determined for intensively and semi-extensively farmed sows (*Klun et al*., 2006, 2011), and particularly for extensively farmed or free-range pigs, such as in Mangalitza breed droves – 67% (*Kuruca et al*., 2016). Understandably, controlling parasitic infections in an extensive husbandry setting is much more challenging; it would perhaps be practically impossible to create a uniform one-fits-all control programme that could enable circumvention of post-harvest mitigation measures. When using meat from free-range animals, vigilance in adhering to these measures is still crucial, be it freezing of meat for 48-72 hours, proper salting or curing, vacuum packing for a minimum of 21 days, cooking to an internal temperature of 66 °C (*Dubey et al*., 1990), or any other means confirmed to kill all viable parasites (tissue cysts) within the meat or meat product.

2. The chicken connection

Similarly, consuming undercooked meat from chickens raised in extensive husbandry conditions, such as free-range or backyard, or from game birds, poses a public health risk. Although it is commonly accepted that chicken meat in general does not pose a risk since it is usually consumed properly cooked, or at least the risk is lower than from other types of meat, specifically from pigs, sheep or goats (*Tenter et al*., 2000), in certain situations the risk may be much higher. Only in one type of dish is chicken meat purposefully used uncooked, and that is in the southern Kyushu region of Japan, where traditional 'torisashi' (e.g. sashimi, or 'carpaccio') is prepared from thinly sliced meat, seared on the surface while remaining raw within, and served with various seasonings. However, in Europe and other parts of the world, chicken meat may still accidentally remain undercooked (with viable *T. gondii* tissue cysts reaching the consumer), due to undue haste in preparing food, for instance when catering for large numbers of people, or in some cases of street served food. Eating undercooked poultry is a known risk factor for developing campylobacteriosis (rev. in *Varrone et al*., 2020). Alternatively, poor standards of hygiene may lead to the use of meat cutting boards for salad (raw vegetables or fruit) preparation immediately afterwards, allowing for cross-contamination, i.e., transfer of particles of infected meat (or tissue cysts) to other food that is naturally eaten raw. Also, chicken liver is sometimes prepared and eaten quickly seared, without thorough cooking to reach an internal temperature of 66 °C; consumption of chicken liver pâté has been implicated in numerous cases of campylobacteriosis and/or salmonellosis outbreaks (*Wensley and Coole*, 2013; *Lahti et al*., 2017). Therefore, it can be inferred that this mode of transmission is also highly probable for *T. gondii*.

Due to their feeding habits, with scratching the ground and pecking, backyard and free-range chickens are frequently exposed to the parasite. They have been used as sources of *T. gondii* strains around the world, and they make for good indicators as well as sentinels for detecting environmental contamination with *T. gondii* oocysts (*Dubey*, 2010; *Dubey et al*., 2020). However, no surveillance for *T. gondii* infection is performed (nor is it mandatory) on chicken

farms, let alone in free-range poultry or wild-caught game birds.

3. Hunting for parasites

The possibilities of setting the stage for a novel and comprehensive surveillance programme for FWP are being explored in a 3-year project funded by the Science Fund of the Republic of Serbia – Environmental Monitoring of Food and Waterborne Parasites – PARASITE_HUNTER (*DIPIS*, 2025). It aims to establish a data-driven surveillance framework for FWP to improve food safety beyond the current standard, by including monitoring of vulnerable groups (humans and animals) not covered by the current food safety legislation. The holistic approach applied is expected to produce information on the presence of FWP of public health significance; therefore, the species targeted by the project are: *Ascaris* spp., *Toxocara* spp., *Trichuris* spp., *Taenia* spp., *Diphyllobothrium* spp., *Echinococcus* spp., *Spirometra* spp., *Opisthorchis felineus*, *Fasciola hepatica, Cyclospora cayetanensis, Cryptosporidium* spp., *T. gondii, Entamoeba* spp., and *Giardia* spp.

The PARASITE_HUNTER project comprises six experimental work packages, and one each of analytical and coordination packages. Experimental packages entail analysis of food (domestic animal tissues, leafy vegetables and herbs), feed (hay, silage), environmental matrices (water and soil), freshwater crustaceans, and wild animals inhabiting urban areas, for the presence of zoonotic parasites using standardised parasitological and molecular techniques. The work also includes exploring the likelihood of interaction of golden jackals as parasite reservoirs with food and feed. In parallel, data is collected on information and knowledge of food producers and suppliers (farmers, fishermen, hunters) about FWP, as well as parasitic diseases and prevention measures. The impact of ecosystem health and wildlife on food safety will be evaluated. All data thus collected will be analysed in the context of assessing food safety and public health risks, while considering potential means of preventing parasite transmission either at the production or distribution level.

For instance, for *T. gondii* surveillance, hearts of backyard or free-range chickens, waterfowl, game birds, as well as lambs have been collected and tested for the presence of *T. gondii* gDNA. The results showed a high molecular prevalence of *T. gondii* in hunted pheasants and backyard chickens, particularly rural (80%), while those raised in peri-urban areas had a considerably lower prevalence of infection (21%), similar to mallards (Table 1) (*Penezić et al*., 2025, *in press*). *T. gondii* has previously been detected and isolated from chickens in Serbia, with two strains successfully propagated in mice (*Uzelac et al*., 2021), so the current results were hardly surprising. However, they show the need for comprehensive and timely surveillance programmes to be applied to extensively farmed, or wild (hunted) animals and birds used for human consumption, as well as to include testing of animal feed, and water and soil from the animals' habitats and environments.

Table 1. *Toxoplasma gondii* infection in edible birds (poultry and game) in Serbia, based on detection of parasite gDNA in heart tissue.

Bird species (type)	*T. gondii* infection (%)	Reference
Domestic chicken (backyard poultry)	41	Penezić et al., 2025 (*in press*)
Ring-necked pheasant (game bird)	29	
Mallard (duck game)	23	

PARASITE_HUNTER is in its second implementation year, and by its completion, it is envisaged that it will give a new viewpoint as well as recommendations that could enhance food safety standards. The resulting proposals are aimed for inclusion in a white paper which will be put forward to the stakeholders – policy makers in Serbia, in the hope of instigating a country-wide, systematic surveillance programme for FWP. Ideally, risk-based surveillance programmes would pinpoint the necessary intervention strategies and keep the costs to a minimum, while not compromising the outcome of effective food safety control measures to reduce the disease burden.

4. Conclusion

Until researched, evidence- and risk-based surveillance programmes for most of FWP are set up, food as well as public health safety will essentially depend on diligent adherence to post-harvest prevention practices to control FWP. These practices involve pre-market processing of meat and meat products by using procedures that inactivate the parasites, as well as educating consumers in safe and hygienic ways of handling and preparing the meat at home.

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Original Scientific Paper

Detection of *Echinococcus* spp. in condemned livers

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ABSTRACT

Echinococcosis, a parasitic zoonosis caused by *Echinococcus* spp. tapeworms, poses a significant public health concern in Europe, but remains under-reported in both humans and animals. Post-mortem examination of slaughtered livestock offers a valuable opportunity for early detection and surveillance of parasitic infections, including echinococcosis, in meat intended for human consumption. In this study, liver samples exhibiting pathological changes were collected from two abattoirs in Serbia. A total of 31 livers—22 from pigs, 7 from lambs, and 2 from bullocks—were analysed microscopically, and molecularly using PCR, to detect *Echinococcus* spp. DNA. The parasite was detected in three pig livers, and three distinct species were identified: *E. granulosus, E. canadensi*s, and *E. multilocularis*. Although the sample size was relatively small, the detection of multiple *Echinococcus* species in pigs suggests a potential role of these animals in the parasite's transmission cycle within Serbia. These findings underscore the ongoing importance of comprehensive meat inspection protocols in abattoirs for zoonotic disease surveillance. They also highlight the need for expanded surveillance efforts and enhanced diagnostic procedures, particularly speciation of the tapeworm, to improve early detection and control of *Echinococcus* infections at the abattoir level.

1. Introduction

Livestock intended for human consumption must undergo slaughter at abattoirs, which consequently serve as valuable places for collecting data on animal health and condition. These data are primarily used to identify meat deemed unfit for human consumption, but they also offer important insights into disease prevalence and potential animal welfare concerns. One such disease, ranked among the top ten foodborne diseases in Europe and therefore of considerable public health significance, is echinococcosis, caused by tapeworms of the genus *Echinococcus* (*Bouwknegt et al*., 2018).

Echinococcus spp. are cestode parasites belonging to the family Taeniidae. Like all taeniid parasites, they possess complex life cycles involving a carnivorous definitive host (typically members of the families Canidae and Felidae) and a herbivorous or omnivorous intermediate host (commonly domestic livestock), in which the larval stage, known as the metacestode, develops. Humans are considered

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intermediate, dead-end hosts in the life cycle of *Echinococcus* spp. In the intestines of Canidae and Felidae, the tapeworm matures into its adult stage and undergoes sexual reproduction, releasing eggs that are subsequently shed in the faeces. Intermediate and dead-end hosts ingest the eggs via food or water, and the hatched larvae reach the various organs where they encyst into the larval stage known as the metacestode. Within these cysts, the parasite reproduces asexually, leading to progressive cyst enlargement, which is the primary cause of symptoms in cystic echinococcosis. The transmission cycle is perpetuated when a definitive host consumes infected tissue from an intermediate host, either through natural predation or the ingestion of raw offal containing viable metacestodes (*WHO Team for Control of Neglected Tropical Diseases*, 2001).

Although reporting of human and animal cases of echinococcosis is mandatory in most endemic countries, including Serbia, global under-reporting remains a major concern (*Casulli,* 2020; *WHO*, 2024). Based on a comprehensive review of human cases in Europe between 2017-2019, it has been estimated that approximately only 25% of cases are actually reported to The European Surveillance System (TESSy), while an earlier study in Germany estimated that between 2003-2005, about 67% of cases of alveolar echinococcosis were never reported (*Casulli et al*., 2023; *Jorgensen et al*., 2008). According to data from the Institute of Public Health of Serbia (IOPHOS) and the official Annual Epidemiological Reports from the European Centers for Disease Control (ECDC), between 2014 and 2019, the number of echinococcosis cases in Serbia exceeded those reported in neighbouring countries. The countries with the highest burden of echinococcosis in Europe are Bulgaria and Germany. In Serbia, 21 new cases were reported in 2023—nearly double the number reported in 2022—suggesting a potential upward trend in the number of reported cases. Since 2022, two confirmed cases of alveolar echinococcosis have been reported in the Vojvodina province, raising significant concern (*Lalošević et al*., 2023; *Milošević et al*., 2024). This may indicate the emergence of an *E. multilocularis* transmission focus in the region—the first documented hotspot in the Balkans. Whether any of the echinococcosis cases reported prior to 2021 were alveolar echinococcosis remains unknown, as the infecting species was not specified.

From an economic perspective, measured by disability-adjusted life years (DALYs), echinococcosis was estimated to account for 569 DALYs per 1000 capita globally in 2021, with just under three thousand reported cases from 30 countries across the Americas and Europe (WHO, Global Health Estimates; *WHO*, 2024). However, considering the significant under-reporting, the actual burden in terms of DALYs is likely substantially higher. The fatality rates of cystic and alveolar echinococcosis approach 100% if left untreated over time. Based on long-term clinical data from several European countries, the cure rate for cystic echinococcosis is estimated at approximately 75%, with a postoperative fatality rate of around 2%. In contrast, the cure rate for alveolar echinococcosis averages 63%, with postoperative survival rates ranging from 50% to 97% (*Budke et al*., 2006; *Salm et al*., 2019). These cure rates are also influenced by relapse rates, which often result from incomplete removal of the metacestodes. Echinococcosis in animals is almost exclusively diagnosed following slaughter. Metacestodes can be found in various organs, with the liver being the most commonly affected. Unlike cystic echinococcosis, morphological changes caused by *E. multilocularis* are more challenging to attribute to parasitic infection due to its infiltrative growth pattern, which results in lesions and extensive tissue damage. Additionally, *E. multilocularis* tends to metastasise to other organs, making alveolar echinococcosis difficult to detect through visual inspection at the slaughter line.

The aim of this study was to detect *Echinococcus* spp. DNA in condemned livers at slaughterhouses/abattoirs and identify the species of the tapeworm in order to gain insight into the occurrence of infection in livestock.

2. Materials and methods

For the purposes of this study, a total of 31 liver samples collected from two major abattoirs were analysed. The analysed samples were 22 pig livers, 7 lamb livers, and 2 bullock livers. All liver samples were pre-selected and removed from the slaughter line due to discernible pathological morphological changes (clearly identifiable cysts, cyst-like formations, discoloration, and scar tissue). To determine the presence of *Echinococcus* spp., cyst contents (liquid or solid) and/or affected tissue areas were sampled by direct isolation using pipette tips and/or tissue excision, then examined microscopically. DNA was rapidly extracted by boiling the samples in 0.02 M NaOH for 15 minutes, followed by amplification of the *cox1* gene using primers capable of

differentiating *E. granulosus*, *E. multilocularis*, and *E. canadensis* via conventional PCR.

3. Results

A detailed overview of the results by groups of analysed animals is presented in Table 1 for pig, Table 2 for lamb, and Table 3 for bovine liver samples. The analyses revealed that *Echinococcus* spp. DNA was present in a total of three livers, all of which originated from pigs (Table 1).

The first positive finding (Pig 1) came from a liver sample that contained several large, fluid-filled cysts. Tapeworm larvae were microscopically confirmed within the cyst content and were subsequently identified by PCR as *E. granulosus*. The second positive finding (Pig 12) was derived from liver tissue, although small fluid-filled cysts were also present. PCR analysis confirmed the presence of *E. canadensis* in the tissue. The third positive finding (Pig 31) was from a liver sample that contained small, fluid-filled cysts, and analysis of the cyst contents identified *E. multilocularis*.

All positive findings originated from livers with fluid-filled cysts; however, not all cyst-like morphological changes in the livers were caused by the presence of *Echinococcus* spp.

Table 1. Analysis of pig livers

Species - sample number(s)	Liver morphology/damage	Presence of cyst fluid	Sample examined	Result
Pig - 1	Large, fluid filled cysts (3)	Yes	Cyst fluid	*E. granulosus*
Pig - 2,5	Flat, button-shaped scar or abscess	No	Tissue sections (2)	Negative
Pig - 3,9	Small cyst or abscess, hard	No fluid but grainy, thick white content	Tissue section and content	Negative
Pig - 4,12	Small, fluid filled cyst	Yes	Cyst fluid and tissue section	*E. canadensis*
Pig - 8	Flat, button-shaped scar or abscess	No	Tissue section	Negative
Pig - 10	Flat, button shaped scar or abscess	No	Tissue section	Negative
Pig - 13	Flat, scar	No	Tissue section	Negative
Pig - 14	Flat, scar	No	Tissue section	Negative
Pig - 15; 20	Flat, button shaped scar or abscess	No	Tissue sections (2)	Negative
Pig - 16	Flat, scar	No	Tissue section	Negative
Pig - 17	Flat, scar	No	Tissue section	Negative
Pig - 18; 19	Flat, button shaped scar or abscess	No	Tissue sections (2)	Negative
Pig - 21	Discoloration, no scarring, no cyst or abscess	No	Tissue section	Negative
Pig - 27	Flat, scar	No	Tissue section	Negative
Pig - 28	Flat, scar	No	Tissue section	Negative
Pig - 29	Flat, scar	No	Tissue section	Negative
Pig - 30	Flat, scar	No	Tissue section	Negative
Pig - 31	Small, fluid filled cyst	Yes	Cyst fluid	*E. multilocularis*
Pig - 32	Small, fluid filled cyst	Yes	Cyst fluid	Negative
Pig - 33	Flat, scar	No	Tissue section	Negative
Pig - 34	Flat, scar	No	Tissue section	Negative
Pig - 35	Flat, scar	No	Tissue section	Negative

Table 2. Analysis of lamb livers

Species - sample number(s)	Liver morphology/damage	Presence of cyst fluid	Sample examined	Result
Lamb - 22	Flat, scar	No	Tissue section	Negative
Lamb - 23	Flat, scar	No	Tissue section	Negative
Lamb - 24	Flat, scar	No	Tissue section	Negative
Lamb - 25	Flat, scar	No	Tissue section	Negative
Lamb - 26	Flat, scar	No	Tissue section	Negative
Lamb - 36	Small, fluid filled cyst	Yes	Cyst fluid	Negative
Lamb - 37	Small, fluid filled cyst	Yes	Cyst fluid	Negative

Table 3. Analysis of bovine livers

Species - sample number(s)	Liver morphology/damage	Presence of cyst fluid	Sample examined	Result
Bullock - 7	Flat, scar	No	Tissue section	Negative
Bullock - 6; 11	Small cyst or abscess, hard	No fluid but thick yellow content	Tissue section and content	Negative

4. Discussion

The abattoir serves as a critical control point for the surveillance and management of zoonotic agents responsible for foodborne infections and intoxications. In addition to pathogen detection, meat inspection provides valuable information on pathological lesions associated with animal health and production diseases. The relevant pathological findings identified in the analysed slaughter by-products underscore the importance of animal health control at the slaughterhouse level and demonstrate its role as a key tool for the surveillance and management of animal diseases at the primary production stage. For example, in the United States, approximately 10–20% of feedlot cattle have presented with liver abscesses at slaughter (*Amachawadi and Nagaraja*, 2016). Similarly, a large-scale study in the Czech Republic reported liver damage in 34% of cows, 17.51% of sows, and 12.97% of ewes (*Valkova et al*., 2023). While liver abscesses were primarily of bacterial or, more rarely, viral origin, parasitic lesions were comparatively rare, accounting for only 1.31% of liver damage in heifers, 7.51% in ewes, and 3.68% in finishing pigs (*Valkova et al*., 2023). These findings along with previous reports clearly show that infections caused by environmentally transmitted parasites are rather uncommon in intensively farmed animals as opposed to extensively farmed and free-range animals (*Betić et al*., 2022; *Kijlstra et al*., 2004). As slaughterhouses and abattoirs in Serbia mostly process animals from intensive farming systems, echinococcosis is expected to be a minor finding during meat inspection. However, as shown here and by *Valkova et al*. (2023), echinococcosis diagnostics for pigs, particularly finishing pigs, may need to be prioritised, as 3/22 were found to be positive. Overall, the results of our study show that the majority of liver changes observed at slaughter are scars, small abscesses and discolorations. Hard cysts with grainy, thick, white contents are likely associated with earlier bacterial infections, while button-shaped scars are, according to literature, most commonly linked to prior *Ascaris* spp. infections. Regarding the detection of *Echinococcus* based solely on liver morphology and visible lesions, a definitive diagnosis cannot be made with absolute certainty or reliability. Smaller fluid-filled cysts may be associated with certain *Echinococcus* species, such as *E. granulosus* and *E. canadensis*, which only rarely infect humans. Unlike *E. granulosus*, which typically forms a single, large, fluid-filled cyst, *E. multilocularis* produces numerous small, infiltrative cysts (locules) that spread throughout the host's organs, mimicking the growth pattern of a malignant tumour (*Macpherson et al*., 2003). Consequently, the cavitations and tissue damage caused by *E. multilocularis* are often difficult to detect visually, particularly in younger animals, which are commonly processed at slaughterhouses/abattoirs, as cyst growth and damage may not have developed sufficiently.

The findings of this study underscore the continued relevance of post-mortem meat inspection at the slaughterhouse level, employing visual assessment, palpation, and incision techniques. This approach remains a valuable tool for the detection of notifiable diseases and zoonoses, identification of production-related conditions, and provision of epidemiological feedback to veterinary practitioners regarding subclinical disease occurrences at the farm level. In particular, palpatory inspection plays a crucial role in identifying internal abnormalities—such as cysts—that may not be visible on the surface of the liver, thereby enhancing diagnostic sensitivity. While conventional meat inspection methods are adequate and successful in detecting *Trichinella* spp., it provides limited information on the presence of other major foodborne parasites, which require laboratory-based molecular diagnostic methods for detection and identification.

5. Conclusion

Although the number of samples examined was limited and *Echinococcus* spp. was not the predominant cause of (morphological) liver changes, the detection of three different tapeworm species remains a cause for concern. While the study shows that most carcasses flagged for conditional use based on liver examination are not infected with *Echinococcus*, the results suggest that pigs can still play a role in the transmission of the parasite in Serbia. These results emphasise the need for more comprehensive studies to obtain reliable data on the prevalence and diversity of *Echinococcus* spp. in pigs. Such studies would provide valuable insights that can support the harmonisation of post-mortem inspection protocols and assist risk managers to develop and implement modernised control strategies in slaughterhouses.

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Original Scientific Paper

Trifur tortuosus in whole Argentine hake

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ABSTRACT

This case report presents the basic parameters used in the identification of the species *Trifur tortuosus*, a parasitic copepod of the genus *Trifur*, family Pennellidae, and relevant literature data on this little-known parasite of marine fish. The parasite was identified in whole Argentine hake, with the fish delivered to our laboratory during the official import control of foodstuffs in 2024. *Trifur* is a relatively large ectoparasite and as such is easy to see, but this case report is the first time *Trifur* were detected in marine fish that were examined in our laboratory. Several species of marine fish in the Atlantic-Pacific region of South America are natural hosts of this parasite. As the import of fish and fish products is increasingly intensive, and *Trifur* spp. could become more frequent in many commercially important fish species, we believe that the presentation of the case can be useful in the practical work of laboratories that conduct examinations of animal origin foods in our region.

1. Introduction

The Food Safety Law in Serbia requires all food business operators, including importers, to ensure that the food they place on the market is safe for human consumption. Local regulations on animal health conditions, on the general and special conditions of hygiene of food of animal origin and on the hygiene of animal slaughter and trade (*Official Gazette RS*, 2011; *Official Gazette RS*, 2014 *Official Gazette RS*, 2023) concern fish and fish products. For these products, one of the requirements is the absence of visible parasites. Epidemiologically, the most important fish parasites are helminths from the group of Nematoda, including *Anisakis* spp. and *Eustrongylides excisus* (*Ćirković et al*., 2013). In our laboratory, the most commonly detected parasite in imported fish is the nematode *Anisakis*.

Over many years of conducting parasitological examinations of fish, parasites of the genus *Trifur*, or those with similar morphology, have not been detected in marine fish samples submitted to our laboratory. The damage caused by *Trifur* spp. is primarily aesthetic in nature, as the parasites leave unsightly scars deep within the flesh of affected fish. *Trifur tortuosus* parasitizes marine fish in the Atlantic-Pacific region of South America *(Oliva & González*, 2004; *Timi et al*., 2008; *Etchegoin et al*., 2009). The presence of this species in a considerable number of phylogenetically unrelated fish species in the same geographic area suggests that *Trifur tortuosus* is a nonspecific parasite (*Etchegoin et al*., 2009).

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2. Materials and methods

Whole Argentine hake (*Merluccius hubbsi*), each weighing between 200 and 350 g, were delivered to the Food Safety Laboratory of the Novi Sad Scientific Veterinary Institute as part of the official inspection of imported fish during summer of 2024. According to the current Food Safety Law, as part of the control of animal origin food during import for safety, both sensory and parasitological testing must be performed on fish samples to ensure safety.

The fish were first identified, which included documenting the quantity, shape of the shipment, and label information. Following identification, parasitological examination was conducted through an external inspection, followed by an internal examination involving dissection of the body, including the abdominal region, digestive tract and other ventral organs.

The collected parasites were fixed in 70% ethanol and dissected using lactic acid for morphological observation.

3. Results

Ectoparasites were observed in the skin of dorsal part of Argentine hake (Figure 1A).

After being fixed in 70% ethanol and dissected with lactic acid, the following body segments were observed in the parasite: a holdfast-bearing head with three horns, a long, thick and cylindrical neck and a sigmoid trunk which includes the abdomen and genital complex. The ventral view is shown in the Figure 1B.

The measured dimensions of the parasite's body segments are presented in Table 1. Using the identification keys by *Wilson* (1917), *Yamaguti* (1963) and *Etchegoin et al.* (2009), the parasite was identified as the species *Trifur tortuosus*.

1A 1B

Figure 1. *Trifur tortuosus* isolated from Argentine hake (*Merluccius hubbsi*); A – Copepod in the skin of the dorsal part of Argentine hake; B – Ventral view of *Trifur tortuosus* showing body shape and segments

Table 1. Body measurements of *Trifur tortuosus* collected from Argentinian hake.

Part of the parasite body	Length (mm)
Total length	35.4
Proboscis length	3.7
Neck length	13
Genital complex length	10.4
Abdomen length	8.3
Dorsal horn length	3.6
Right horn length	2.8
Left horn length	2.7

4. Discussion

Parasitic copepods in the family *Lernaeidae* are known for their high morphological diversity and wide distribution among both marine and freshwater fish species. The classification within this group has evolved considerably over time as knowledge about their biology improved. In 1917, in the collection of the United States National Museum, Charles B. Wilson performed the thirteenth revision of the *Lernaeidae* family, pointing out the wrongly interpreted genera of that oldest family of parasitic copepods, due to incomplete understanding of their life cycles (*Wilson*, 1917). He noted that some of these copepods show highly unusual and distinctive forms. That author also recommended a method for examining their internal anatomy by thoroughly dehydrating specimens in alcohol and clearing them in clove oil, which provides clear visualization without the need for sectioning. Based on a careful study of external and internal morphological characteristics and their life history, Wilson proposed reorganizing the family Lernaeidae into 17 genera—introducing three new ones, including *Trifur* and described 80 species, of which fifteen were new to science. Among these new species was *Trifur tortuosus*, originally described from two females (one missing the head), found on the body of Patagonian cod (*Salilota australis*) from near South America's southern tip (Wilson, 1917). More recently, *Etchegoin et al*. (2009) revisited the species description and confirmed its taxonomic status based on modern morphological methods.

Regarding classification, different data can be found in the literature. World taxonomic databases, the World Register of Marine Species (WoRMS), the Global Biodiversity Information Facility (GBIF), the Interim Register of Marine and Nonmarine Genera (IRMNG), and the Ocean Biodiversity Information System (OBIS), currently place *Trifur tortuosus* in the family *Pennellidae*. Classification according to WoRMS) is presented in the Table 2.

The family *Pennellidae* contains copepod parasites commonly found in a wide range of marine fish species. Members of this family are typically distinguished based on the shape and proportions of the cephalothorax, neck, trunk and abdomen (*Castro-romero et al.*, 2016). However, due to their pronounced morphological plasticity and considerable intra-species variability, identifying species solely on the basis of morphology remains highly challenging and often controversial.

The genus *Trifur* Wilson, 1917 includes relatively large and conspicuous parasitic copepods that infect the body surface and gills of several marine fishes. Several species have been described so far, such as *T. tortuosus* (1878, 1917), *T. merluccii* (1936), *T. puntaniger* (1963), *T. chlorophthalmi* Yamaguti (1939), *T. physiculi* Heegaard (1962) and *T. lotellae* (1890). After several changes in systematization, the genus now includes five species: T. tortuosus, T. merluccii, T. puntaniger, T. physiculi and T. lotellae (*Etchegoin et al*., 2009).

T. tortuosus has been reported the body surface of several marine fish species, including *Salilota australis*, *Merluccius gayi*, *M. hubbsi*, *M. australis*, *Percophis brasiliensis*, *Pinguipes brasilianus* and several host species inhabiting the Chilean coasts. The main characteristics of *T. tortuosus* according to *Wilson* (1917) are: chitinized cephalothorax with small

Table 2. Classification of *Trifur tortuosus* according to the World Register of Marine Species (*WoRMS*, 2008)

Kingdom	Animalia
Phylum	Arthropoda
Subphylum	Crustacea
Superclass	Multicrustacea
Class	Copepoda
Infraclass	Neocopepoda
Superorder	Podoplea
Order	Siphonostomatoida
Family	*Pennellidae*
Genus	*Trifur*
Species	*Trifur tortuosus*

lateral horns and a long, slightly curved posterior horn; curved neck, twice as long as the trunk, abdomen bent into a semicircle, color a brownish yellow, more of a brown on the head and neck, more of a yellow on the trunk; egg strings cinnamon-brown. The original description of *T. tortuosus* made by *Wilson* (1917) was based on only two ovigerous females—one of females lacking the head. For this reason, cephalic appendages and legs were not described in detail. By comparing the original description with the description given by *Etchegoin et al*. (2009), it was determined that the second antenna is three-segmented (not two-segmented) and leg 3 is uniramous (not biramous). Based on specimens collected from three fish species from the Argentine coast, the description of the species *Trifur tortuosus* was refined by *Etchegoin et al*. (2009). The refined description is presented in Table 2.

Variations in the shape and size of the horns and trunk in *T. tortuosus* are likely influenced by differences in host species and the specific microhabitats they inhabit, such as the fish body surface, fins or branchial cavities. The resistance provided by host tissue (bone or connective tissue) influences the variability of the parasite's shape, by truncating or deviating the process of its development (*Etchegoin et al*., 2009). In most cases, only one parasite colonizes a single host. The resulting tissue damage is usually visible as deep scars on the skin, and in cases where the parasite dies *in situ*, abscesses measuring 2 cm or more long can develop (*Garcia-Sepulveda*, 2017).

In Germany, the sale of fish fillet batches with >5% parasite infestation is prohibited, and these fish are used for the production of animal feed and pet food (*Garcia- Sepulveda*, 2017). The fact that *T. tortuosus* has been found in a wide range of phylogenetically unrelated fish species suggests that it is not host-specific. Moreover, species from the genus *Trifur* have been recorded in geographically distant regions, including Australia and New Zealand (*Etchegoin et al*., 2009).

Due to its pronounced morphological variability, traditional identification methods based on morphology alone are insufficient. Therefore, modern approaches such as molecular techniques, including DNA sequencing (*Munoz et al.*, 2015) and DNA barcoding are increasingly used to achieve accurate species identification (*Castro-Romero et al*., 2016).

Tabel 2. Refined description of *Trifur tortuosus* (Etchegoin et al., 2009) removed from *Merluccius hubbsi* in the Argentine Sea

Body	Divided into holdfast-bearing head, long and cylindrical neck and sigmoid trunk (genital complex + abdomen)
Total length (from tip to head to tip of trunk)	37.5 (31.6 - 46.3) mm
Head	Nearly at right angle to neck, with a cylindrical anterior proboscis
Head length	1.71 (1.34 - 1.95) mm
Head width	1.4 (1.22 - 1.83) mm
Proboscis length	3.9 (3.7 - 4.5) mm
Horns	Three cylindrical to conical posterior horns
Dorsal horn length	3.7 (3.1 - 4.2) mm
Right horn length	2.9 (2.4 - 3.9) mm
Left horn length	3.0 (2.5 - 3.5) mm
Neck	Moderately thick, longer than trunk, and smooth.
Neck length	12.0 (7.5 - 15.9) mm
Neck width	0.97 (0.69 - 1.3) mm
Trunk	Swollen, bent into a sigmoid curve at right angles to neck
Genital complex length	13.8 (11.6 - 17.4) mm
Abdomen length	7.7 (6.4 - 9.3) mm

5. Conclusion

Considering the increased volume of imported fish that is a potential host of this type of parasite, this case report is significant as a useful source of information for the identification of the *Trifur* parasites. The presence of such ectoparasites causes consumer disgust and affects sensory properties, although the parasites themselves do not pose a risk to the health of consumers. This case report summarizes important data for *Trifur* genus identification and the practical work of food safety laboratories in the region.

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Original Scientific Paper

Production techniques and microbial stability of traditional pasta filata cheese throughout its shelf life

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ABSTRACT

Traditional rolled *pasta filata* cheese is a distinctive product valued for its elastic texture, mild flavour and handcrafted appearance. This research aims to show the traditional production technique of rolled *pasta filata* cheese and assess the evolution of its microbial stability throughout shelf life. The cheese is typically produced by stretching the curd in hot water, followed by manual rolling and shaping. During storage, significant changes occur due to moisture migration, proteolysis, lipolysis, and microbial activity. This paper summarizes findings on microbial changes during cold storage under vacuum packaging. Understanding the behaviour of traditional rolled *pasta filata* cheese during its shelf life is essential for maintaining product quality, safety and authenticity, especially as consumer demand for artisanal and minimally processed dairy products grows. The future research can contribute to the preservation and valorisation of traditional cheesemaking heritage, while meeting modern consumer demands for natural, authentic and high-quality dairy products.

1. Introduction

Rolled cheeses are traditionally produced in Vojvodina, the northernmost part of Serbia, and represent *pasta filata* cheeses, which are mainly made from raw cow's milk, raw sheep's milk, or a combination of those two. The production process of rolled cheeses is specific and requires knowledge and skills that are passed down through generations (*Popović-Vranješ et al*., 2017; *Vidaković Knežević et al*., 2024).

The production of rolled cheeses typically begins with the spontaneous fermentation of raw milk. Once the milk reaches a certain level of acidity, a small amount of coagulating enzyme is added. The formed curd is then cut, processed, and the whey is separated. After that, the curd is thermally treated in warm whey or brine, resulting in a stretchable cheese mass that is hand-stretched, salted, and shaped into rolls (*Popović-Vranješ et al.*, 2017). In addition to salt, creative producers in Vojvodina often use various spices and additives, such as paprika, pepper, oregano, basil, chives, sesame, coconut, nuts and chocolate, to enhance the flavour of rolled cheese.

Rolled cheeses are characterized by the regular shape of the rolls. The texture is smooth and elastic, while the consistency is usually semi-hard. The cross section is layered. The colour of rolled cheese is white with yellowish shades. Rolled cheese has a milky, not too sour or salty taste, and a slight milky-sour smell (*Vidaković Knežević et al*., 2024).

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These types of cheeses are complex microbial ecosystems. The sources of microorganisms in rolled cheeses are diverse. Primarily, they include the microbiota of raw milk and of the surfaces and hands that encounter the cheese.

The natural microbiota of raw milk is heterogeneous in composition, but is mainly rich in lactic acid bacteria, such as *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., and *Enterococcus* spp., which enable the spontaneous fermentation of milk and contribute to the flavour development of the final product (*Quigley et al*., 2013). In addition to these bacteria, raw milk also contains various representatives of both Gram-positive (*Bacillus, Clostridium, Staphylococcus, Micrococcus, Microbacterium, Corynebacterium*) and Gram-negative (*Pseudomonas, Flavobacterium, Acinetobacter, Aeromonas, Enterobacteriaceae*) bacteria (*Tilocca et al*., 2020). Additionally, many yeasts and moulds can contribute to the flavour, aroma and texture of some types of cheese. However, in the case of rolled cheeses, they can be the cause of spoilage and the development of undesirable sensory characteristics (*Kure and Skaar*, 2019).

Coagulase-positive staphylococci, *Enterobacteriaceae* and *Escherichia coli* are highlighted as process hygiene criteria in the raw milk cheese production process, while *Listeria monocytogenes* and *Salmonella* spp. are considered food safety criteria (*Official Gazette of the Republic of Serbia*, 2024). Since rolled cheese is made from raw milk and belongs to the category of fresh *pasta filata* cheeses (*Official Gazette of the Republic of Serbia*, 2014), it has a short shelf life.

The objectives of this research were to present the production technology of traditional rolled cheese and to determine its microbiological quality during cold storage under vacuum packaging.

2. Materials and methods

2.1. Cheese production

Raw cow's milk was left to spontaneously ferment using its natural microbial population at 20 °C for 24 hours. The resulting curd was cut and then separated from the whey by the action of its own weight through a cotton cloth. After the curd draining process was completed, the curd was steamed in water at 70 °C to 80 °C, with constant mechanical stretching using a wooden spoon. Once the cheese dough reached a plastic consistency, further kneading, shaping, and stretching were performed by hand on the table. The warm dough was stretched to 5 mm thickness and by folding the stretched cheese dough inwards, the required width was achieved. At this stage, the cheese dough was hand salted. To form rolls of approximately equal size, cheese dough was then rolled inwards from both sides. The cheese rolls were then wrapped in cotton cloth and stored at 4 °C for 24 hours to allow the excess whey to drain. Then, the rolled cheeses were vacuum-packed and stored at 4 °C.

2.2. Microbiological analysis

Following the reference methods, total viable count (TVC) were counted on plate count agar (PCA, CM0325, Oxoid, United Kingdom) according to the SRPS EN ISO 4833-1 (*ISO*, 2014); *Enterobacteriaceae* were counted on violet red bile glucose (VRBGA, CM1082, Oxoid, United Kingdom) according to the SRPS EN ISO 21528-2 (ISO, 2017a); *Escherichia coli* were counted on tryptone bile X-glucuronide medium (TBX, CM0945, Oxoid, United Kingdom) according to the SRPS ISO 16649-2 (*ISO*, 2008); coagulase-positive staphylococci were counted on Baird-Parker agar (Biokar Diagnostics, France) according to the SRPS EN ISO 6888-1 (*ISO*, 2021); *Salmonella* spp. were detected according to SRPS EN ISO 6579-1 (*ISO*, 2020); and *Listeria monocytogenes* were detected according to SRPS EN ISO 11290-1 (*ISO*, 2017b).

2.3. Statistical analysis

Data were analysed using R statistical software (version 3.2.2; R Foundation for Statistical Computing, Vienna, Austria). One-way analysis of variance (ANOVA), followed by Duncan`s multiple range test, was performed to evaluate differences in microbial populations, with a significant level set at $p < 0.05$.

3. Results and discussion

The microbial status of the rolled cheese is presented in Table 1. During storage of rolled cheese at 4°C over 60 days, there was a significant ($p < 0.05$) decrease in the number of aerobic mesophilic bacteria by 0.28 $\log_{10}$ CFU/g, *Enterobacteriaceae* by 1.34 $\log_{10}$CFU/g, and *Escherichia coli* by 1.97 $\log_{10}$ CFU/g. The only increase was observed in the number of coagulase-positive staphylococci, which rose by 0.7 $\log_{10}$ CFU/g.

Table 1. Microbial status of traditionally produced rolled *pasta filata* cheese vacuum-packed and stored at 4 °C over 60 days

Microorganisms	Day 0	Day 20	Day 40	Day 60
Aerobic mesophilic bacteria (log_{10} CFU/g)	8.03 ± 0.11^{a}	7.89 ± 0.11^{ab}	7.35 ± 0.19^{c}	7.75 ± 0.21^{b}
Enterobacteriaceae (log_{10} CFU/g)	2.87 ± 0.16^{a}	3.06 ± 0.57^{a}	1.81 ± 0.16^{b}	1.53 ± 0.61^{b}
Escherichia coli (log_{10} CFU/g)	3.12 ± 0.47^{a}	2.42 ± 0.11^{b}	1.32 ± 0.23^{c}	1.15 ± 0.17^{c}
Coagulase-positive staphylococci (log_{10} CFU/g)	1.40 ± 0.61^{b}	2.28 ± 0.18^{ab}	2.83 ± 0.43^{a}	2.10 ± 0.83^{ab}
Salmonella spp. (in 25 g)	Not detected	Not detected	Not detected	Not detected
Listeria monocytogenes (in 25 g)	Not detected	Not detected	Not detected	Not detected

Legend: Values within the same row marked with different superscript letters ($^{a, b, c}$) indicate statistically significant differences ($p < 0.05$).

Compared with the current microbial counts, our previous research showed lower counts of *Enterobacteriaceae*, *Escherichia coli* and coagulase-positive staphylococci in rolled cheeses made with cow's and donkey's milk (*Vidaković Knežević et al*., 2025). This could be attributed to the slightly different production method of the rolled cheeses, as previously, the milk was heated to 40 °C before the addition of rennet.

The aerobic mesophilic bacteria can be used as part of a general assessment of food quality during its shelf life. Aerobic mesophilic bacteria levels below 6 log_{10} CFU/g indicate a diverse microbiota, while numbers above this level indicate the dominance of a particular microorganism which, depending on the type of food, could affect the organoleptic properties of the food. Although aerobic mesophilic bacteria levels above 7-8 log_{10} CFU/g are often considered as indicators of unsatisfactory microbiological quality, this level cannot be generalized. In the case of fermented dairy and cheese products, aerobic mesophilic bacteria are not suitable as quality indicators. For products containing high levels of these bacteria, additional testing should be carried out to determine the number of yeasts, Gram-negative bacteria and lactic acid bacteria (if they are not intentionally added during the manufacturing process). In the case of rolled cheese, the product is not satisfactory if it contains >6 log_{10} CFU/g of yeasts, >7log_{10} CFU/g Gram-negative bacteria and >8 log_{10} CFU/g lactic acid bacteria (*Health Protection Agency*, 2009).

Escherichia coli is a Gram-negative, rod-shaped bacterium that belongs to the coliform bacteria, a group of bacteria in the family *Enterobacteriaceae* (*International Dairy Federation*, 2016). Ruminants excrete these bacteria through their faeces, which via milking teats can then contaminate the udder and, thus, the milk. A previous study showed a high contamination rate of *Escherichia coli* in traditionally-produced cheeses in the Banat region of Romania (*Imre et al*., 2022). In fermented dairy products, however, relatively low numbers of *Enterobacteriaceae* and *Escherichia coli* can be due to the antagonistic activity of lactic acid bacteria. It is believed that lactic acid bacteria prevent the multiplication of bacteria from the *Enterobacteriaceae* family, which includes *Escherichia coli*, through several control mechanisms. Firstly, these two groups of bacteria (lactic acid bacteria and *Enterobacteriaceae*) compete for nutrients. Additionally, during the fermentation process, lactic acid bacteria produce acids that are considered potential inhibitors of *Enterobacteriaceae* (*Mladenović et al*., 2021).

Subclinical staphylococcal mastitis is a common source of the coagulase-positive staphylococci (*Kousta et al.*, 2010). Some strains of coagulase-positive staphylococci can produce enterotoxins, which are heat-stable proteins. The ingestion of enterotoxins through food leads to foodborne intoxication, manifested by vomiting and diarrhoea (*Katić*, 2008). The prevalence of coagulase-positive staphylococci varies among different types of cheese, and up to 80% of raw milk cheeses contain these bacteria (*Johler et al*., 2018). The slight increase in coagulase-positive staphylococci in the rolled cheeses during storage in the current study was likely the result of an ideal environment, namely a high content of proteins and fats, a relatively acidic medium and low salt content (*Al-Nabulsi et al*., 2020).

In this study, neither *Salmonella* or *Listeria monocytogenes* were detected in the rolled cheeses. Previously, *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Dublin and *Salmonella* Montevideo were detected in cheeses made with raw milk (*Yoon et al*., 2016). Also, *Listeria monocytogenes* was detected in up to 37.5% of fresh cheeses, in up to 55% of soft and semi-hard cheeses, and in up to 5.5% of hard and extra-hard cheeses (*Gérard et al*., 2018).

4. Conclusion

Traditional rolled *pasta filata* cheese is distinctive artisanal product, appreciated for its texture, mild flavour, and handcrafted appearance. This study presented the traditional production method and examined microbial changes during refrigerated storage under vacuum packaging. The results showed a general decline in the populations of aerobic mesophilic bacteria, *Enterobacteriaceae* and *Escherichia coli* during storage, while a slight increase was observed in the number of coagulase-positive staphylococci.

Understanding these microbial dynamics is essential for ensuring the safety, quality and authenticity of traditional dairy products. Preserving and promoting traditional cheesemaking practices, alongside meeting modern consumer preferences for natural and minimally processed foods, remains an important direction for future research and development.

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Original Scientific Paper

Health risk assessment of metal intake from fish species caught from the Danube River in Serbia

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ABSTRACT

The aim of the present study was to assess the potential health risks associated with the consumption of metals accumulated in fish meat of common carp, Wels catfish, and silver carp caught from 2010 to 2021 from the Danube River in Serbia. Therefore, estimation of daily intake rate (EDI), percentage of provisional tolerable weekly intake (% PTWI), target hazard quotient (THQ), hazard index (HI), and target cancer risk (TR) were calculated. Results of health risk assessments showed that the highest values of EDI were found for Hg in common carp, for As, Hg, and Pb in silver carp, while in Wels catfish the highest EDI was detected for Pb and Hg. In all three fish species, the highest % PTWI values were determined for Hg, while % PTWI for As, Cd, and Pb were lower than 5%. HIs higher than 1 were detected in common carp and Wels catfish during 2013 and in Wels catfish during 2010, indicating a potential for adverse non-carcinogenic health effects. In silver carp, HIs were lower than 1. The TR for As from 2010 to 2021 was regarded as acceptable, except in common carp during 2013, when it was defined as an unacceptable carcinogenic risk (higher than 10^{-4}). The TR for Cr ranged from 10^{-6} to 10^{-4} in all three fish species, so was regarded as acceptable, while the TR for Pb was indicated as negligible (lower than 10^{-6}).

1. Introduction

In previous decades, emerging attention has been paid to environmental pollution with metals that have detrimental effects both to fish and human health (*Cordeli et al*., 2023). The increase of metal level in aquatic environments has been due to different anthropogenic activities, like mining, accidental chemical waste discharge, agriculture drainage, industrial and sewage discharge (*Milošković and Simić*, 2023). Metals are non-biodegradable, persist in the environment for long periods, and accumulate in different tissues of the body, impairing health (*Cordeli et al*., 2023). Heavy metals like arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg) do not have any physiological role and are very toxic at low levels (*Vardhan et al*., 2019). Fish accumulate metals by absorption through gills, guts, and skin, and the level of tissue deposition depends on the content in water and sediment, fish species, time of

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exposure, and type of feeding behaviour (*Milošković and Simić*, 2023). Since fish are very sensitive to aquatic pollution (*Colin et al*., 2016), they are appropriate as bioindicators of environmental metal pollution (*Milošković and Simić*, 2023). Moreover, consuming fish contaminated with metals poses a risk to human health and can have detrimental effects on the nervous system, gastrointestinal tract, kidneys, and also can lead to anaemia, hypertension, and increased risk of cancer (*Cordeli et al*., 2023). Recently, various methods have been developed to assess health risks in order to assess the adverse effects of consuming fish contaminated with metals on human health (*USEPA*, 2000; *JECFA*, 2011; *Li et al*., 2013; *Jovic and Stankovic*, 2014; *Ahmed et al*., 2015; *Griboff et al*., 2017; *Malavolti et al*., 2020).

In Serbia, only 16% of wastewater is treated (*NALED*, 2019) and the Danube River, as the second longest river in Europe, is exposed to numerous pollutants (*Cordeli et al*., 2023). Furthermore, reviews by *Cordeli et al*. (2023) and *Milošković and Simić* (2023) presented detected levels of metals in the meat of different fish species caught from the Danube River in the past 15 years. Since in the performed studies, the health risks associated with fresh-water fish consumption were not established, the aim of the present study was to determine the potential risk associated with the consumption of metals accumulated in fish meat of common carp, Wels catfish, and silver carp caught from 2010 to 2021 from the Danube River in Serbia.

2. Materials and methods

2.1. Material

Data regarding concentrations of arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), lead (Pb), and zinc (Zn) in fish muscles published in the past 15 years were included for health risk assessment. The studies performed on three fish species (common carp, Wels catfish, and silver carp) caught from the Danube River were considered. For determination of health risks after consumption of common carp, the data used were presented by *Lenhardt et al*. (2012), *Subotić et al*. (2013a), *Subotić et al*. (2013b), *Ivanović et al*. (2016), *Milanov et al.* (2016), *Jovanović et al.* (2017), and *Aleksić et al.* (2025). For Wels catfish, studies used were performed by *Lenhardt et al.* (2012), *Ivanović et al.* (2016), *Milanov et al.* (2016), *Jovičić et al.* (2016), *Milošković et al.* (2016), *Jovanović et al.* (2017), and Aleksić et al. (2025). Data regarding silver carp were from studies by *Lenhardt et al.* (2012), *Ivanović et al.* (2016), *Milanov et al.* (2016), and *Aleksić et al.* (2025).

2.2. Methods

Assessment of health risks (estimation of daily intake rate – EDI, percentage of provisional tolerable weekly intake – % PTWI, target hazard quotient – THQ, hazard index – HI, and target cancer risk – TR) was performed according to the equations presented by Aleksić et al. (2025). Before calculation, concentrations of metals originally presented in dry weight were transformed to wet weight (wet weight refers to the weight of the sample including its water content) according to *Subotić et al.* (2021).

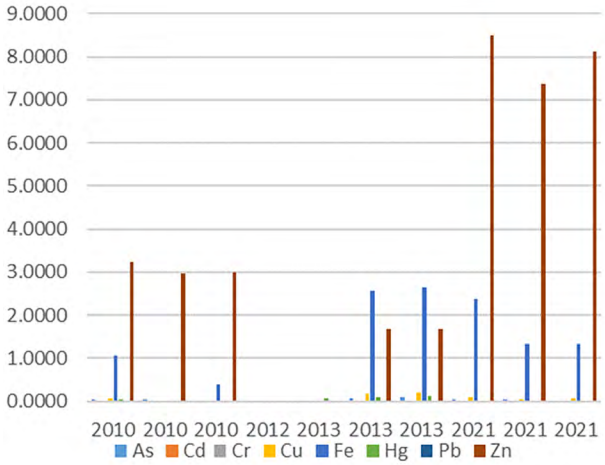
3. Results and discussion

3.1. Estimation of daily intake rate (EDI) and percentage of provisional tolerable weekly intake (% PTWI)

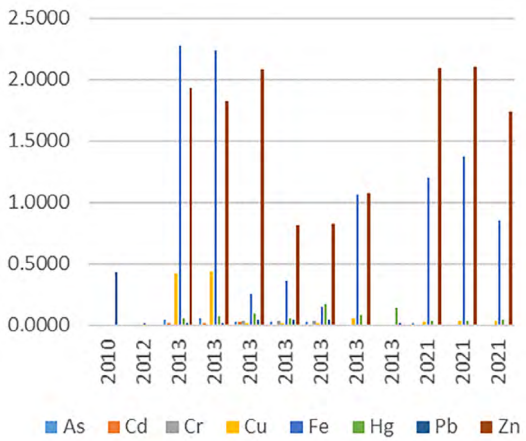
Figures 1 and 2 illustrate the estimated daily intake (EDI) (μg/kg of body weight (BW) per day) and percentage of provisional tolerable weekly intake (% PTWI) for common carp, Wels catfish, and silver carp caught from the Danube River in Serbia in the past 15 years. EDI levels were the highest for Fe and Zn in common carp (2.65 and 8.49 μg/kg BW per day, respectively), followed by Wels catfish (2.28 and 2.11 μg/kg BW per day, respectively), and silver carp (5.77 and 2.90 μg/kg BW per day, respectively). Regarding toxic elements, in common carp, the highest EDI levels were found for Hg (0.13 μg/kg BW per day; *Jovanović et al*., 2017), As (0.05 μg/kg BW per day; *Aleksić et al*., 2025), and Hg (0.04 μg/kg BW per day; *Ivanović et al*., 2016), in silver carp the highest EDI was for Pb (0.04 μg/kg BW per day; *Ivanović et al.*, 2016), while in Wels catfish, the highest EDIs detected were for Pb (0.43 μg/kg BW per day; *Lenhardt et al.,* 2012) and Hg (0.17 μg/kg BW per day; *Milošković et al.*, 2016).

The highest % PTWI were found for Hg in common carp (22.34%) (Jovanović et al., 2017), Wels catfish (29.73%) (*Milošković et al*., 2016), and silver carp (7.67%) *(Ivanović et al*., 2016). Furthermore, % PTWI for As, Cd, and Pb in fish meat of common carp, Wels catfish, and silver carp were lower than 5%.

(a) Common carp



(b) Wels catfish



(c) Silver carp

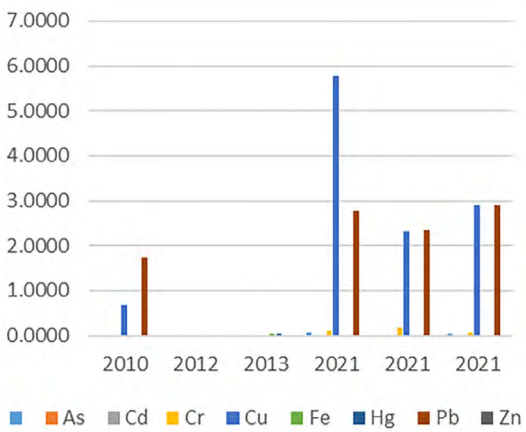
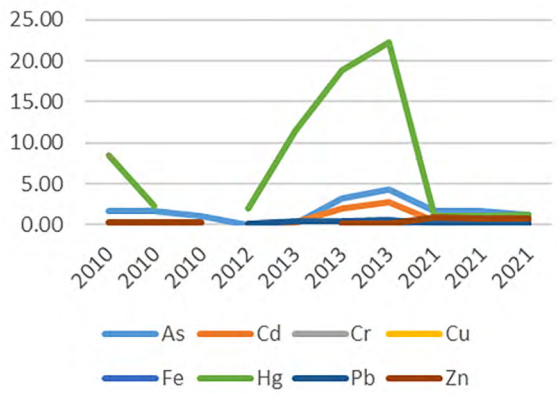
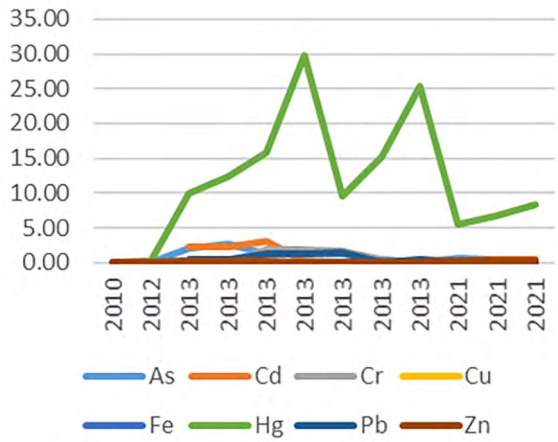


Figure 1. Estimation of daily intake rate (EDI; µg/kg BW/day) in fish meat of common carp, Wels catfish, and silver carp caught from the Danube River in Serbia for the period 2010-2021

(a) Common carp



(b) Wels catfish



(c) Silver carp

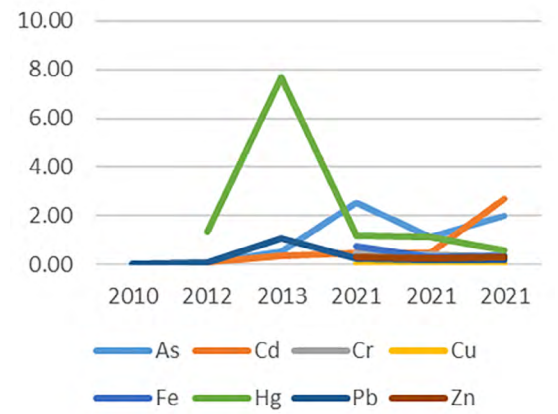


Figure 2. Percentage of tolerable weekly intake (% PTWI) of metals in fish meat of common carp, Wels catfish, and silver carp caught from the Danube River in Serbia for the period 2010-2021

3.2. Target hazard quotient (THQ) and hazard index (HI)

In Figure 3, we present results of calculated target hazard quotient (THQ) and hazard index (HI) for the meat of common carp, Wels catfish, and silver carp caught from the Danube River in Serbia in the past 15 years. The highest HIs were found in common carp caught in 2013 from Grocka (2.18; *Jovanović et al*., 2017) and Zemun (1.75; *Jovanović et al*., 2017). In those studies, the THQ of Hg largely contributed to HI. In Wels catfish, the highest HI was found during 2010 (5.27; *Lenhardt et al*., 2012) due to the high contribution of lead to the THQ. Moreover, in Wels catfish caught in Zemun and Grocka during 2013 (*Jovanović et al.*, 2017), Novi Sad, Zemun, and Radujevac during 2011-2013 (*Milošković et al*., 2016), and in Vinča during 2013 (*Ivanović et al*., 2016), HIs was higher than 1. The consumption of those fish posed a significant health hazard to consumers (*USEPA*, 2010), mainly due to high contribution of Hg to the THQ. In silver carp, observed HIs were lower than 1, although in 2013 (*Ivanović et al*., 2016), HI was slightly below 1 (0.99), indicating potential adverse non-carcinogenic health effects (*USEPA*, 2010). The latest research (*Aleksić et al*. 2025) performed in 2021 showed that HIs of common carp, Wels catfish, and silver carp caught from the Danube River have decreased in the past decade.

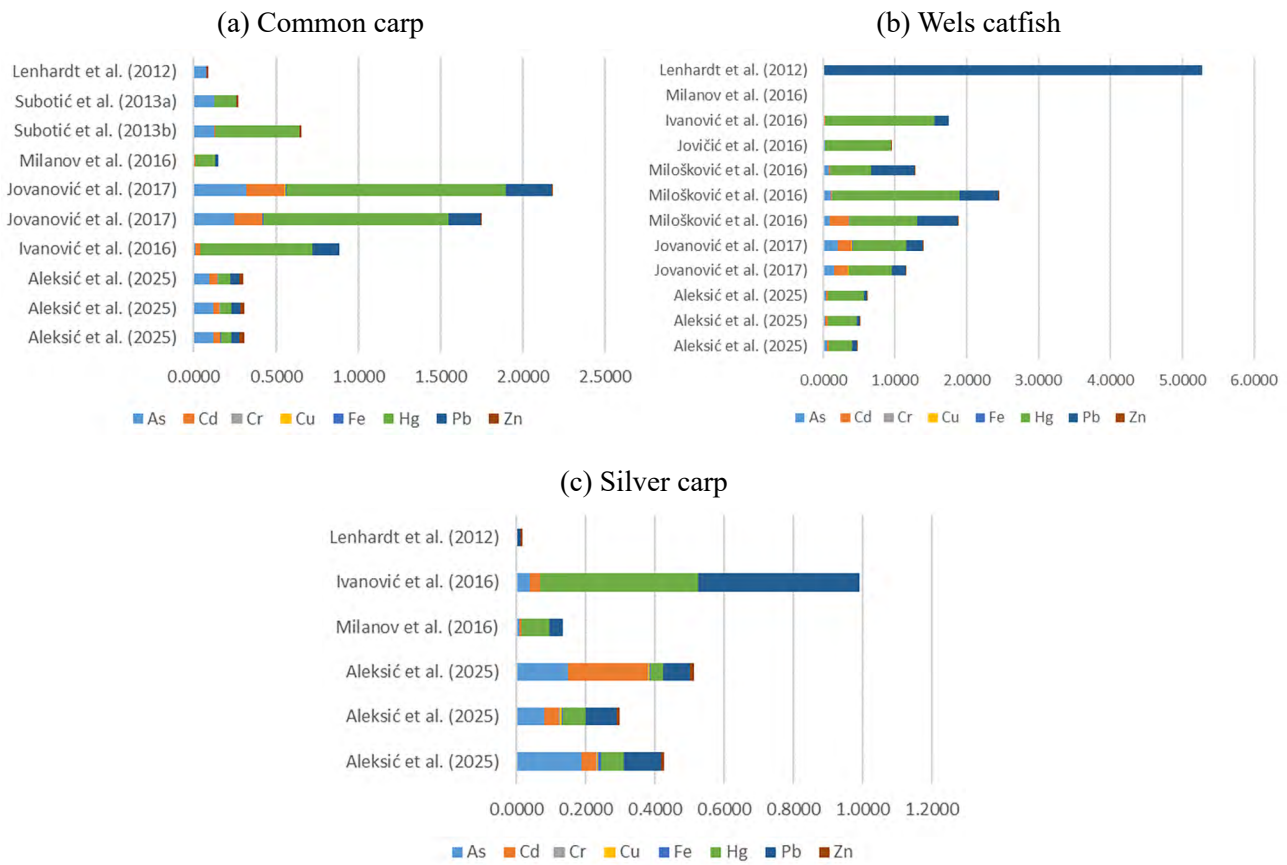


Figure 3. Target hazard quotient (THQ) and hazard index (HI) in fish meat of common carp, Wels catfish, and silver carp caught from the Danube River in Serbia for the period 2010-2021

3.3. Target cancer risk (TR)

Table 1 shows the target cancer risk (TR) calculated for fish meat of common carp, Wels catfish, and silver carp caught from the Danube River in Serbia for the period 2010-2021. The TR for As ranged in common carp from 1.11E-06 (*Milanov et al*., 2016) to 1.43E-04 (*Jovanović et al*., 2017), in Wels catfish from 1.29E-06 (*Ivanović et al*., 2016) to 9.04E-05 (*Jovanović et al*., 2017), and in silver carp from 3.09E-06 (*Milanov et al*., 2016) to 8.43E-05 (*Aleksić et al*., 2025). The TR for As in previous studies was regarded as acceptable, except in common carp (*Jovanović et al*., 2017) when was higher than 10^{-4} and recognised as unacceptable carcinogenic risk. The TR for Cr ranged from 10^{-6} to 10^{-4} in all three fish species,

Table 1. Target cancer risk (TR) in fish meat of common carp, Wels catfish, and silver carp caught from the Danube River in Serbia for the period 2010-2021

As	Cr	Pb	Region	Year of sampling	Reference
			Common carp		
5.41E-05	3.14E-06	3.45E-08	Novi Sad	2021	Aleksić et al. (2025)
5.43E-05	3.29E-06	4.01E-08	Zemun	2021	Aleksić et al. (2025)
4.32E-05	3.71E-06	4.01E-08	Grocka	2021	Aleksić et al. (2025)
4.29E-06		1.17E-07	Vinča	2013	Ivanović et al. (2016)
1.11E-04		1.43E-07	Zemun	2013	Jovanović et al. (2017)
1.43E-04		2.04E-07	Grocka	2013	Jovanović et al. (2017)
1.11E-06		1.70E-08	Belgrade	2012	Milanov et al. (2016)
5.66E-05	2.86E-07		Belgrade	2010	Subotić et al. (2013b)
5.66E-05			Belgrade	2010	Subotić et al. (2013a)
3.39E-05			Belgrade	2010	Lenhardt et al. (2012)
			Wels catfish		
2.43E-05	2.26E-06	3.81E-08	Novi Sad	2021	Aleksić et al. (2025)
1.56E-05	2.76E-06	2.60E-08	Zemun	2021	Aleksić et al. (2025)
1.68E-05	2.61E-06	2.26E-08	Grocka	2021	Aleksić et al. (2025)
6.90E-05		1.41E-07	Zemun	2013	Jovanović et al. (2017)
9.04E-05		1.68E-07	Grocka	2013	Jovanović et al. (2017)
4.29E-05	2.07E-05	4.13E-07	Novi Sad	2011-2013	Milošković et al. (2016)
4.71E-05	2.00E-05	3.89E-07	Zemun	2011-2013	Milošković et al. (2016)
3.86E-05	1.86E-05	4.37E-07	Radujevac	2011-2013	Milošković et al. (2016)
1.12E-05	3.94E-06	2.91E-09	Belgrade	2013	Jovičić et al. (2016)
1.29E-06		1.46E-07	Vinča	2013	Ivanović et al. (2016)
		3.84E-06	Belgrade	2010	Lenhardt et al. (2012)
			Silver carp		
8.43E-05	5.11E-06	7.65E-08	Novi Sad	2021	Aleksić et al. (2025)
3.65E-05	4.36E-06	6.56E-08	Zemun	2021	Aleksić et al. (2025)
6.68E-05	3.60E-06	5.76E-08	Grocka	2021	Aleksić et al. (2025)
3.09E-06		2.72E-08	Belgrade	2012	Milanov et al. (2016)
1.71E-05		3.40E-07	Vinča	2013	Ivanović et al. (2016)
		7.29E-09	Belgrade	2010	Lenhardt et al. (2012)

and so is regarded as acceptable. Furthermore, the TR for Pb in all observed studies was lower than 10^{-6} and indicated as negligible, except in the study by *Lenhardt et al*. (2012) (3.84E-06).

4. Conclusion

Results of health risk assessment of fish meat of common carp, Wels catfish, and silver carp caught from the Danube River in Serbia for the period 2010-2021 showed that regarding toxic elements, the highest values of EDI were found for Hg in common carp, for As, Hg, and Pb in silver carp, while in Wels catfish, the highest EDI was detected for Pb and Hg. Moreover, the highest % PTWI were determined for Hg in all three fish species reaching 29.73%, while % PTWI for As, Cd, and Pb were below 5%. HIs higher than 1 were detected in common carp and Wels catfish during 2013 due to high contribution of Hg to the THQ, indicating a potential for adverse non-carcinogenic health effects. In Wels

catfish, the highest HI was found during 2010, due to high contribution of Pb to the THQ. However, in silver carp, observed HIs were lower than 1. The TR for As in previous studies was regarded as acceptable, except in common carp during 2013, when was higher than 10^{-4} and recognised as an unacceptable carcinogenic risk. The TR for Cr ranged from 10^{-6} to 10^{-4} in all three fish species, and so is regarded as acceptable. Furthermore, the TR for Pb in all observed studies was lower than 10^{-6} and indicated as negligible, except during 2010 when it was indicated as acceptable.

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Original Scientific Paper

Comparation of validation results of HPLC-UV/PDA and LC-MS/MS methods for the determination of sorbates and benzoates in food

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ABSTRACT

Sorbates are GRAS (Generally recognized as safe) additives, while benzoates are more of concern and can be converted to benzene through decarboxylation. Preservatives like sodium benzoate and potassium sorbate may contribute to intestinal dysbiosis and inflammatory bowel diseases by altering gut microbiota. Animal studies show teratogenic and neurotoxic effects on zebrafish embryos and chromosome aberrations in human lymphocytes The health benefits of sorbates and benzoates have been explored in scientific literature, particularly in the context of their therapeutic potential beyond their antimicrobial properties. Sodium benzoate, a metabolite of cinnamon, upregulates neurotrophic factors, suggesting potential benefits in treating neurodegenerative disorders such as Alzheimer's and Parkinson's disease. The clinical administration of sodium benzoate is well-established for treating urea cycle disorders, where it helps manage hyperammonaemia by facilitating ammonia excretion. The aim of this study was comparing suitability of two methods, HPLC-UV/PDA and LC-MS/MS. High-Performance Liquid Chromatography (HPLC) is the most widely used method due to its high sensitivity, specificity and ability to separate and quantify sorbates (e.g., sorbic acid) and benzoates (e.g., benzoic acid) simultaneously. For trace-level detection, complex matrices, or multi-residue methods, LC-MS/MS is superior due to its unmatched sensitivity and selectivity, albeit at higher cost and complexity. Comparison, using paired sample t-test, using T-distribution (two-tailed), of the sorbate content of 36 foodstuffs analysed by the two methods, showed a non-significant small difference between the results of HPLC (mean = 358 mg/kg, SD = 378.5) and results of LC/MS-MS (mean = 335.6 mg/kg, SD = 336.6), t (35) = 1.9, p = 0.071.

1. Introduction

Sorbates (e.g., potassium sorbate) and benzoates (e.g., sodium benzoate) are food preservatives used to inhibit mould, yeast, and bacterial growth. Common foods that are preserved by adding sorbates are cheese, yogurt, dried fruits, baked goods, beverages, soft drinks, fruit juices, sauces, and pickled products (*Choyam et al*., 2019). Sorbates are generally recognized as safe by regulatory agencies like the Food and Drug Administration (FDA) and European Food Safety Authority (EFSA) when used within acceptable daily intake (ADI) limits (*Chen et al*., 2020). Both sorbates and benzoates are rapidly metabolized and excreted, minimizing accumulation in the body. Adverse effects are rare at typical dietary levels but can occur in sensitive populations

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or with excessive consumption (*Piper and Piper*, 2017). Individuals with compromised liver function or specific allergies may be at higher risk. Regulatory bodies (FDA, EFSA) monitor usage levels to ensure safety. Safe daily intake (ADI) for sorbates is considered to be 11 mg/kg body weight (*EFSA*, 2019; *FAO/WHO*, 2021). Sensitive populations, people with allergies, asthma, or histamine intolerance may be more prone to adverse reactions (*Abily-Donval et al*., 2015a). Cumulative exposure risk may increase with frequent consumption of multiple preserved foods (*Udovicki et al*., 2024). The aim of this study was to compare suitability of two methods for determination of sorbates in food. Samples of different food were analysed by high performance liquid chromatography HPLC UV-PDA and liquid chromatography with tandem mass spectrometry (LC/MS-MS) methods.

1.1 Sorbates as food additives

Potassium sorbate ($C_6H_7KO_2$) and sorbic acid ($C_6H_8O_2$) are two forms of this preservative in food. The metabolism pathway of sorbic acid starts in the gastrointestinal tract and ends metabolized primarily in the liver. It undergoes β-oxidation, similar to fatty acids, breaking down into carbon dioxide and water. The process involves conjugation with coenzyme A, followed by oxidation to smaller molecules excreted via urine or exhaled as CO_2 (*Chen et al*., 2020). Minor amounts may be conjugated with glycine to form urocanic acid derivatives, excreted in urine. Potential adverse effects of sorbates if used in high doses can cause several issues. Potassium sorbate can contribute to intestinal dysbiosis and inflammatory bowel diseases (IBD) by altering gut microbiota (*Jarmakiewicz-Czaja et al*., 2022). Allergic reaction, including skin irritation, urticaria, or contact dermatitis in sensitive individuals and gastrointestinal issues, i.e., nausea, diarrhoea, or stomach discomfort in rare cases (*Trasande et al.*, 2018). High doses can cause glycine depletion, impair memory, or increase attention deficit hyperactivity disorder (ADHD) symptoms in sensitive individuals (*Abily-Donval et al*., 2015b). Animal studies show teratogenic and neurotoxic effects on zebrafish embryos and chromosome aberrations in human lymphocytes.

1.2. Benzoates as food additives

Benzoates are food additives in form of acid and salts with chemical formula sodium benzoate ($C_7H_5NaO_2$) and benzoic acid ($C_7H_6O_2$). Sodium benzoate can also alter the gut microbiota and contribute to health issues (IBD), much like sorbates (*Jarmakiewicz-Czaja et al*., 2022). The benzoate metabolism pathway starts with absorption in the stomach and small intestine. In the liver, benzoate is conjugated with glycine to form hippuric acid (benzoyl glycine), catalyzed by acyl-CoA synthetase and glycine N-acyltransferase. Hippuric acid is excreted in urine, typically within 6-12 hours (*Ogbadu*, 2014).

1.3. Analytical methods for preservatives in food

Determination of sorbates (and benzoates) involves various analytical methods to ensure accurate detection and quantification. HPLC is the most widely used method due to its high sensitivity, specificity, and ability to separate and quantify sorbates (e.g., sorbic acid) and benzoates (e.g., benzoic acid) simultaneously (*Pylypiw and Grether*, 2000). Gas chromatography with flame ionization detection (GC-FID) is used for quantification of volatile derivatives of sorbates and benzoates, often after derivatization (e.g., esterification to increase volatility). Gas chromatography-mass spectrometry (GC-MS) is used for confirmation and trace-level detection (*Tungkijanansin et al*., 2020). Capillary electrophoresis (CE) separates charged molecules like benzoates and sorbates based on their electrophoretic mobility in a capillary tube. Detectors used are UV or diode array detector (DAD), typically at 200–230 nm (*Mesquita et al*., 2018). Due to its lower sensitivity, CE is less common than HPLC, but since organic solvents are not used in CE, it is a green chemistry method. Spectrophotometric UV-Vis methods measure absorbance of sorbates and benzoates at specific wavelengths (e.g., 250 nm for sorbic acid, 225 nm for benzoic acid). These methods are cost effective, much like CE, but have weak specificity. LC-MS/MS combines HPLC with mass spectrometry for highly sensitive and selective detection, especially in complex matrices like dairy or meat products (*Gören et al*., 2015). This method has advantage for trace level detection, but the disadvantage of being expensive because of the required equipment and maintenance. A reversed-phase C18 column is commonly used due to its ability to separate polar compounds like sorbates and benzoates. The hydrophobic nature of the C18 stationary phase effectively retains and separates these acidic preservatives in food matrices. Thin-layer chromatography (TLC) is sometimes

used for qualitative or semi-quantitative analysis of sorbates and benzoates (*Khan et al*., 1994). Ion chromatography (IC) separates sorbates and benzoates based on ionic interactions. It is a practical method for aqueous matrices like beverages (*Iammarino and Taranto*, 2013). Regulatory standards set by organizations like the FDA, EFSA, or Codex Alimentarius often specify HPLC or LC-MS/MS, while for official testing, HPLC with UV or MS detection is the gold standard for routine analysis of sorbates and benzoates in food due to its versatility and reliability. GC and CE are alternatives for specific applications, while spectrophotometry and TLC serve as cost-effective screening tools.

2. Materials and methods

Thirty-six samples of milk products (soft cheese products, milk spreads, seasoned milk fermented products), bakery products (soft cookies, fruit coated pies), fruit and vegetable products (spreads, sauces, brined fruit), fish products and pasteurized egg products were analysed for sorbates using both HPLC and LC-MS methods. Student's t-test was used for comparison of results for two different methods (XLSTAT 2025).

2.1. LC-MS/MS method for sorbate and benzoate quantification

Sample preparation was matrix dependent, so for samples with certain protein amounts, simple protein precipitation with Carezz I and II solutions was used, or liquid-liquid extraction with methanol or acetonitrile to extract sorbates and benzoates, respectively, from food matrices like beverages, jams, and yogurt (*Hatton and Warr*, 2024). The temperature range 30–40°C ensured consistent retention times and peak shapes because it minimizes viscosity of the mobile phase and improves reproducibility. The column used was C18 100 Å, 50 × 2.1 mm; 2.6 µm. The mobile phase consisted of: A: 0.1% formic acid in water (to enhance ionization in MS) and B: Methanol with 0.1% formic acid. The gradient started with 10% of organic phase, reaching 90% at 10-12 minutes, and re-equilibrating to 15 minutes of total run. Formic acid enhanced negative ion formation for MS detection, and the gradient ensured separation of sorbates, benzoates, and potential matrix interferences (*Gören et al*., 2015). Electrospray Ionization (ESI) was in negative ion mode. Multiple reaction monitoring (MRM) transitions were with a collision energy of 10eV.

Table 1. Transitions of sodium benzoate and potassium sorbate for Shimadzu LCMS-8045

Component	Precursor ion: m/z	Product ion: m/z	Product ion: m/z
Sodium Benzoate	121.0	77.0	93.0
Potassium Sorbate	111.10	67.0	83.0

These transitions are specific and sensitive for quantification and confirmation. Thirty-six samples of different food with added preservatives were analysed and content of sorbates was quantified, using both HPLC-UV/PDA and LC-MS/MS.

1.2. HPLC- UV/PDA method for sorbate and benzoate quantification

The Waters Alliance HPLC 2695 model is a robust, modular system designed for reliable and reproducible separation, while the 2996 PDA detector provides UV-Vis spectral data across a wide wavelength range (190–800 nm). The method is in the scope of accreditation according to ISO 17025. Mobile phase was 20 min gradient of acetonitrile and potassium dihydrogen phosphate buffer, pH 2.5 (Sigma Aldrich). The column used for separation was C18, 150 × 4.6 mm, with particle size 5 µm. Sorbates and benzoates, which have strong chromophores, show maximum absorbance of λ_{max} ~260 nm and λ_{max} ~230 nm for sorbic and benzoic acid, respectively. The main parameters of validation for the HPLC method were: reproducibility RSDr=10.1; trueness: 96.6% and 99.7%; limit of detection/quantification LOD/LOQ=1.63/2.25 mg/kg and 0.66/1.23 mg/kg; linearity=0.9999, for benzoates and sorbates respectively. Paired sample T-test, using T distribution (two-tailed) from Microsoft Excel was used to assess data obtained from the two different techniques used for sorbate analysis of foods.

3. Results

According to the HPLC UV PDA method, LODs were 0.66–1.63 mg/l for sorbates and benzoates, but using LC-MS/MS, LODs were significantly lower 0.132–0.175 mg/l. Nonetheless, recovery

was between 97% and 104%, for both methods and analytes. The measurement uncertainty for HPLC/UV-PDA method was 10% for both analytes, sorbates and benzoates. During the validation period of the LC-MS/MS method, measurement uncertainty—including combined uncertainty from proficiency test data—resulted in Uc=14%. Figure 1 shows results of sorbate quantitative analysis of the 36 food samples by the two methods, HPLC-UV/PDA and LC-MS/MS.

Paired sample T-test, using T distribution (two-tailed) results indicated that there was a non-significant small difference between the results from HPLC (M = 358.3 mg/kg, SD = 378.5) and LC/MS-MS (M = 335.6 mg/kg, SD = 336.6), t(35) = 1.9, p = 0.071. Since the p-value > α, H0 can not be rejected. The test statistic T was -1.864, which was in the 95% region of acceptance: [-2.0301, 2.0301]. The subtraction difference (-22.64) was also in the 95% region of acceptance: [-24.6575, 24.6575]. The 95% confidence interval of subtraction difference was [-47.2978, 2.0172]. The observed effect size (d) was small, 0.31. Results of sorbate analysis by the HPLC UV PDA and LC/MS-MS methods, according to foodstuff groups, are presented in Tables 2-6.

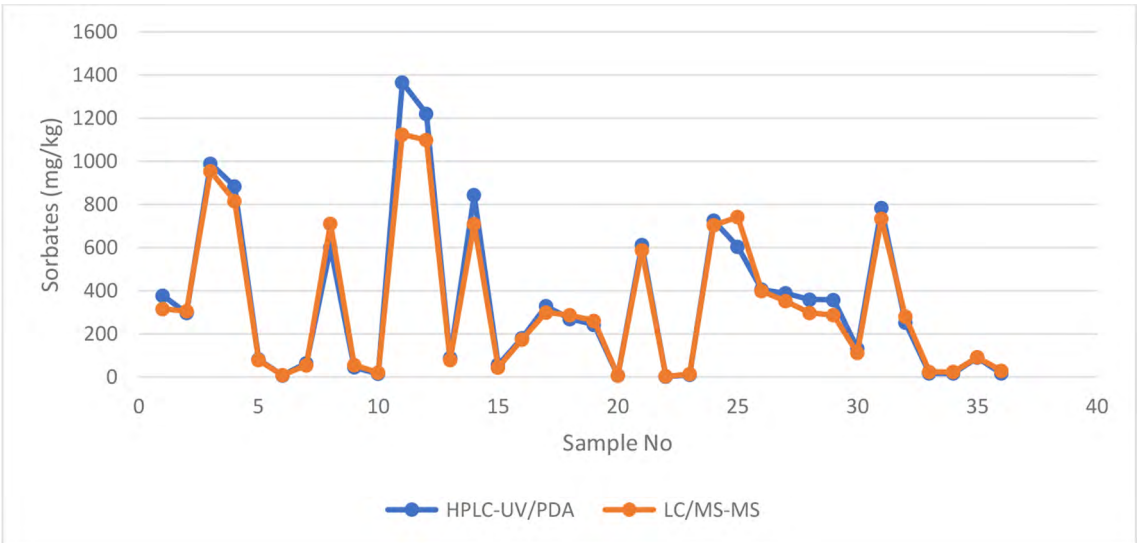

Figure 1. Sorbate (mg/kg) content in 36 foods, analysed by HPLC-UV/PDA and LC-MS/MS

Table 2. Sorbates (mg/kg) in milk products

	MILK PRODUCT GROUP	Result HPLC	Result LC/MS-MS
S1	BURGER CHEDDAR	989.37	953.42
S2	TRAPIST CHEESE SLICE	82.32	79.22
S3	SOFT CHEESE	8.19	9.12
S4	FULL FAT CHEESE	64.64	54.95
S5	COTTAGE CHEESE	601.59	711.22
S6	GOUDA CHEESE SLICE	45.55	56.55
S7	TRAPIST CHEESE PIECE	15.46	21.25
S8	MIXED CHEESE	784.31	734.22
S9	SOFT CHEESE	252.32	280.21
S10	YOGHURT SALAD PICKLES	612.58	587.23
S11	YOGHURT SALAD HERBS	2.81	3.03

Table 3. Sorbates (mg/kg) in fruit and vegetable products

	FRUIT AND VEGETABLE PRODUCT GROUP	Result HPLC	Result LC/MS-MS
S12	BLACK OLIVE IN BRINE	88.79	80.25
S13	GREEN OLIVE IN BRINE	843.41	709.11
S14	CANDIED DATES	180.46	174.545
S15	SAUERKRAUT	329.35	300.11
S16	KETCHUP HOT	604.62	742.11
S17	KETCHUP MILD	405.53	399.12
S18	KETCHUP PIZZA	388.79	352.44
S19	KETCHUP SPICY	359.85	298.41
S20	TOMATO SAUCE	357.98	287.45
S21	BBQ SAUCE	132.98	112.21
S22	BBQ COLESLAW SALAD	7.6	7.75
S23	SAUERKRAUT LEAF	725.17	704.00

Table 4. Sorbates (mg/kg) in bakery products

	BAKERY PRODUCTS	Result HPLC	Result LC/MS-MS
S24	APPLE PIE	378.31	316.51
S25	CHERRY PIE	297.33	305.54
S26	COOKIES	57.64	45.265
S27	CHERRY CAKE	269.61	269.8273
S28	APPLE CAKE	243.55	261.25

Table 4. Sorbates (mg/kg) in vegetable products

	VEGETABLE PRODUCT	Result HPLC	Result LC/MS-MS
S29	VEGETABLE SWEETENED CREAM	1365	1124.55
S30	FRUIT AND VEGETABLE SPREAD	1365	1098.74
S31	VEGETABLE SPRAY CREAM	10.84	14.21

Table 5. Sorbates (mg/kg) in fishery products

	FISHERY PRODUCTS	Result HPLC	Result LC/MS-MS
S32	BREADED FISHBURGERS	17.39	25.23
S33	SURIMI	17.39	24.18

Table 6. Sorbates (mean mg/kg) in egg products

	EGG PRODUCTS	Result HPLC	Result LC/MS-MS
S34	MAYONAISE	884.23	816.47
S35	PASTEURIZED LIQUID CHILLED EGG WHITE	90.8	92.54
S36	PASTEURIZED LIQUID CHILLED EGG YOLK	17.1	29.77

3. Discussion

Paired sample t-test, using the T distribution (two-tailed) indicated there was a non-significant small difference between results (sorbate content, mg/kg) obtained with the different methods, LC-MS/MS and HPLC-UV/PDA, for 36 foods. Validation parameters of both methods meet requirements of Serbian (*Serbia*, 2018) and European regulations (*European Commission*, 2011). The sorbate contents of 36 foodstuffs were within the permitted values stipulated for these groups of food. The observed effect size (d) was small, 0.31. This indicates that the magnitude of the difference between the average of the differences and the expected average of the differences is small. Validation measurements, such as LOD, LOQ, and measurement uncertainty, as well as repeatability, reproducibility and recovery were satisfactory for both methods, HPLC and LC-MS, as other authors also found in their comparative research (*Gören et al*., 2015; *Horiyama et al.,* 2010; *Tungkijanansin et al*., 2020). Generally, LC-MS/MS is preferred for trace analysis and regulatory compliance (*Horiyama et al*., 2010), while HPLC UV/PDA is suitable for high throughput of samples with limits up to 2000 mg/kg of sorbates.

4. Conclusion

Comparing the Waters Alliance HPLC with UV/PDA 2996 and the Shimadzu LCMS-8045 LC-MS/MS for analysing sorbates and benzoates involved evaluating their performance based on sensitivity, specificity, sample preparation, cost, and suitability for the specific method. The choice of method depends on the food matrix, required sensitivity, and available instrumentation.

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Original Scientific Paper

Acrylamide content in potato chips in 2024: monitoring on the production site

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ABSTRACT

The presence of acrylamide, a probable human carcinogen, in thermally processed potato products remains a significant food safety concern. This study monitored acrylamide levels in 336 potato chips (crisps) collected throughout 2024 directly from the production site. A subset of 41 samples with known production parameters was used for screening to evaluate the impact of chemical and processing variables. Statistical analysis using contrast screening revealed that, among the factors examined, the content of reducing sugars had the strongest linear correlation with acrylamide levels. Moreover, synergistic quadratic effects between reducing sugars and both frying and blanching temperatures were observed, significantly influencing acrylamide formation. Contingency analysis showed no substantial difference in acrylamide content outcomes between the two predominant potato cultivars, Opal and Sinora. However, a notable shift in acrylamide levels occurred after August 2024, correlating with a change in the potato lot, suggesting the strong influence of agronomic and storage conditions. The study underscores the importance of continuous monitoring and comprehensive traceability in chips production to control acrylamide levels, in line with EU Regulation 2017/2158. These findings can support mitigation strategies, including the selection of potato varieties, temperature control and process optimization, ultimately contributing to enhanced food safety and regulatory compliance.

1. Introduction

Potatoes are the fourth field crop in the world in terms of production, right behind wheat, rice and corn, and are of great importance in human nutrition. China, with 32% of the area under cultivation and with 25% of the total production at the world level, is the leading country in the production of this field crop (*Li et al*., 2024). For human consumption, most potatoes are processed into products, such as dehydrated flakes, dough, semi-prepared and fried products. One of the most popular products is potato chips (crisps), primarily because of their unique taste and crunchy texture. The production of chips and related snack products from potatoes is a significant branch of the food industry with a turnover that is measured in billions of dollars at the world level.

Acrylamide is formed by the Maillard reaction in products that are rich in carbohydrates, such as potato products—chips, French fries, but it is also present in coffee/coffee substitutes, biscuits, bakery products—bread, baby food and tobacco smoke (*EFSA*, 2015). This reaction occurs at temperatures >120°C and in low humidity conditions, as a result of the interaction of reducing sugars and free asparagine. Acrylamide's presence in food probably dates back to the first thermal processing, but it has

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attracted attention since 2002 (*Adani et al.*, 2020). When consuming the aforementioned foods, thereby introducing acrylamide into the digestive tract, it is converted into glycidamide and is quickly absorbed by all organs. It is classified as a carcinogenic compound in animals and as a probable carcinogen in humans (*Marques et al*., 2024). It is believed that acrylamide and metabolites cause neurotoxic, hepatotoxic, nephrotoxic, genotoxic and carcinogenic changes in the body (*Khoshbin et al*., 2024; *Yan et al*., 2023; *EFSA*, 2015).

The European regulation 2158 (*Commission Regulation*, 2017; *Juncker* (2017)) references values for acrylamide concentrations in specific foods of up to 750μg/kg for potato chips, 400μg/kg for crackers and 350μg/kg for biscuits. According to reports by the European Food Safety Agency (EFSA), amounts of acrylamide from 170μg/kg to 430μg/kg of body weight on a daily basis represent a safety limit beyond which pathological changes occur (*EFSA*, 2015a; *Calabrese et al*., 2024). The International Agency for Research on Cancer (IARC) classified this organic compound in the A2 group, which includes all agents that are "probably carcinogenic to humans" (*Adani et al*., 2020).

In order to prevent and reduce the formation of acrylamide during the production of chips, it is necessary to pay attention to the selection of the potato variety. Also, the transport should not be carried out at temperatures below 5°C, and the storage temperature should not be below 8°C, because at lower temperatures during storage, the sugar level rises. Humidity and air circulation should be adapted to the potato variety to prevent subsequent germination. Immediately before frying, and after cutting the potatoes, there are recommendations for soaking the potato chips in water or a solution of citric acid, which will reduce the amount of acrylamide produced after frying by up to 40% or 75%.

Also, the effect of reducing the temperature and extending the frying time should be investigated (*FDE*, 2019). *Jana et al*. (2024) reduced the amount of acrylamide in food by adding the enzyme L-asparaginase. *Santiago-Mora et al*. (2024), using a pulsed electric field (PEF) after peeling potatoes, ensured easier slicing while reducing energy and water consumption. The device was primarily used in the production of French fries, but after treating potatoes with the PEF, they were used for the production of chips. The frying time was shortened by 10%, and the produced chips contained 28.9% less acrylamide than controls.

The aim of this study was to monitor the formation of acrylamide in chips on the production line during 2024, and to consider the factors that potentially contribute to the formation of acrylamide beyond the permitted prescribed levels.

2. Materials and methods

2.1. Samples

Chips were obtained from the manufacturer. A total of 336 samples of chips in 2024 were included in the research. A subset of 41 samples manufactured under various processing conditions were employed for screening for contrast analysis since their production conditions data were available (Table 1).

2.2. Chemical determination

Analysis of acrylamide in potato chips was performed using an in-house method accredited according to ISO/IEC 17025. Previously homogenized samples were extracted with acetonitrile and purified with QuEChERS technique. Analysis was performed by liquid chromatography mass spectrometry LC MS-8040 (Shimadzu, Kyoto, Japan). The chromatographic separation of acrylamide was obtained on a reversed phase Kinetex C18 column, 50 mm × 2.1 mm, particle size 2.6 μm (Phenomenex, Torrance, USA). Isocratic elution was used with 2% methanol and 1% acetonitrile in 0.1% formic acid. The flow rate was 0.3 mL/min. Acrylamide was analyzed using electrospray ionization in positive ion mode. Multiple reaction monitoring mode (MRM) was employed with the characteristic fragmentation transitions m/z 72>55 and 72 >27. As a control, FAPAS QC Material/Potato crisps/T30148QC (Fera Science Ltd, York, Great Britain) was used. The results for quality control samples were within the range of certified values.

Content of sugars, reducing and total, was conducted using ion chromatography with pulse amperometric detection. The system consisted of an 858 Professional sample processor, 930 Compact IC Flex with Oven/Deg, IC Amperometric Detector, all from Metrohm (Herisau, Switzerland). The separation column was Metrosep Carb 2 250/4, also from Metrohm, and separation of mono- and di-saccharides was isocratic in accordance with original method reported by Metrohm (Riverview, FL, USA).

The dry matter content was determined by the ISO reference method (*SRPS EN ISO*, 2024).

2.3. Statistics

Arrangement of data for statistical processing and preliminary filtering of results was performed using the Excel program from the Microsoft Office package (Microsoft, Richmond, VA, USA). For evaluation of the influence and significance of the physico-chemical properties of the raw material and technological process parameters on the acrylamide content in chips, the data processing software JMP Statistical Discovery 10 (SAS Institute, Cary, NC, USA) was used. Screening for contrast was used for data validation and assessment of correlation between input data, as well as assessment of the significance of the influence of individual parameters on the acrylamide content in the final product. Also, contingency analysis was performed for comparison of categorical data.

3. Results and discussion

The potato chips' chemical parameters along with frying and blanching temperatures are shown in Table 1. The presented data were used for screening and estimation of chemical and processing parameters' effects on the resulting content of acrylamide. Screening design results of the given parameters' effects on acrylamide quantity in chips confirmed that the amount of reducing sugars was the main linear factor in direct correlation with the acrylamide amount in chips. Also, frying and blanching temperatures had a synergistic effect on the reducing sugar content. These results are consentaneous with previously reported data (*Muttucumaru et al.,* 2017; *Liyanage et al.*, 2021; *Passos et al.*, 2018; *Sanny et. al*., 2012; *Ferreira et al.*, 2025; *Bachir et al.*, 2023).

Table 1. Acrylamide, sugar and dry matter contents and processing temperatures of potato chip samples in the study

No	Acrylamide (µg/kg)	Reducing sugars (g/100g)	Total sugars (g/100g)	Dry Matter (g/100g)	Frying T (°C)	Blanching T (°C)
1	1320.5	0.41	0.57	22.0	165	70
2	1266.7	0.41	0.57	21.4	162	68
3	820.0	0.14	0.24	21.0	161	75
4	1086.0	0.12	0.53	22.2	162	77
5	853.3	0.08	0.45	22.5	160	74
6	1193.5	0.09	0.70	23.0	153	78
7	440.7	0.05	0.10	20.0	161	55
8	594.4	0.05	0.30	21.4	163	58
9	1019.7	0.04	0.13	21.0	165	65
10	926.0	0.03	0.28	22.7	165	65
11	913.1	0.14	0.32	23.0	162	64
12	902.7	0.09	0.28	22.6	164	64
13	620.1	0.08	0.26	22.3	162	65
14	409.3	0.08	0.03	21.0	165	65
15	485.8	0.36	0.81	21.6	161	65
16	464.0	0.03	0.10	22.3	161	69
17	372.0	0.03	0.17	22.0	163	65
18	487.0	0.05	0.21	22.4	162	69
19	553.7	0.02	0.13	22.3	161	68
20	820.8	0.02	0.74	22.0	160	70

No	Acrylamide (µg/kg)	Reducing sugars (g/100g)	Total sugars (g/100g)	Dry Matter (g/100g)	Frying T (°C)	Blanching T (°C)
21	684.4	0.01	0.05	22.0	161	72
22	720.0	0.01	0.09	22.1	163	68
23	726.7	0.03	0.23	22.1	163	71
24	531.0	0.04	0.27	22.1	162	74
25	491.2	0.03	0.15	22.0	164	70
26	644.0	0.34	1.31	22.0	161	70
27	226.7	0.01	0.17	22.1	155	77
28	667.1	0.03	0.09	20.0	161	55
29	624.9	0.03	0.12	20.7	162	49
30	788.4	0.05	1.05	21.0	163	54
31	739.0	0.01	0.11	22.7	161	52
32	666.3	0.02	0.07	21.0	160	53
33	569.5	0.08	0.18	21.8	159	53
34	696.4	0.13	0.71	21.4	160	52
35	1335.3	0.29	0.80	21.5	163	74
36	517.8	0.02	0.26	22.8	163	76
37	687.0	0.01	0.95	22.7	161	69
38	472.0	0.25	0.56	22.1	157	70
39	749.1	0.06	0.14	22.3	165	71
40	941.0	0.13	0.20	21.5	163	68
41	582.0	0.04	0.05	21.8	162	68

The contingency analysis of potato cultivars and corresponding grouped categories of acrylamide content is shown in Figure 1. Two varieties of potato were predominantly used for chips manufactured in 2024. The Opal variety accounted for almost 3/4 (72%) of the total number of samples tested. Figure 1 shows all the results, but only the varieties Opal and Sinora were analyzed in sufficient numbers to draw a reliable conclusion about the relative ratio of acceptable and unsatisfactory acrylamide content results. From Figure 1, it can be concluded that the ratios of the acrylamide result categories (good from 0 µg/kg to 700 µg/kg, satisfactory from 700 µg/kg to 750 µg/kg and bad over 750 µg/kg) for both varieties are almost identical.

Figure 2 presents the results of the contingency analysis for the number of analyses by month in 2024 and the corresponding distribution of the result categories. The number of analyses by month was relatively uniform. However, a clear difference in acrylamide content was observed during the year. In the period from January to July, bad results accounted for about and less than 20%, while in the latter part of the year from August to December, this number increased sharply to about 50%. According to information received from the manufacturer, the potato lot was changed in August 2024. This information is very intriguing, because the answer to such a sudden change in results most likely lies in the way the potatoes for chip production were grown and stored. The conclusion is unequivocal; in order to achieve uniform and acceptable acrylamide content, information on the potato's origin, growing method and storage must be available in further research. At the time the research was conducted, this information was not complete and available for all samples.

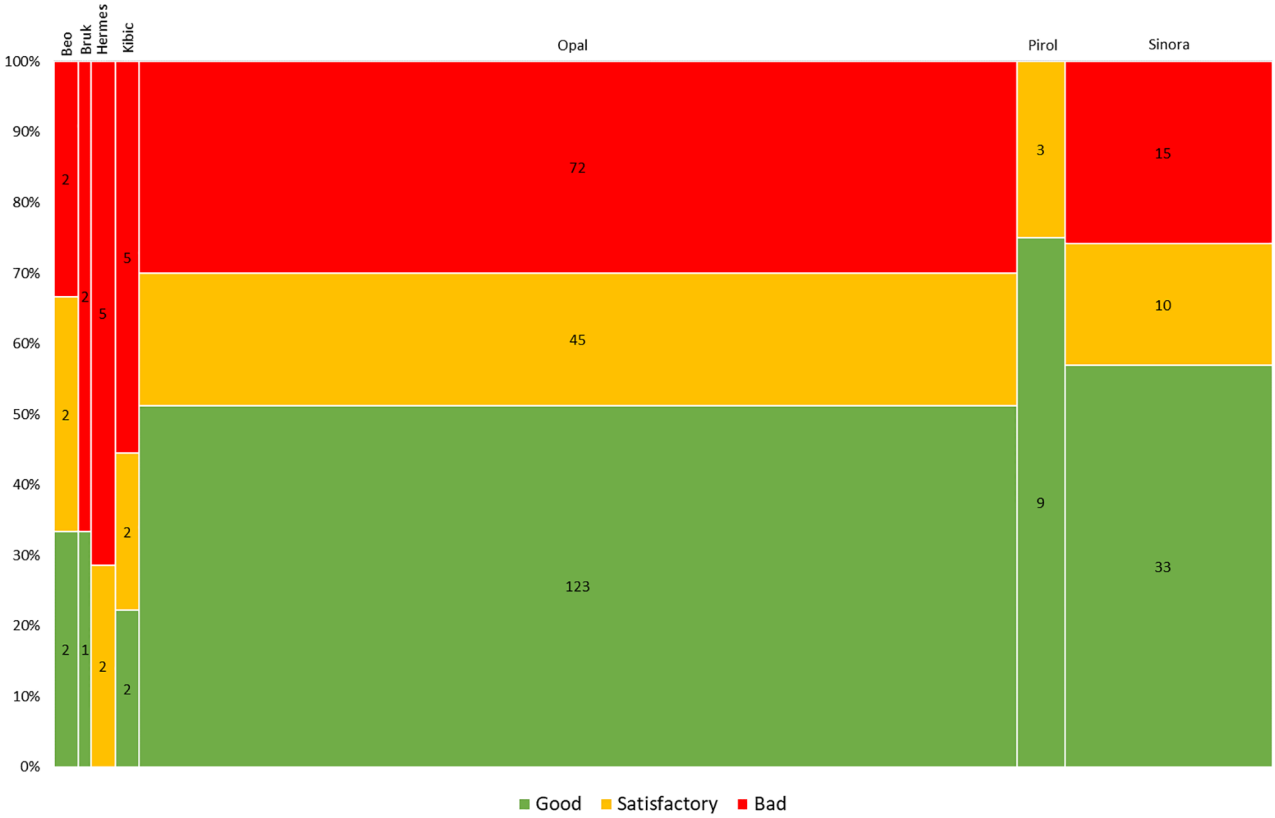

Figure 1. Distribution of acrylamide content category and potato cultivar in 2024

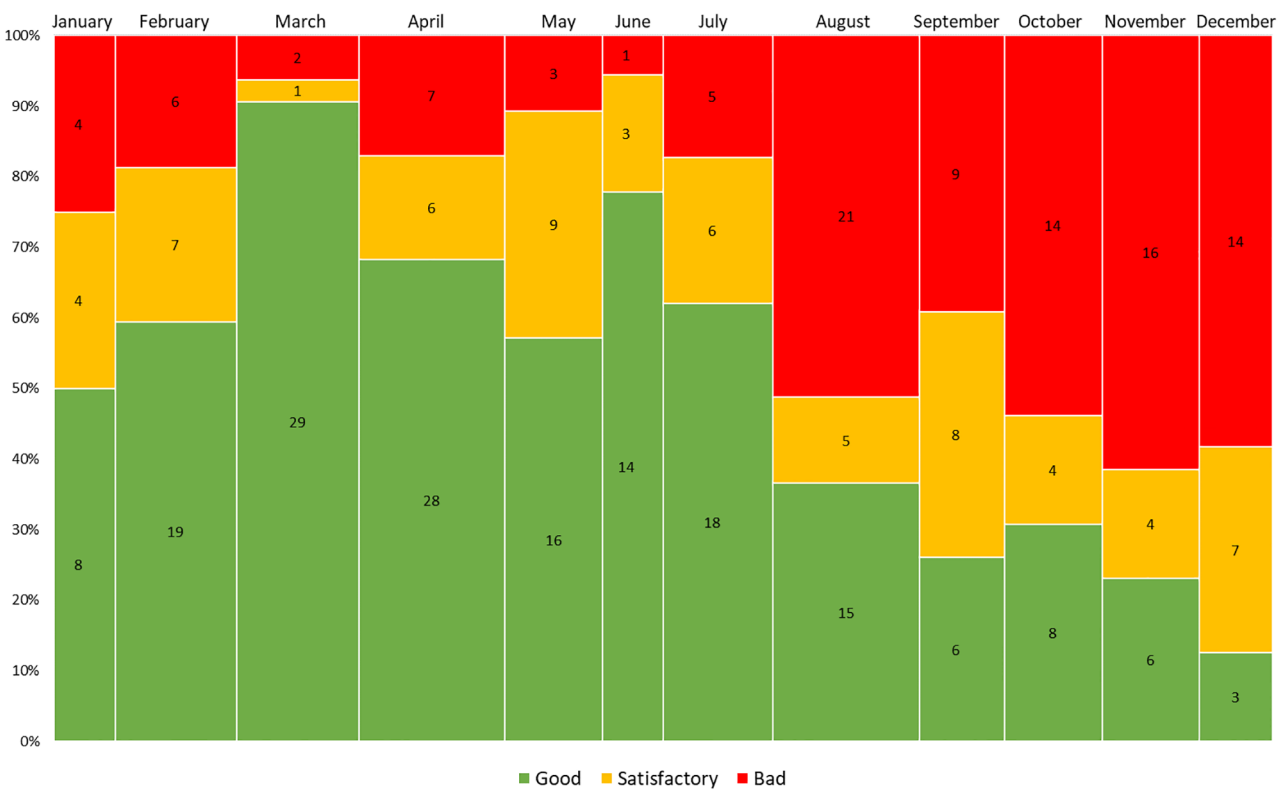

Figure 2. Acrylamide content result category by month in 2024

4. Conclusion

Screening design was applied to estimate the effect of some parameters (reducing and total sugar contents, dry matter, and frying and blanching temperatures) on acrylamide levels in potato chips. Results showed that the content of reducing sugars is the main linear factor in direct correlation with the acrylamide amount in chips. It was confirmed by screening that frying and blanching temperatures had a synergistic effect on the content of reducing sugars in the chips.

Two varieties of potato, Opal and Sinora, were predominantly used for chips manufactured in 2024. They had a sufficient number of analyses to draw a reliable conclusion about the relative ratio of acceptable and unsatisfactory acrylamide contents. The ratios of the result categories (good, satisfactory and bad) for both varieties are almost identical.

Through the whole research period in 2024, the number of analyses by month was relatively uniform. The greatest number of analyses was in April and August, with fewer analyses in January and June. A distinctive difference in acrylamide content was observed during the year. From January to July, the bad category accounted for about and less than 20%, while from August to December, this number increased to approximately 50%. Such a sudden change in results most likely lies in the way the potatoes for chip production are grown and stored. In order to achieve as low an acrylamide content as possible, information on the origin, growing method and storage must be available in further research.

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Review Paper

Antimicrobial stewardship for sustainable meat production in pig farming from One Health perspectives

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ABSTRACT

The widespread use of antimicrobials in pig farming has played a key role in improving animal health, reducing mortality, and ensuring productivity. However, inappropriate or excessive antimicrobial use contributes to the global challenge of antimicrobial resistance (AMR), posing risks to both public health and the sustainability of meat production. The increasing global concern over AMR has positioned livestock farming at the centre of sustainability debates. Pig farming, as one of the most intensive livestock production systems, plays a critical role in shaping antimicrobial use (AMU) patterns and in mitigating risks associated with AMR. This paper explores antimicrobial stewardship (AMS) in pig farming from regulatory, practical, and sustainability perspectives. It also highlights strategies that link AMS with sustainable meat production, including biosecurity measures, vaccination, and feed optimization. Drawing on regulatory frameworks, farm-level strategies, and the One Health approach, the study emphasizes the importance of aligning industry practices with global sustainability goals. The analysis highlights how AMS contributes to sustainable meat production and food system resilience.

1. Introduction

Antimicrobial resistance (AMR) poses an unprecedented global health and economic challenge, threatening human, animal, and environmental health (*Alhassan et al.*, 2025). The profound consequences of AMR on human health are well established (*Cassini et al.*, 2019; *Salehi et al.*, 2022), while in contrast, precise data quantifying the direct effects of AMR on animal health remain limited (*Emes et al.*, 2022). Even so, the veterinary field recognizes AMR's detrimental role in increased disease burden, higher mortality, and decreased productivity *(White & Hughes*, 2019; *Palma et al.*, 2020; *Martins et al.*, 2024), emphasizing the urgency of coordinated strategies to address this growing challenge.

Antimicrobial stewardship (AMS) refers to coordinated interventions designed to improve and measure the appropriate use of antimicrobials. Its primary objectives are to optimize therapeutic efficacy, reduce the emergence of AMR, and ensure animal welfare and food safety (*Guardabassi & Prescott*, 2015) through a coordinated set of actions. The need for AMS arises from the widespread antimicrobial use (AMU) in livestock production, often as growth promoters or for disease prevention, which has contributed significantly to the development of AMR (*Van Boeckel et al.*, 2015).

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In pig farming, AMU is particularly high due to intensive production systems that expose animals to increased disease pressure. The European Medicines Agency (EMA) has repeatedly emphasized that pigs are among the top consumers of veterinary antimicrobials within the European Union (EU) (*EMA*, 2020). The consequences extend beyond animal health, as resistant pathogens and resistance genes may spread to humans and the environment, threatening public health and ecosystems (*Ferri et al*., 2017). Acknowledging this reservoir, veterinarians are key actors in the One Health framework for AMS (*McEwen & Collignon*, 2018). AMS is closely aligned with the One Health concept, which recognizes the interdependence of human, animal, and environmental health. Thus, addressing AMR requires multisectoral cooperation, with livestock farming serving as a critical intervention point (*OIE*, 2019). Moreover, since many antimicrobial classes are shared between human and veterinary medicine, their use in animals can increase AMR in pathogens relevant to public health *(Marshall & Levy, 2011; Thanner et al*., 2016). Pig farming in Southeast Europe, particularly in countries where pork is a dominant component of the diet, presents both a challenge and an opportunity: while AMU remains relatively high, regulatory alignment with EU standards offers a pathway toward sustainable and responsible production.

This paper examines AMS in pig farming by analyzing regulatory frameworks, practical implementation strategies, and sustainability outcomes. By doing so, it highlights the central role of AMS in transitioning toward sustainable meat production systems. The present paper builds upon our earlier published work (*Kovačević et al.,* 2026), expanding the discussion toward sustainable meat production and regional implementation of AMS principles in pig farming through a One Health perspective.

2. Policy environment supporting sustainable antimicrobial use

EU has established a robust regulatory framework to ensure the prudent use of antimicrobials in animals, primarily through the EU Regulations 2019/4 (*Regulation, 2019/4,* 2019) and 2019/6 (*Regulation, 2019/6,* 2019), which replaced Directive 2001/82/EC (*Regulation, 1831/2003/EC,* 2003). These acts define clear obligations for veterinarians, producers, and pharmaceutical companies, including strict control of prophylactic use and a complete ban on growth promotion. A detailed overview of these EU regulatory foundations has been presented previously (*Kovačević et al*., 2026), providing the groundwork for the present discussion focused on their sustainability and implementation aspects. Beyond their legal intent, these measures reflect a broader policy shift toward integrating AMS into the EU's sustainability and *One Health* agenda (*WHO*, 2017).

At the international level, the World Health Organization (WHO), Food and Agriculture Organization (FAO), and World Organisation for Animal Health (WOAH, formerly OIE) provide guidance through the Global Action Plan on AMR (*WHO*, 2015). These frameworks emphasize the importance of monitoring, reporting, and reducing AMU in livestock, while fostering innovation in disease prevention strategies. In the regional context, Southeast European countries aspiring to EU accession have adopted harmonized regulations. National strategies now include restrictions on antimicrobial prophylaxis, requirements for veterinary prescriptions, and obligations for AMU reporting. These measures reflect a convergence with EU standards but also expose challenges in enforcement, particularly at the farm level where compliance depends on farmer awareness and veterinary capacity. They promote responsible prescription practices and encourage alternative strategies such as improved biosecurity, vaccination, and precision livestock farming to reduce antimicrobial dependence without affecting productivity (Lhermie et al., 2017). EMA, the European Food Safety Authority (EFSA), and the European Centre for Disease Prevention and Control (ECDC) jointly monitored AMU and resistance through the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) program, providing data for evidence-based policymaking and benchmarking across Member States (*EMA*, 2022). Within the *Farm to Fork Strategy* and the EU *One Health Action Plan*, AMS is recognized as a core element of sustainable agricultural policy rather than an isolated veterinary issue (*WHO*, 2015; *OECD*, 2022).

3. Translating policy into sustainable farm practices

Effective AMS in livestock depends on translating policy into farm-level action through practical, science-based management. AMS should be seen not as a regulatory burden but as a pathway to

greater productivity, animal welfare, and sustainability. Veterinarians play a central role by guiding diagnostics-based prescribing and ensuring responsible use of critically important antimicrobials (*WHO*, 2017; *EMA*, 2019). In particular, third- and fourth-generation cephalosporins, fluoroquinolones, and colistin are tightly restricted in animals to safeguard their effectiveness for people (*ECDC, EFSA and EMA*, 2017). Farms that implement all-in/all-out systems, maintain strict sanitation, and adopt vaccination strategies consistently report lower AMU levels. Beside vaccination programs, alternatives to antimicrobials, such as prebiotics, probiotics, and feed additives (organic acids and essential oils), are alternatives that improve gut health and resilience (*De Lange et al*., 2010; *Xu et al*., 2020; *Nhara et al*., 2024). Educational campaigns and incentive schemes have proven effective in improving compliance with AMS programs (*Hardefeldt et al*., 2018; *Gozdzielewska et al*., 2020). Ultimately, on-farm AMS implementation embodies the One Health vision, linking responsible veterinary practice with environmental protection and public health objectives.

4. Antimicrobial stewardship in a sustainability context: One Health and regional perspectives

AMS contributes directly to sustainable meat production by aligning animal health management with environmental and public health goals. By reducing AMU, AMS decreases the risk of resistant pathogens entering the food chain, protects ecosystems from antimicrobial residues, and ensures the long-term efficacy of essential medicines. Actually, successful AMS in veterinary medicine has shown reduced AMU without harming productivity. Denmark and the Netherlands stand out, implementing policies focused on veterinary training, regulation, and surveillance (*Robinson et al.*, 2016). Denmark's "Yellow Card" system, introduced in 2010, set usage thresholds and achieved a 27% drop in livestock antibiotic consumption *(Time & Veggeland*, 2020; *WOAH*, 2022). Figure 1 provides an overview of the main elements of AMS in pig farming, highlighting the interaction between management practices, AMU, and sustainability outcomes.

In Southeast Europe, where pork production is a cornerstone of the agricultural economy, AMS also has socio-economic significance. Aligning local practices with EU frameworks enhances competitiveness in international markets while supporting national food security. Importantly, AMS is integral to the One Health approach, which integrates the interests of veterinary, human health, and environmental sectors (*Hibbard et al*., 2024). According to James et al. (2025), the key drivers of effective AMS include strong governance, education, access to diagnostics, and digital surveillance all of which require context-specific adaptation across different production systems. Recent evidence from Uganda further illustrates that farm-level factors such as hygiene and the intensity of production directly influence the spread of resistance, with pigs in semi-intensive systems being more than twice as likely to harbor resistant bacteria compared to those in free-range settings (*Muwonge et al.*, 2025).

AMS should also be viewed in relation to the Sustainable Development Goals (SDGs) which promotes reduced AMU, supports SDG 3 (Good Health and Well-being), SDG 12 (Responsible Consumption and Production), and SDG 15 (Life on Land) (*Alhassan et al*., 2025). Thus, AMS is not only a veterinary or regulatory concern but a broader sustainability imperative.

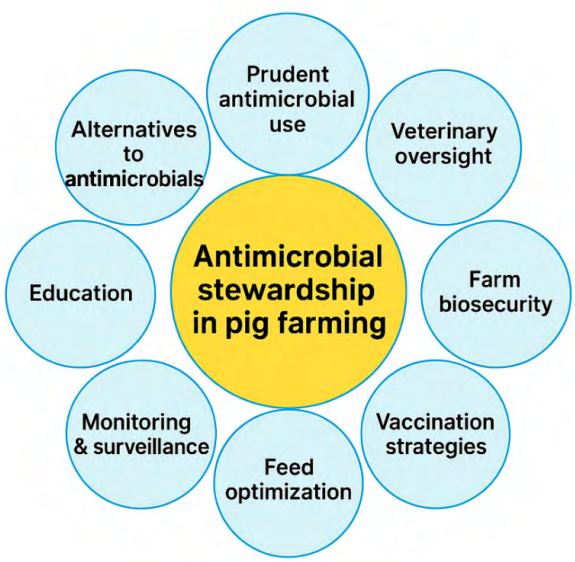

Figure 1. Key components of antimicrobial stewardship in pig farming.

5. Conclusion

AMS is no longer optional but an essential component for sustainable meat production. While regulatory frameworks establish necessary restrictions and monitoring requirements, real progress depends on farm-level implementation supported by veterinary oversight, farmer engagement, and preventive

health strategies. The One Health perspective further underlines the interconnectedness of AMS with human health and environmental protection. In the regional context of Southeast Europe, aligning pig production practices with EU regulatory frameworks presents both a challenge and an opportunity for ensuring sustainable, competitive, and resilient food systems. The path forward requires cross-sectoral collaboration, continuous surveillance, and global commitment to responsible AMU.

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Review Paper

Transformation of the meat production chain towards sustainability

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ABSTRACT

The global demand for meat is projected to rise significantly by 2050, necessitating a transformation in traditional meat production systems to address environmental, economic, and ethical concerns. Industrial livestock farming contributes to some extent to greenhouse gasses emissions, deforestation, biodiversity loss, and water pollution, highlighting the urgent need for sustainable practices. Concerns over animal welfare, food safety, public health, environmental health, and economic inequalities further emphasize the need for transformation of the meat chain. This paper briefly explores strategic solutions, such as sustainable farming practices, point-of-care food safety control methods, valorization of animal by-products, regulatory reforms, and consumer awareness initiatives. Addressing industry resistance through financial and technological support is also crucial for a successful transition. A multi-level approach integrating innovative technologies, policy support, and consumer engagement is essential to achieving transformation to resilient, sustainable, and ethically responsible meat production system.

1. Introduction

The global demand for meat is expected to rise significantly by 2050, putting immense pressure on existing traditional meat production systems to transform towards sustainability due to its significant environmental, economic, and ethical impacts (*FAO*, 2017). This presents an urgent need to develop more sustainable practices that reduce greenhouse gas (GHG) emissions, minimize resource consumption (land, water), and ensure ethical treatment of animals (*Dopelt et al.*, 2019).

Two declarations, the Dublin Declaration (2022) and the Denver Call for Action (2024), related to the interconnection between agriculture, livestock and food systems, were issued by a group of scientists advocating for the importance of livestock in sustainable food systems advocating for a more balanced and science-based perspective on livestock's role (*Leroy and Ederer*, 2023). The Dublin Declaration (https://www.dublin-declaration.org/) emphasizes that livestock contributes positively to nutrition, livelihoods, ecosystems, and food security. It argues that eliminating or drastically reducing meat consumption could have unintended negative consequences on human health and rural economies. The Denver Call for action (https://www.dublin-declaration.org/the-denver-call-for-action) advocates recognizing the complex roles that well-managed livestock play in enhancing nutrition, supporting livelihoods, and providing essential ecosystem services such as biodiversity maintenance and soil fertility. It calls for

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policies grounded in comprehensive, evidence-based assessments that consider ethical, economic, social, and environmental dimensions of sustainability. Both declarations advocate a balanced, science-guided discussions on livestock's role in sustainability, rather than blanket reductions in meat consumption. Achieving sustainability in the meat production chain requires systemic changes involving production methods, such as adoption of responsible farming practices, innovation, shortening supply chains, new regulatory frameworks, and including changing consumer behavior (*Caccialanza et al*., 2023). This paper explores the current challenges, barriers, and strategic solutions necessary for creating a more sustainable meat production system.

2. Current challenges in the meat production chain

The conventional meat industry is dominated by large-scale industrial farming, which presents several sustainability challenges, as presented below.

2.1. Environmental impact

The sources of GHG emissions from livestock sector have been divided to seven modules: (i) from livestock biological processes (CH_4 from enteric fermentation), (ii) from land use change, (iii) from livestock feed production, (iv) from manure management (N_2O, CH_4, NH_3), (v) from cultivated soils (N_2O from fertilizer application), (vi) from land degradation and desertification, and (vii) from animal respiration. Industrial livestock farming is believed to be responsible for approximately 11.1% of global anthropogenic GHG emissions based on data from the ruminant supply chain, assuming its contribution to global warming (*FAO*, 2023). Furthermore, emissions of N_2O and CH_4 from livestock manure contribute together around 10% of the total non-CO_2 GHG emissions (*Møller et al*., 2022). A potential solution can be found in effective recycling of livestock manure to reducing the negative impact of livestock-associated GHG emissions (*Liu et al.*, 2023). Deforestation to make way for pastureland and feed crop production further exacerbates carbon emissions, contributing to 17.4 % of all GHG from anthropogenic sources (*IPCC*, 2007). Excessive use of chemical fertilizers and pesticides in feed production contaminates water sources, leading to eutrophication and the degradation of aquatic ecosystems (*Akinnawo*, 2023). Intensive farming leads also to severe biodiversity loss due to habitat destruction and land-use change (*Machovina et al*., 2015). Large-scale meat production operations require vast amounts of land (integrated supply chain 'feed-to-fork'), often obtained by clearing forests, wetlands, and grasslands, displacing wildlife, and disrupting ecosystems (*Kraham et al*., 2017). Water consumption in industrial meat production is also a critical concern, with around 13.16 m^3 of H_2O needed to produce just one kilogram of beef (*Navarrete-Molina et al.*, 2019). The high concentration of animal waste in confined animal feeding operations (CAFOs) contaminates soil and water bodies, causing harmful algal blooms and dead zones in coastal regions (*Burkholder et al*., 2006).

2.2. Animal welfare concerns

Intensive livestock farming ('factory farming') is designed to maximize production efficiency while minimizing costs. This involves keeping a large number of animals in confined spaces, often in restrictive cages or overcrowded barns (*Anomaly*, 2014), limiting natural behaviors such as foraging and social interactions, and leading to discomfort among animals. Selective breeding for rapid growth and high yield can have severe health issues, such as skeletal disorders in poultry and lameness in cattle (*Hartcher and Lum*, 2019). The transportation of livestock from farms to slaughterhouses is a critical phase that significantly impacts animal welfare. Long journeys, overcrowded conditions, and improper handling can cause severe stress, injuries, and fatalities among transported animals. Rough handling by workers and the use of electric prods can cause pain and fear among animals (*Ljungberg et al*., 2007; *Lambooij*, 2024). Re-establishing short meat supply chains, by promoting investment in local slaughterhouses, can reduce the need for long-distance transport minimizing the stress experienced by livestock (*Kneafsey et al*., 2013).

2.3. Public health risks

Foodborne hazards are significant concern in industrial meat production, with public health risks from low-level farm biosecurity, poor heard health management, improper handling and unhygienic processing at slaughter, and cross-contamination during further stages along meat supply chain (deboning, meat-processing, distribution, retail). Pathogens such as *Salmonella*, *Campylobacter*, Shiga

toxin-producing *E. coli* (STEC), and *Listeria monocytogenes* are commonly associated with meat products and can lead to severe foodborne illnesses (*Lianou et al.*, 2017), in particular in vulnerable populations including children, the elderly, pregnant women, and immunocompromised individuals (YOPI). Chemical residues from pesticides, hormones, dioxins, and heavy metals in meat products raise additional health concerns (*Onyeaka et al*., 2024). The overuse/misuse of antibiotics in animal farming is another pressing public health risk, as it contributes to the emergence of antimicrobial-resistant (AMR) bacteria and reduced therapy response in livestock and people. To mitigate these risks, policymakers and industry stakeholders must enforce stricter regulations on antibiotic use in livestock production and promote alternative disease prevention strategies (*Nastasijević et al*., 2023).

2.4. Food safety control

Conventional culturing methods, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) are widely used for detecting foodborne pathogens in the meat chain to meet the regulatory food safety requirements, but they are often time-consuming, labor-intensive, and require specific laboratory facilities. This emphasizes the need for the cheap and point-of-care devices, e.g., biosensors offering rapid, accurate (sensitive and specific), and on-site food safety diagnostics to reduce contamination risks (*Nastasijević et al*., 2025).

2.5. Economic inequality

Industrial meat production often outcompetes small and medium enterprises (SMEs), creating monopolization and reducing diversity in meat production. Large corporations produce meat at lower costs, benefiting from vertical integration, controlling multiple stages of the supply chain, from feed production to meat processing and retail (feed-to-retail continuum), also having the financial strength to fulfil the livestock environmental regulations (*Jiang et al*., 2023). In attempts to find the best way to organize meat production between large-versus small-scale production systems or long-versus short meat supply chain, it would be wise to adopt the attitude that coexistence of both ways can be effective and expected in the long run (*Schulze et al*., 2006).

3. Sustainable transformation strategies

To overcome mentioned barriers and drive a sustainable transformation of the meat production chain to lessen the environmental footprint, a multilayer approach is needed as presented below.

3.1. Advancing sustainable farming practices

Agro-ecological approaches, regenerative agriculture techniques, and valorization of animal by-products can reduce reliance on chemical inputs, improving animal welfare standards, promoting alternative protein sources, and enhancing the circular economy. An agroecological approach involves practices, such as pasture-based and rotational grazing systems, to reduce environmental impact (*Marchegiani et al*., 2025). Regenerative agriculture supports soil health, reducing chemical inputs and enhancing biodiversity (*Borsari*, 2020). Valorization of animal by-products from slaughter can be done by incorporating high-protein and micronutrient-rich materials into food products, such as Ready-to-use Supplementary Food (RUSF) and Ready-to-use Therapeutic Food (RUTF) (*Fetriyuna et al*., 2023).

3.2. Enhancing food safety testing

As food safety testing can be time-consuming and expensive (costs for collection of samples, shipment to the accredited laboratory and labor), the development of alternative methods that are cheaper and can be applied on-site is needed, such as biosensors. Biosensors are devices which offer real-time monitoring to detect foodborne hazards at selected critical points along the meat chain (farm-retail continuum) by recognizing a target 'biomarker' characteristic for a particular pathogen or chemical contaminant via an immobilized sensing element called a 'bioreceptor' (monoclonal antibody, RNA, DNA, aptamer, glycan, lectin, enzyme, tissue, whole cell). By integrating biosensors into the meat safety assurance system, early and rapid (response 20-60 min) quantitative detection of foodborne pathogens, toxins, and food spoilage markers becomes possible, reducing reliance on time-consuming traditional methods, e.g., 5-7 days for culturing for *Salmonella* and *Listeria*, respectively, or 24 h for ELISA and PCR. Biosensors transform the meat chain by enabling proactive risk mitigation, enhancing food safety management and compliance with food safety regulations, reducing food waste through early

intervention, and increasing consumer confidence in meat products (*Nastasijević et al*., 2025).

3.3. Investment in alternative proteins

Re-using the proteins from animal by-products leads to reducing the environmental and ethical issues and promoting the circular economy (*Mylan et al*., 2023). Food supplements from proteins sourced from animal by-products present a sustainable means of utilizing nutrient-rich materials that would otherwise be wasted, offering high-quality protein sources for human and animal nutrition. This presents a foreseeable future in transformation of the meat chain (*Fetriyuna et al*., 2023). Further, 'hybrid products' (blending plant-based proteins with meat) can act as a transitional solution for reducing meat consumption without drastic dietary changes (*Profeta et al.*, 2021). Governments and private investors should accelerate funding for lab-grown (cultured) meat technologies to enhance scalability and affordability (*Mancini and Antonioli*, 2022).

3.4. Policy and regulatory support

Effective policy support is essential for driving sustainability in the meat chain. The governments should find ways to simplify complex compliance requirements, such as certification processes for organic and sustainable meat production, to encourage small-scale farmers while providing the platform for ensuring food safety (*Home et al.,* 2017). Subsidies should be revised by redirecting financial support from conventional industrial livestock farming more to sustainable and alternative protein production systems (RUSF, RUTF). This helps the meat sector to transform itself more efficiently and maintain its position in a global market (*Sutton et al*., 2024).

4. Consumer awareness and behavioral change

Educational campaigns should actively promote the benefits of sustainable meat production (*Ramsing et al*., 2021). Consumers should be encouraged to adopt flexitarian diets (more frequent consumption of hybrid or plant-based products) advocating moderate use of good quality meat and meat product programs (*Dagevos*, 2021). Since consumers tend to be resistant to fundamental changes in their diet, a rapid transition is not foreseen (*Siegrist et al*., 2024). Pricing and labeling strategies and affordable prices for sustainable meat products should guide consumer choices (*Amman et al*., 2023). Overcoming industry resistance rooted in emotional ties to conventional practices requires coordinated support from governments and stakeholders through technology, incentives, and education (*Hübel and Schaltegger*, 2022).

5. Conclusion

Transforming the meat production chain is both a pressing necessity and a complex, multifaceted challenge requiring multi-sectorial efforts across environmental, economic, ethical, and public health dimensions. Addressing animal welfare concerns, public health risks, innovations in food safety testing, valorization of animal waste proteins, adjustment of regulatory framework, addressing economic inequalities, and changing consumer behavior are crucial for this transformation, which will support a more ethical, resilient, and sustainable meat chain to ensure food security and public health protection for future generations.

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Original Scientific Paper

Raising broilers without antibiotics: challenges and alternatives

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ABSTRACT

Intensive poultry production has long relied on the use of antibiotics to improve animal health and yield. However, growing concerns about the emergence of antimicrobial resistance have led to restrictions and bans on the use of antibiotics as growth promoters in many countries. "Raised without antibiotics" production can be described as the production of broilers that have not come into contact with antibiotics during the production cycle, whether for prophylactic, therapeutic or growth-stimulating purposes.

The aim of this study was to demonstrate the opportunities and challenges in raised without antibiotics poultry production, focusing on the available alternative measures such as probiotics, phytobiotics, organic acids, improved biosecurity, hygiene and management. The data presented were obtained based on observational and survey-based methodologies. Test matrices were water, feed and litter. The technique used to determine the presence of antibiotics was ultra-high performance liquid-chromatography-mass spectrometry (UPLC-MS/MS).

From January 2020 to June 2025, antibiotic use on the studied farms steadily declined from 20.62 to 4.70 mg/kg of live weight. In the total of 3320 broiler production cycles examined on 200 farms, it was found that 62.14% of production runs met the requirements for antibiotic-free rearing. From such facilities, 59.36% of slaughtered broilers met the requirements for the Raised Without Antibiotics certificate.

The results indicate that it is possible to achieve satisfactory production indicators and maintain good animal health, but with strict adherence to preventive measures and precise management.

1. Introduction

"Raised without antibiotics" production means the production of broiler chickens that have not come into contact with antibiotics during the production cycle, whether for prophylactic, therapeutic or growth-stimulating purposes. In addition to this term, the term antibiotic-free is also often used. Unlike this production, traditional broiler chicken production allows the use of antibiotics for therapeutic purposes. For the avoidance of doubt, it should be noted that neither antibiotic-free nor traditional production allows the presence of antibiotics in the final product, i.e., in the meat and organs of the slaughtered chicken.

Poultry production is one of the most dynamic sectors of livestock production, with high produc-

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tion intensity and relatively fast return on investment. Traditionally, broiler farming has relied on the use of antibiotics not only for therapeutic purposes, but also as growth stimulants, thereby contributing to more efficient feed conversion, lower mortality and better economic profitability (*Castanon*, 2007). However, a growing body of evidence suggests a link between the use of antibiotics in animal production and the development of antimicrobial resistance in pathogens that can be transmitted to humans, which represents a serious global health challenge (*World Health Organization - WHO*, 2022). In response to these threats, the European Union banned the use of antibiotics for growth promotion in 2006 (*Regulation (EC) No. 1831/2003*, 2003). Similar measures have been introduced in the USA, China and Canada according to the Global Research on Antimicrobial Resistance (GRAM) report (*Murray et al*., 2022), while many other countries are implementing similar measures or planning a transition to a raised without antibiotics production model. All this should be integrated into the One Health concept, which implies the joint action by human and veterinary medicine to combat zoonoses, reduce antimicrobial resistance, and protect the environment and animal welfare (*Nedic et al.,* 2023). In this context, there is a need to develop effective and safe alternatives that will enable the preservation of animal health and meet technological and market requirements.

The use of antibiotics in poultry farming began in the mid-20th century, when it was discovered that certain subtherapeutic doses could significantly improve growth and feed efficiency in broilers. Antibiotics soon became an integral part of industrial poultry production, not only for therapeutic and prophylactic purposes, but also as so-called growth promoters (*Gadde et al*., 2017). However, the widespread and often uncontrolled use of antibiotics has led to the emergence of resistant strains of bacteria, both in animal and human populations. According to the *World Health Organization - WHO* (2022), antimicrobial resistance is among the ten greatest global threats to public health. In addition, antibiotic residues in meat and poultry products pose an added risk to consumer health. In the EU, around 33,000 human deaths due to antimicrobial resistance are recorded annually (*Cassini et al*., 2019), and estimates indicate that antimicrobial resistance could cause up to 10 million deaths per year by 2050 (*O'Neill*, 2016).

The use of antibiotics in poultry is regulated by a series of regulations with the aim of preventing the presence of residues in meat consumers. According to the European Union regulations and the Codex Alimentarius standard, it is prohibited to place on the market animal products that contain antibiotic residues above the prescribed maximum permitted levels (Maximum Residue Limits – MRLs) (Regulation (EC) No. 1831/2003). In order to avoid the presence of antibiotics in tissues, the implementation of a withdrawal period is crucial – this is the period that must elapse between the last application of antibiotics and the slaughter of the animal. Some antibiotics have a longer withdrawal period, especially those that accumulate in the liver or muscles. Failure to comply with the withdrawal period is a violation and directly threatens food safety.

In addition to antimicrobial resistance, antibiotic residues in food can cause other health problems in sensitive consumers. The most common consequence includes allergy reactions – especially to antibiotics such as penicillins and sulfonamides, even at very low concentrations (*Pawankar et al*., 2013). The presence of antibiotics can disrupt the microbiome and lead to digestive problems. Long-term exposure to residual amounts can lead to the development of hypersensitivity. Some trace amounts of antibiotics can affect the immune system response (immunosuppressive effects) (*Sharma et al*., 2018). The long-term effects in children and pregnant women are unknown because there is insufficient data on the effects of chronic exposure.

Studies show that improving animal welfare through ventilation, natural lighting, environmental enrichment and stress reduction leads to a lower incidence of disease and, therefore, a reduced need for antibiotics (*Broom*, 2010). Poultry production also encourages fair treatment of animals in accordance with the principles of the five freedoms, which can also influence consumer perception and increase the value of the product.

Reducing or eliminating the use of antibiotics in poultry production contributes to reduced contamination of soil, water and feed with drug residues. Antibiotics are often excreted unchanged from animals, entering ecosystems where they can disrupt the microbiological balance and promote the development of resistant bacteria in nature (*Kemper*, 2008).

Veterinary drug residues are tested using various screening (STAR protocol; PremiTest, four-plate, five-plate, and six-plate ELISA) and confirmatory methods such as LC-Orbitrap HRAM, UPLC-MS/MS, HPLC-FLD, and HPLC-UV. Certain studies

highlight the importance of simultaneous sampling of multiple matrices (feed, water, litter) in order to detect the possible presence of antibiotics on the farm (*Cortés et al*. 2025; *Davis et al*., 2021). Laboratory analyses involve several types of different analyses that are recommended in raised without antibiotics production (*Kemper*, 2008). The research objective of this study was to demonstrate the opportunities and challenges in raised without antibiotics poultry production, focusing on the available alternative measures such as probiotics, phytobiotics, and organic acids, along with improved biosecurity, hygiene, and management.

2. Materials and methods

The research was conducted in cooperation with a company from Bosnia and Herzegovina that markets chicken under the label Raised Without Antibiotics. They market chicken carcasses, fillets, wings and drumsticks, packaged on trays with plastic foil wrap and labelled as "Raised Without Antibiotics". Continuous education of farmers is carried out regularly, conditions and hygiene are improved, alternative means for improving immunity are given if necessary, quality food and water are used and the entire system is monitored. The same company previously produced and marketed chickens in bulk, without prior labeling.

The research was conducted in the period from January 2020 to June 2025 on a total of 3320 production cycles in 200 facilities. The certification body prepared a sampling plan based on the company's business volume, which included the type of samples, locations and sampling dynamics. Test matrices were water, feed and litter. Samples from these were selected because of the possible route of antibiotic administration, i.e., orally, as this is the only way to distribute the antibiotic to a large number of broilers. Unannounced sampling was carried out and 660 analyses were performed for 10 antibiotics most commonly used in poultry production. The sampling was unannounced for reasons of impartiality, i.e., the impossibility of any possible influence on the quality and integrity of the samples.

The technique used for the detection of antibiotics was ultra-high performance liquid chromatography-mass spectrometry (UPLC-MS/MS). The samples were analyzed for the presence of antibiotics identified as most commonly used in poultry farming, including those that are banned: amoxicillin (β-lactam), enrofloxacin (fluoroquinolone), doxycycline, oxytetracycline and tetracycline, sulfadiazine + trimethoprim (sulfonamides), flumequine (quinolone), florfenicol and chloramphenicol (amphenicol), nitrofurans and malachite green.

For water, animal feed and litter samples, the regulation does not prescribe the MRL. The detection limit of the method used was <0.1 µg/kg. A sample was considered positive if the presence of any of the target substances was detected above the detection limit of the method.

3. Results

No antibiotic residues were detected in any of the feed, water and litter samples. Of the total of 3,320 production cycles, it was found that 2,063 production cycles or 62.14% were raised without the use of antibiotics, and 1,257 or 37.86% were raised in the traditional way with the use of antibiotics for therapeutic purposes (Figure 1).

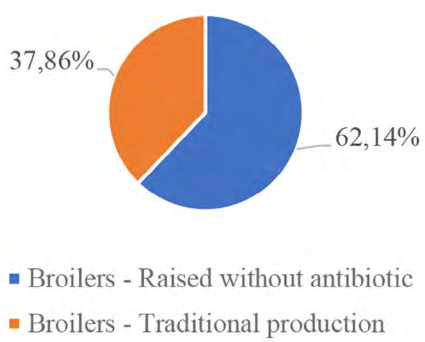

Figure 1. Proportion of broiler farming production cycles with (traditional production) and without the use of antibiotics

The share of slaughtered broilers raised without antibiotics was 59.36%, while 40.64% of the animals had experienced antibiotic use (Table 1).

Table 1. Overview of the number of slaughtered broilers raised without the use of antibiotics and traditional farming (using antibiotics)

Total number of slaughtered broilers	Traditional production (No)	Traditional production (%)	Raised without antibiotics (No)	Raised without antibiotics (%)
55,661,670	22,622,267	40.64	33,039,403	59.36

Table 2. Feed conversion ratio (FCR) and number of production cycles by broiler production types from 2020 to 2025

Type of production	Production cycles													
	2020		2021		2022		2023		2024		2025		Total	
	No.	FCR	No.	FCR	No.	FCR	No.	FCR	No.	FCR	No.	FCR	No.	FCR
Traditional production	30	1.83	278	1.82	269	1.82	262	1.80	300	1.79	118	1.79	1257	1.81
Raised without antibiotics	36	1.83	363	1.84	458	1.82	480	1.80	528	1.80	198	1.80	2063	1.81

Table 2 provides an overview of the number of breeding cycles and the average feed conversion ratio in the two types of broiler farming. No significant difference in feed conversion was found in the raised without antibiotics and traditional production systems.

Mortality and technological scrap of chickens is a very important parameter in broiler farming (Table 3). The measured percentage of mortality and technological scrap was higher in traditional farming than in the raised without antibiotics system because even with therapy, significant mortality of broilers occurs. This is also explained by the fact that antibiotics are used as a last resort, respecting the welfare of the animals. When therapy is applied at any stage of production, the entire batch cannot obtain the status of "Raised Without Antibiotics".

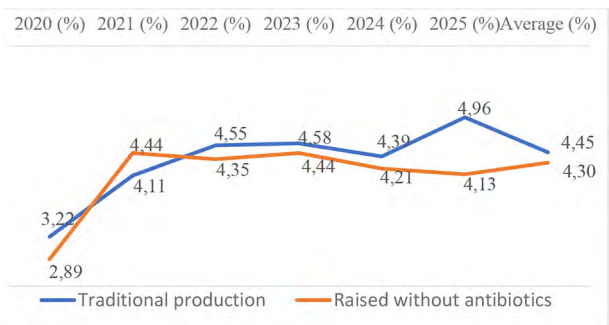

Figure 2. Percentage of mortality by batch in the studied facilities

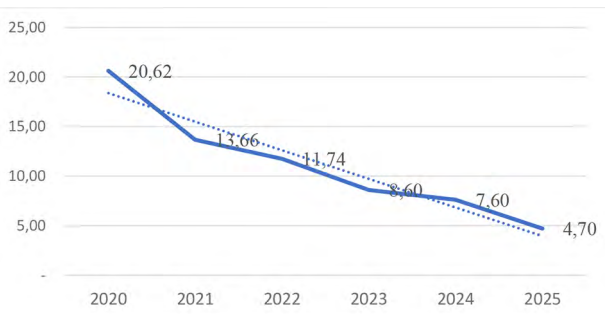

Figure 3. Antibiotic consumption on the 200 studied broiler farms (mg/kg of live weight)

On farms where broilers were not raised without antibiotics, the use of antibiotics was often necessary. In spite of that, however, Figure 3 shows that antibiotic consumption per kilogram of live weight in broiler farming has been in a constant decline over the last six years.

4. Discussion

The World Health Organization (WHO), the Food and Agriculture Organization (FAO) and the World Organization for Animal Health (WOAH) jointly recommend reducing the unreasonable use of antibiotics, respecting withdrawal periods and developing national residue monitoring programs in food of animal origin (*WHO*, 2022). Several countries have effective control systems, which include regular sampling of chicken meat in shops and slaughterhouses.

Transition to the raised without antibiotics broiler farming model directly affects animal welfare practices. Raised without antibiotics production requires better housing conditions, lower stocking density, adequate microclimate, ventilation, hygiene and general supervision, all in order to reduce the risk of disease (*Iannetti et al*., 2021). In such conditions, the need for the introduction of animal health monitoring programs is increased, with an emphasis on prevention rather than intervention.

Raised without antibiotics broiler production requires a multidisciplinary approach. Scientific research indicates the effectiveness of probiotics, prebiotics, phytobiotics and organic acids as alternatives to antibiotics. Probiotics stabilize the intestinal microbiota (*Mountzouris et al*., 2010), while phytobiotics such as oregano and thyme have antimicrobial effects (*Hashemi & Davoodi,* 2011). Organic acids lower pH and inhibit the growth of pathogens (*Dibner & Buttin*, 2002). In addition, biosecurity – disinfection, entry and vector control – plays a key

role. Genetic selection for resistance is also gaining importance (*Gogolin-Ewens et al*., 1990).

Raised without antibiotics, thus, is an important element in a sustainable poultry production strategy. This antibiotic-free production system requires more efficient waste management, better animal handling and adapted feeding systems that reduce nitrogen and phosphorus emissions to the environment (*Thanner et al*., 2016). This is particularly important in the context of climate change and biodiversity conservation.

Denmark and the Netherlands were among the first to reduce antibiotic use in farmed animals (*Aarestrup*, 2012; *Speksnijder et al*., 2015). The USA is developing raised without antibiotic production models, motivated by consumer demands. Antibiotic-free production conditions show a slight decrease in growth and increased mortality, but with good nutrition and hygiene, these effects are mitigated (*Hoelzer et al.*, 2018). Production costs are higher, but they are compensated by a higher product price and better market positioning (*Salois et al*., 2016).

In our study, the proportion of broiler production cycles from antibiotic-free rearing was 62.14%, while the proportion of broilers slaughtered from antibiotic-free rearing was 59.36%. No significant difference in FCR was observed between the two production systems over the years, indicating that it is possible to maintain productivity with a strong preventive approach. Raised without antibiotics production had 0.15% lower total mortality than the antibiotic-treated broilers from traditionally raised birds. In broiler farming, antibiotics are used only when preventive measures cannot prevent the spread of disease and bird deaths. By using antibiotics, that production cycle loses its "Raised Without Antibiotics" status. Treated broilers remain on the farm until the antibiotic is eliminated from the birds' bodies.

The observed decreasing trend in antibiotic consumption (mg/kg live weight) is consistent with European and American experiences in raised without antibiotics programs, where key effects are achieved through a combination of biosecurity, vaccination and nutritional strategies. For example, in Denmark, the share of antibiotics in broiler production was reduced by more than 50% between 2010 and 2020 (*DANMAP*, 2021). Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2022 (MARAN) has recorded a reduction of over 70% in total consumption of antibiotics in the raised without antibiotics production in the period 2011–2022 (MARAN, 2023). In the USA, according to *USDA* (2021), more than 60% of commercial broilers are raised in no antibiotics ever (NAE) systems. *Singer et al.* (2020) point out that the implementation of biosecurity measures and vaccination is crucial for maintaining performance in the raised without antibiotics system, while *Aarestrup* (2012) emphasizes the importance of an integrated approach in reducing antimicrobial resistance.

Our results show that it is possible to produce broilers without antibiotics by using alternatives with improved and controlled farm conditions. Alternatives, such as probiotics and phytobiotics, have the potential to replace antibiotics in maintaining health and improving productivity. The literature supports this approach, and the economic aspects can be positive under market support conditions (*Salois et al*., 2016).

5. Conclusion

The study confirms that a raised without antibiotics broiler production model is feasible with appropriate biosecurity measures and improved farm conditions. The results on a large sample of broiler production cycles confirm that raised without antibiotics broiler production is sustainable: we measured a stable feed conversion ratio (FCR) over time, higher shares of flocks and of slaughtered birds in raised without antibiotics systems than in traditional systems, lower mortality/technological scrap rates of the antibiotic-free flocks and a clear decrease in antibiotic consumption over time.

Consumer awareness, regulatory enforcement, and a developed market contribute to the expansion of antibiotic-free broiler production. The results presented are an encouragement and basis for the expansion of the program and its implementation in other companies in Bosnia and Herzegovina and in the region. Raising broilers without antibiotics has many benefits, but insufficient laboratories and expensive laboratory methods are a complicating factor. It is necessary to continuously educate farmers and raise consumer awareness about the benefits of rearing without antibiotics, specifically reducing antimicrobial resistance, strengthening animal welfare and protecting the environment (the One Health concept). Future research should contribute to a more precise assessment of the efficiency and cost-effectiveness of this type of farming.

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Original Scientific Paper

Detection of *Salmonella spp.* in clinical and food samples from poultry (2020–2025): a broiler farm outbreak case study - highlighting biosecurity and economic implications

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ABSTRACT

This study analyzed the positive rate of *Salmonella* spp. in chicken primary production and food samples examined at the Scientific Institute of Veterinary Medicine of Serbia between 2020 and 2025 and evaluated biosecurity measures on a broiler farm during an outbreak. A total of 2,728 primary production samples and 6,289 food samples were tested. *Salmonella* was detected in 3.23% of the primary output and 9.76% of food samples, with S. Enteritidis being the most common serovar. A detailed biosecurity assessment of the affected farm revealed an overall score of 90%, surpassing national and global averages, yet it identified critical gaps in broiler depopulation, carcass removal, and cleaning protocols. An economic analysis showed a loss exceeding 58.2% per production cycle of 10,000 broilers due to market restrictions following *Salmonella* detection. These findings highlight the need for continuous biosecurity improvements and rigorous surveillance to mitigate *Salmonella* risk and reduce financial impacts in poultry production.

1. Introduction

Salmonella spp. is globally recognized as a significant zoonotic foodborne pathogen, posing major public health concerns and causing substantial economic losses in both animal production and human healthcare systems (*Majowicz et al*., 2010). Foodborne illness remains a pressing issue in Serbia. In 2023, salmonellosis was the most frequently reported bacterial enteric disease in humans in Serbia, with 1,217 confirmed cases. Food was identified as the transmission route in 14 of the 21 recorded outbreaks that year (*Institute of Public Health of Serbia "Dr Milan Jovanović Batut"*, 2024). However, this represents the number of reported cases, while the actual number is assumed to be significantly higher due to underreporting. In response, both veterinary and human health sectors are actively working to control and reduce the presence of *Salmonella* along the food production chain. Collaboration between these two sectors is of particular importance in addressing the issue of antimicrobial resistance. A particularly serious concern is the rise of multidrug-resistant *Salmonella* strains (*Petrović et al*., 2015; *Nikolić et al*., 2017; *Kureljušić et al*., 2017; *Kureljušić et al*., 2021). Emerging antimicrobial-resistant variants continue to pose a significant threat to both animal and public health (*Wigley*, 2024). In the Serbian poultry industry, the predominant serovars are *Salmonella* Enteritidis, *S.* Infantis, and *S.* Typhimurium (*Velhner et*

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al., 2011; *Spalević et al*., 2023). Poultry is considered a primary source of human salmonellosis, primarily transmitted through the consumption of contaminated meat and eggs (*Shaji et al*., 2023). Birds can become infected during production through various routes, including contaminated feed, litter, water, aerosols, and contact with vectors such as rodents, insects, or other animals (*O'Bryan et al*., 2022). Complete eradication of *Salmonella* is deemed unrealistic. Therefore, effective control strategies rely on vaccination—particularly with multivalent vaccines—combined with strict biosecurity measures and robust surveillance systems capable of identifying production risks, antimicrobial-resistant strains, and serovars not covered by vaccines (*Wigley*, 2024). To reduce the incidence of human salmonellosis, veterinary authorities must implement control measures at all stages—from poultry rearing and production to slaughter and processing, as well as consumer education (*Velhner et al*., 2005; *Official Gazette of RS*, 2024).

This study aimed to analyze the positive rate of *Salmonella* spp. and its serotypes in primary production and food samples collected between 2020 and 2025, to investigate potential transmission pathways on broiler farms, and to assess the associated financial impact in the event of an outbreak.

2. Materials and methods

At the Scientific Institute of Veterinary Medicine of Serbia, a total of 2,728 chicken primary production samples were analyzed for *Salmonella* over five and a half years. These samples were chicken droppings from broiler, laying hens, and breeder flocks; incubated eggs (both unhatched eggs at the end of the hatching process and hatched eggshells); transport crates and carcasses from the transport and the first three days of life. Primary production specimen sampling was conducted per local regulations (*Official Gazette RS*, 2018; *Official Gazette RS*, 2024).

Additionally, 6,289 food samples from broiler slaughterhouses—broiler meat, meat products, neck skin, and environmental swabs from food-processing surfaces (such as working tables, knives, cutting boards, and equipment in contact with raw poultry)—were included in the study. The *Salmonella* spp. detection, isolation, and serotyping were performed according to respective parts of *ISO 6579* (2017).

To explore potential transmission pathways of *Salmonella* on broiler farms and the associated financial costs, a case study was conducted on a farm that experienced an outbreak in the early months of the year. This farm houses 10,000 broiler chickens in a single production unit and completes approximately 4.5 production cycles annually, introducing new birds every 11 to 12 weeks. Broilers are typically slaughtered between 42 and 56 days of age, resulting in a downtime of at least 14 days between cycles. The pathogen was detected in fecal samples collected within three weeks before slaughter. Farm observations and assessments of biosecurity measures were conducted using a standardized broiler farm checklist. The Biocheck.UGent risk-based scoring system (http://www.biocheck.ugent.be) was employed to evaluate broiler farm biosecurity. The overall score was calculated as the average of the internal and external biosecurity scores. The veterinarian and farm owner were informed of the study's purpose and process before filling out the questionnaire. The farm owner provided written informed consent for data collection, sharing, and publication. During the visit, their responses were compared with actual on-farm observations, and the correct answers were recorded accordingly.

To assess the financial impact of the *Salmonella*-positive finding, a partial budget analysis (*Cai et al*., 2024) was conducted based on actual production costs and market prices. The farm raised 10,000 broilers in a single cycle using its facilities (i.e., no rental costs). Costs included the purchase of one-day-old chicks (0.42 €/chick), electricity (593 €), water (150 €), heating (590 €), vaccines and vitamins (491 €), laboratory analyses (200 €), labor for night shifts (1,200 €), labor for bird loading (700 €), and feed (18,870 €). An average mortality rate of 4% was assumed based on previous flock data. At the time of the *Salmonella* detection (boot swab sampling), the birds had reached an average body weight of 3,270 g. Due to the positive result, broilers could only be sold to slaughterhouses authorized to process meat for thermal treatment, at a significantly reduced price of 0.64 €/kg, compared to the regular market price of 1.10 €/kg. The economic loss was calculated as the difference between potential and realized income, adjusted for actual mortality.

3. Results

Detection rates of Salmonella spp. (2020-2025)

During the examined period (2020-2025), out of a total of 2,728 chicken primary production samples, *Salmonella* spp. was found in 88 (3.23%)

samples (Table 1). The highest positive rate was recorded in 2022 (10.29%), while the lowest was in 2023 (0.75%). Serotyping revealed 5 *Salmonella* serovars. The most frequently isolated serovar was *S.* Enteritidis (69.31%), followed by *S.* Typhimurium (21.59%), *S.* Infantis (4.54%), *S.* Mbandaka (2.27%), and *S.* Senftenberg (2.27%), as shown in Table 1.

The highest number of positive samples, serovar *S.* Enteritidis, was detected in the samples originating from broiler flocks (Table 2).

Concerning the broiler flock, besides *S.* Enteritidis, *S.* Typhimurium, and *S.* Infantis were also isolated (Fig. 1).

Table 1. Positive rate of *Salmonella* spp. and *Salmonella* serovars in primary production samples in the period 2020-2025.

	2020	2021	2022	2023	2024	2025	Total
Number of analyzed samples	856	624	379	266	405	181	2,728
Number of positive samples	20	19	39	2	4	4	88
Positive rate (%)	2.33	3.04	10.29	0.75	0.99	2.21	3.23
S. Enteritidis	16	4	37	-	-	4	61
S. Typhimurium	2	11	-	2	4	-	19
S. Infantis	-	4	-	-		-	4
S. Mbandaka	2	-	-	-	-	-	2
S. Senftenberg	-	-	2	-	-	-	2

Table 2. Number of S. Enteritidis within the flocks in the period 2020-2025.

Category	2020	2021	2022	2023	2024	2025
Parent flock	4	0	0	0	0	0
Layers	0	0	2	0	0	2
Broilers	12	4	35	0	0	2

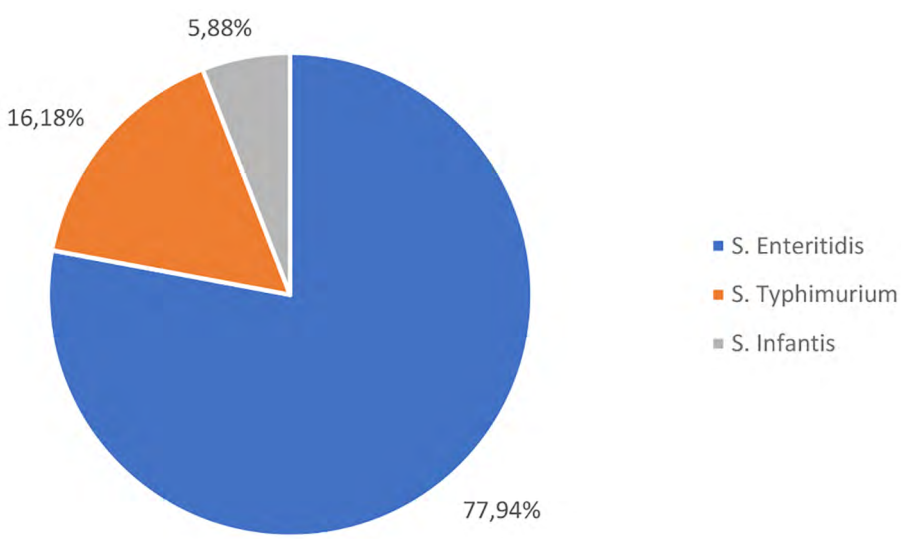

Figure 1. *Salmonella* serovars identified in the samples originating from broiler flocks 2020–2025

Table 3. Positive rate of *Salmonella* spp. in food samples from broiler slaughterhouses in the period 2020-2025.

	2020	2021	2022	2023	2024	2025	Total
Number of analyzed samples	848	1151	1323	1225	1330	412	6289
Number of positive samples	65	145	92	178	90	44	614
Positive rate (%)	7.66	12.60	6.95	14.53	6.77	10.69	9.76

During the period 2020-2025, out of a total of 6,289 food samples, *Salmonellae* spp. were detected in 614 (9.76%) samples (Table 3). The highest positive rate was recorded in 2023 (14.53%), and the lowest in 2024 (6.77%).

Biosecurity assessment result (case farm)

During the farm visit, it was noted that the farm had a biosecurity plan in place, with defined measures related to both external and internal biosecurity. Summarized results of the biosecurity evaluation is presented in Table 4.

The overall biosecurity score was 90%, which was higher than the national and global average. The national average presents the biosecurity scores that were taken from the Biochek.UGent database, and were obtained by the completion of 46 questionnaires.

The external biosecurity score and internal biosecurity score for the farm with *Salmonella* contamination were both 90%, with the following specific deficits. Notably, broiler depopulation, manure, and carcasses are subcategories within the category of external biosecurity, and had the lowest mean score for this farm compared to other categories. Usually, depopulation of flocks is performed in three to four steps. Farmworkers and part-time, paid workers from outside who are involved in the poultry depopulation process were not provided with specific clothing, shoes, or gloves—so no measures were in place at the beginning of the process. The loading truck arrived empty, cleaned, and disinfected; however, the catching process was carried out by a large group of workers who failed to adhere to biosecurity protocols during the operation. The carcass storage facility was enclosed and equipped with refrigeration; however, it is not situated within a designated dirty area of the farm and lacks adequate separation from the farm's production units. Also, we noticed there was no explicit signage or formal notice restricting access to the poultry houses for individuals (employees or visitors) without prior registration. While such a rule was assumed, it was neither visibly communicated nor consistently enforced.

Table 4. Biosecurity evaluation results on the observed farm

Biosecurity assessment	Farm (%)	National Average* (%)	Global Average (%)
One-day-old chick purchasing	90	62	67
Broiler depopulation	69	48	65
Feed and water supply	92	60	62
Manure and carcass removal	88	39	67
Farm workers and visitors	100	73	76
Material supply	100	81	70
Infrastructure and biological vectors	89	81	82
Farm location	100	73	68
External biosecurity score	90	65	70
Disease management	88	77	80
Cleaning and disinfection	87	54	71
Measures between compartments	100	71	75
Internal biosecurity score	90	66	75
Overall biosecurity score	90	65	72

*National Average - taken from the Biochek.UGent database, average obtained by completing 46 questionnaires.

Concerning internal biosecurity, the lowest score was obtained for the cleaning and disinfection (87%) subcategory. The farm implemented its own cleaning, disinfection, and rodent control procedures without engaging a professional service provider. While a specific protocol was followed after each production cycle, the effectiveness of these measures was not verified—no microbiological testing of surface swabs or water and feed lines was conducted. At the beginning of each production cycle, before one-day-old chicks were housed, the farm routinely introduced and then disinfected the bedding material. However, to maintain litter quality, the farm periodically added straw during the cycle. In such cases, the supplemental straw was introduced into the production unit without prior disinfection.

The total production cost for the cycle was estimated at 26,994 €. Assuming a 4% mortality (9,600 marketable birds), the expected live weight yield was approximately 31,392 kg (9,600 birds × 3.27 kg). Under normal conditions, the expected gross income from the sale would have been approximately 34,531.20 € (31,392 kg × 1.10 €/kg). Despite an expected net profit of approximately 7,537 € (≈27.9%) under normal market conditions, the confirmed detection of *Salmonella* and subsequent market restrictions led to a net financial loss of around 6,903 € for the production cycle. This corresponded to a total negative financial impact of approximately 14,440 € (≈58.2%) compared to normal conditions, underscoring the critical economic importance of effective biosecurity measures and preventive strategies in broiler production (Table 5).

Table 5. Partial budget analysis of a 10,000-bird broiler flock affected by *Salmonella*: Estimated costs and revenue losses

Category	Value (EUR)
Total production cost	26994
Marketable birds*	9600
Total live weight (kg)	31392
Expected income (normal market)	34531.2
Actual income (Salmonella restriction)	20090.88
Income loss due to Salmonella	14440.32
Net result (normal conditions)	7537.2
Net result (Salmonella case)	-6903.12

*Assumed mortality rate is 4%, meaning that 4% of the birds did not survive or were not marketable: 10,000 × (1 - 0.04) = 10,000 × 0.96 = 9,600 birds

4. Discussion

This study revealed a dynamic pattern of *Salmonella* positivity rates in the broiler production chain from 2020 to 2025. Notably, in 2021, a significant discrepancy was observed between primary production and food chain contamination: while the *Salmonella*-positive rate in samples from broiler farms was elevated, the highest incidence throughout the entire study period was found in food samples. In contrast, in 2022, the situation reversed, with a higher positive rate at the farm level compared to food products. The data also indicate that in 2022, the positive rate of *Salmonella* in food samples was low, while the highest positive rate in primary production samples—originating from broiler farms—was recorded in the same year. Notably, in 2023 and 2024, *Salmonella* was not detected in any samples from broiler farms. However, in 2023, food samples collected from broiler slaughterhouses showed the highest *Salmonella* positive rate recorded during the entire 2020–2025 period.

These fluctuations could have partly arisen from the effects of the COVID-19 pandemic. Concerns about the transmission of infection through the food system led to measures such as surface sanitation, improved hygiene in workplaces, safe food handling and distribution practices, and physical distancing protocols (*Rizou et al*., 2020). According to *FAO* (2020), consistent implementation of hygiene and food safety practices—regardless of the production system—can significantly reduce the risk of contamination with harmful pathogens. Strengthening such measures, as outlined in Codex guidelines (*FAO*, 2020), likely contributed to the reduced

contamination levels in food products observed in 2022, while also alleviating pressure on public health systems during the pandemic. Additionally, the observed discrepancy could stem from differences in sample origin—clinical samples were predominantly collected from the Belgrade area, whereas food samples originated from a broader geographic region across Serbia, which could influence the representativeness and comparability of the data.

Concerning the results from the case farm and established biosecurity protocols, all materials entering a poultry house during the production cycle—including bedding—should undergo proper hygiene measures to minimize the risk of introducing pathogens. The introduction of non-disinfected straw during the cycle, as observed on the farm, created a potential biosecurity gap. Best practices recommend that any additional litter material be stored in a clean, protected area and disinfected before entry, or that litter management strategies be designed to avoid the need for mid-cycle additions (*Ivanov*, 2001; *Voss-Rech et al*., 2017; *Course et al.*, 2021). Additionally, the farm should verify the efficacy of the applied protocol through methods such as bacteriological testing, as visual inspection alone is not a reliable indicator of hygiene status in broiler houses (*Luyckx et al*., 2015; *Maletić et al*., 2023).

A partial budget analysis demonstrated the severe economic consequences that *Salmonella* outbreaks can have on broiler production. These findings underscore the crucial importance of implementing and maintaining effective biosecurity measures. Preventive actions protect animal health and preserve the farm's economic viability by minimizing the risk of costly disease outbreaks (*Maletić et al*., 2023; 2025).

5. Conclusion

This study confirmed the continued presence of *Salmonella* spp. in broiler production and food samples in the samples received between 2020 and 2025, with *S.* Enteritidis as the dominant serotype. Although the assessed farm had a high overall biosecurity score, critical weaknesses were identified. The substantial financial losses observed underline the importance of continuous surveillance and strict adherence to biosecurity protocols as essential strategies for reducing *Salmonella*-related risks in poultry farming.

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Original Scientific Paper

Bioinformatic discovery of novel collagen-binding aggregation factors in lactic acid bacteria

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ABSTRACT

Lactic acid bacteria (LABs) are a unique group of microorganisms found in the diet of nearly all humans and form an integral part of the healthy human microbiome. Some LAB strains exhibit a remarkable ability for autoaggregation, mediated by Snowflake Forming Collagen Binding Aggregation Factors (SFCBAFs)—a fascinating group of proteins described so far only in LABs. To date, only five SFCBAFs have been characterized in detail: AggL from *Lactococcus lactis*, AggE from *Enterococcus faecium*, AggLb from *Lacticaseibacillus paracasei*, AggLr from *Lactococcus raffinolactis*, and AggA from *Tetragenococcus halophilus*. In this study, we present bioinformatically predicted novel SFCBAF candidates and demonstrate their widespread distribution among LAB species. Furthermore, we provide evidence that such proteins may not be exclusive to LABs, as homologous sequences were also identified in phylogenetically distant bacteria such as *Staphylococcus aureus*, *Oceanobacillus* spp., *Bacillus* spp., and others, expanding our understanding of this unique protein family.

1. Introduction

Lactic acid bacteria (LAB) represent a unique group of microorganisms that are found in foods, such as meat products (*Carneiro et al.*, 2024), consumed by nearly every human on the planet and are also present in the microbiome of healthy individuals. In nature, LAB are not limited to food products—they can also be found in soil, on plants, and on or within both terrestrial and marine organisms. Thus, this group of bacteria is truly ubiquitous and is represented across nearly all ecological niches (*Akpoghelie et al.*, 2025).

One of the remarkable properties of certain LAB strains is autoaggregation, ability to adhere among themselves or to other strains, forming visible aggregates *(Trunk, S. Khalil, & C. Leo*, 2018). There is substantial evidence suggesting that aggregation is closely linked to biofilm formation, adhesion, colonization, and host physiological functions *(Burgain et al., 2014; Du et al.*, 2022; *Kragh et al.*, 2016; *Miljkovic et al.*, 2015).

Among wide range of different aggregation factors, there is a distinct group that is referred to as Snowflake Forming Collagen Binding Aggregation Factors (SFCBAFs). These are large proteins that contain adhesion domains in the first part of the protein, followed by an immunoglobulin-like domain and then repeats domains. They are exported from the cell to the surface and subsequently

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covalently anchored to the cell wall through a well-characterized mechanism involving the LPXTG cell wall anchor domain (*Siegel, Reardon, & Ton-That*, 2017). While the aggregation mechanism is still under investigation, it is believed that the adhesive properties are conferred by collagen-binding and immunoglobulin-like domains, whereas the repeat regions serve to spatially project the protein away from the cell surface (*Miljkovic et al*., 2016). At present, only five representatives of this group have been well characterized: AggL from *Lactococcus lactis*, AggE from *Enterococcus faecium*, AggLb from *Lacticaseibacillus paracasei*, AggLr from *Lactococcus raffinolactis*, and AggA from *Tetragenococcus halophilus (Endo et al., 2023; Kojic et al., 2011; Miljkovic et al., 2018, 2015; Veljović et al., 2017)*. In this study, we predict the presence of novel proteins from this group and perform their analysis.

2. Materials and methods

2.1. Selection of representatives for phylogenetic tree construction

To retrieve protein sequences for analysis, we used the local alignment tool BLASTP 2.16.0. Searches were conducted against NCBI databases using amino acid sequences of known SFCBAFs as queries. (*Sayers et al*., 2022). The presence of signal peptides in the retrieved proteins was predicted using SignalP 6.0 (*Teufel et al.*, 2022). Duplicate sequences were removed prior to further analysis.

2.2. Multiple sequence alignment and phylogenetic tree construction

Multiple sequence alignment of the protein sequences was performed using Clustal Omega, which employs hidden Markov models (HMMs) for improved accuracy (*Madeira et al*., 2024). The resulting phylogenetic tree was saved in Newick format (*Junier & Zdobnov*, 2010) and used for downstream clustering analysis.

2.3. Clustering of the phylogenetic tree

To assess diversity and select representatives from distinct evolutionary lineages, we calculated pairwise evolutionary distances between all taxa based on the phylogenetic tree generated by Clustal Omega. Using the resulting distance matrix, we applied KMeans clustering (Steinley, 2006), specifying ten clusters. From each cluster, we selected the most distant taxon from the cluster centre, i.e., the taxon showing the greatest divergence from the cluster's average profile. This approach allowed us to compile a set of phylogenetically diverse representatives for further analysis.

2.4. Domain structure analysis

To analyse the domain architecture of the proteins corresponding to the selected taxa, we used the online tool InterPro (*Paysan-Lafosse et al.*, 2023).

3. Results and discussion

3.1. Phylogenetic analysis of potential SFCBAF representatives

In order to assess the distribution of aggregation factors, we performed BLASTP 2.16.0 searches using sequences of previously described SFCBAF-type factors (AggL, AggE, AggLb, AggLr, and AggA) against the NCBI protein database (Sayers et al., 2022). All predicted protein sequences were subsequently screened for the presence of signal peptides required for protein cell export using SignalP 6.0, and sequences lacking a signal peptide were excluded. The remaining candidates were then analysed for the presence of LPxTG anchor motifs, taking into account known motif variants as described previously (*Malik et al.*, 2023) and our internally obtained data. As a result, we assembled a dataset of 246 proteins, including the previously described ones, each of which had less than 100% sequence identity to any other in the set. A phylogenetic tree was constructed based on Clustal Omega alignment (results shown in Supplementary). During our research we identified novel SFCBAF-like factors in non-LAB bacteria, including *Mycoplasma sp. P36-A1*, *Oceanobacillus* spp., *Virgibacillus* spp., *Gracilibacillus* spp., *Jeotgalicoccus halotolerans*, *Aliicoccus persicus*, *Pseudogracilibacillus* spp., *Ornithinibacillus* sp., *Bacillus* spp., *Amphibacillus* sp., *Atopostipes* spp., *Irregularibacter muris*, *Eubacterium nodatum*, *Corticicoccus populi*, *Coprococcus* spp., *Staphylococcus agnetis*, *Staphylococcus aureus*, *Mammaliicoccus stepanovicii*, and *Lentibacillus sp. JNUCC-1*. Interestingly, aside from *Mycoplasma sp. P36-A1*, all other bacteria were Gram-positive, which may indirectly suggest a conserved mechanism of cell surface anchoring for these proteins.

3.2. Domain structure analysis of potential SFCBAF representatives

To understand the diversity of newly predicted SFCBAF representatives, we clustered the phylogenetic tree into 10 groups based on pairwise distances between its leaves and selected one most "central" representative from each cluster. In addition, we included the longest (5511 amino acids) and the shortest (1063 amino acids) SFCBAF representatives. We also re-verified the presence of leader and anchor sequences. The list of the selected representatives is provided in Table 1.

Table 1. Selected representatives of SFCBAF

ID	Number of amino acids	Leader peptide	LPxTG anchor motifs
*Oenococcus oeni*_ WP_032822309.1	1875	MNKWFRRLSAVLMALVISLQYVAAGA	LPNTG
*Lacticaseibacillus paracasei*_WP_211757376.1	1963	MNKRKLGQQLYSLVLIILLFLQSSAQVVGA	MPNTG
*Lactococcus raffinolactis*_ WP_412921610.1	1667	MKKLWQFFALIILLMQSVSPGIVVA	LPQTS
*Lactococcus lactis*_ WP_058224032.1	1793	MEVFLKKHMRKIVSIFFILLQFIQPITAIA	LPKTG
*Streptococcus pacificus*_ WP_199575997.1	1063	MSKSIKLFFKSTLIAIFAIIGMIIASANTVEA	LPKTG
*Enterococcus faecium*_ WP_002335623.1	1918	MNRKKIDQFEGLKRVMPLIMLIVLLLQSVFSPVISVA	LPTKE
*Oceanobacillus sojae*_ WP_260053073.1	2523	MKKRAKRVSIFMIFLLVMQSFATGFAPIQTAYA	LPGTA
*Irregularibacter muris*_ WP_257533483.1	1700	MNRCKGKSRYNIALIVVLIMLFQMIIPSTLAVA	LPKTG
Candidatus *Enterococcus huntleyi*_WP_161843597.1	5511	MERLRKVLTIIGLLGILANIMPVNAFA	LPKTG
*Mammaliicoccus stepanovicii*_ WP_095088808.1	1458	MGKKLVVFSVFMLLLNLFSPFVNGEKVFA	LPQTG
Lactococcus lactis BGKP1_ AggL	1768	MEKKSRYATKFYVVLMMLSLVSQLFMPFLQVAA	LPATG
Enterococcus faecium BGGO9-28_AggE	1636	MENKSRYATKFYVVLMMLSLVSQLFVPVLQVAA	LPATG
Lacticaseibacillus paracasei BGNJ1-64_AggLb	2997	MNKKKIGQQIYSLVLIFLLFLQSSAQVVGA	MPNTG
Lactococcus raffinolactis BGTRK10-1_AggLr	1774	MKKLSKSSIFILMAVIILLQYVSPILA	LPKTS
*Tetragenococcus halophilus*_AggA	2298	MTFNHVKKVAMVFMLVVLVFQSFVSPLSAVA	LPKTG
*Enterococcus italicus*_ WP_251870029.1	2822	MENKSRYATKFYVVLMMLSLVSQLFVPVLQVEA	LPATG
*Tetragenococcus halophilus*_ WP_317915830.1	3346	MTFNHVKKVAMVFMLVVLVFQSFVSPLSAVA	LPKTG

We aligned the selected representatives once again relative to each other (Figure 1, A) and performed domain structure analysis using InterPro (Figure 1, B). All potential representatives showed a similar structural organisation to previously described SFCBAF-type proteins: following the leader peptide, all contained adhesion domains (InterPro ID: IPR008966) with collagen-binding regions. The bacterial adhesion domain consists of a β-sandwich formed by 9 β-strands arranged in 2 layers with a Greek-key topology, representing a subclass of the immunoglobulin-like fold. Such domains are commonly found in surface-associated proteins and are essential for bacterial adhesion to host cell surfaces (*Symersky et al*., 1997). The number of adhesion domains varied from 1 (in the longest protein) to 7 (in several representatives). These domains are followed by an immunoglobulin (Ig)-like domain (InterPro ID: IPR013783) characterized by a prealbumin-like fold. Ig-like domains are among the most widely distributed protein modules found in diverse organisms. They frequently mediate interactions, often through β-sheet pairing with other Ig-like domains. Ig-like folds are present not only in immunoglobulins but also in a variety of other proteins, including T-cell antigen receptors, cell adhesion molecules, MHC class I and II antigens, muscle proteins such as titin, and others*(Bork et al., 1994; Halaby et al.*, 1999; *Potapov et al.*, 2004; *Teichmann & Chothia*, 2000). We hypothesize that adhesion domains, together with Ig-like domain, are crucial for aggregation. After the Ig-like domain, two organizational variants are observed: in one group, the Ig-like domain is repeated 6 to 19 times (excluding the first domain); in the other group, the repeats consist of a Cna protein B-type domain (InterPro ID: IPR008454). This domain has been well-characterized in the Cna protein of *Staphylococcus aureus*. Most probably if forms an extended stalk structure that positions the ligand-binding domain away from the bacterial surface. Cna is a collagen-binding MSCRAMM (Microbial Surface Component Recognizing Adhesive Matrix Molecules) and is necessary for *S. aureus* cells to bind to cartilage (*Foster & Höök*, 1998; *Shimoji et al*., 2003). The number of such repeats among the selected representatives ranges from 3 to 50. Finally, only one SFCBAF representative, *Enterococcus faecium* WP_002335623.1, displays a hybrid structure: after the initial Ig-like domain, there is another Ig-like domain followed by six Cna protein B-type domains. At the C-terminus of each protein, an LPxTG anchor motif is present, although in some cases, it deviates from the canonical form. For example, in *Lactobacillus paracasei* WP_211757376.1 and *L. paracasei* BGNJ1-64 AggLb, it is represented as MPNTG, while in *E. faecium* WP_002335623.1, it appears as LPTKE.

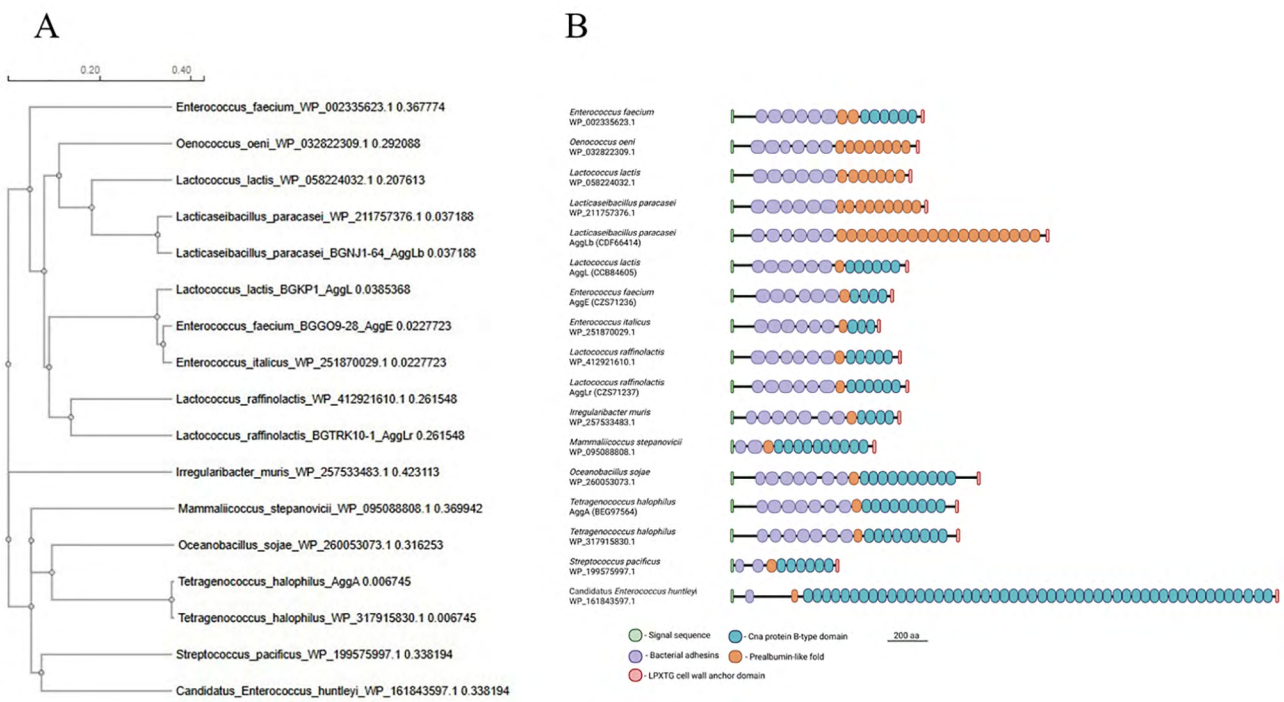

Figure 1. Analysis of SFCBAF representatives. **A** – Phylogenetic tree constructed based on Clustal Omega alignment of the selected SFCBAF representatives. **B** – Domain organisation of SFCBAF representatives.

4. Conclusion

Lactic acid bacteria represent a unique group of microorganisms closely associated with the food industry, making them of particular interest for research. The aggregation of lactic acid bacteria gives them a distinctive phenotype and new properties, including potential adhesion to the human gastrointestinal tract and the displacement of other pathogenic microorganisms. In this study, we demonstrated the prevalence of SFCBAF-type aggregation factors. Investigating such bacteria may help improve the properties of LAB used in the food industry, including in the meat industry.

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Review Paper

Beyond recyclability: a holistic framework for sustainable meat packaging, balancing circularity, shelf life and consumer acceptance

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Consumers

ABSTRACT

The intersection of material science (bio-based solutions), functionality (active and smart packaging) and systemic approaches (circular supply chains) is given to indicate sustainable meat packaging. This packaging should be regarded as a process balancing environmental goals with practical constraints (cost, performance) and consumer willingness to pay for sustainable packaging.

1. Introduction

This short review emphasizes a multi-dimensional approach (environmental, technical, and social) to sustainability in meat packaging, addressing gaps in current research that often focuses narrowly on recyclability or material substitution without integrating consumer behaviour or systemic trade-offs between sustainability, shelf life and cost (*Spada et al*., 2024; *Saini et al*., 2025). Current packaging relies on non-recyclable, multi-layered plastics, but sustainability is a driving force for meat packaging, including the development of packaging based on green materials in order to minimize environmental impact (*McMillin,* 2017*; Baskar et al.,* 2023*; Tandon et al.,* 2025).

The research objective was to evaluate emerging sustainable materials and technologies and propose a holistic framework integrating circular design, supply chain efficiency, and consumer perception to advance sustainable meat packaging.

2. Current challenges in meat packaging sustainability

Replacing commonly used plastic films derived from fossil fuel-based feedstock in the meat packaging sector is a complex challenge due to the exceptional properties of these films, such as high barrier performance, mechanical strength, and thermal resistance (*Caccialanza et al.,* 2023). However, growing environmental concerns, regulatory pressures, and consumer demand for sustainable packaging are driving the search for alternatives (*Ibrahim et al.,* 2022). Another challenge is the infrastructural gap embodied in the lack of composting/recycling facilities for bio based materials (e.g., only 2% of

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the U.S. population has curbside composting) (*Cruz Foam,* 2024). There is also consumer misalignment in, e.g., their willingness to pay for sustainable packaging (82% of consumers) compared to low recycling rates (21% of recyclable materials collected) (*Harris,* 2023). The mismatch between eco-conscious claims, actual recycling behaviour, and premium pricing of sustainable options limits their adoption and scalability (*Donkor et al.,* 2023).

3. Emerging sustainable solutions

Recent advancements in sustainable meat packaging focus on reducing environmental impact while maintaining food safety and shelf life. Biodegradable materials like polylactic acid (PLA), polyhydroxyalkanoates (PHA), polybutylene adipate terephthalate (PBAT), and chitosan- and starch-based films are replacing conventional plastics, offering compostability and antimicrobial properties (*Saini et al.,* 2023). Circular economy approaches for mono-material designs, such as recyclable polypropylene (PP) and amorphous *polyethylene terephthalate* (APET) trays, simplify recycling processes (*Fellegara et al.,* 2023), while active packaging, incorporating oxygen scavengers and moisture absorbers, extends product freshness (*Cenci-Goga et al.,* 2020). Additionally, smart labels with pH-sensitive indicators provide real-time spoilage alerts, helping to minimize food waste (*Nat,* 2024).

4. A proposed framework for sustainable meat packaging

The reviewed literature shows the main directions that define the framework for the development and application of sustainable packaging in the meat industry, namely: circular packaging design, integration of supply chains, consumer engagement, arising pressure from regulatory bodies. The main goal of circular design is to prioritize mono-materials and compostable/biobased options where feasible (*Ibrahim et al.,* 2022). The integration of supply chains implies centralized processing near markets to reduce transport emissions and shelf life loss (*Harris,* 2023). Consumer engagement is achieved by companies that practice transparent and clear product labelling to gain consumer trust and loyalty (*Spada et al., 2024*). Here, we should also mention the definition of metrics that evaluate the success of this process: cost savings, reduction in material use, increased shelf life, and improved recycling rates (*Morashi et al.,* 2022).

The sustainable meat packaging market is projected to reach US$12.4 billion by 2035, with a compound annual growth rate of 4.1% (*FMI, 2025*). The sustainable packaging market will grow from $276 billion in 2023 to $558 billion by 2034 (Figure 1) (*Statista,* 2025). That is more than doubling in just over a decade.

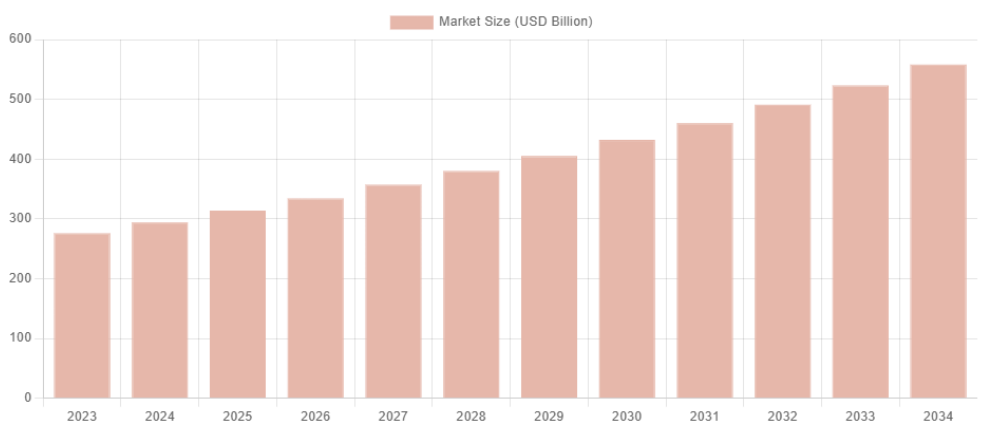

Figure 1. Global sustainable packaging market size projections (2023-2034)

https://stampedwithlovexoxo.com/blogs/love-letters/sustainable-packaging-statistics?srsltid=AfmBOoqQaBPseGssODgJZkFQFhkpNQ-shuTW9whVHaNSjbc9_epnD7-k

5. Conclusion

The meat industry is undergoing a transformative shift toward sustainable packaging, driven by environmental concerns, regulatory pressures, and consumer demand for eco-friendly solutions. Replacing widely adopted materials in the meat packaging sector is possible, but will take time. In the short term, hybrid materials and recycled materials will dominate as transitional solutions. Over the next 10–20 years, advances in biodegradable polymers, active

packaging, and circular economy models are expected to enable the widespread adoption of sustainable alternatives. The timeline will depend on technological progress, cost reductions, regulatory support, and consumer acceptance. While complete replacement may not happen immediately, the industry is moving steadily toward more sustainable packaging solutions.

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Original Scientific Paper

Nutritional strategy in production vitamin D fortified eggs

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ABSTRACT

Vitamin D is a liposoluble vitamin that regulates calcium homeostasis and is important for animal and human health. Lack of vitamin D in humans can cause rickets, osteomalacia, and decreased immunity. In laying hens, vitamin D deficiency negatively affects egg production (decrease in egg production, thin-shelled eggs, poor feed conversion rate, etc.). Farm animal requirements for vitamin D depend on age, sex, health status and production category. Foods that contain vitamin D in human nutrition include salmon, red meat, liver, tuna and eggs, but due to an unbalanced diet, people often cannot meet their needs in this way. In animals, the daily needs can be met by adding vitamin D through premixes into the feed. The Department of Nutrition and Botany, Faculty of Veterinary Medicine, University of Belgrade, conducted research on a commercial laying hen farm, where an active form of vitamin D was added through premix into the feed of 20-week-old laying hens. The nutritional reference value for vitamin D is 5 µg/day, and an enriched egg contained an average of 2.56 µg/100 g of egg mass. By consuming food or feed enriched with vitamin D, various diseases can be prevented, and it is easier to meet the daily needs of both humans and animals.

1. Introduction

Chicken eggs belong to the group of products of animal origin that are most often consumed around the world. According to the reports of the Food and Agriculture Organization (FAO), the consumption of eggs increased sharply in the period from 2000 to 2018 (by as much as 50%) (*FAO*, 2020). The demand for chicken eggs is expected to continue to grow due to their widespread acceptance by consumers and population growth (*FAO*, 2018). The perfect balance and variety of nutrients, together with high digestibility and an affordable price, put the egg at the top of the list of foods used in human nutrition. Eggs are considered as nutritionally the best source of protein (*Domingo*, 2014; *Lesnierowski and Stangierski*, 2018). They contain an optimal ratio of essential amino acids, fatty acids, fat-soluble vitamins (A, D, E, K) and B group vitamins (B1, B2, B5, B6, B7, B9, B12, choline). In addition to the above, they contain minerals: calcium, iron, magnesium, phosphorus, selenium, sodium and zinc, as well as antioxidants that reduce free radicals produced by cellular metabolism (*Rodriguez-Gonzalo et al.* 2017). Table 1 shows the chemical composition of the whole edible hen's egg.

With the increase in egg production for the world market (*FAO*, 2020), consumer awareness of egg quality is increasingly developing (*Duman et al.*, 2016), which includes information on the origin of

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the product, type of production, nutrition of the laying hen, animal welfare and environmental conditions on the farm. For these reasons, ways to improve and adapt development to market requirements are being explored, while respecting all relevant regulations and meeting consumer expectations for high-quality products (*Gracia et al*., 2014). Consumer culture, traditions, lifestyles and attitudes about egg consumption are carefully considered in order to meet the demands and expectations set by the market (*Patricia and Hester*, 2017). Factors affecting the quality of chicken eggs are very complex and are mainly generated in the production phase (*Zaheer*, 2015). Research by the European Consumer Association indicates that table eggs are increasingly recognized as a quality product, highlighting the most important parameters: nutritional value and sensory characteristics.

Table 1. The basic chemical composition of the whole edible hen's egg

Nutrient	g/100g
Protein	12.56
Fat	9.51
Carbohydrate	0.72
Moisture	76.15
Ash	1.06

However, the egg still faces long-standing recommendations by nutritionists aimed at limiting egg consumption in order to reduce the incidence of cardiovascular disease. Human nutrition in developed countries is specific because it involves an excessive intake of protein, cholesterol, saturated fatty acids and n-6 polyunsaturated fatty acids (PUFA), while the intake of n-3 PUFA, fibre and antioxidants is insufficient. Such an unbalanced diet is partly responsible for the high frequency of illness and the occurrence of chronic diseases, of which cardiovascular diseases are the leading cause of death in the world (*Miranda et al.,* 2015). But most experimental, clinical and epidemiological studies have concluded that there is no evidence of a correlation between dietary cholesterol provided by eggs and an increase in total plasma cholesterol. Egg remains a food product of high nutritional quality for adults, including the elderly, and children (*Godbert et al.,* 2019). Today, great efforts are invested in the development of innovative technologies, which create a product with added value (enriched product or functional food). In addition to increasing the content of desirable functional ingredients in the product (eggs), they also have a positive effect on animal health, improve performance and, thus, affect the sustainability and quality of the product (*Wang et al.*, 2017). The most common functional ingredients or nutrients used in food fortification (meat and eggs) are: omega-3 fatty acids, selenium, vitamin E, vitamin A, vitamin D and others. These listed ingredients are of interest since they are factors in the biggest problems of the modern lifestyle (*Kralik and Lovreković*, 2012).

Vitamins are a group of complex diverse organic compounds essential for the development of basic physiological and metabolic processes in humans and animals. These are physiologically active food micro-ingredients required in minimal amounts for health, life support, growth and reproduction. In case of deficiency (insufficient amount in food, insufficient synthesis), signs of deficit and health disorders are manifested.

Vitamin D (calciferol) belongs to a group of compounds with anti-rachitic activity, the most important of which are ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3). Calciferol is necessary for optimal growth and reproduction, as well as for maintaining a normal state of health, while a possible function in the immune status of animals is also indicated (*Clark et al.*, 2021). The main function of calciferol is to, together with calcitonin and parathormone, ensure the optimal concentration of calcium in the blood necessary for normal physiological processes in which calcium is involved. Calciferol affects resorption (gut), deposition and mobilization (bones), as well as calcium excretion (kidneys) (*Jokić et al.*, 2024).

Poultry have higher requirements for calciferol than do other animal species. Actively laying hens, due to the high content of calcium in the eggs, have significantly higher needs for vitamin D compared to the needs for life support in non-laying hens. The calciferol requirements for laying hens, 1500-1800 IJ/kg of feed, were determined on the basis of the material balance and biological nutritional trials. This amount meets the hen's needs for optimal growth, ossification, reproduction and normal health maintenance. Apart from the absolute amounts of calciferol in the diet necessary to meet the needs of the laying hen, the form is also unusually important. Namely, cholecalciferol is a physiologically more active form of the vitamin than ergocalciferol. In poultry, the relative biological activity of ergocalciferol is only 28% compared to cholecalciferol. Most feeds used in the

diet of laying hens contain small amounts of calciferol, although provitamins are widely distributed. Cereal feeds, as well as industrial by-products, are extremely poor sources of calciferol or do not contain it at all. Conversion of ergosterol to ergocalciferol occurs only in the dead parts of plants exposed to UV rays. Foods of animal origin are a relatively poor source, and only fishmeal and fish oil from marine fish contain extremely large amounts of cholecalciferol. Considering the role of calciferol in the regulation of calcium and the importance of calcium in the process of muscle contraction and neuromuscular excitability, paresis and paralysis occur in cases of deficiency. This occurs especially in animals that have extremely high calcium needs, such as laying hens, in which, if experiencing a deficit, the inability to stand and paralysis can occur (cage paralysis of laying). Also, apart from the above, a lack of calcium causes a drop in carrying capacity, eggs with shell deformities (thickness, mass, firmness, shape) or eggs without a shell. In poultry, poor feathering occurs with feather pigmentation disorder (achromotrichia) (*Šefer and Sinovec*, 2008).

Due to the low concentration of vitamin D in feed, in the animal feed industry, vitamin D is added to feed mixtures for laying hens through vitamin-mineral premixes. The vitamin D additive used in these vitamin-mineral premixes is present in one of two basic forms: conventional vitamin D3, at levels in feed of at least 500,000 I.U./g of vitamin D3 and 1,25-dihydroxycholecalciferol – the active form of vitamin D3. Metabolism of the active form of vitamin D3 in laying hens takes place in the kidneys, where, bypassing the metabolic processes in the intestines and liver, it enters the bloodstream directly. The addition to feed of the active form of vitamin D3 enables its proper absorption, which, facilitates better assimilation of Ca and P, improvement of carrying capacity, and transfer and deposition of vitamin D into the egg mass (*Šefer and Sinovec*, 2008).

Eating two eggs a day covers about 10% of people's vitamin needs. It is important to note that the content of fat-soluble vitamins (vitamins A, D, E and K) in the yolk largely depends on the diet of the hen.

2. Materials and methods

The research was carried out in cooperation with the Department of Nutrition and Botany of the Faculty of Veterinary Medicine, University of Belgrade, and the company Fish Corp 2000, by adding the active form of vitamin D3 through a premix, to mixtures for laying hens for 4 weeks. The study lasted six months and was carried out on a commercial egg-laying hen farm in Srbobran, Serbia, with a capacity of 13,000 hens, 20 weeks old. Vitamin-mineral premix, in floury form and intended for use at a level of 1% in the finished feed mixture for egg-laying hens, contained: Vitamins: 1,000,000 I.J. Vitamin A (3a672a), 200,000 I.J. Vitamin D3 (3a671), 11.25 mg 25-hydroxycholecalciferol (3a670a), 1,500 mg Vitamin E (3a700), 200 mg Vitamin K3 (3a711), 200 mg Vitamin B1 (3a821), 400 mg Vitamin B2/riboflavin, 200 mg Vitamin B6 (3a831), 1.2 mg Vitamin B12 /cyanocobalamin, 2,000 mg Vitamin C/ascorbic acid, 10 mg Biotin (3a880), 2,000 mg Niacin (3a314), 500 mg Calcium D-pantothenate (3a841), 40 mg Folic acid (3a316), 50,000 mg Choline chloride (3a890); Microelements: 5,000 mg iron as iron (II) sulphate monoch. (3b103), 1,500 mg copper as copper (II) sulphate pentah. (3b405), 6,000 mg zinc as zinc oxide (3b603), 8,000 mg manganese as manganese sulphate monoch. (3b503), 50 mg iodine as calcium iodate (3b202), 20 mg cobalt as cobalt carbonate (3b302), 15 mg selenium as sodium selenite (3b801).

The content of vitamin D3 in eggs was determined by the 02I.01.022 method (Ložnjak Švarc et al. 2021).

3. Results and discussion

With conventional nutrition, egg-laying hens contain 1 µg of vitamin D3 per 100 g of egg mass. Based on the statement of the local Serbian Zdravlje Health Cooperative, an egg can be declared as rich in vitamin D provided that 100 g of the product contains a minimum of 30% of the nutritional reference value (NRV) for vitamin D. Given that the NRV for vitamin D is 5 µg, an egg rich in vitamin D should contain a minimum of 1.5 µg of vitamin D in 100 g of egg mass. Addition of the active form of vitamin D via premix to layers for 4 weeks resulted in a product that contained an average of 2.56 µg of vitamin D3 per 100 g of egg mass, shown in Figure 1. These eggs contain more than 50% of the NRV for vitamin D and can be declared as a Vitamin Egg.

Daily consumption of vitamin D-enriched eggs produced by hens that receive appropriate pre-mix is important for maintaining optimal health, especially for people with mobility difficulties and reduced outdoor time. Eggs enriched with vitamin D can provide adequate intake of the vitamin, which is important in a state of stress or illness (*Persia et al*., 2013).

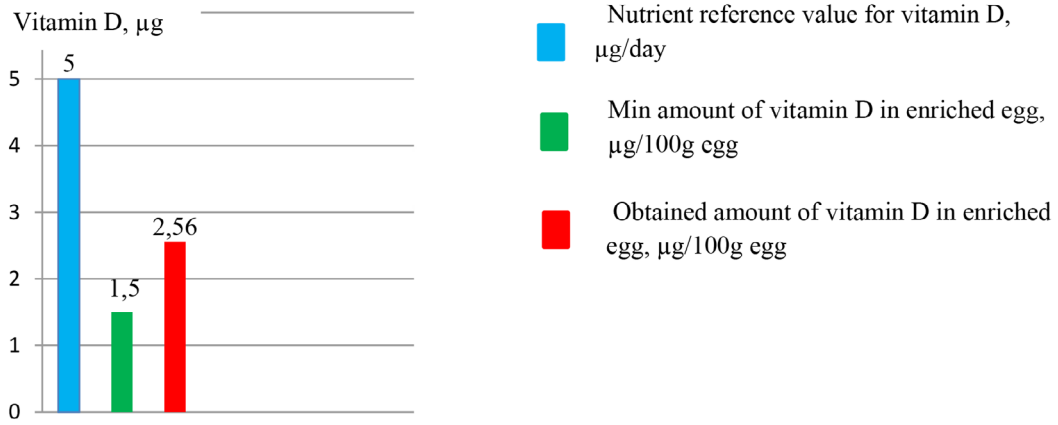

Figure 1. Nutritional reference value for vitamin D, minimum amount of vitamin D in an enriched egg and mean vitamin D level per 100g of egg mass after hens received vitamin-mineral premix.

Finnish scientists from the Agricultural Research Center came to similar conclusions. They fed 30-week-old hens a conventional diet and a diet supplemented with vitamin D. Hens that consumed feed with 3.5 times more vitamin D produced eggs with seven times more vitamin D3 than eggs from hens fed a standard diet. They also observed that increased supplementation of this vitamin has an effect on the health of chickens: healthy reproductive system, properly formed egg shell, maintenance of strong bones and a well functioning immune system (*Matilla et al.*, 1999).

Multiple studies in the UK have shown that adding vitamin D3 to the hen diets results in a significant improvement in the vitamin D content of eggs (*Browning and Cowieson*, 2014). These studies have shown that yolk saturation with vitamin D is achieved within about 6 weeks of consuming fortified feed, and that the total vitamin D content of eggs can potentially increase by up to 78% *(Matilla et al.*, 2003).

4. Conclusion

For centuries, eggs have been considered a food of high nutritional value for humans. It is predicted that their consumption will continue to grow in the future, given the increasing number of consumers who are starting to adopt a meat-free diet (vegetarians) or who are significantly reducing their meat intake. Demands are increasing as consumer awareness of the quality of the food they eat is growing.

The Vitamin Egg is a product of the collaboration between the Department of Nutrition and Botany, Faculty of Veterinary Medicine, University of Belgrade and the company Fish Corp 2000. With these joint efforts that aim to reduce the difference between the intake of vitamins through food and human nutritional needs, eggs enriched with vitamin D (2.56 µg/100g of egg mass) were obtained, which contain more than 50% of the nutritional reference value for vitamin D (5 µg/day).

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Original Scientific Paper

Determination of the efficiency of energy sources in feed mixtures for broilers

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ABSTRACT

Adding fat to poultry feed mixtures increases the energy value of feed, absorption of liposoluble vitamins, reduces feed spilling, enhances feed palatability, slows down the passage of feed through the digestive tract of animals, and thus, improves the absorption of nutrients and utilization of metabolic energy. In areas where corn is used as the main source of energy, high-energy mixtures are usually used, which also provide the best economic effects. However, good results (especially if the goal is a meaty carcass) can also be achieved by using a feed mixture with a lower energy level that includes nutrients of lower energy value compared to corn. Today, the physiological mechanisms by which poultry react to different energy levels have not yet been fully clarified. In addition to an overview of the most important sources of energy in poultry feed mixtures, this paper presents research organized by the Department of Nutrition and Botany, University of Belgrade, with the aim of examining the influence of different fat sources in broiler feed on production results, health status, fatty acid composition and quality of broiler meat. The selection of energy nutrients that optimizes broiler production results and the meat quality is presented and discussed.

1. Introduction

Poultry meal is mainly composed of concentrated granular nutrients, soybean meal or cake, fats or vegetable oils, macrominerals, and vitamin-mineral supplements with the addition of some synthetic amino acids. In addition to the usual nutrients, various additives are included in poultry feed in order to improve production results and product quality. In poultry feeds, nutrients that serve as a source of energy are the most prevailing components (75%), followed by proteins (20%), minerals (about 5%), and vitamins and other supplements at a low percentage (*Jokić et al*., 2004).

The addition of fat to poultry feed mixtures increases the energy value of feed, the absorption of liposoluble vitamins, reduces feed spilling, enhances feed palatability, slows down the passage of feed through the digestive tract of animals, and thus, improves the absorption of nutrients and the utilization of metabolic energy (*Ševković et al*., 1980). The values of metabolic energy (ME) and net energy (NE) both vary, and they are conditioned by other factors, such as the type of poultry, breed, hybrid, physiological state and age of the poultry, in addition to the composition of the feed mixture. In areas where corn is used as the main source of energy,

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high-energy feed mixtures are usually used, which also provide the best economic effects. However, good results (especially if the goal is a meaty carcass) can also be achieved by using a feed mixture with a lower energy level that includes nutrients of lower energy value than corn (*Marković and Baltić*, 2018). Today, the physiological mechanisms by which poultry react to different energy levels have not yet been fully clarified, despite the fact that several of them have been proposed. Namely, the energy needs of poultry depend on several factors at the same time: their body weight, ambient temperature, daily gain, egg production, etc. Based on the equation given by the United States National Research Council (*NRC*, 1984; 1994), the required amount of feed consumed for layers and broilers can be accurately predicted (*Jokić et al*., 2004).

Fats are, in addition to proteins, an important nutrient that poultry efficiently digest and utilize. These are esters of trihydroxyl alcohol of glycerol and higher fatty acids. The presence of a high amount of saturated fatty acids in a fat molecule refers to solid fats, while oils are predominantly unsaturated fatty acids (with one, two or more double bonds). In the feed mixtures for poultry and other types of domestic animals, fats are the richest source of energy, given that their energy value is 2.25 times higher than that of carbohydrates. In fact, fats have a constructive role and are necessary in intermediate metabolism. Fats are a good source of essential fatty acids, fat-soluble vitamins dissolve in them (*Šefer and Sinovec*, 2008), and they can be deposited in large quantities in the animal body. Depending on the category of poultry and other factors (genetics, structure and composition of the feed mixture, ratio between nutrients), the amount of deposited fat ranges from a few percent (in chickens) to 30% (in ducks). A higher percentage of fat in mixtures (more than 10%) effects more efficient use of feed, but also leads to serious technological and nutritional problems. Such mixtures are difficult to manipulate, because the high proportion of fat requires high levels of protein, some amino acids and other nutrients important in fat metabolism (choline, folic acid, vitamin E and B12). The inclusion of a comparably larger amount of fat in complete poultry mixtures today can be solved technologically successfully by using a special spray technique—applying fat to hot pellets as they exit the pellet mill or extruder.

When using fats in feed mixtures, care must be taken regarding their source and the age of the poultry. Feeds for poultry contain 2-5% fat, except for oilseeds where the fat ranges from 17-30% (soybeans) and 40% (sunflower and rapeseed). Of all cereals, the highest percentage of crude fat is found in corn and oats (4-5%). Sorghum, wheat and barley contain 2-3% crude fat. Somewhat higher levels of 5-10% crude fat are found in protein of animal origin (*Šefer and Sinovec*, 2008).

Corn is used as a basic energy feed in poultry mixtures. In poultry meal, it is found at concentrations of 40-70%, depending on the category of poultry. Corn grain is characterized by a high content of easily digestible starch (about 80%), and low contents of protein of poor biological value and mineral substances. Of all cereals, it has the least cellulose (2-2.5%) and the most fat (about 4%), so it has the highest concentration of energy (14.1 MJ/kg).

Wheat is similar in nutritional value to corn, but with a lower energy/protein ratio. It has a higher protein content and 10% lower energy value because it contains less fat (about 2%) and more non-starchy polysaccharides than does corn. The protein content in wheat is 10-14%, depending on the variety. Wheat proteins are poor in the amino acids lysine and methionine. The ME value of wheat used in poultry nutrition, depending on the variety, is 10.6-12.9 MJ/kg. In poultry meal, wheat serves only as a partial substitute for corn, where the percentage share should not exceed 20% for poultry aged 1-4 weeks, or 25% if the animals are older than 4 weeks. The nature of the protein in wheat and the fineness of the grinding leads to its sticking around the beak, and when used in a higher percentage, to wet and sticky excrement.

Barley contains more proteins (barley grain contains 10-11%) and amino acids (lysine, tryptophan, methionine, cystine) than corn. Due to its lower fat content (1.7%), it has a lower energy value than corn (11.34 MJ/kg). It contains more crude fibre (about 5%) and can only be used as a partial substitute for corn and wheat in poultry feed mixtures. However, barley grain has a low level of essential amino acids, especially lysine. Barley is not widely used in poultry nutrition because it has sharp seeds, so poultry do not like to consume it. That is why it is necessary to free the grain of barley from the axils and cover it before use. Due to its lower energy content, barley is used for the energy dilution of poultry feed mixtures, especially in the last phase of feeding young poultry. This lower energy content has a favourable effect on the quality and deposition of fat, which has a firmer consistency and lighter color compared with the fat laid down in birds consuming higher energy feed.

Oats are characterized by a high content of coarse cellulose fibre in the grain envelope (7-11%), and oats have a low energy value (1.46MJ/kg), so they are of little importance in poultry nutrition. The protein content in this feed is 10-11%, and it is of poor biological value. An oat grain contains about 5% fat, 0.08% calcium and 0.34% phosphorus. In the feed mixtures for young poultry categories, oats should not constitute more than 10-15%, and in feed mixture for laying hens, no more than 20-25%. Oats in broiler feed mixture affects feathering, while if in the form of whole grains, oats prevent feather pecking and cannibalism.

Rye has a similar chemical composition to wheat. It contains about 12% protein, 1.5% fat, 2.2% crude fibre and has an energy value of 12 MJ/kg. Rye proteins have relatively low biological value, because they contain low amounts of lysine, methionine, threonine, leucine and isoleucine. The large amount of pentosan in rye reduces its energy value and utilization of other nutrients. Rye also contains higher amounts of alkyl resorcinol compared to wheat and triticale, and this gives it a bitter taste and limits its use in poultry nutrition.

Triticale is a cereal produced by crossing rye and wheat. It has a higher content of protein (15.8%) and lysine (0.39%) compared to the species from which it was produced and a lower energy value. The content of fat in this feed is 1.5% and raw cellulose is 4%. In the case of young poultry, the share of triticale in the feed mixture should not be higher than 2%, and in the case of breeding stock and layers, 2-15%.

Sorghum is also used in poultry nutrition as an energy feed. Its chemical composition is similar to corn, but it has 10% less nutritional value. It contains more crude protein (10-11%) and ash (1.7-2%), and less fat than corn. Sorghum proteins are of poor biological value with a marked lack of lysine. The energy value of sorghum is 13.6MJ/kg in poultry nutrition. The nutritional value of sorghum really depends on its tannin content. The amount of sorghum in poultry feed mixture, regardless of the category, should not exceed 40-50% (*Ševković et al*., 1980).

Fats and oils can be used to meet the energy requirements of poultry. Soy and sunflower oil can be used from the group of vegetable oils, and tallow, pork and poultry fats and fish oil from the animal fats. These suitable fats and oils are an excellent source of energy (32.2-36.8MJ/kg), which is the reason for their mandatory inclusion in poultry feed mixture if the mixture of other components is not satisfactory in terms of energy. The effect of added fat depends on the source and age of the poultry. Young poultry categories cannot digest saturated fatty acids, so the optimal solution is to include a mixture of fats and oils in the feed mixture (*Attia et al*., 2020). Fats are a good source of essential linoleic acid, the amount of which in poultry rations should be at least 1%. All fats and oils should be treated with approved antioxidants to prevent their oxidation. At least 1% and at most 5% of poultry fat, fish or vegetable oil or a mixture of vegetable oil and animal fat can be included in feed mixture for young poultry (1-4 weeks). The same amount can be used in the diet of breeding young (4-18 weeks), with the possibility of including 1-5% tallow or pork fat and more poultry fat (up to 7%). Vegetable oil should constitute up to 8% of the ration produced for laying and breeding hens, while a mixture of vegetable oil and animal fat can make up to 7% of the ration. Using the new technology of applying fat (spray method) to the pellets when they come out of the pellet mill enables the inclusion of larger amounts of fat in poultry feed. In doing so, one must take into account the level of protein and certain vitamins, which are important for the efficient utilization of fat. Using this spray technology, production results can be improved, especially feed conversion rates (*Ševković et al*., 1980).

A diet high in fat is thought to lead to better digestion and absorption of other nutrients in the feed mixtures. This can be explained by the prolonged retention time of fat in the intestines of poultry (*Baião and Lara*, 2005). Adding lipids affects the composition of lipids laid down in the bird's bodies, that is, the content of fatty acids in muscle tissue and eggs. The fatty acid composition of broiler feed fat has a direct influence on the fatty acid composition of fat reserves in broilers. The main goal of this research was to examine the influence of different fat sources in broiler feed on production results, health status, fatty acid composition and quality of broiler meat.

2. Materials and methods

In the study, 240 broilers of Cobb 500 provenance and both sexes were used, divided into four experimental groups. Broilers in all four experimental groups (K, O-I, O-II and O-III; see below) received feed of standard raw material and chemical composition (NRC, 1994) for the given provenance and production stage (complete broiler fattening mixture I from days 1 to 10; complete broiler

fattening mixture II from days 11 to 21 and; complete broiler fattening mixture III from days 22 to 42). Linseed oil and lard were added to the feed for the diet of the experimental groups in different amounts. Feed mixtures for group O-I contained 1%, 2.5% and 5% lard in starter, grower and finisher feed, respectively. Feed mixtures for group O-II contained 1%, 2.5% and 5% linseed oil in starter, grower and finisher feed, respectively. Feed mixtures for group O-III contained 0.5%, 1.25% and 2.5% lard and 0.5%, 1.25% and 2.5% linseed oil in starter, grower and finisher feed, respectively (so this latter group's feed contained a combination of two fat sources).

At the end of each fattening phase, each animal's body weight and feed consumption were measured, and then feed conversion rate and body weight gain were calculated. During the study, the health condition of the animals was monitored. At the end of the feeding trial, birds were slaughtered and the carcasses were cut into the main parts: breast, drumstick with thigh, wings, neck and back. After that, the parts of the carcass were measured, and six drumsticks were taken from each experimental group for chemical analysis.

Fatty acid composition was determined in the broiler drumstick meat. The determination of fatty acids was done using an internal standard (heneicosanoic acid, C23:0). Fatty acid content is presented as a percentage share (%) of the total identified fatty acids. After that, lipid indexes were calculated according to the formula (Ulbricht and Southgate, 1991), which includes in the calculation only those fatty acids that are proven to influence that parameter. For the determination of malondialdehyde (MDA), the test was based on the spectrophotometric determination of the pink complex formed after the reaction of MDA with two molecules of 2-thiobarbituric acid. The test determines the so-called thiobarbituric acid reactive substances (TBARS), and the test result is collectively expressed as the TBARS-number (*Tarladgis et al.,* 1960). The meat samples were stored at -20 °C for 6 months, after which the MDA was determined again.

3. Results and discussion

Statistically significantly higher ($p<0.05$) body weight gain at the end of the study were recorded in the O-II group (Figure 1), while the feed conversion rate was significantly better ($p<0.05$) in groups O-I and O-II (Figure 3). Accordingly, feed consumption was the lowest in group O-II (Figure 2). According to the Cobb 500 broiler performance and nutrition guide, the average weight of broilers at the end of fattening (day 42) should be higher (Cobb 500 Broiler Guide, 2018) than obtained in this study. The resulting difference between the expected body weight gain according to the guide and the lower average body weight gain obtained in the control and experimental groups could have arisen for several reasons. These include differences in feed quality, differences in diet with palletized or mealy feed, length of starvation before slaughter, transport to the slaughterhouse, stress, housing conditions, differences in genetic material, etc. The most likely cause

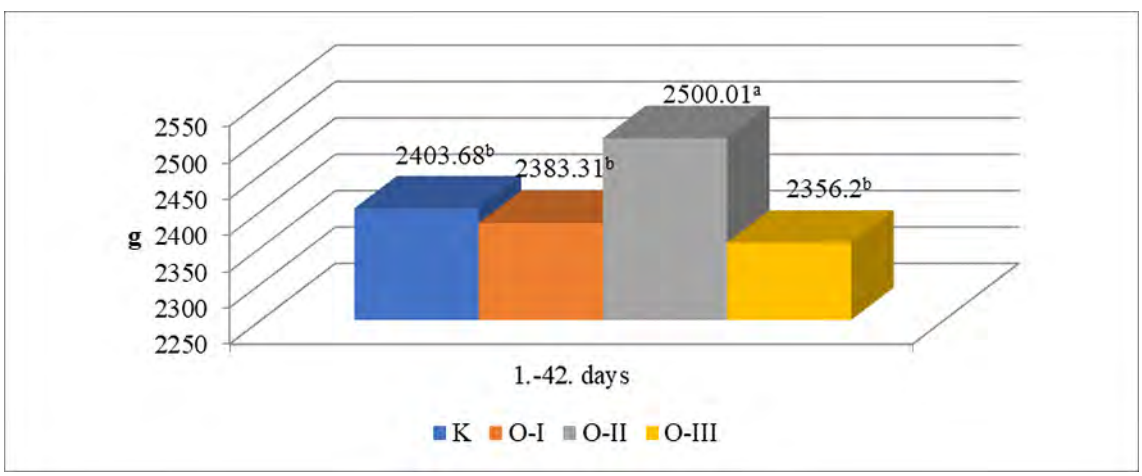

Figure 1. Average body weight gain (g) of broilers during fattening (n=60). O-I; 1%, 2.5% and 5% lard in starter, grower and finisher feed, respectively. O-II; 1%, 2.5% and 5% linseed oil in starter, grower and finisher feed, respectively. O-III; 0.5%, 1.25% and 2.5% lard and 0.5%, 1.25% and 2.5% linseed oil in starter, grower and finisher feed, respectively. Different letters, ^{a,b}, indicate significant difference at $p<0.05$.

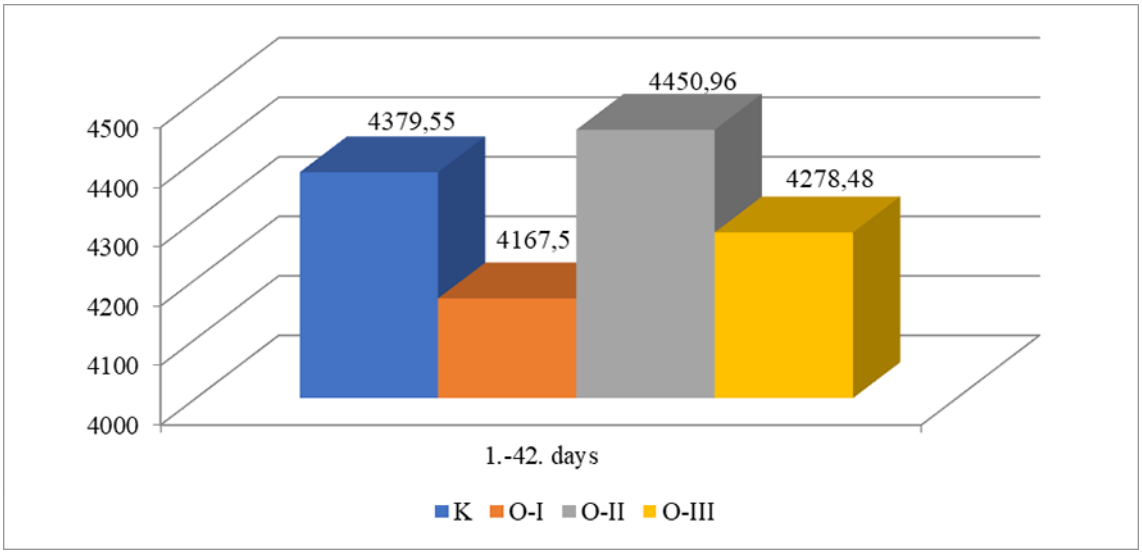

Figure 2. Mean total feed consumption (g) during fattening (n=60). O-I; 1%, 2.5% and 5% lard in starter, grower and finisher feed, respectively. O-II; 1%, 2.5% and 5% linseed oil in starter, grower and finisher feed, respectively. O-III; 0.5%, 1.25% and 2.5% lard and 0.5%, 1.25% and 2.5% linseed oil in starter, grower and finisher feed, respectively.

in this case could be the quality of the feed and the form of the feed. In our study, mealy feed was used, and it is known that when using such feed, generally worse production results are obtained due to the way broilers take the feed and the greater spilling of feed. Other possible reasons for the low body weight gains achieved were the length of starvation before slaughter and the transport of broilers to the place of slaughter.

According to the Cobb 500 standard (*Cobb 500 Broiler Guide*, 2018), the feed conversion rate on the 42nd day of fattening should be 1.61, which is better than the feed conversion rate achieved in the control group and group O-III by 0.21% and 13.04%, respectively. The feed conversion rate according to the Cobb 500 standard is better than the conversions achieved in group O-I by 0.14 or 8.69%, and by group O-II by 0.17 or 10.56%.

The average content of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in the drumstick meat of group O-I was higher ($p<0.05$), and the content of polyunsaturated fatty

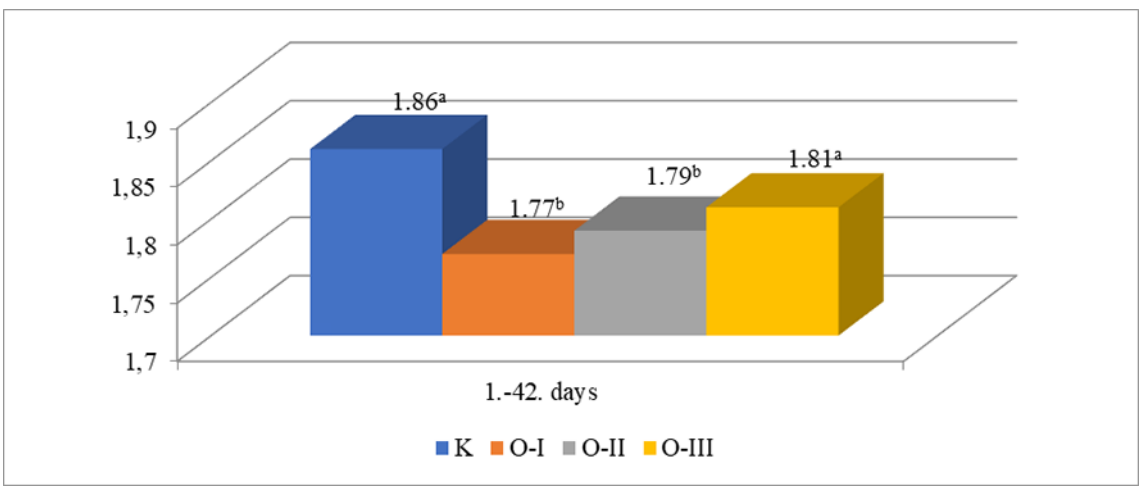

Figure 3. Mean total feed conversion rate (g) during fattening (n=60). O-I; 1%, 2.5% and 5% lard in starter, grower and finisher feed, respectively. O-II; 1%, 2.5% and 5% linseed oil in starter, grower and finisher feed, respectively. O-III; 0.5%, 1.25% and 2.5% lard and 0.5%, 1.25% and 2.5% linseed oil in starter, grower and finisher feed, respectively. Different letters, ^{a,b}, indicate a significant difference at $p<0.05$.

acids (PUFA) was lower ($p<0.05$) compared to the content of these acids in the drumstick meat of the other tested groups (Figure 4). The fatty acid composition of meat is one of the most important characteristics of meat considered in the development of functional foods. The most nutritionally valuable fatty acids, in this sense, are n-3 fatty acids, and especially long-chain n-3 fatty acids: eicosapentaenoic acid 20:5n-3, docosopentaenoic acid 22:5n-3 and docosahexaenoic acid 22:6n-3. These fatty acids have an important influence on the general development of the body and the nervous system and have a cardioprotective effect. One of the first indications of their cardioprotective action was observed in Inuit, who, although they had a high cholesterol content in their blood, had very few cardiovascular diseases (*Desnoyers et al*., 2018). A later analysis of their diet found that they consumed significantly more long-chain n-3 fatty acids than other peoples. Many studies after that showed the cardioprotective effect of n-3 fatty acids (*Keys et al*., 1967; *de Lorgeril et al*., 1998). Also, it was shown that it is possible to influence the fatty acid composition of meat by changes in the composition of complete feed mixtures for broilers, i.e., to obtain a fatty acid composition of the meat very similar to the fatty acid composition of the mixture (*Starčević et al*., 2014). *Desnoyers et al*. (2018) tried to explain why in some studies on humans, the cardioprotective effect of n-3 fatty acids is absent, and they came to the conclusion that this could be due to a large intake of n-6 fatty acids through the usual diet of modern times. Moreover, in order to achieve favourable results, it is not enough to add n-3 fatty acid supplements to the animal diet, but it is also necessary to reduce the animal intake of n-6 fatty acids. For this reason, it is very important to monitor the ratio of n-6/n-3 fatty acids in the obtained meat in every nutritional study on this topic.

The most favourable n-6/n-3 ratio ($p<0.05$) was in groups O-II and O-III (Figure 5). Zelenka et al. (2008) calculated from their experiments with linseed oil that the maximum favourable n-6/n-3 ratio in favour of n-3 fatty acids could be achieved using the inclusion of 5.8-5.9% of linseed oil in the mixture and would be 0.93/1 for drumstick meat. Any inclusion of linseed oil higher than this would exceed the enzyme capacity of the broiler. *Starčević et al*. (2014) achieved a ratio of 1.47/1 with the inclusion of 5% linseed oil, from the 25th to the 52nd days of fattening, which coincides with the results for white meat (*Chen et al*., 2014). In our study (Figure 5), as expected, the lowest ratio was in the group with the addition of 5% linseed oil (2.67/1), and it is interesting that the ratio was higher in the control group (12.18/1) than in the group with the addition of 5% lard (11.24/1), which clearly shows how negative an impact grains can have on the n-6/n-3 ratio.

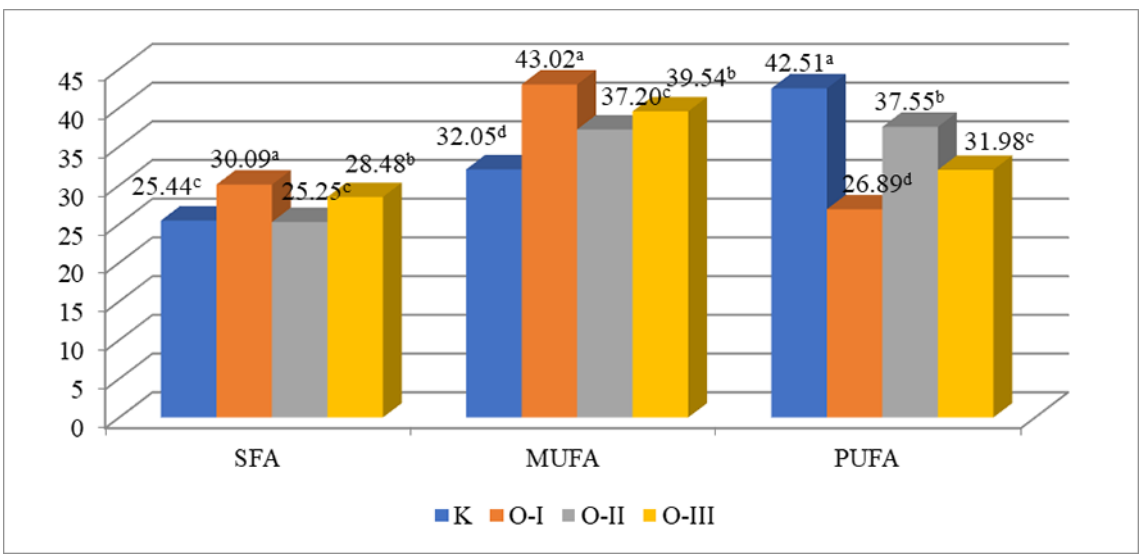

Figure 4. Mean content (%) of SFA, MUFA and PUFA in drumstick+thigh meat from the examined broiler groups (n=6). O-I; 1%, 2.5% and 5% lard in starter, grower and finisher feed, respectively. O-II; 1%, 2.5% and 5% linseed oil in starter, grower and finisher feed, respectively. O-III; 0.5%, 1.25% and 2.5% lard and 0.5%, 1.25% and 2.5% linseed oil in starter, grower and finisher feed, respectively. Different letters, ^{a,b,c,d}, in a fatty acid category indicate significant differences at $p<0.05$.

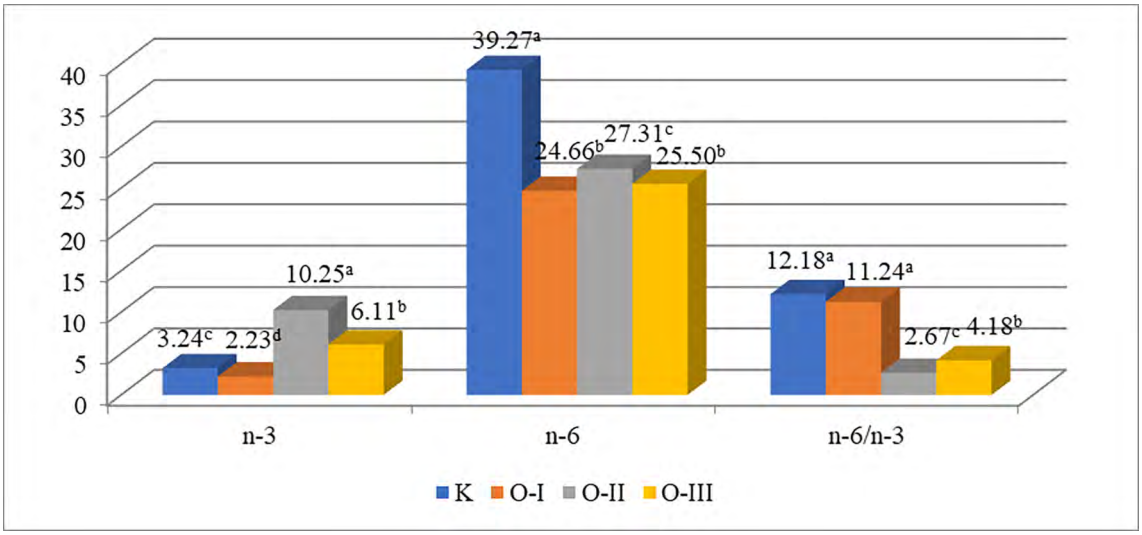

Figure 5. Mean content (%) of n-3 and n-6 fatty acids and n-6/n-3 ratio in drumsticks of the examined broiler groups (n=6). O-I; 1%, 2.5% and 5% lard in starter, grower and finisher feed, respectively. O-II; 1%, 2.5% and 5% linseed oil in starter, grower and finisher feed, respectively. O-III; 0.5%, 1.25% and 2.5% lard and 0.5%, 1.25% and 2.5% linseed oil in starter, grower and finisher feed, respectively. Different letters, ^{a,b,c,d}, in a category of lipid indicate significant differences at $p<0.05$.

Lipid indexes include the atherogenic index (AI), thrombogenic index (TI) and hypo/hypercholesterolemic index (h/H). The concentrations of C14:0 and C16:0 are important for calculating the AI and h/H indexes, while the concentration of C18:0 from saturated fatty acids is also important for the calculation of the TI index. The concentration of n-3 fatty acids is very important for obtaining the most favourable value of lipid indexes, and especially for obtaining a favourable TI. Among our four groups, lower AIs ($p<0.05$) in drumstick meat were found in the control and O-II groups (Figure 6). TI was lower ($p<0.05$) in the O-II group the TIs in the drumsticks meat of the other experimental

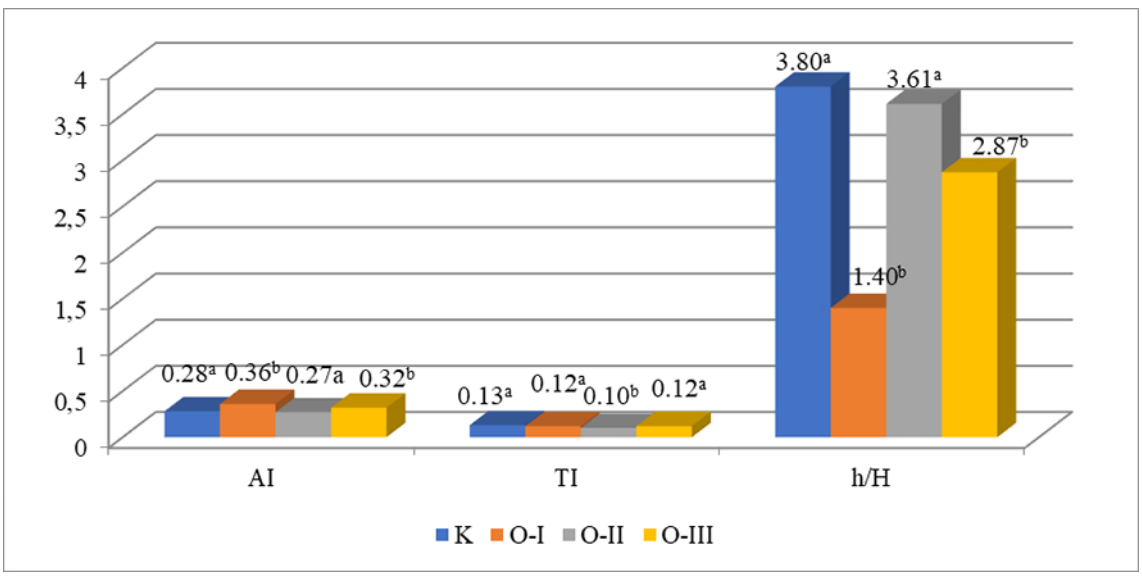

Figure 6. Mean lipid indexes in . AI—atherogenic index; TI—thrombogenic index and h/H—hypo/hypercholesterolemic index. O-I; 1%, 2.5% and 5% lard in starter, grower and finisher feed, respectively. O-II; 1%, 2.5% and 5% linseed oil in starter, grower and finisher feed, respectively. O-III; 0.5%, 1.25% and 2.5% lard and 0.5%, 1.25% and 2.5% linseed oil in starter, grower and finisher feed, respectively. Different letters, ^{a,b,c,d}, in a lipid index indicate significant differences at $p<0.05$.

groups. The h/H of the control and the O-II group were higher ($p<0.05$) compared to those of the other two groups of broilers.

It has been shown that an increased intake of saturated fatty acids, cholesterol and food with an increased AI and TI has negative consequences on human health (*Vitina et al*., 2012; *Attia et al*., 2020). By replacing red meat with chicken meat, the risk of developing cardiovascular diseases is reduced by 19% (*Hu*, 2005). However, *Attia et al*. (2017) determined that the h/H index of broiler meat is influenced by whether the meat is fresh or frozen. Fresh meat, compared with frozen, had a larger, i.e. more favourable h/H index, as well as higher MUFA, PUFA, and n-6 MK contents, MUFA/SFA ratio, and lower content of malondialdehyde (MDA) and SFA (Attia et al., 2017). In this study, the mixture with added pork fat (1%, 2.5% and 5%) had the highest content of ZMK. The h/H index of this O-I mixture was significantly lower than those of the other groups.

The mean content of MDA on day 0, i.e., before frozen storage, in the drumstick with thigh meat of the control group was higher ($p<0.05$) compared to the mean content of MDA of the experimental groups of broilers. After six months of storage, the mean content of MDA in the meat of the O-I group was lower ($p<0.05$) compared to the other groups (Table 1).

Different unsaturated fatty acids are differently sensitive to the process of lipid peroxidation. PUFA undergo this process easier and faster than MUFA, so linoleic acid (C18:2) oxidizes 10 times faster than oleic acid (C18:1), while linoleic acid (C18:3) oxidizes 20 to 30 times faster. Ions of iron and copper, as well as myoglobin, can further increase the degree of lipid peroxidation. The process of lipid peroxidation of the cell membrane occurs at the moment when the level of free radicals exceeds the antioxidant capacity of the cell, which results in the formation of primary peroxides (Glišić, 2020).

Table 1. Mean content of malondialdehyde (MDA; mg/kg) in drumstick+thigh meat (n=6) from control and experimental broiler groups after six months of frozen storage

	MDA ($\overline{X}$)	Measures of variation				
		Sd	Se	X_{min}	X_{max}	C_v (%)
K	0.40[a]	0.02	0.01	0.37	0.44	**6.11**
O-I	0.30[b]	0.03	0.01	0.26	0.34	**9.00**
O-II	0.39[a]	0.03	0.01	0.35	0.43	**6.80**
O-III	**0.38**[a]	**0.03**	**0.01**	**0.34**	**0.41**	**7.11**

O-I; 1%, 2.5% and 5% lard in starter, grower and finisher feed, respectively. O-II; 1%, 2.5% and 5% linseed oil in starter, grower and finisher feed, respectively. O-III; 0.5%, 1.25% and 2.5% lard and 0.5%, 1.25% and 2.5% linseed oil in starter, grower and finisher feed, respectively. Different letters, [a,b], ascribed to means indicate significant differences at $p<0.05$.

4. Conclusion

The best weight gain in the study was achieved by broilers consuming mealy feed with the addition of linseed oil, while the best feed conversion parameters were achieved with the addition of linseed oil and lard to the feed. Also, the best meat yield and the most favourable fatty acid profile in the meat was achieved through broilers consuming mealy feed with added linseed oil as an energy source, which means that the quality of the meat can be influenced by the correct choice of energy source.

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Review Paper

A brief review of hay or silage use in dairy cow diets and their effect on milk and dairy products

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ABSTRACT

Hay versus silage and their effect on the diet and performance of high-yield dairy cows, as well as on milk production and the quality of cheese has been the subject of research and a brief review of the issue is given here. Hay production and silage are the two most common methods of preserving green mass today and are important nutrients in the diet of cows. However, there has been much controversy about which feed is better and more useful in feeding highly productive cows, hay or silage? Based on studies that have dealt with this issue, information indicates that in recent years in European alpine regions of Switzerland, hay is returning to use in dairy cow diets, not so much because of the yield and quality of milk, but because of the dairy products that are of better quality and tastier if they are made from the milk of hay-fed rather than silage-fed cows. It was also found that the milk of cows with hay in their diet has a higher content of essential fatty acids, which have a beneficial effect on human health.

1. Introduction

Feed has a significant role in animal husbandry and is the main source of carbohydrates, proteins, vitamins and minerals. The choice of feed depends on the nutritional requirements of animals, the availability of land and water resources, and the climatic conditions of a particular area. Cattle feed can be fodder plant-based in the form of hay, silage or fresh green mass, and can be supplemented with other feed sources such as concentrated feeds, minerals and vitamins. Hay and silage are two common methods of preserving fodder plants. Hay is obtained by mowing and drying grasses or legumes, and the quality depends on the type of plant, stage of maturity and environmental conditions. Suitable plants for hay production are with thin stems and more leaves, because they dry quickly and have more protein than cellulose (most types of meadow grasses), and legumes (clover and alfalfa) that are mowed when the flowering phase begins. On the other hand, silage is obtained by fermenting green plants. Fresh green plants are used for silage, primarily cereals (mainly corn and oats) and legumes, which are cut and stored under cover, such as in silos or silo pits. During the storage period, chopped crops in anaerobic conditions undergo a natural fermentation process, which preserves nutrients and extends the shelf life of silage. However, silage has a higher moisture content so it is prone to spoilage if it is not properly preserved and stored. Silage production is the predominant method of preserving fodder crops today,

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as modern silage production technology allows for less dependence on weather conditions. Therefore, silage production has been successively replacing hay production since the 1970s, even on dairy farms in European alpine regions (*Gruber et al*., 2015). However, in the last twenty years, hay feeding has begun to reappear in these regions. The reason for this change is, on the one hand, the successful marketing and promotion of "hay milk", as a specialized product, and on the other hand, technological developments that allow farmers to dry green fodder mass indoors, using energy-efficient and powerful ventilation systems with dehumidifiers.

2. The effect of hay or silage on feed consumption and performance of dairy cows

On examining the effects of hay and silage on feed consumption, different results were obtained. Depending on the study, feed intake was higher when cows were fed hay (*Fasching et al*., 2015), remained unchanged (*Schulze et al*., 2014), or was lower (*Shingfield et al*., 2002) compared to silage. In wetter silages, a lower pH and higher amounts of fermentation end products (lactic acid, short-chain fatty acids, esters, biogenic amines) can reduce feed intake (*Grant & Ferraretto*, 2018). In their study, *Haselmann et al*. (2020) compared the effect of hay and silage from similar home meadows on the diet, feed consumption, and performance of dairy cows. Forage was obtained from meadows dominated by grasses (65% grasses, mainly *Lolium multiflorum* and *L. perenne*; and 35% legumes, mainly *Trifolium pratense* and *T. repens*) and from sowing meadows consisting of 75% grasses (mainly *Dactylis glomerata*, *L. perenne*, *L. multiflorum*, *Trisetum flavescens*, *Alopecurus pratensis*, *Anthoxanthum odoratum*), 20% legumes (mainly *Trifolium repens*, *T. pratense*) and 5% other plants (mainly *Taraxacum officinale* and *Plantago lanceolata*). The results (as indicated in Table 1) showed that cows fed hay rather than silage had greater intake of dry matter (18.3 vs. 17.8 kg/day, respectively) and consumed a higher amount of water-soluble carbohydrates (+1.2 kg/day). Also, hay-fed cows showed a trend towards higher energy excretion via their milk than did silage-fed cows (30.1 and 28.5 kg of energy-corrected milk/day, respectively). The higher content of water-soluble carbohydrates improved the fermentation processes in the rumen, which in turn provided the hay-fed cows with additional amounts of nutrients and energy. In cows that received hay rather than silage, a higher milk fat content was observed (+2.4 g/kg). In terms of milk protein and lactose, no significant differences were observed between the feeding regimes, while the urea content of milk was 1.4 mg/100 mL higher when cows were fed hay. Cows on silage had a significantly thinner consistency of faeces, but the faecal dry matter content did not differ from that of cows fed on hay. The efficiency of feed conversion was almost the same in both groups, while the efficiency of nitrogen conversion was 2% higher in the cows fed with hay. The daily energy yield of milk was 5.26 MJ/NEL higher in the hay group compared to the silage group. The authors concluded that the hay diet improved the efficiency of feed use in dairy cows.

3. The effect of hay or silage in the diet of cows on the quality of milk and cheese

Milk production has always been an important link in the food supply chain. That is why a constant supply of high-quality nutrients is crucial for milk producers. Silage is more flexible and economical compared to hay; however, poorly preserved silage can develop unwanted microorganisms that cause spoilage and a decrease in nutritional value, and even worsen the quality of milk and dairy products, especially mature cheeses. Poor quality silage feeding carries the risk of contamination of raw milk with clostridial endospores, which can cause late-maturation defects in hard and semi-hard cheeses in the later stages of ripening (*Brandle et al*., 2016). *Schaeren et al*. (2005) analyzed milk samples from 18 farms where 12 farms fed silage and six farms operated without silage, finding a higher prevalence of *Clostridium tyrobutyricum* spores in milk

Table 1. Result indicators derived from a study on the effect of hay and silage on dairy cow feed intake and milk components (Haselmann et al., 2020)

	Dry matter intake	E in milk	Milk carbohydrates	Milk urea	Milk lipids	Milk proteins	Milk lactose
Hay	+	+	+	+	+	=	=
Silage	-	-	-	-	-	=	=

from farms where silage was used, with no differences in gross milk composition. Even well-preserved silages can affect the composition and sensory characteristics of cheeses (*Cosentino et al*., 2016). That is why rules were introduced regarding the silage-free diet of dairy cows. The European Union has registered hay milk as a "traditional speciality guaranteed" product, whereby strict criteria regarding milk production must be met (silage must not be used as animal feed) (*European Commission*, 2016; *European Parliament and Council of the European Union*, 2012). This is supported by *Baars et al*. (2012), who found a lower content of phytanic acid in milk from farms that used hay compared to farms that used silage. Moreover, hay milk is described as milk that is suitable for the production of hard cheese. *Van den Oever et al.* (2021) did a significant study that directly provides a comparison of milk from cows fed on hay and milk from cows fed with silage produced from the same plant material. Forage plants from two types of meadows were used, a combination of grass and clover (65% grass, 35% legumes) and a combination of grass, legumes and other herbs (75% grass, 20% legumes, 5% plants). Fodder plants are mowed at the same time and loaded into wagons, either for ensiling in a horizontal silo or fed into a hay crate for drying. The results showed that there are some significant differences between milk from hay-fed cows and milk from silage-fed cows (indicated in Table 2). Linoleic acid, α-linolenic acid, lysine and putrescine showed higher concentrations in the milk of cows fed on hay, while vitamin B2 and spermine were higher in the milk of cows fed on silage. The milk yield of cows averaged 28.8 kg per day. Compared with the hay-fed cow milk, the content of whey protein was higher (+14.3%) in the milk of cows fed with silage, as was the concentration of free amino acids (+20.4%). A slightly higher protein intake in cows fed silage could be partly responsible for the higher concentration of free amino acids in their milk. Compared with the hay-fed cow milk, a higher content of vitamin B2 in the milk of cows fed with silage (+15.8%), as well as vitamin B1 (+3.8%) and vitamin B12 (+12.3%) was found. However, concentrations of vitamins B1 and B12 dropped significantly as the cows produced more milk. Interestingly, in the milk of cows fed on hay, the concentration of vitamins B1 and B12 increased as the milk yield of the cows increased. The vitamin E content was 1.9 times higher in the milk of the cows that received silage than in milk of hay-fed cows. It is assumed that silage offers cows smaller amounts of polyunsaturated fatty acids than does hay, which in turn could increase the concentrations of vitamin E in the milk of cows on silage. The effect of hay or silage on the sensory properties of milk indicates that the attributes (color, consistency and smell) were significantly higher in the milk of cows fed with silage. Other attributes, such as the separation of sour cream, sweetness, or taste, showed almost no differences between the two types of milk.

Manzocchi et al. (2020a) compared the effect of hay and silage obtained from the same plot on the milk and cheese components. The most common plant species on the plot were: 65% grass (*Lolium perenne*, *Phleum pratense*, *Bromus hordeaceus*, *Poa pratense*, *Dactylis glomerata*, *Festuca pratensis*), 15% legumes (*Trifolium repens*) and 20% others (*Taraxacum officinale*, *Veronica chamaedrys*, *Lamium album*). In addition to fodder plants, late-lactating cows received 3 kg/day of dry matter from concentrates. The results showed (Table 3) that the milk of hay-fed cows had a higher protein content and a higher concentration of P (+0.1 g/L) than the milk of silage-fed cows, while the fat and lactose content was similar in both groups. The milk of cows fed on hay had a more intense color compared to the milk of cows fed with silage. A weak yellow tint of milk and cheese was observed in both groups and coincided with the previously observed lower content of carotenoids in the milk of cows fed silage, and especially in milk from cows fed hay. Carotenoids are depleted by photooxidation during wilting and plant preservation, and the carotenoid content of milk is directly related to dietary carotenoid intake (Nozière et al., 2006). The milk of cows fed with silage had

Table 2. Result indicators derived from a study on the impact of hay and silage on milk quality (Van den Oever et al., 2021)

	Linoleic acid	α linoleic acid	Lysine	Putrescine	Vit.B2	Spermine	Whey Proteins	Free amino acids	Vit.E
Hay	+	+	+	+	-	-	-	-	-
Silage	-	-	-	-	+	+	+	+	+

Table 3. Result indicators derived from a study on the impact of hay and silage on milk and cheese quality (*Manzocchi et al*., 2020a)

	Milk proteins	Milk phosphorus	Milk fat	Milk lactose	Milk colour	Sour cream smell	Cheese yield	Cheese pH	Dry matter content of cheese	Salt content of cheese
Hay	+	+	=	=	+	-	+	=	=	=
Silage	-	-	=	=	-	+	-	=	=	=

a stronger smell of sour cream than did the milk of cows fed with hay, which is the result of a higher proportion of PUFAs in the lipids of the silage. The yield of ripe cheese was higher when cows were fed with hay (+128 g/100 kg of milk) rather than silage. The cheeses did not differ in pH, or dry matter, fat or salt contents.

Serrapica et al. (2020) investigated the effect of hay and silage on volatile compounds and sensory properties of hard cheese during ripening. The hard cheese was a traditional semi-hard cheese that is usually produced in the hilly southern part of Italy in small artisanal factories or farms, using milk obtained from animals fed a combination of concentrates, grazing and canned feed. A hybrid of *Sorghum sudanense* × *Sorghum sudanense* (Piper) Stapf. was grown on the plot and after mowing, half of it was ensiled into plastic silo bags, and half was dried to hay. The lactating cows were divided into two groups, one using sorghum hay and the other using sorghum silage. Both groups were given both isonitrogen and isoenergetic mixed feed. Milk from both groups was used to produce three batches of cheese that were 30, 60 and 90 days old. As expected, ensiling increased the solubility of nitrogen so that higher NPN values were observed in the milk of cows fed silage than in milk from hay-fed cows (as indicated in Table 4). There was no difference in milk yield (26.8 vs. 27.0 kg/day) or milk fat (4.07 vs. 4.27). However, milk protein was higher in hay-fed cow milk than in silage-fed cow milk (3.24 versus 3.50). This result, which was also noted by *Verdier-Metz et al.* (2005), may be due to a decrease in rumen microbial protein synthesis in silage-fed cows, which is associated with higher solubility of silage proteins (*Huhtanen et al*., 2003). Consequently, *Vaga and Huhtanen* (2018) observed that dried grass contributes to the provision of ammonia for microbial protein synthesis to a greater extent than silage grass. In cheese made from milk from cows fed with silage, higher amounts of ketones and fatty acids were observed than in the hay-fed counterpart. In contrast, cheese made from milk from hay-fed cows showed the presence of the terpene α-pinene, which was not detected in milk cheese derived from silage-fed cows. The composition of milk, and the chemical and fatty acid composition of the cheese were not significantly different between the feeding regimes. However, cheese ripening provoked changes in sensory characteristics. The silage-based diet induced a greater perceived intensity of color, smell, flavour and texture which allowed the trained panellist to differentiate products and determine the increased consumer preference for "hay" cheese after 30 and 90 days of ripening, thus supporting the marketing strategy for hard cheese to be sold with the Hay milk label.

Manzocchi et al. (2020b) investigated the feeding of dairy cows with hay and different types of silage on feed intake, milk composition, and coagulation properties. They aimed to examine whether the milk of hay-fed cows differs from the milk

Table 4. Result indicators derived from a study on the effect of hay and silage on milk and cheese quality (*Serrapica et al*., 2020)

	NPN	Milk yield	Milk lipids	Milk Proteins	Ketones in cheese	FA in cheese	α-pinene in cheese	Colour of cheese	Smell of cheese	Taste of cheese
Hay	-	=	=	+	-	-	+	-	-	-
Silage	+	=	=	-	+	+	-	+	+	+

Table 5. Result indicators derived from a study on the impact of hay and silage on milk and cheese quality (*Manzocchi et al*., 2020b)

	Milk yield	Milk Lipids	Milk proteins	Lactose	Urea	Acidity of milk	Dry matter intake
Hay	+	+	=	+	-	=	+
Grass silage	-	+	=	-	+	=	+
Corn silage (10mm)	+	+	=	-	=	=	+
Corn silage (30mm)	+	-	=	-	=	=	-

of silage-fed cows and whether the type of silage also has an important influence. Hay, grass silage, conventional short-chopped corn silage and long-chopped corn silage were used in the experiment. All four types of feeds had the same estimated milk production potential and a nutrient-to-concentrate ratio of 0.85:0.15. The first experimental feed was warm-ventilated hay from the first sowing from a permanent meadow dominated by ryegrass. The second forage was silage from ryegrass and clover, harvested during the second and third mowing. The third and fourth feeds were silages from the whole maize plant, both harvested at the stage of milky maturity from the same field and cut to lengths of 10 mm (short-cut) or 30 mm (long-cut) and stored in the same silo container. Absolute milk yield was numerically lower, but not significantly, in cows fed grass silage than cows on the other diets. Among the diets, the milk fat content was the lowest in the milk of cows fed long-chopped corn silage (indicated in Table 5). The milk protein and casein content, as well as the ratio of casein to total milk protein, did not differ between diets. The lactose content was highest in the milk of cows fed with hay compared with the other diets. The urea content was highest in the milk of cows fed grass silage, i.e., urea was twice as high as in the milk of cows fed with hay, and almost the same in the milk of cows fed on corn silage. The acidity of the milk and the parameters of coagulation of rennet did not differ between the diets. The lower dry matter intake from long-shredded corn silage can be explained by the longer retention time of longer particles in the rumen (*Kononoff et al*., 2003), which could slow down rumen emptying and, thus. limit the intake capacity. Despite this lower feed intake, milk yield was not affected by the diet of long-chopped corn silage. However, reduced milk yield was observed with prolonged exposure, as shown by *Mahlkow et al*. (2005) when they fed cows long-shredded corn silage for seven months.

4. The effect of hay or silage in the diet of cows on the concentrations of fatty acids in milk

The composition of fatty acids plays an important role in milk quality and has a long-term impact on human health (*Van Vliet et al*., 2021; *Haug et al*., 2007). Many studies have confirmed the role of fatty acids in the prevention of several chronic human diseases, such as cardiovascular disease, some forms of tumours, obesity and diabetes (*Pereira*, 2014; *Haug et al*., 2007). Increasing the proportion of maize silage in cow diet increased economic efficiency, but reduced the relative contents of fatty acids, such as CLA and n-3 PUFA, in raw milk (*Van Vliet et al*., 2021; *Riuzzi et al*, 2021; *Liu et al*., 2020) and cheese (*Serrapica et al.* 2020). Based on the fatty acid profile, a distinction can be made between grass hay milk and corn silage milk (*Riuzzi et al*., 2021; *Paredes et al*. 2018).

Wang et al. (2023) investigated the effect of hay and silage on the fatty acid status of dairy cow milk. Cows weighing 650 kg producing about 30 kg/day of milk were fed a variety of feeds (dry-based): (1) 46% corn silage as the sole source of feed, (2) a mixture of 23% corn silage and 14% meadow hay (6% alfalfa hay and 8% oat hay) as a feed source, and (3) 28% meadow hay (12% alfalfa hay and 16% oat hay) as the sole source of feed. Hay feeding increased the content of SCFA, CLA, and n-3 PUFA in milk compared to milk from cows fed corn silage (indicated in Table 6). The lower amount SCFAs in the milk of cows fed with maize silage is predominantly due to a decrease in acetate production during rumen fermentation and absorption by the mammary glands. Increasing the proportion of hay in the diet increased intestinal digestion and arteriovenous differences in n-3 PUFAs and total CLA, and, together with higher Δ9-desaturase activity in the mammary glands, resulted in higher relative n-3 PUFAs and total CLA in milk.

Table 6. Result indicators derived from a study on the effect of hay and silage on fatty acid concentrations in milk (*Wang et al*., (2023)

	SCFA	CLA	n-3 PUFA
Hay	+	+	+
Mix	-	-	-
Silage	-	-	-

Schaeren et al. (2005) analyzed the gross milk composition as well as vitamins and minerals in milk from cows fed with or without silage. Although the farms may have had similar production conditions (same region, similar growing conditions, and levels of milk production), the plants came from different plots. There was no significant difference in gross milk composition between the silage-fed and non-silage-fed groups, but the concentrations of conjugated linoleic acid and omega-3 fatty acids were slightly lower in the milk of silage-fed cows. *Paredes et al.* (2018) reported that commercially pasteurized hay milk had a higher content of α-linolenic acid (+37%) and CLA (+22%) than non-hay milk. The study results altogether as summarized in this section show that a higher proportion of hay in the diet of dairy cows increased the relative content of short-chain fatty acids (SCFA), CLA and n-3 PUFA.

5. Discussion

The European Union has registered hay milk as a *traditional speciality guaranteed* product, and therefore, strict criteria must be met regarding production of this milk, i.e., silage must not be used as animal feed (*European Commission*, 2016; *European Parliament and Council of the European Union*, 2012). Studies have confirmed that a hay compared to a silage diet increased feed consumption and energy production in milk in dairy cows (*Haselmann et al*., 2020). Many studies have investigated the effects of feeding dairy cows with hay or silage produced from the same parent plant material, which was important in order to ensure direct comparability, on several components of milk, such as protein fractions, fatty acids, and certain vitamins. The analyzed milk from hay-fed cows and silage-fed cows differed significantly in several components. Higher concentrations of the essential fatty acids, lysine and putrescine, were found in the milk of hay-fed cows, while the milk of silage-fed cows showed higher concentrations of vitamin B12, vitamin E, and spermine (*Van den Oever et al*., 2021). Because of this, as well as intensive marketing campaigns, farmers receive premium prices when hay is the only canned feed used by cows (silage-free meals). Traditionally, in European alpine areas, only milk from hay-fed cows is used to produce semi-hard and hard cheeses from raw milk. In Switzerland, farms that do not use silage account for one-third of total milk production. Cheeses made from raw milk without silage do not show unwanted later swelling during ripening and do not spoil with butyric acid, which has a negative effect on smell and taste (*Wyss & Goy*, 2012). The Swiss government supports the production of milk and cheese without the use of silage in the diet of cows and labels it as hay milk. The products are marketed and sold at a much higher price in several Central European countries (Austria, Germany, Switzerland, France and Italy). In 2016, the term hay milk was included in the European Register of Traditional Specialities Guaranteed and is, thus, protected throughout the EU (*European Commission*, 2016). The product specification hay milk stipulates that the annual diet of dairy herds consists of hay-based feed with at least 750 g/kg dry matter (SM) and that it contains no more than 100 g of concentrate/kg SM (*Verein Heumilch Schweiz*, 2017). In addition to hay and fresh grass, artificially dried grass is also allowed, while the use of all industrial wet feed products is prohibited.

Finally, however, it should be noted that some of the analyzed components in milk from cows fed with silage did not differ significantly from the components in the milk from cows fed with hay. Further research is needed to investigate other effects and possibilities of using silage in the diet of dairy cows.

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Review Paper

In ovo technique—a new promising tool for improving growth production results, yield and meat quality of broilers

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ABSTRACT

The perinatal period (the last few days prior and after hatching) is currently recognized as the most crucial time in the development of a young chick. During this time, the chicks undergo metabolic and physiological shifts from the utilization of egg nutrients to exogenous feed. Unlike mammals, embryonic development in avian species is an external process that occurs in the absence of continuous maternal energy supply. Especially in the final third of incubation, embryo development in birds becomes limited by the nutrient content present in the egg. *In ovo* technology is defined as the direct application of various feed additives (amino acids, hormones, vitamins, carbohydrates, minerals, prebiotics, probiotics), vaccines and nanoparticles into different internal parts of the egg during the incubation period. This technique has been recognized for its potential to improve digestive capacity, increase growth rate and feed efficiency, reduce post-hatch mortality and morbidity, improve immune response to enteric antigens, reduce incidence of developmental skeletal disorders, and increase muscle development and breast meat yield. Nutrients like carbohydrates, amino acids, vitamins and trace minerals were found to be most effective for the purpose of *in ovo* feeding.

1. Introduction

Large selection centres dealing with the genetic improvement of broilers have created fast-growing hybrids (provenances) that reach their final slaughter weight at the age of 35-39 days, with minimal feed consumption per unit of growth. It is an interesting fact that the body weight of the boilers at the end of fattening (42nd day after hatching) in the period from 1957 to 2014 grew by 3.30% every year, while at the same time, the consumption of food per unit of growth (food conversion) decreased by 2.55% (*Kucharska-Gaca et al*., 2017). However, the intensive growth of genetically improved hybrids caused the appearance of numerous health disorders, such as ascites, sudden death syndrome, improper development of the skeletal system (leg problems), and increased sensitivity to pathogenic microorganisms (*Radulovic et al*., 2023). Genetic progress has put

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more stress on the fast growing and high meatiness bird, which resulted in histological and biochemical modifications of the muscle tissue by impairing some meat quality traits (*Petracci and Cavani*, 2012).

In addition to all these previously mentioned problems, it is considered that the amount of nutrients contained in the egg is no longer sufficient for the optimal development of embryonic tissues. Unlike in mammals, embryonic development (inside the egg) in poultry is an "external process", during which there is no possibility of supplying energy and other nutrients from the mother, and which makes the nutritional status of the embryo crucial for the success in the production of chicks. In relation to the total lifespan of modern broiler hybrids (35-39 days), the embryonic phase (21 days) represents about 33-38%. Bearing in mind that the formation and early development of tissues (muscles and digestive tract) takes place precisely in this phase, there is a strong connection between embryonic development and the production results that broilers can achieve during the fattening period. Physiological and metabolic imbalances at the end of the embryonic phase and the initial period of development after incubation of chicks negatively affect their subsequent growth and carcass characteristics (*Retes et al*., 2018).

At the same time, it should be noted that in industrial production, after hatching, chicks generally remain without food and water for 24-72 hours. This occurs due to different hatching times, handling of chicks (separation by sex, vaccination, packaging) and the time required to transport them to the farm (*Willemsen et al*., 2010). As a result, the starvation of chickens during this period has a negative effect on the morphometric characteristics of the mucous membrane of the digestive tract (shortening of the height of the intestinal villi and reduction of the depth of the crypts). In this way, the ability to digest and absorb nutrients (from the complete mixtures that the broilers will receive upon arrival at the farm) is reduced and the time required to reach slaughter weight is increased by 1-2 days. Due to the impossibility of food intake, peristaltic movements (which are activated by exogenous feeding) are absent, which results in slower transport of the contents of the yolk sac into the intestines. The impossibility of access to food additionally limits the use of nutrients that are present in the yolk, which leads to the consumption of energy reserves in the body. Under these conditions, the proliferation of satellite cells is reduced and muscle growth is inhibited, which further reduces the initial performance of broilers (*Zhao et al*., 2017, *Noy and Slan*, 1999). For all these reasons, embryonic nutrition is a key factor for muscle and digestive tract development during late embryonic development and in the first days after hatching.

2. *In ovo* technique

In order to overcome all the mentioned problems, and primarily to increase the availability of nutrients to the embryo, the *in ovo* nutrition technique was developed. With this technique, nutrients can be delivered (inoculated) to different internal parts of the egg. *In ovo* technology (Lat. in the egg) is defined as the direct application of feed additives (amino acids, hormones, vitamins, carbohydrates, minerals, prebiotics, probiotics, vaccines and nanoparticles) during the egg incubation period. By timely supply to the embryo of nutrients, positive effects on its growth, d evelopment, and resistance are achieved, but at the same time, long-term effects are accomplished, which become visible only during the next period, i.e., rearing and fattening of chicks (*Saeed et al.*, 2019). The main goals of this technique are reflected in treated individuals by the following:

1) Lower embryo mortality (by strengthening immunity and defence against pathogenic microorganisms),
2) Higher body mass at hatching,
3) Stimulated development of the gastrointestinal tract before and after hatching,
4) Growth stimulation,
5) Better utilization of feed, and
6) Higher meat yield (*Bhanja et al*., 2013).

The entire process of this technique can be applied manually or automatically. Both types of application have proven to be effective, but their choice depends primarily on the number of eggs that need to be treated, the age of the embryo, and types of inoculants that are used. This technique was first applied on a larger scale in the 1990s in the United States during the automatic vaccination of poultry against Marek's disease and Gumboro disease. After that, the possibility of its much wider application was recognized, primarily in the stimulation of embryonic development, immune response and the development of beneficial types of bacteria in the digestive tract of the embryo (*Kadam et al*., 2013). *Jokić et al*. (2023) described in detail the entire procedure of the

in ovo technique, where the first step in the practical work is the selection of a specific hybrid of laying hens that will be examined or the collection of eggs from local producers. It is necessary to first examine all eggs, then discard the damaged ones and weigh only the ones that will be further used. The eggs are then laid in an incubator with controlled temperature and humidity (37.5°C and 60.0%, respectively, for Cobb 500). After a certain period (most often on the 6th day of incubation), the eggs are lamped and the non-fertile ones are discarded, while the fertile eggs are distributed into the appropriate groups. After a few days (generally on the 18th day of life) the following procedure is applied to evaluate the success of this technique:

1) The first group of eggs is treated with the prepared inoculum,
2) The second group is not treated and serves as a control, while
3) The third group is treated with normal (physiological) solution.

In this way, it is possible to compare the third with the first group, considering that handling eggs and piercing the egg shell with a needle can have negative effects on the development of the embryo. The solutions used for *in ovo* treatment must be heated to a temperature of 30°C before use. At the same time, it is very important to use a new sterile needle for each egg, which can be of different dimensions (most often 13 × 0.3 mm or 18.4 × 1.27 mm) and a syringe with a volume of 1 ml. The injection site on the egg must be disinfected with iodine, alcohol or another disinfectant, after which the contents of the syringe are applied to the desired part of the egg, usually the amnion. In order to ensure success in the work, a certain number of eggs must be broken immediately after the treatment in order to check the accuracy (precision) of the application of the inoculum in the desired region. The needle penetration site is sealed with paraffin, and all eggs are then returned to the incubator. The room where the incubator is located must be regularly disinfected and a constant temperature and air humidity must be maintained. After hatching, the time elapsed from the beginning of incubation, percentage of hatchlings, and the body mass of chicks are measured. The hatched chicks can be sacrificed immediately or they are assigned further to rearing, during which they can be subjected to histological, biochemical and microbiological tests. At the end of the 25th day from the start of incubation, all unhatched eggs are opened to assess the cause of embryonic death and then record their number. Various factors, such as the site and rate of injection, the volume of the inoculum, and even the dimensions of the needle used, can have a significant impact on the success of the *in ovo* feeding technique.

3. *In ovo* administration of the inoculum

There are five basic egg regions into which inoculation can be conducted:

1) The body of the chicken embryo,
2) Amniotic fluid,
3) Allantoin membrane,
4) Air sac, and
5) Yolk sac (*Saeed et al.*, 2019).

In the initial stage of embryonic development, the inoculum is injected into the egg whites at a depth of 12 mm, as close as possible to the germinal disc. Only at a later stage of development are embryos inoculated into the yolk sac. This site is suitable for injection, primarily due to its large surface area and ability to digest nutrients. After the 17th day of incubation, the yolk sac is resorbed (but not completely), so the application is made into the air sac or amnion. It is considered that in the late stage of embryo development, *in ovo* administration is best carried out into the amniotic fluid, which then comes into contact with the cells of the digestive tract and serves the embryo as a source of nutrients. Therefore, the material injected into this area will be used together with the amniotic fluid (*Kucharska-Gaca et al*., 2017). It is interesting to note that when vaccine is administered into the amniotic fluid, regardless of the day of vaccination, more than 90% protection is achieved, while injecting the same vaccine into the allantoin fluid or the air sac creates less than 50% protection. Special attention must be paid to the depth of injection. If the needle is not applied deep enough into the egg, dispersion of the inoculum into the allantoic fluid or air sac may occur, while inserting the needle too deeply can result in trauma to the embryo. The injection site is determined based on the characteristics of the injected substance, the procedure of its introduction, the age of the embryo, etc. (Roto et al., 2016).

4. Nutrients with highest potential for in ovo administration

Carbohydrates

Among the nutrients most often examined in the *in ovo* technique, carbohydrates are in first place, because inside the egg they occur only in very low concentrations (amounting to < 1% of total nutrients, while only 0.3% is free glucose). An additional reason for the use of carbohydrates in this diet is the fact that increasing the amount of available carbohydrates can reduce the use of amino acids in gluconeogenesis and, thus, increase protein synthesis in the muscles of the embryo (*Retes et al*., 2017). In this way, by using carbohydrates and stimulating the proliferation of satellite cells, long-term effects on muscle growth can be achieved (increased myofibril diameter and muscle weight on the 35th day after hatching). By injecting carbohydrates (sucrose + maltose), it is possible to increase glycogen reserves in the liver and muscles of the embryo, thus creating favourable conditions for overcoming the energy deficit in the last days of incubation (*Neves et al.*, 2016; *Saeed et al*., 2019). An important effect of *in ovo* application of carbohydrates is the achievement of a higher body mass of chicks at hatching. This can be the result of an increase in the mass of the yolk sac, considering that with an additional supply of carbohydrates, the embryo has less need to use nutrients from the yolk. However, an increase in the mass of the yolk sac can also be a consequence of an increase in water, i.e., the amount of water present in the yolk, which has a negative effect on the transport of nutrients from the yolk sac to the body of the embryo. In order to avoid this effect, it is necessary to use adequate amounts of inoculum (using too little inoculum does not induce the desired effect, while using too much inoculum reduces the percentage of hatching) (*Kucharska-Gaca et al*., 2017). Using carbohydrates with the *in ovo* technique ensures:

1) Greater presence of these nutrients in the intestinal lumen,
2) Stimulation of intestinal villi development,
3) Stimulation of the activity of brush coat enzymes, and consequently
4) Improved intestinal capacity for digestion and absorption after hatching (*Tako et al*., 2004).

Today, there is an opinion that with *in ovo* nutrition, the digestive tract of the treated individuals, in terms of their functionality on hatching day, approaches the digestive tract of a two-day-old chicken. The digestive tract does not have the same ability to absorb all nutrients, nor is it always the same (constant). Thus, the absorption of carbohydrates and proteins is low at the time when chicks hatch, and gradually increases during the first four days of life. On the other hand, fatty acid absorption is high at hatching and remains at this level during the first four days after hatching. Numerous studies have shown that when eggs are inoculated with carbohydrates, this additional source of energy increases the development of goblet cells (the presence of carbohydrates in the lumen of the intestine is a trigger for the proliferation of goblet cells) and increases the surface of the intestinal villi, which creates better conditions for the absorption of nutrients *(Jokić et al*., 2023).

Amino acids

Amino acids play a significant role in the body's immune response, growth and development of embryonic tissues, while, in the absence of carbohydrates, they can also be used as sources of energy. In this case, the application of amino acids can improve embryonic survival and increase embryo resistance to pathogenic microorganisms. It was established that during the early period of its development, the embryo uses the amino acids glycine, proline, lysine and arginine to a great extent. The needs for proline and glycine increase during embryonic development, so that in the last third of incubation, the contents of these two amino acids are not sufficient for normal embryo growth (*Cardeal et al*., 2015; *Saeed et al*., 2019). In overcoming this problem, exogenous (*in ovo* technique) addition of the amino acids in this phase is useful. By adding the amino acids proline and glycine in the last third of embryonic development, it is possible to limit antibodies from using them as a source of energy or as a source of protein. In this way, the basic (immunological) purpose of antibodies can be preserved, and thus, the conditions for improving production results during the rearing period can be provided. In addition to the amino acids proline and glycine, numerous studies have examined the effects of using arginine and threonine, either individually or in combination with other amino acids. Arginine is considered an essential amino acid for embryo growth, with its primary function in protein synthesis, although it can also be used for the synthesis of glucose (glucogenic amino

acid). Also, this amino acid is used to create biologically active components that stimulate the secretion of growth hormones. Arginine also has the role of an immunomodulator, because it participates in the production of nitric oxide and in the increasing the function of the thymus over time. The amino acid threonine is the main component of intestinal mucin and participates in the synthesis and control of the secretion of pancreatic enzymes, which promotes the development of the digestive tract and the intake of food after hatching. In addition, threonine plays an important role in the immune protection of the body, given that it is part of gamma globulin (*Kucharska-Gaca et al*., 2017). After *in ovo* application (20–30 mg) of threonine into the yolk sac of embryos, the titre of antibodies against sheep red blood cells increases in broilers. Similar results can be achieved if other amino acids, such as glycine and serine, are used simultaneously with threonine (*Toghyani et al.*, 2018).

Vitamins

The only source of vitamins for the development of the chicken embryo is a well-balanced diet of the mother hen. Mistakes made when formulating the ration intended for the parent flock often result in deficits and inadequate embryo development, as well as increased embryonic mortality (most often in the middle or later stages of incubation). In this way, the application of vitamins and minerals is aimed at:

1) Improving the antioxidant status of the embryo,
2) Increasing embryo immune response,
3) Improving the bone system, and
4) Improving production results of chicks after hatching (Jokić et al., 2023).

During the utilization of lipids from the yolk sac, long-chain polyunsaturated fatty acids (PUFA) are formed in the embryonic tissues. A high concentration of PUFA in cell membranes increases the sensitivity of the embryo to the action of free radicals, which results in tissue breakdown and cell death (*Selim et al*., 2012). Antioxidants present in the egg yolk control this process by reducing or deactivating free radicals. However, a special problem is caused by the fact that when hatching, chicks switch from the so-called chorioallantoic respiration to the pulmonary type of respiration. This switch causes the concentration of oxygen in the tissues to increase, and due to intensive oxidative processes, the production of free radicals increases significantly (*Araujo et al.*, 2019). Therefore, *in ovo* inoculation of antioxidants can significantly improve the antioxidant capacity of embryonic tissues, hatching, quality of chicks, as well as the production results that they will achieve during fattening. The effects of the use of vitamin E have been mostly studied in numerous *in ovo* studies. Its use can provide adequate protection of intestinal cells in the fight against free radicals and, thus, increase cell renewal and stimulate the growth of intestinal villi in the duodenum (*Bhanja et al*., 2012). At the same time, by increasing the antioxidant capacity (with the use of vitamin E) in the yolk sac, both the embryo and the chick in the first days after hatching are given the opportunity to more fully use the available nutrients. In addition to vitamin E, positive effects have been noted with the use of other vitamins, such as vitamins B1 and B2 in stimulating growth, as well as vitamins A, B1 and B6 in improving the immune response of broilers (*Saeed et al*., 2019).

Mineral substances

The role of minerals in the development of the skeletal, muscular, immune and cardiovascular systems is well known. Their optimal deposition in different parts of the egg is crucial for the proper development of the embryo. The hen (mother) deposits minerals in the egg in two ways:

1) Through the ovary to the yolk, and
2) Through the oviduct to the albumen, shell and shell membrane (Yair et al., 2015).

During the last days of incubation, the amount of Cu, Mn, P and Zn in the yolk is very low, so the embryo uses these minerals very little or not at all during this period (Yair and Uni, 2011). *In ovo* use of organic forms of Cu, Fe, Mn and Zn can increase their contents in the yolk and, thus, their use by the embryo. In this way, a long-term positive effect on mineralization and bone strength of broilers is achieved. Likewise, when using the aforementioned microelements, but in inorganic forms, their concentration in the yolk increases, which indicates that the form of the used minerals is not crucial for this transport (*Oliveira et al*., 2015). In addition to the abovementioned forms, in recent years, nanoparticles have been attracting more attention, i.e., nano forms of minerals are interesting considering the possibility that they bypass normal transport and undergo facilitated passage through the cell membrane. The use of nano forms of Zn, Cu and Se through *in ovo* feeding improves the feed conversion in broilers during

fattening, as well as increasing the yield of breast muscle (*Joshua et al*., 2016). Using sodium selenite (Na_2SeO_3) as a source of Se improves the immune response and antioxidant capacity of chickens, especially if they are previously exposed to *Eimeria maxima* and *Clostridium perfringens*—the causative agents of necrotic enteritis (Lee et al., 2014).

Other substances suitable for *in ovo* feeding

In addition to all the listed and described nutrients, specific potential is also shown by other supplements, such as:

1) Growth hormone (IGF I)—stimulation of growth and development of muscle tissue (Bhanja et al., 2014),
2) Grapefruit seed extract—improved degree of hatching, improved daily gain and reduction of the population of *Escherichia coli* in the ileum (Hayati et al., 2014),
3) L-carnitine—improvement of the transport of fatty acid acyl residues from the yolk sac to embryonic tissues (Cardeal et al., 2015), and
4) In recent years, the possibility of early management of the microbial composition of the digestive tract in chickens has been highlighted as a special segment of *in ovo* nutrition. The concept itself does not represent anything new in poultry production, but the possibility that the management of microbiota, instead of in chicks during the rearing and fattening period, can already be started during embryonic development, represents a great innovative progress. The aforementioned concept of nutrition in modern literature is referred to as "early programming" or "early programmed development", and implies the use of feed supplements such as probiotics, prebiotics, symbiotics and phytogenic preparations. Their common goal in the body of the treated individual is the establishment of a desirable and stable relationship between beneficial and harmful bacteria in the digestive tract. In this way, a eubiotic effect and a positive impact on the health of the digestive tract, as well as the health of the whole organism, is achieved, which is a basic prerequisite for achieving high production results (*Pruszynska-Oszmalek et al*., 2015; *Tavaniello et al*., 2018).

5. Future approaches regarding the in ovo technique

With the introduction of the *in ovo* feeding technique in poultry production, a new chapter has opened in the development and nutrition of broilers, and farmers are given the opportunity to manage this process before the birds arrive on the farm. The *in ovo* technique offers great potential for improving the animal's immune system and production results. Given that *in ovo* vaccination has already become a common practice in modern poultry production, it is also desirable to enable the simultaneous application of *in ovo* nutrition and vaccination. In this way, the negative impact of additional egg handling is reduced and its practical application is facilitated, with lower overall costs. New trends in the application of *in ovo* nutrition are mainly aimed at examining the influence of the used inoculum on the expression of genes that control the creation of hormones and enzymes (as part of the modern concept of nutrigenomics). A number of substances used in the *in ovo* technique have shown potential to improve embryo growth and development, but the results obtained have often been inconsistent. For these reasons, further research is necessary in which only those substances with a constant effect in application will be singled out, and the mechanisms underlying the obtained results will be explained. Taking into account the fact that in a large number of *in ovo* feeding experiments, an unsuitably low percentage of hatched chicks resulted, it is necessary to define and establish less aggressive procedures which will avoid or at least reduce this effect and keep the hatching rate above 90%. In order to motivate producers for the wider application of *in ovo* nutrition, more detailed assessment of the long-term effects that this technique provides, primarily through the improvement of production results during the exploitation of individuals, will be necessary. A new aspect of this diet, which is developing intensively, points to the possibility of early programming, i.e., management of the microbiota of the embryo with the aim of establishing a desirable bacterial population, which will be maintained after hatching and during the first contact with pathogenic microorganisms from the environment. It is important to keep in mind that the development of the *in ovo* diet should not be any obstacle for the continuation of the development of a precisely formulated diet for chicks during the first week after hatching (the so-called pre-starter mixture). In fact, both

mentioned feeding techniques should be developed in a synchronized manner to enable the best possible results during both production phases (incubation and fattening periods).

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Original Scientific Paper

Organic trace minerals as an innovative nutritional solution for advancement of production performance and intestinal microbiota in piglets

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ABSTRACT

After weaning, piglets are usually stressed by nutritional, psychological, environmental, physiological and social factors. During this period, piglets must rapidly adapt to a change in the type of feed they consume, from liquid sow's milk, which is easily digestible, tasty and evenly distributed throughout the day, to solid dry feed, which is less digestible and tasty. As a consequence, feed consumption usually decreases in the first days after weaning with bad influence on composition on intestinal microbiota. Nutrient additives belong to the basic group of feed additives, and the main aims in using them are to increase the overall nutritional value of feed and to ensure the safety of food of animal nutrition through feed. Due to the reduced usability, potential toxicity and negative impact on the environment of inorganic forms of trace elements, the attention of the scientific public in recent years has been focused on finding forms of minerals that will neutralize the potential harmful effects of use and at the same time ensure better mineral usability and economy in livestock production. The Department of Animal Nutrition and Botany, Faculty of Veterinary Medicine, University of Belgrade, organised a trial that aimed to test the effects of using organic trace elements in the diet on the health status, production performance measures and intestinal microbiota of the piglets. The use of new forms of trace elements showed a beneficial effect on piglet gut health, and consequently, on the yield of meat.

1. Introduction

The gastrointestinal tract has multiple functions, such as digesting and absorbing nutrients and electrolytes, maintaining the balance of body fluids, and secreting digestive enzymes, mucins, immunoglobulins and other components. In addition, it fulfils a barrier role for the host with the aim of protection from harmful pathogens and other antigens (*Gao et al.*, 2019). After weaning, piglets are usually stressed by nutritional, psychological, environmental, physiological and social factors. During this period, piglets must rapidly adapt to a change in the type of feed they consume, from liquid sow's milk, which is easily digestible, tasty and evenly distributed throughout the day, to solid dry feed, which is less digestible and tasty. As a consequence, feed

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consumption usually decreases in the first days after weaning and the piglets become malnourished with a reduced growth rate, entering an energy and protein deficit. Piglets lose about 100-250 g of body weight on the first day after weaning and recover this loss of body weight in about four days (*Le Dividich and Seve*, 2000). Understanding the challenges and impact of low feed intake associated with weaning and the consequent impact on piglet performance can help nutritionists implement modern feeding strategies using various supplements that have been proven to directly or indirectly increase feed intake and digestibility.

2. The influence of the weaning process on the composition of the intestinal microbiota of piglets

Over recent decades, the interaction between nutrition and the gut microbiota, as well as its impact on health, has great attention. Initial studies have primarily focused on the classification of microorganisms that form the gut microbiota and the relationship of the composition of microorganisms to the health status of the host (*Hermann-Bank et al.*, 2013). The primary intestinal microbiota of piglets after birth is formed because of the milieu favouring the lactic acid bacteria in the sows' milk (*Zhao et al*., 2019). However, the transitional period of nutrition during the weaning period reduces the relative abundance of bacteria of the *Lactobacillus* and increases the abundance of *Clostridium* spp., *Prevotella* spp., *Proteobacteriaceae* and *Escherichia coli*, which increases microbial diversity in the gut (*Williams et al.*, 2005). The composition of the gut microbiota of piglets, in addition to the stressors that occur during the weaning process, is largely influenced by the levels and sources of protein and fibre in the feed mixtures during this period (*Hammer et al*., 2012). During the weaning period, piglets, despite the use of pre-starters, sharply reduce their feed intake due to their transition from a liquid diet to solid feed with a complex composition, and due to other stressors that were previously described in detail. Thus, the nutrients for the survival and reproduction of bacteria are also limited. Pathogenic bacteria are able to take advantage of special nutrients (e.g., ethanolamine, a compound that is a weak base and has an odour similar to ammonia), thereby enhancing the expression of their virulence factors. For example, both *Salmonella* and enterohemorrhagic *E. coli* can use ethanolamine as a source of carbon or nitrogen in order to gain a nutritional advantage in competition with other bacteria (*Ni Lochlainn et al.*, 2018). Enterohemorrhagic *E. coli* can also use the hexose deoxy sugar, fucose, for the purpose of activating the type III secretion system, which facilitates the adhesion of pathogenic bacteria to host enterocytes (*Bäumler and Sperandio*, 2016).

Considering the importance of the composition of the intestinal microbiota for piglets, the proper establishment of a complex and well-balanced ecosystem in the gut is currently recognized as a key point in any program to reduce the use of antibiotics in animal husbandry (*Liao and Nyachoti*, 2017). One of the main causes of low body weight of slaughtered piglets and variable production results of piglets during the suckling period is the high prevalence of digestive disorders associated with opportunistic pathogens. Among them, *E. coli* is the main pathogenic agent that causes neonatal diarrhoea and post-inlet diarrhoea, and consequently is also responsible for the use of large amounts of antibiotics (*López-Colom et al.,* 2020). In recent years, various feeding programs, as well as the use of supplements, have been investigated as a way to combat this pathogen and reduce the incidence of diarrhoea in piglets.

3. Using innovative nutritional strategies in piglet nutrition

Due to the increasing demands of the global consumer lobby, and respecting the production priorities (better use of feed, longer sustainability, easier manipulation) with the ultimate aim of increasing production and improving the quality of food of animal origin, in addition to the basic nutrients, a large number of additives with different purposes are added to the feed mixtures (*Šefer et al.*, 2014). Nutrient additives belong to the basic group of feed additives, and their main aim is to increase the overall nutritional value of feed. Inorganic forms of trace elements in the form of salts have been used for years as additives in mixtures for piglets: oxides, carbonates, chlorides and sulphates. The bioavailability of minerals from these sources differs, with sulphates typically having higher relative bioavailability values than oxides. The bioavailability of inorganic forms of micronutrients is limited, and high doses are required to meet the requirements of animals, often leading to nutrient imbalances and potential toxicity problems (*Ma et al*., 2020).

Due to the low usability, potential toxicity and negative impact on the environment of inorgan-

ic forms of trace elements, the attention of the scientific public in recent years has been focused on finding forms of minerals that will neutralize the potential harmful effects of mineral use and, at the same time, ensure better usability and economy in livestock production. The formation of a complex molecule of trace elements with organic components can increase passive absorption in the intestine by reducing the interaction between minerals in the intestinal lumen and, thus, preventing the formation of insoluble complexes with substances, such as hydroxides, carbonates, phosphates, oxalates and phytates, which would make copper, manganese or zinc unavailable for absorption (*Peters and Mahan*, 2008). Mineral complexation with an organic component can also result in a more favourable water-lipid partition coefficient that favours absorption over a wide pH range. The strength of the bond between the organic ligand and the trace element during complex formation can prevent dissociation while passing through the gastrointestinal tract and improve the bioavailability of the mineral (*Byrne et al*., 2021). Organically bound trace elements are recognized as bioavailable sources of trace elements that are more usable than their traditional inorganic analogues, such as sulphates and oxides *(To et al*., 2021). The aim of the research was to investigate the justification and effects of using of organic forms of copper, manganese and zinc in the diet on the health status and production performance of piglets, as well as the on the microbiota composition in chosen segments of the piglet gastrointestinal tract.

4. Materials and methods

The experimental protocol was approved by the Veterinary Directorate of the Serbian Ministry of Agriculture, Forestry and Water Management and the Ethics Committee of the Faculty of Veterinary Medicine, University of Belgrade (Resolution number: 23/2020).

During the formation of the trial, an individual clinical examination of the chosen piglets was carried out, so that all the selected individuals were healthy and vital. During the formation of experimental groups, all piglets were uniform in terms of origin, sex balance and body weight (± 10%). The health condition of the test subjects was monitored daily. The experiment was conducted on 48 weaned piglets of the same origin, with an equal sex ratio. Piglets aged 28±1 days were randomly assigned to two experimental groups (C and E-I). Each experimental group consisted of six pens (two male and two female individuals per pen). The study lasted 42 days and was divided into two phases. The first phase lasted from 0-21 days (starter phase) and the second phase from 21-42 days (grower phase). From the beginning (28-day-old piglets) to the end (70-day-old piglets) of the study, experimental groups of animals were fed with complete mixtures of standard chemical and raw material composition, and the mixtures were formulated to meet the recommended nutrient requirements according to the *NRC* (2012).

The composition of the premix at the starter stage for the control group was (in kg of complete mixture): 20,000 IU vitamin A, 2,000 IU vitamin D3, 80 mg vitamin E, 2.4 mg vitamin K3, 2.4 mg vitamin B1, 6 mg vitamin B2, 6 mg vitamin B6, 0.4 mg vitamin B12, 0.3 mg biotin, 32 mg niacin, 14 mg Ca-pantothenate, 5 mg folic acid, 3 mg I, 0.4 mg Se, 0.6 mg Co, 550 mg choline chloride, 240 mg Fe, 130 mg Cu, 120mg Mn, 100 mg Zn, 1000 mg phytase, 100 mg antioxidant BHT. In the first phase, in the complete mixture for feeding group E-I, trace elements Cu, Mn and Zn of inorganic origin were replaced by trace elements Cu, Mn and Zn of organic origin (130 ppm Cu, 60 ppm Mn and 60 ppm Zn), in organic form bound to methionine. The composition of the premix at the grower stage for the control group was (in kg of complete mixture): 20,000 IU vitamin A, 1,800 IU vitamin D3, 120 mg vitamin E, 2.8 mg vitamin K3, 4 mg vitamin B1, 8.8 mg vitamin B2, 6.8 mg vitamin B6, 0.04 mg vitamin B12, 0.28 mg biotin, 28 mg niacin, 16 mg Ca-pantothenate, 0.8 mg folic acid, 0.8 mg I, 0.4 mg Se, 0.6 mg Co, 500 mg choline chloride, 200 mg Fe, 80 mg Cu, 120mg Mn, 100 mg Zn, 1000 mg phytase, 40 mg antioxidant BHT.

In the second phase, in the complete mixture for feeding group E-I, trace elements Cu, Mn and Zn of inorganic origin were replaced by trace elements Cu, Mn and Zn of organic origin (80 ppm Cu, 60 ppm Mn and 60 ppm Zn), in organic form bound to methionine.

Production results (piglet body weight, body weight gain and feed conversion rate) were measured at the beginning if necessary and the end of the study, on day 42. Immediately after slaughter and evisceration of piglet organs, the contents of the jejunum and ileum were sampled (six samples of each intestinal segment from each group – one pig per subgroup) to determine the total number of aerobic

bacteria (PCA), the total number of anaerobic bacteria (PCA an), and the numbers of *Enterococcus* (ECC), *E. coli*, and *Lactobacillus* (MRS). Samples for bacteriological tests were taken directly from the intestine with a sterile swab, 1 g of a sample of intestinal contents. Dilutions were formed in sterile saline solution for bacteria that were later incubated aerobically and in thioglycolate broth for bacteria that were incubated anaerobically.

5. Results and discussion

The health status of the individuals during the study was satisfactory, and no signs of disease were observed. The E-I group of piglets that consumed feed with added organic forms of copper, manganese and zinc achieved better production results, i.e., better growth and feed conversion rate, while consuming a smaller amount of feed per achieved unit of growth.

There were no differences in the two piglet groups from the point of view of statistical analysis at the beginning of the study. However, in percentage terms, the mean body weight of the E-I piglets was 6.06% greater compared to the control group at the end of study (Figure 1). Our results are in line with another study that proved that piglets fed with 50% copper, manganese, zinc and iron in organic form achieved better production results than piglets fed with similar concentrations of trace elements exclusively from inorganic sulphate forms (*Veum et al.*, 1995). The replacement of part of the inorganic trace elements with organic forms also improved the feed utilization in those piglets (*Veum et al.*, 1995). Additionally, piglets fed protein forms of copper and zinc had higher concentrations of these micronutrients in the liver than pigs fed sulphate forms (*Schiavon et al.*, 2000). This indicates a more efficient use of organic forms of copper and zinc compared to inorganic sources.

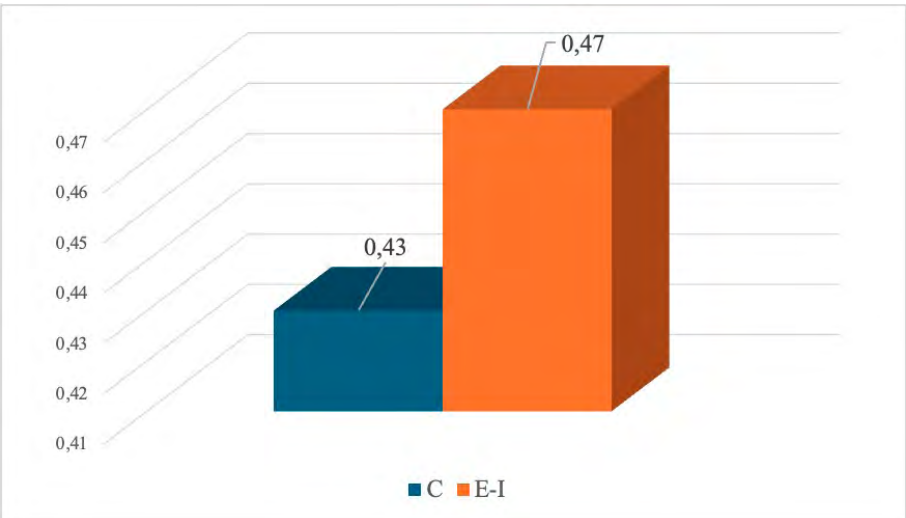

Figure 1. Mean body weight (kg) of control (C) (inorganic trace elements) and E-I (organic trace elements) groups of piglets (n = 24 per group) on days 1 (left side of figure) and 42 (right side of figure) of the study.

Analyzing the entire study period, the E-I group achieved a higher daily body weight gain compared to the control group (Figure 2). After calculating the feed conversion rate (Figure 3), and statistically analyzing the results (data not shown), significant differences were clearly shown between the examined nutritional treatments ($p<0.01$). *Mullan et al.* (2004) also described an improvement in the feed conversion rate following the inclusion of an organic source of trace elements in piglet feed mixtures. The authors of studies in which identical results were obtained generally attribute this effect to greater digestibility of nutrients, an increase in the height and width of the intestinal villi, modulation of the intestinal microbiota, but also to the antimicrobial properties of chelates that lead to an improvement in the immune function of the intestine, achieving to a greater extent the above effects (*Kong et al.*, 2010; *Han et al.*, 2014).

The numerical status ($\log_{10}$ CFU/g) of total aerobic bacteria, *E. coli*, *Enterococcus* spp., anaerobic bacteria and *Lactobacillus* spp. in the intestinal contents of the jejunum and ileum is shown in Figures 5 and 6, with the results indicating that the levels were within the physiological framework (*Dibner and*

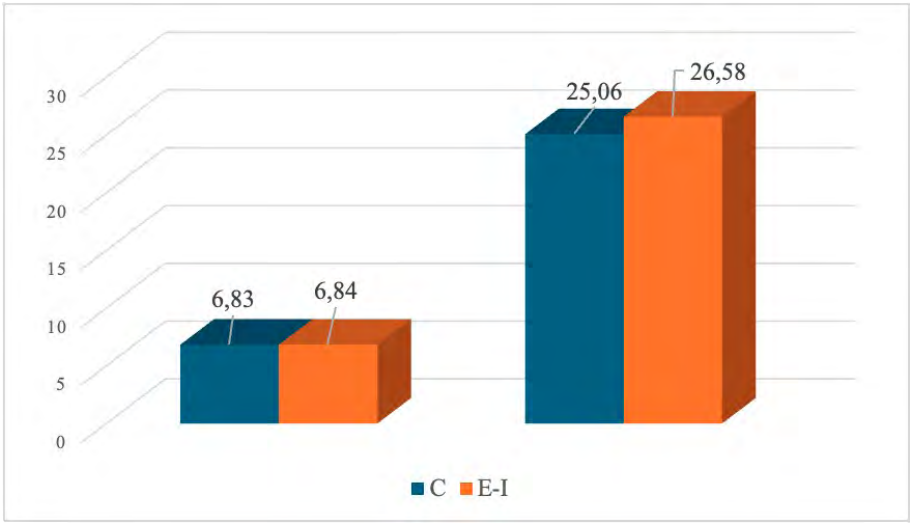

Figure 2. Mean daily body weight gain (kg) of control (C) (inorganic trace elements) and E-I (organic trace elements) groups of piglets (n = 24 per group) during the 42-day study

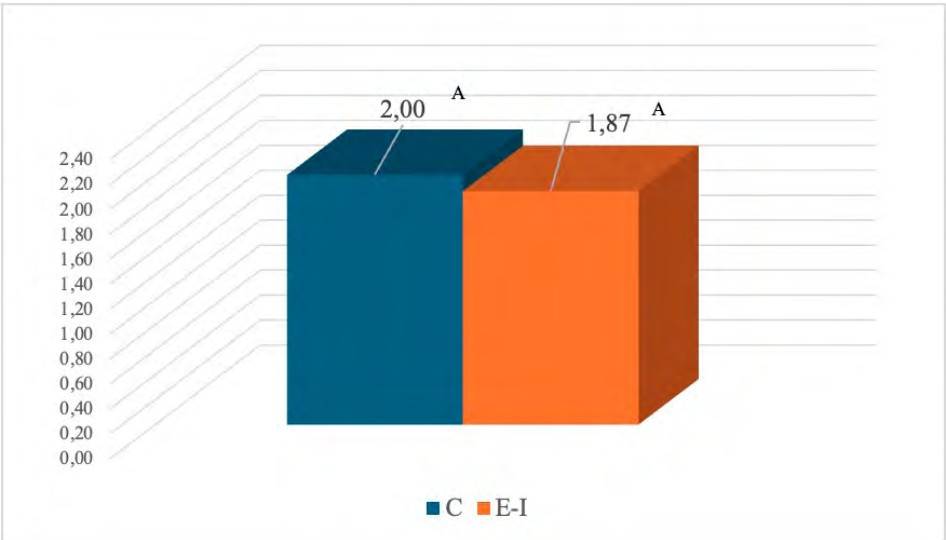

Figure 3. Mean feed conversion rate of control (C) (inorganic trace elements) and E-I (organic trace elements) groups of piglets (n = 24 per group) during the 42-day study

Buttin, 2002; *Ahmed et al*., 2014; *Diao et al.*, 2014). Analyzing the jejunum microbiota of the piglets, the highest mean (and statistically significant) total number of aerobic bacteria was found in the control group, compared to the E-I piglets ($p<0.01$). The effect in our study of adding organic forms of trace elements is consistent with the results of *Wang et al.* (2004). They conducted an experiment on piglets and demonstrated a reduction in the number of *E. coli*. However, the results of *Broom et al.* (2006) showed that the use of zinc oxide reduced the number of lactic acid bacteria, but had no effect on the number of *E. coli* in the intestines of piglets, probably due to an inorganic source of zinc in the mixtures. With the change to an organic source of zinc, a positive effect on *E. coli* numbers (lower in E-I piglets than in controls) was shown in our study. The *Enterococcus* spp. counts in our E-I group (i.e., no significant change with the use of organic trace elements compared with the control group; Figure 6) were not consistent with the results of *Broom et al.* (2006), which was expected because the oxide form of zinc was used in their study. However, the effect we measured on *Enterococcus* spp. counts was similar to that in a study by *Castillo et al.* (2008) with the use of the organic form of zinc in combination with mannan-oligosaccharides.

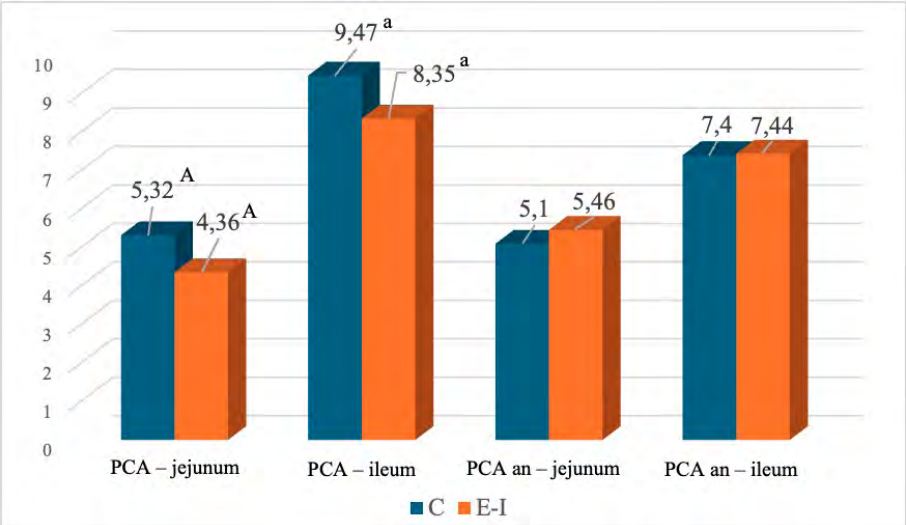

Figure 5. Total aerobic count (PCA) and total anaerobic count (PCA an) (log_{10} CFU/g) in jejunum and ileum of control (C) (inorganic trace elements) and E-I (organic trace elements) groups of piglets (n = 6)

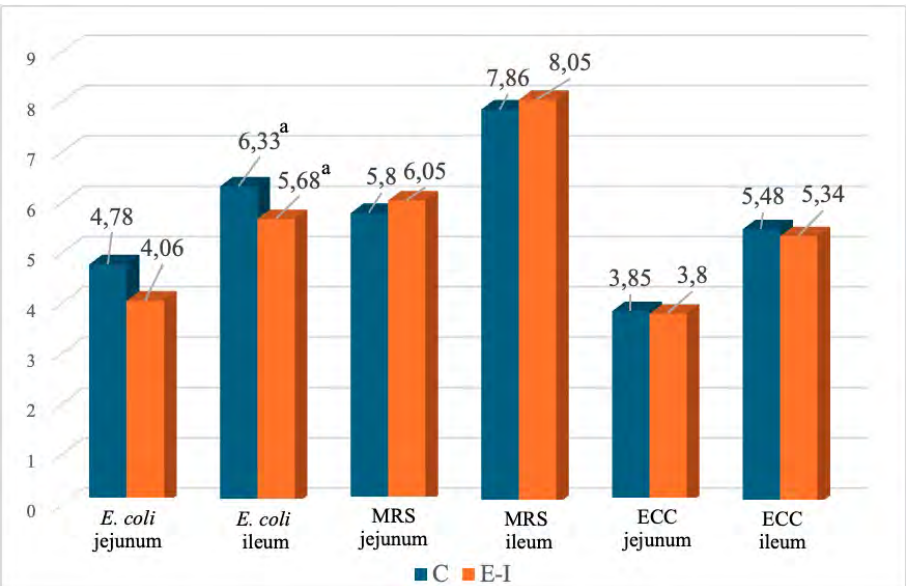

Figure 6. *Escherichia coli*, lactic acid bacteria (MRS) and *Enterococcus* (ECC) numbers (log_{10} CFU/g) in jejunum and ileum of control (C) (inorganic trace elements) and E-I (organic trace elements) groups of piglets (n = 6)

6. Conclusion

Based on the obtained results, we can conclude that the use of organic forms of copper, manganese and zinc in piglet nutrition has its own nutritional, medical and economic justification. Improving the ratios between intestinal microbiota led to better performance measures of piglets.

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Review Paper

Former food in animal feed

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ABSTRACT

With the global population on the rise and growing demand for animal products, competition between food sources for humans and animals is intensifying. As a result, the livestock industry is actively seeking cost-effective feed alternatives that are not suitable for human consumption. One promising option is the use of former food products (FFPs), which are derived from leftovers and residues from food processing. Available FFPs offer a potential substitute for traditional cereals and concentrates in animal diets. This review examines the relevant regulatory framework alongside recent findings on the application of FFPs in animal nutrition, focusing on their capacity to replace energy-rich feeds while addressing the associated risks and benefits. Overall, existing research supports the use of FFPs as a sustainable practice in animal feeding, contributing to global efforts to reduce food waste.

1. Introduction

The United Nation's 2024 report projects world population to be about 10.2 billion in 2100 (*UN*, 2024). In addition, according to recent data from the Food and Agriculture Organization (*FAO*, 2024), with only six years left until 2030, current trends in hunger and food insecurity are not on track to meet the goal of ending hunger by the target date. Similarly, progress indicators for global nutrition targets show the world is falling behind in efforts to eliminate all forms of malnutrition. Billions of people still lack access to adequate, safe, and nutritious food. The escalating nutritional demands of a growing global population, along with climate challenges, are exerting significant pressure on both animal husbandry and the animal feed production sector. This situation underscores critical concerns regarding the availability and sustainable use of essential resources, such as land and water, which are fundamental to food production systems. Consequently, competition between human and animal food requirements is intensifying. In response, there is a sustained effort within the scientific and agricultural communities to identify and implement innovative strategies that can mitigate these challenges.

Among several alternatives, the idea of using so-called former food in the diet of domestic animals stands out, which practically represents recycling within the food chain. As defined within the EU Catalogue of Feed Materials (*European Union*, 2017), "*former foodstuffs* mean foodstuffs, other than catering reflux, which were manufactured for human consumption in full compliance with the EU food law but which are no longer intended for human consumption for practical or logistical reasons or due to problems of manufacturing or packaging defects or other defects and which do not present

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any health risks when used as feed". *Luciano et al*. (2020) indicate that examples of former food products (FFPs) include a range of food industry leftovers such as pasta, bread, cereals, savoury snacks, biscuits, sweets, and chocolate bars. These foods are high in sugar, starch, oil, or fat, so they have a high energy content.

The European Feed Manufacturers' Federation (*FEFAC*, 2023) reported that processed former foodstuffs represent a nutrient-rich alternative ingredient to cereal grains and vegetable oils in a compound feed formulation and that this approach also supports the circular economy and reduces environmental impact by requiring fewer natural resources than traditional crop-based feed. The importance of this practice is evidenced by the fact that the European Former Foodstuff Processors Association (*EFFPA*, 2025) was founded in 2014 as a non-profit EU trade network with the aim of connecting food manufacturing with the animal feed production sector. One of the main goals is to make an important contribution to relevant sustainability targets in terms of resource efficiency.

2. Regulatory framework

Former foodstuffs incorporated into animal feed are subject to specific legislative requirements, whereby both former foodstuff processors and food business operators are classified as feed business operators. They are responsible for maintaining feed safety, ensuring the quality and integrity of both sourced and processed materials, and preserving the feed status throughout the supply chain. These materials must only be sourced from the manufacturing, distribution, and retail sectors of the food and beverage industry, explicitly excluding catering establishments. Additionally, sourced former foodstuffs must be free of any prohibited animal by-products (*FEFAC*, 2023).

Feed safety is the foremost priority in the use of former foodstuffs as feed ingredients. To maintain trust in the sector, both suppliers and customers must be assured that former foodstuff processors operate responsibly and uphold stringent standards of safety and traceability. Therefore, these processors are required to comply with a range of key EU Regulations and Directives governing feed safety, hygiene, and traceability within the feed supply chain (*EFFPA*, 2025).

Arranged in chronological order, the following EU regulations apply:

- Regulation (EC) No 178/2002 laying down the General Principles and Requirements of Food Law (*European Union*, 2002a). Producers of feed for food-producing animals are an integral part of the food chain and are subject to the principles outlined in the General Food Law. Crucially, feed must be safe and must not pose any risk to animal or human health, as stipulated in Article 15. Furthermore, Article 18 requires that feed be fully traceable and identifiable at all stages of the supply chain. In cases where non-compliant products are identified, Article 20 mandates that appropriate measures be taken to withdraw such products from the market, ensuring the continued integrity and safety of the food and feed system.
- Directive 2002/32/EC on Undesirable Substances in Animal Feed (*European Union*, 2002b). To ensure the delivery of safe feed to the market, former foodstuff processors must fully comply with the legislation that sets maximum allowable limits for undesirable substances in animal feed.
- Regulation (EC) No 882/2004 on Official Controls performed to ensure the Verification of Compliance with Feed and Food Law (*European Union*, 2004). This regulation establishes the framework for official feed controls, to which former foodstuff processors, as feed business operators, are fully subject.
- Regulation (EC) No 183/2005 laying down Requirements for Feed Hygiene (*European Union*, 2005). These regulatory requirements fully apply to former foodstuff processors, as they do to all operators within the animal feed sector. Consequently, former foodstuff processors are obligated to implement Hazard Analysis and Critical Control Points (HACCP) principles as part of their feed safety management systems.
- Directive 2008/98/EC on Waste (*European Union*, 2008). To emphasize that former foodstuffs are not legally considered waste, former foodstuff processors refer to Article 5 of this Directive, which outlines the criteria for by-products.
- Regulation (EC) No 767/2009 on placing on the Market and Use of Feed (*European Union*, 2009a). Former foodstuff processors, as entities placing feed on the market, are required to fully comply with all aspects of this regulation. This includes, among other provisions, adherence to specific feed labelling requirements.

- Regulation (EC) No 1069/2009 laying down Health Rules as regards Animal By-Products and Derived Products not intended for Human Consumption (*European Union*, 2009b). Former foodstuffs that contain or are derived from animal by-products, such as milk, eggs, honey, or gelatine, are classified as Category 3 animal by-products, as defined in Article 10 of this Regulation. This category includes materials considered low risk and suitable for use in feed, subject to specific handling and processing requirements to ensure safety.
- Regulation (EU) No 2017/1017 on the Catalogue of Feed Materials (*European Union*, 2017). The Catalogue serves as a voluntary, co-regulated reference for feed materials used in livestock farming within the EU. As mentioned before, in Annex, Part A(3), it provides the official definition of former foodstuffs. Notably, the third update of the Catalogue, released in 2017, introduced *mechanical packaging removal* as a recognised process within the glossary, further clarifying the handling of former foodstuffs intended for feed use.
- Commission Notice — Guidelines for the Feed Use of Food no Longer Intended for Human Consumption (*European Union*, 2018). Although these Guidelines do not have legal status, they have been accepted by EU Member States and provide valuable clarifications regarding the eligibility of former foodstuffs, including those that have fallen on the floor and those past their food labelling expiration dates.

3. Implementation for food-producing animals

Depending on the target animal species, processed FFPs are typically included in compound feed formulations at inclusion rates ranging from 5% to 30%. They are generally available across most European feed markets at costs comparable to those of cooked cereal grains and oil-rich feed components (*FEFAC*, 2023). These materials can be broadly classified into two primary categories: residues from the food industry predominantly comprising bakery products (e.g., bread, pasta) and those principally consisting of confectionery products (e.g., chocolates, biscuits). Bakery products, including bread and savoury cakes or snacks, due to the prolonged baking process, constitute a macerated and readily digestible source of energy characterized by high starch content. Confectionery products, such as chocolate, dry cakes, biscuits, waffles, and muesli, are considered supplemental feeds, available throughout the year, and are rich in simple sugars, lipids, and energy. Given these compositional attributes, FFPs are primarily targeted at young animals, including piglets, chicks, and calves, owing to their elevated levels of digestible carbohydrates, particularly cooked starch (*Luciano et al*., 2020).

Pinotti et al. (2021) summarized the available data and provided an overview of the nutritional composition of bakery and confectionery products analyzed as FFPs (Table 1). They concluded that these values are variable and affected by processing, and although they pose a challenge for feed

Table 1. Nutritional composition (g/Kg of dry matter or MJ/Kg of dry matter) of former food as reviewed by *Pinotti et al* (2021)

Component	Bakery products		Confectionery products	
	Range	Average	Range	Average
Crude protein	2.10 - 16.70	11.40	7.30 - 13.20	10.60
Ether extract	0.30 - 12.20	6.50	4.80 - 15.00	9.80
Crude fibre	0.50 -13.40	3.60	0.50 - 5.20	2.60
Neutral detergent fibre (NDF)	2.10 - 50.50	20.50	5.40 - 22.60	12.10
Acid detergent fibre (ADF)	0.40 - 22.10	7.90	1.20 - 6.80	3.20
Ash	0.70 - 8.60	4.90	1.40 - 8.20	3.40
Non-structural carbohydrate	60.10 - 78.90	65.70	50.60 - 79.30	64.70
Starch	24.00 - 86.30	44.70	41.90 - 73.40	50.90
Nitrogen free extractives	75.50 - 77.90	76.70	60.80 - 79.00	69.40
Metabolizable energy (MJ/Kg of dry matter)	11.40 - 19.00	14.60	14.50 - 18.20	16.40

formulation, they also provide valuable flexibility in adjusting ratios to meet the nutrient and energy needs of the target animals.

Srikanthithasan et al. (2024a) studied the inclusion of dietary FFPs in broiler diets, assessing growth performance, digestibility, haematobiochemical profiles, and liver gene abundance. They concluded that these foodstuffs could be incorporated into nutritionally balanced diets at up to 25% substitution levels for 33 days without negatively affecting growth performance in male broilers raised under commercial conditions. However, they emphasized the need for further research to confirm the haematological trait findings. *Luciano* (2021) suggested that a percentage of inclusion from 27% to 30% of FFPs in weaning pig diets does not affect growth performance or health of animals, proposing FFPs as a valuable alternative feed ingredient. *Termatzidou et al.* (2023) later examined the inclusion of bakery meal at 15% and 20% (w.w.) in the diet of post-weaned piglets, reporting no significant negative effects on growth parameters, welfare, or behavioural indicators. According to existing *in vitro* and *in vivo* studies on the inclusion of FFPs in ruminant feeding, FFPs appear to support adequate rumen function and pH, while promoting favourable ruminant performance and health (*Tretola et al*., 2025). The use of FFPs as alternative energy sources—replacing conventional ingredients, such as cereal grains, fats, oils, or molasses—could potentially alleviate the current feed-food competition, without negatively affecting animal productivity or welfare in lactating dairy cows or fattening cattle (*Tretola et al*., 2025). However, achieving this objective requires careful regulation of the chemical composition and inclusion rates of FFPs, ensuring their integration with other feed components. The nutritional composition of the final diet is crucial, as it directly influences rumen microbial communities, which in turn impact animal performance, health, and related products. Despite the promising potential, the limited number of studies available and the variability in *in vitro* and *in vivo* findings highlight the need for further validation to ensure the safe and effective use of FFPs in ruminant diets from a nutritional perspective.

4. Safety remarks

As highlighted by *Srikanthithasan et al.* (2024b), further research focusing on safety aspects across various animal species is essential. The critical need for farmers, nutritionists, industries, and governments is to prioritize animal feed production, as the quality and safety of feed are fundamental to ensuring human food safety. One key concern, as noted by *Luciano* (2021), is the potential presence of packaging material residues, which must adhere to regulatory standards governing feed constituents. Furthermore, *Tretola et al.* (2025) identified a significant gap in the existing literature regarding the potential effects of undesirable substances present in FFPs, including theobromine (a principal alkaloid of the cacao bean), packaging residues, and chemicals associated with packaging materials. Additionally, it is recognized that all other requirements pertaining to the safety of feed and feedstuffs must be fully satisfied.

5. Conclusion

The utilization of FFPs contributes to achieving international targets for food waste reduction and supports the broader objectives of developing a sustainable food system. Nevertheless, further multidisciplinary *in vivo* studies are essential to validate the potential inclusion of FFPs in the diets of various livestock species at different inclusion levels—particularly for those that have so far been largely overlooked, such as beef cattle, goats, lambs, laying hens, and ducks. These studies should aim to identify the optimal or maximum inclusion rates that meet the specific nutritional requirements of each species, while also thoroughly assessing all safety aspects. Overall, this is a promising strategy that warrants continued research and development in pursuit of effective solutions for the collective benefit. Consequently, it is essential to promote its recognition and encourage its implementation within animal nutrition practices through appropriate domestic regulatory frameworks.

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Review Paper

Pet food industry – current state and perspectives

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ABSTRACT

Pet food is more than simply a basic meal that contains the components required for an animal's growth and development. Nevertheless, it takes a human-oriented approach to food, adhering to the guidelines of a well-balanced diet that includes all necessary ingredients and supplements, being aesthetically pleasing, and concentrating on the owner who wishes to provide the best for their pet. A pet is now a part of the family with the same status as the other family members, not just an animal.

The use of natural ingredients, sustainability, and product and recipe personalization are the three main global trends in pet food production. Customers are likely to consider their pets and explore the healthiest food options for them as they continue to seek ways to customize and improve their health However, the use of natural ingredients free of artificial additives remains popular in dog and cat food, just as it is in human food. The largest obstacle to achieving "naturalness" in pet food is the distribution, storage, and transportation of frozen or chilled food. Sustainability is becoming ever more important in the pet food sector, particularly regarding packaging. In keeping with the trends in human nutrition, there are now pet food companies that focus on regenerative agriculture.

1. Introduction

The pet food industry has experienced huge growth over the last two decades, caused by increasing pet ownership, humanization of pets, and rising awareness of animal health and nutrition. Once a niche market, it has evolved into a dynamic global sector that competes with human food trends. According to data from the European Pet Food Industry Trade Association (FEDIAF) for 2022, there are 352 million pets in Europe with an annual pet food production of 9.9 million metric tons. The latest research shows that profits from the production of pet food have reached extremely high values, and it is predicted that by 2030, this market will generate earnings of $300 billion. North America remains the dominant market, followed by Europe and the Asia-Pacific region. Growth in emerging economies is particularly notable due to rising disposable incomes and changing attitudes toward pet care. The range of pet food products available on the market is widening due to differentiation based on animal attributes (e.g., size, age, species, etc.), nutritional values, and branding (e.g., private labels, multinationals). Sustainability is becoming a key element of product branding, not only for environmental protection, but also social sustainability in pet food production and animal health (*Sparacino et al.*, 2025). Similar to the human food industry, some pet food companies are accelerating their efforts towards the

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green economy and are actively promoting several initiatives via their means of communication.

In today's culture, pets are an increasingly significant part of our daily lives. According to a number of studies, people can live with animals and view them as "just an animal", but they can also develop relationships with them that are comparable to those they would have with family members (*Fantechi et al*., 2024). Many families buy presents for their pets and regularly celebrate their birthdays, even though some may only see their dog as a home protector. The primary areas where the pet food industry can make changes to lessen its environmental impact are the use of sustainable ingredients in diet formulations, resource optimization, improved waste management, and the decrease of overfeeding and excess nutrient intake (*Acuff et al.*, 2021; *Djuragic et al.*, 2023).

1.1 Market overview and product categories

The pet food markets in North America and Western Europe are still the most developed, while those in Asia-Pacific and Latin America have significant growth potential. Economy and standard products are giving way to premium, organic, and functional formulations, which is what defines the industry. Due to logistics and cost-effectiveness, dry extruded food continues to dominate the market, although wet, raw, and freeze-dried products are gaining ground. Pet food is typically divided into:

- **Dry food** (extruded kibble)
- **Wet food** (canned, pouch)
- **Raw/fresh food** (BARF – Biologically Appropriate Raw Food)
- **Treats and snacks**
- **Functional diets** (e.g., for renal disease, obesity, allergies, skin issues)
- **Nutraceuticals and supplements**

The nutrition profile, processing level, and shelf life of these categories differ (*Swanson et al.*, 2013). The Association of American Feed Control Officials (AAFCO) and FEDIAF requirements need to be adhered to by complete pet foods in order to ensure that the essential nutrient demands of different species and life stages are met. Yet with homemade and alternative diets that often do not have scientific formulation, the risk of nutrient imbalances or deficiencies exists (*Freeman et al.*, 2013). There are established health benefits associated with some modern pet food ingredients, such as probiotics and prebiotics for gut health, omega-3 fatty acids in cognitive and dermatological functioning, joint-supporting ingredients such as chondroitin and glucosamine, and antioxidants to combat oxidative stress.

1.2 Sustainability challenges and innovations

1.2.1. Environmental footprint

The production of pet food has a substantial negative impact on the environment because of its high meat content and energy-intensive processing. Research indicates that 20% of the world's meat and fish are consumed by pets, which raises questions about resource efficiency (*Okin*, 2017). The production of dry pet food worldwide is linked to 56–151 Mt CO_2 equivalent emissions (1.1%–2.9% of global agricultural emissions), 41–58 Mha of agricultural land use (0.8–1.2% of global agricultural land use), and 5–11 km^3 of freshwater use (0.2–0.4% of agricultural water extraction). According to *Alexander et al.* (2020), these effects amount to an environmental footprint that is roughly twice the land area of the United Kingdom. Among the sustainable tactics are:

- Using co-products and by-products
- Utilizing insect protein (such as *Hermetia illucens*)
- Investigating plant-based and cultured meat substitutes

1.2.2. Circular economy and waste reduction

The pet food industry is investigating the concepts of the circular economy, specifically in the area of repurposing agro-industrial waste for the extraction of functional compounds, protein, or fibre (*Aleisa & Alsaleh*, 2024). Cereals, fruits, and vegetables are usually thrown away along with their skins, peels, seeds, and leaves. Because of their poor nutritional value, the presence of dangerous substances, or their unfavourable sensory attributes, these materials are frequently rejected as human food. The enormous amounts produced each year present environmental hazards, and their high moisture content encourages microbial growth. Because they contain beneficial substances, like dietary fibre, polyphenols, and antioxidants, fruit by-products are nevertheless receiving more attention in the fields of human and animal nutrition (*Čolović et al.*, 2019; *Aït-Kaddour et al.*, 2024).

1.2.3. Technological advances and industry 4.0

The growing global population and increasing consumer demand present major challenges for the feed industry, particularly in reducing feed costs while maximizing production efficiency. Important areas for advancement in the animal feed industry include the search for alternative raw materials, the creation of technological equipment, the integration of new technologies, the use of natural additives (as alternatives to antibiotics), and the automation and digitization of feed management. Predictive analytics, automation, and real-time monitoring are all integrated into modern pet food production to optimize the process. Nutrient bioavailability and consistent quality are guaranteed by sophisticated extruders, drying systems, and coating technologies (*Cannavacciuolo et al.*, 2023; *Aït-Kaddour et al.*, 2024).

1.2.4. Personalized pet nutrition

Emerging pet food trends focus on precision feeding based on breed, activity, genetics, and microbiome profiles. Start-ups are developing digital feeding tools and custom diet plans using artificial intelligence (AI) and big data analytics. For decades, pet food formulation has been generic and based on market trends and historical data—overlooking a particular pet's specific or essential health needs. The most important lesson learned has been the advantage of creating recipes using real pet health data that yield measurable health outcomes (*Zhang et al.*, 2023). This approach is timely, especially as pet owners are increasingly looking for the highest calibre ingredients and observable health benefits. In the pet industry, differentiation increasingly relies on the demonstrable impact diet has on improved measurable health outcomes (*Buff et al.*, 2014).

1.2.5. Regulatory landscape and quality assurance

The pet food industry is regulated by national and international rules to ensure food safety, traceability, and proper nutrition. The pet food regulatory landscape includes various agencies and organizations that set and enforce standards for pet food. Important bodies include the Food and Drug Administration (US FDA) in the US, the European Food Safety Authority (EFSA) in Europe, and AAFCO. Quality assurance in pet food manufacturing requires strict controls throughout the entire production process. This includes sourcing and processing ingredients, inspecting the final product, and labelling it to meet regulatory requirements and consumer needs. Major challenges include the lack of consistency, unclear labelling, and oversight of new ingredients (*Buchanan et al.*, 2011).

2. Future perspectives

The future of the pet food industry is dependent on ongoing innovation in product, packaging, and distribution. To capture more sophisticated consumers, brands are pursuing new protein sources, functional ingredients (prebiotics, probiotics), and eco-friendly packaging options. The experience will also be elevated by technological innovations like AI-driven customer service and customized dietary recommendations based on pet analytics. There are issues, nonetheless, such as managing customer expectation for delivery timescales, ensuring quality control over disparate supply chains, and being able to compete favourably with established brands with large retail chains. The pet food industry is at a point of intersection where science, consumer demand, and sustainability must meet. Cross-disciplinary collaboration between nutritionists, food technologists, veterinarians, and environmental scientists is necessary to provide safe, nutritious, and environmentally friendly pet food systems. Continued investment in research and regulatory consistency will be critical to bringing the full potential of this industry to fruition.

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Review Paper

Mild technologies in meat processing: balancing nutritional quality and safety

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Nutritional quality

ABSTRACT

This paper discusses the application of mild processing technologies in the meat industry, with emphasis on their role in preserving nutritional quality and ensuring product safety. Given the highly perishable nature of meat, conventional processing techniques, such as chilling, freezing, curing, smoking, drying and heat treatment, have been employed to extend its shelf life. However, these approaches, especially thermal processing, often compromise sensitive nutrients. In response, optimization of conventional methods, hurdle system application, as well as modern non-thermal and low-intensity technologies, including high-pressure processing (HPP), pulsed electric fields (PEF), ultrasound, and cold plasma, have been developed as effective alternatives. These methods achieve microbial inactivation while minimizing nutritional and sensory degradation. Furthermore, the combination of mild technologies with optimized conventional methods (e.g., sous-vide cooking, vacuum drying, modified atmosphere packaging, biopreservation etc.) has demonstrated synergistic effects in enhancing product quality and satisfying shelf life. This integrated approach supports the production of clean-label, minimally processed meat products that align with contemporary consumer expectations. The findings underscore the potential of mild technologies to contribute significantly to the future of meat processing through improved product safety, functionality, and nutritional retention.

1. Introduction

Meat is a highly nutritious food, valued for its rich content of high-quality proteins, essential amino acids, vitamins (especially B-complex), and bioavailable minerals such as iron and zinc (*Toldrá et al*., 2012). However, due to its high water activity and nutrient density, meat is also highly perishable and susceptible to microbial spoilage as well as enzymatic and oxidative deterioration (*Zhou et al*., 2010). Traditionally, to extend shelf life and ensure safety, a range of processing methods, such as chilling, freezing, curing, drying, smoking and heat treatment, have been developed (*Feiner,* 2006). While effective, many of these conventional techniques, and especially heat treatment, often lead to degradation of sensitive nutrients, sensory changes, and even to the formation of undesirable compounds, such as heterocyclic aromatic amines and others (*Leroy et al*., 2023).

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In response to the growing consumer demand for minimally processed foods with preserved nutrients, the food industry and researchers have developed a range of mild or non-thermal preservation technologies. These include high-pressure processing (HPP), pulsed electric fields (PEF), ultrasound, cold plasma, and others, which aim to inactivate spoilage and pathogenic microorganisms without the use of excessive heat (*Campus*, 2010; *Barba et al*., 2018). The main advantage of these approaches lies in their ability to maintain the nutritional and sensory integrity of the product while ensuring microbial safety (*Bolumar et al*., 2020).

Moreover, conventional processing techniques in meat industry consume a lot of energy, while innovative solutions, including mild heat treatment and non-thermal methods, offer significant potential to lower energy use, reduce production costs and enhance the overall sustainability of food production (*Selvaraj et al*., 2025). Thus, mild technologies in meat processing represent a modern solution to challenges of preserving food quality and ensuring safety, while also offering potential for sustainable production (*Ahmed et al*., 2023).

2. Optimization of conventional processing methods

In the meat processing industry, the greatest loss of nutrients is certainly in sterilized canned meat, but nevertheless, it is necessary to avoid any further excessive loss of the already compromised nutritional value of such products. Namely, the sterilisation process should guarantee that *Clostridium botulinum* spores are destroyed and the manufacturers, aware of the risks involved in such production, often sterilize products too intensively. Testing the F_0-value in several facilities, although $F_0 \geq 3$ is needed to ensure safety, values of more than 8, in some cases even over 10 were determined (*Vasilev et al*., 2019). Such values are unnecessarily high from a safety perspective, but also significantly reduce the nutritional value of the product and unnecessarily waste energy resources (*Vasilev et al*., 2019). Simple optimization of the existing heat treatment procedures according to the F_0 value, a very useful tool in assessing the microbial lethality obtained during the heat treatment, can contribute not only to the better preservation of products nutritional value but also to savings in facility energy consumption (*Rašeta et al*., 2018).

In contrast to sterilized cans, from the nutritive and safety point of view, much more attention is paid to combining mild thermal treatments with other preservation strategies. Traditional processes such as heat treatment, drying, smoking and fermentation can be adapted using lower temperatures or shorter processing times to reduce nutrient loss while still achieving desirable preservation effects (*Toldrá et al*., 2012). This approach, well known as *hurdle technology*, represents the simultaneous action of several antimicrobial parameters (F-, pH-, water activity (a_w-)value, biopreservation, etc.), thanks to which it is possible to achieve adequate product safety even though milder conservation procedures are used, and is a good path to enhance both shelf life and nutritional integrity (*Leistner & Gould*, 2002).

For example, mild heat treatments (e.g., pasteurization at 55–65°C) can be combined with vacuum drying or modified atmosphere packaging (MAP) to inhibit microbial growth and oxidative degradation, while minimizing thermal damage to heat-sensitive nutrients, such as thiamine and vitamin B12 (*Zhou et al*., 2010). Similarly, sous-vide cooking, which is typically done at 55–65 °C for extended time followed by chilling and vacuum packaging, preserves meat texture and nutritional quality more effectively than conventional cooking (*Roldán et al*., 2013). In fermented and cured meat products, relatively low concentrations of salt and nitrites are being used in combination with bioprotective cultures and controlled drying to maintain safety while aligning with clean label demands (*Toldrá & Reig*, 2011). Mild heat treatment in combination with previous marinating and mild smoking, with additional short drying, contributes to producing a product without the use of chemical preservatives and flavour enhancers, which is aromatic and nutritionally satisfactory, but also safe due to the adjusted F- and a_w-value (*Vasilev et al*., 2025). Similarly, essential oils (e.g., citral) combined with sublethal heat (53°C) have demonstrated synergistic *Escherichia coli* O157:H7 inactivation in meat model systems, indicating potential for lower thermal loads and improved nutrient retention (*Berdejo et al*., 2023). Finally, the whole group of so-called *refrigerated processed foods of extended durability* has been developed, which are focused on mild heat, preserved nutritive value, but also extended shelf life (*Vasilev et al*., 2019). These combinations illustrate how classical techniques can be re-engineered with milder parameters, but also they could be combined with emerging technologies to meet modern standards of quality, nutrition, and safety, which is described in the following section.

3. Emerging non-thermal technologies in meat processing

Several non-thermal technologies have been developed and studied for their ability to ensure microbial safety while preserving the nutritional and sensory attributes of meat products.

High-pressure processing (HPP) is one of the most extensively researched non-thermal technologies. It involves the application of pressures up to 600 MPa, which can effectively inactivate pathogenic and spoilage microorganisms without significantly raising the temperature of the product (*Campus*, 2010). HPP-treated meat retains its natural colour, texture and nutrient profile better than thermally processed counterparts (*Bolumar et al*., 2020).

Pulsed electric fields (PEF) apply short bursts of high voltage to food placed between two electrodes. This method permeabilizes microbial cell membranes, leading to cell death, with minimal thermal impact. PEF has shown promising results in improving tenderness in meat and accelerating marination, all while preserving vitamins and other heat-sensitive compounds (*Toepfl et al*., 2006).

Pulsed light (PL) uses short, high-intensity flashes, mainly in the UV spectrum, to inactivate microorganisms by damaging their DNA. In the meat industry, PL can decontaminate carcasses, knives, sliced fermented sausages, and improve the safety of fresh products like beef or tuna carpaccio. However, excessive intensity can alter meat's colour and aroma, so treatment conditions must be carefully optimized (*Mahendran et. al*., 2019).

Ultrasound involves the use of high-frequency sound waves to create cavitation in meat systems, disrupting microbial cells and enhancing mass transfer processes like marination and tenderization. Even more, low-intensity ultrasound has been found to improve meat texture without adverse effects on nutritional value (*Chemat et al*., 2011).

Cold plasma technology relies on ionized gas at room temperature to generate reactive species that inactivate microorganisms on meat surfaces. It is particularly useful for surface decontamination, with minimal impact on product quality. Though still under development for commercial use in meat processing, cold plasma has shown great potential for ready-to-eat products. Many cold plasma systems are suitable for food applications; however, its toxicity requires further study, and regulatory aspects remain obstacles to its adoption in the food sector (*Laroque et al*., 2022).

Ozonation offers several advantages, including cost-effectiveness, the absence of chemicals, environmental friendliness, and ease of use. Nevertheless, its application in the meat industry presents challenges due to ozone's strong oxidative capacity, which can potentially damage cellular fatty acids and proteins in meat (*Roobab et al*, 2024).

Irradiation uses ionizing radiation (gamma rays, electron beams, or X-rays) to improve food safety, extend shelf life, and keep product quality. The absorbed dose is measured in Greys (Gy). When properly applied, irradiation is effective, cold, penetrative, and does not compromise food's sensory or nutritional qualities, although lipid oxidation in fatty foods could be an issue. Irradiation is now used commercially in about 30% of countries worldwide, especially for spices, grains, fruits, vegetables, and to a lesser extent meats and seafood. Wider adoption is limited by consumer acceptance, which could be improved through education and clear labelling (*Vasilev et al.*, 2019).

Table 1 provides a comparative overview of conventional, mild, and emerging non-thermal technologies in meat processing, highlighting their main advantages and limitations.

Collectively, non-thermal mild technologies offer promising alternatives to conventional heat-based methods, aligning with consumer preferences for cleaner labels, fresher-tasting products, and preserved nutritional value (*Barba et al*., 2018). As mild heat is often insufficient on its own to inactivate resistant pathogens or spores, non-thermal hurdles, such as high-pressure processing, antimicrobial packaging, cold plasma or natural antimicrobials, can be effectively integrated to achieve substantial microbial inactivation while minimizing physicochemical degradation. HPP paired with vacuum packaging can be used to eliminate pathogens and spoilage organisms while keeping the nutritional value and freshness of raw or lightly processed meats intact (*Campus*, 2010; *Bolumar et al*., 2020). High-pressure thermal processing (HPTP), which applies HPP at pasteurization temperatures (~600 MPa, 70 °C), has been shown to inactivate *Bacillus cereus* spores in cooked and fermented sausages while preserving sensory and nutritional quality (*Kaur et al*., 2022). Similarly, the application of HPP at refrigeration temperatures to dry-cured Iberian ham significantly reduced microbial loads without adverse effects on colour stability or lipid oxidation, maintaining the characteristic attributes valued in such premium products (*Carrapiso et al*., 2023).

Beyond pressure-based approaches, combinations such as cold plasma with HPP offer complementary modes of action, achieving 2–5 log reductions in pathogens across various ready-to-eat meats while preserving vitamins, pigments, and lipid profiles (*Zhang et al*., 2025).

Table 1. Mild and non-thermal technologies in meat processing

Technology	Pros	Cons
Optimized heat treatment	Preserves nutrients; Saves energy; Safe if controlled.	Needs precise monitoring; Risk if under-processed.
Hurdle technology	Synergistic safety; Mild processing; Clean-label potential.	Complex design; Product-specific; Higher cost.
Sous-vide cooking	Good texture and flavour, Less nutrient loss; Longer shelf life.	Long cooking; Strict cold chain.
Modified atmosphere packaging	Slows microbes and oxidation; Extends shelf life.	Needs special gases and materials; Fails if cold chain breaks.
Vacuum drying	Less oxidation; Less nutrient loss; Energy-efficient.	Costly; Not for all meat types.
Biopreservation	Natural; Fewer artificial additives; Clean-label friendly.	Variable effect; Can change taste; Regulatory limits.
High-pressure processing	Kills microbes without heat; Keeps nutrients and texture.	Expensive; Weak on spores; Batch process.
High-pressure thermal processing	Inactivates spores; Preserves quality.	Costly; Limited use.
Pulsed electric field	Low-heat antimicrobial effect; Keeps vitamins; Improves tenderness.	Poor penetration in solids; Scaling issues.
Pulsed light	Fast surface decontamination; No chemicals.	Can alter colour/flavour; Only surface action.
Ultrasound	Improves marination and tenderness; Little nutrient loss.	High power harms texture; Uneven effect.
Cold plasma	Strong surface decontamination; Eco-friendly; Preserves nutrients and pigments.	Surface only; Safety and regulatory concerns.
Ozonation	Cheap; Eco-friendly; Chemical-free.	Oxidizes fats/proteins; Needs strict control.
Irradiation	Effective, penetrative; Preserves nutrients if optimized.	Consumer stigma; Lipid oxidation risk.

4. Conclusion

Mild technologies in meat processing represent a significant advancement toward producing safer, nutritionally superior, and consumer-friendly meat products. By minimizing heat exposure and combining modern non-thermal approaches with optimized traditional techniques, processors can enhance product shelf life and safety while preserving valuable nutrients. Careful selection and optimization of mild thermal processing within a multi-hurdle framework can achieve regulatory safety targets while sustaining the nutritional and sensory qualities of processed meats. The success of such approaches depends on the compatibility of hurdles with the product matrix, the nature of target microorganisms, and the desired shelf life, making process validation essential for each application. Ongoing research and innovation could further refine these methods, ensuring they remain sustainable, effective, and widely applicable.

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Review Paper

Pre-slaughter conditions and related effects on welfare and meat quality of slaughter horses: a mini review

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ABSTRACT

In recent years, horsemeat has gained recognition as a valuable dietetic food due to its low caloric content, high-quality protein, rich vitamin and mineral composition and favourable fatty acid profile, making it a suitable alternative meat for individuals with anaemia, obesity, atherosclerosis, hypertension, organ diseases and compromised immunity. As with other livestock species, minimising pre-slaughter stress is essential for ensuring animal welfare and achieving high meat quality, as stress can result in bruising and/or other injuries. Therefore, the aim of this review was to provide insights into the pre-slaughter conditions in the horsemeat production chain and related effects on welfare and meat quality, which will in turn expand knowledge in this area and determine directions for future research. Throughout the pre-slaughter period, horses encounter multiple potential stressors that can compromise their welfare and negatively affect carcass and meat quality. These stressors can arise at various stages, including conditions at the point of purchase, loading, transport, unloading, lairage, stunning and exsanguination. Pre-slaughter welfare conditions are significantly affected by animal characteristics (age, gender, breed, temperament), the infrastructure at both the point of purchase and the slaughterhouse, vehicle design, environmental conditions and the quality of human-animal interactions. Since current recommendations on minimum floor space in transport vehicles are insufficient and also clear guidelines on optimal lairage duration for horses at slaughterhouses are lacking, further research is required to enhance horse welfare across the meat production chain.

1. Introduction

In recent years, horsemeat has gained recognition as a valuable "dietetic" food due to rising demand for alternative meats (*Lorenzo et al*., 2019). It is low in calories compared to other red meats (*Jastrzębska et al*., 2019; *Kołodziejczyk et al*., 2019), rich in protein with a complete amino acid profile, and particularly suitable for immunocompromised individuals (*Stanisławczyk et al*., 2020). High vitamin, mineral and iron content and a favourable fatty acid profile makes horsemeat beneficial for people with anaemia, obesity, atherosclerosis, hypertension and various organ diseases (*Jastrzębska et al*., 2019; *Kołodziejczyk et al*., 2019; *Seong et al*., 2016; *Stanisławczyk et al*., 2020). As a specific product, horsemeat has a specific group of consumers. Italy leads Europe in per capita horsemeat consumption,

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averaging 0.88 kg per person per year, followed by Belgium with about 0.5 kg (*Kołodziejczyk et al*., 2019).

As with other slaughter species, minimising pre-slaughter stress is crucial to ensure animal welfare and obtain high-quality meat, as stress can lead to bruises and/or injuries (*Consortium of the Animal Transport Guides Project*, 2018; *Marlin et al*., 2011; *Pawshe et al*., 2016). During the pre-slaughter period, horses are exposed to numerous potential stressors that adversely affect their welfare and carcass and meat quality, starting from conditions at the point of purchase, loading, transport, unloading, lairaging, stunning and exsanguination (*Driessen et al*., 2022; *EFSA*, 2022). Therefore, the aim of this review was to provide insights into the pre-slaughter conditions in the horsemeat production chain and related effects on welfare and meat quality, which will in turn expand knowledge in this area and determine directions for future research.

2. Place of purchase

Considering that the primary role of most horses is not meat production, acquiring a sufficient number of animals for slaughter transport involves sourcing individuals from various locations—such as farms, auctions, livestock markets and assembly centres—usually through intermediaries, dealers or traders (*Driessen et al*., 2022). To a lesser extent, slaughter horses can originate from farms where they are raised under extensive conditions (e.g., the USA, South America and Spain), but the majority are sport, recreational or working horses sent for slaughter after the end of their productive use, most often due to locomotor disorders (musculoskeletal diseases) or chronic illnesses (*Driessen et al*., 2022).

As a result, horses are frequently transported to slaughter in poor body condition, making it difficult for them to maintain balance during transport, which leads to quick fatigue and increases the risk of developing transport fever (*Driessen et al*., 2022; *EFSA*, 2022). Additionally, horses sourced from different locations can be subclinically infected and act as carriers for various infectious agents, while the mixing animals from diverse origins increases the risk of respiratory diseases (*Driessen et al*., 2022; *EFSA*, 2022). As a consequence of subclinical respiratory infections, *postmortem* examination can reveal lung lesions, which negatively affect carcass and meat quality in slaughter horses (*Čobanović et al*., 2023). Signs of parascarosis in horses are non-specific, can develop over a prolonged period, or may not be clinically apparent at all. However, *postmortem* inspection can reveal scarring in the form of white spots on the liver caused by larval migration of *Parascaris equorum*. These lesions are the result of the liver's fibroplastic response to the presence of these nematodes (*Boyle and Houston*, 2006).

3. Loading

Loading horses into a lorry is one of the most stressful events during pre-slaughter handling, as individuals are transferred from a familiar environment into a new setting, come into direct contact with unfamiliar people, corridors, compartments and vehicles, are subjected to spatial restrictions and are mixed with unfamiliar conspecifics (*Driessen et al*., 2022; *EFSA*, 2022). During this stage, animal welfare can be compromised due to rough handling, exposure to unfavourable climatic conditions (e.g. rain, wind, intense sunlight, noise, etc.) and inadequate infrastructure (*Driessen et al*., 2022). During loading, horses have shown elevated heart rates—on one hand, as a result of increased physical effort while negotiating overly steep loading ramps (>20°), and on the other hand, due to emotional fear triggered by sudden and unfamiliar sounds and visual stimuli, which can cause horses to stop, back away, sidestep or flee (*Driessen et al*., 2022; *Tateo et al*., 2012; *Waran*, 1993). This reaction is even more pronounced in farmed slaughter horses, who, unlike sport or recreational horses, have little or no previous experience with transport (*Driessen et al*., 2022). Excessive use of force during loading, including the use of whips, electric prods, sticks and dogs, causes fear and distress in horses, which can lead to slipping, falling and injury (*Dai et al*., 2021; *Grandin*, 1999; *Nivelle et al*., 2020).

4. Transport

The transport of horses can negatively impact their health, welfare and performance, as well as carcass and meat quality (*Čobanović et al*., 2023; *Driessen et al*., 2022; *EFSA*, 2022). During transit, horses face multiple stressors, including vehicle design (e.g. flooring, compartmentalisation, restraint), feed and water deprivation, driving style, ambient conditions, floor space, transport duration, season, and social disruption due to mixing of unfamiliar individuals (*Driessen et al*., 2022; *EFSA*, 2022).

Within the EU (*European Commission*, 2005), unbroken horses can be transported for a maximum

of eight hours and only in small groups (≤4 per compartment), considering their poor adaptation to restraint and novel environments. Both excessively long and short transport durations have been shown to induce stress responses—elevated levels of cortisol, lactate, glucose, and creatine kinase—and compromise welfare through increased carcass bruising (*Driessen et al*., 2022; *EFSA*, 2022; *Werner & Gallo*, 2008). Insufficient floor space (e.g. 1.28 m²/horse) increases the risk of loss of balance, slipping, falling, fighting and carcass bruising (*Collins et al*., 2000; *Iacono et al*., 2007; *Knowles et al*., 2010). Furthermore, high loading densities (>200 kg/m²) during transport impair welfare, particularly in stallions, who display heightened stress responses and more bruising than geldings (*Božić Jovanović et al*., 2024). These conditions also negatively affect meat quality, resulting in higher ultimate pH and darker meat colour (*Božić Jovanović et al*., 2024).

Recommended floor space allowances for long-distance transport (>8 hours) of adult slaughter horses vary (e. g. 1.75 m²/horse in the EU vs. 1.2 m² in Australia) (*Australian Animal Welfare Standards and Guidelines*, 2021; *European Commission*, 2005), but are often not adhered to in commercial practice, where overloading is common to increase profit (*Driessen et al.*, 2022; *EFSA*, 2022). No consensus currently exists on the optimal floor space in lorries, considering animal size, age, weight, transport duration and environmental conditions, that would simultaneously ensure profitability, animal health and welfare, and especially high carcass and meat quality (*Driessen et al*., 2022; *EFSA*, 2022). Data on how these factors influence horsemeat quality remain scarce, highlighting the need for further research. During transport, slaughter horses from different groups are often mixed, which disrupts the social hierarchy and can lead to fighting (biting and kicking) as animals attempt to establish new social structures, resulting in carcass bruising (*Driessen et al*., 2022; *EFSA*, 2022). Council Regulation No 1/2005 (*European Commission*, 2005) mandates separate transport for horses of different sizes, ages or sexes (especially stallions and mares), unless reared together. Stallions are more difficult to manage during transportation than mares and geldings (*Roy et al*., 2015), while foals are especially vulnerable to transport stress and unfamiliar social contact (*Driessen et al*., 2022; *EFSA*, 2022).

Ambient temperature, humidity, solar radiation and wind speed inside the lorry also affect horse behaviour, health and welfare during transport (*Driessen et al*., 2022). When temperatures deviate from the thermoneutral zone (5–25 °C), horses expend additional energy to maintain homeostasis (*Morgan*, 1998). Cold stress (<5 °C) leads to shivering or huddling, while heat stress (>25 °C) triggers skin vasodilation, sweating and panting (*Consortium of the Animal Transport Guides Project*, 2018; *Luz et al.*, 2015). In commercial conditions, heat and cold stress during transit can only be detected through close observation of horses by trained personnel (*Driessen et al*., 2022).

5. Unloading

The unloading of horses has been largely overlooked in scientific research, despite being one of the most stressful events on the day of slaughter. Exposure to an unfamiliar environment and unknown handlers during this phase can induce stress, adversely affecting animal welfare and carcass and meat quality (*Driessen et al*., 2022; *EFSA*, 2022). The welfare of horses during unloading can be compromised due to inadequate conditions at the slaughterhouse (e.g. steep unloading ramps, corridor design, lighting, unloading equipment), poor human-animal interactions and exposure to adverse weather conditions (e.g., snow, rain, wind, intense sunlight) (*Driessen et al*., 2022; *EFSA*, 2022).

During unloading, horses should be given sufficient time to explore the unfamiliar environment (e. g. the unloading ramp) and exit the lorry calmly, to minimise the risk of injuries (*Messori et al*., 2016). It has been found that excessively steep unloading ramps (>20°) can cause stress (evidenced by elevated concentrations of lactate, glucose and cortisol) and increased heart rate (*Tateo et al*., 2012; *Werner & Gallo*, 2008). Improper handling of horses during unloading, such as yelling, whistling, sudden movements, hitting with hands or feet and the use of whips, electric prods, sticks or dogs, leads to fear and distress in the animals, which can result in slipping, falling, and injuries to both the horses and slaughterhouse personnel (*Driessen et al*., 2022; *EFSA*, 2022).

6. Lairage

Lairage is the holding area in slaughterhouses where animals are temporarily kept prior to slaughter to ensure a continuous slaughter line and to allow recovery from the stress of loading, transport and unloading, thereby increasing meat quality (*Driessen et al*., 2022; *EFSA*, 2022). While intended to

provide rest and protection from adverse weather, lairage can also negatively impact animal welfare and carcass and meat quality if not properly managed. It is, therefore, essential to avoid errors at this stage that could undermine welfare improvements achieved during earlier production phases (*Driessen et al*., 2022; *EFSA*, 2022).

During lairage, attention should be paid to pen design, duration of stay, feed and water deprivation and mixing of animals from different groups (Driessen et al., 2022). Currently available scientific literature provides very limited data on the recommended optimal lairage duration for horses at slaughterhouses (*Driessen et al*., 2022; *EFSA*, 2022; *Farm Animal Welfare Council*, 2003). Under commercial conditions, horses can either be slaughtered immediately upon arrival or kept in lairage for several hours (~six hours), overnight or for several days (*Werner & Gallo*, 2008; *Driessen et al*., 2022). However, from an animal welfare standpoint, some authors advocate for immediate slaughter, as extended lairage could elevate stress levels (e. g. due to noise, fear, thirst, hunger), ultimately compromising meat quality (*Werner & Gallo*, 2008). Lower horse meat quality arises as a consequence of severe stress, bruising or injuries sustained during extended lairage periods (C*onsortium of the Animal Transport Guides Project*, 2018; *Nivelle et al*., 2020; *Pawshe et al*., 2016). In addition, it has been found that mixing horses from different origins during lairage leads to fights for the establishment of new social hierarchies and for limited resources in the lairage pens (e. g. space, feed and water), which results in carcass bruising and consequently lower meat quality (*Miranda-de la Lama et al.*, 2021).

7. Stunning

The process of moving horses from lairage to the stunning box, as well as the stunning itself, can induce significant stress, thereby compromising animal welfare and carcass and meat quality. To minimise stress, careful attention must be given to the stunning box design, animals' temperament, stunning method and slaughterhouse personnel training (*Driessen et al*., 2022). Restraint within the stunning box can provoke agitation, fear and stress; therefore, the duration of restraint should be minimised. Horses should not be led into the box until the operator responsible for stunning and bleeding is fully prepared to perform the procedure without delay (*Driessen et al*., 2022). Extended restraint in the stunning box has been associated with behavioural signs of distress—such as nervousness, muscular rigidity and tremors—as well as elevated blood concentrations of lactate and creatine kinase at exsanguination (*Werner & Gallo*, 2008).

In Europe and South America, stunning is typically performed using a penetrating captive bolt (powered by compressed air or explosive cartridges), while in Canada, a long rifle is used (*Algers & Atkinson*, 2014; *Gibson et al*., 2015). Malfunction of cartridge-fired captive bolts can occur due to damp cartridges (*Grandin*, 2015), and pneumatic devices can be compromised by inadequate air pressure (*Bulens et al*., 2013), both resulting in ineffective stunning and serious animal welfare issues. When using firearms for stunning horses, risks such as ricochet necessitate strict adherence to safety protocols, including correct operator positioning (*Gallo et al*., 2022; *Hultgren*, 2022; *Needham & Hoffman*, 2022; *Shearer & Ramirez*, 2013).

8. Exsanguination

Delayed or improper exsanguination can compromise horse welfare and negatively affect meat quality (*Werner & Gallo*, 2008). The procedure is typically performed using a sharp, rigid, long-bladed knife to sever the jugular veins, carotid arteries and trachea (*Woods & Shearer*, 2015). To enhance bleeding efficiency, thoracic sticking—severing major vessels near the heart, such as the brachiocephalic trunk and artery—can also be employed (*Woods & Shearer*, 2015). To ensure animal welfare and optimal bleeding, exsanguination should occur within 60 seconds of stunning (*Werner & Gallo*, 2008).

During the interval between stunning and exsanguination, and throughout the bleeding process, horses must be monitored for signs of inadequate stunning, including the presence of the corneal reflex, spontaneous blinking, rhythmic breathing and righting reflex (*Bulens et al*., 2013). Carcass processing and *postmortem* inspection can only proceed once the absence of vital signs has been confirmed (*Bulens et al*., 2013). *Werner and Gallo* (2008) reported that in 95.2% of horses, the interval between stunning and bleeding exceeded one minute (range: 1–4 minutes), and 57.2% of animals exhibited signs of sensibility post-stunning and bleeding. The most frequent signs of sensibility were rhythmic breathing (47.6%), eye movement (42.7%), vocalisation (14.7%), head lifting (12.2%) and attempts

to rise (9.5%) (*Werner & Gallo*, 2008). Additionally, elevated blood concentrations of lactate, glucose, creatine kinase and cortisol were observed under conditions of delayed or ineffective exsanguination, indicating stress and compromised welfare (*Werner & Gallo*, 2008).

9. Conclusion

The welfare of horses can be compromised at any stage of the meat production chain – from the place of purchase, through loading, transport, and unloading, to lairage and the slaughter process itself. Animal characteristics, infrastructure at the point of purchase and slaughterhouse, vehicle design, environmental factors, and the quality of human-animal interactions significantly influence pre-slaughter welfare conditions. Considering that the current recommendations on minimum floor space in transport vehicles are inadequate, and that there are no clear guidelines regarding the optimal lairage duration for horses at slaughterhouses, further research is needed to improve horse welfare throughout the meat production chain.

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Original Scientific Paper

Biogenic amines in meat and meat products: markers of quality and factors affecting formation

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ABSTRACT

Biogenic amines (BAs) can be found in a wide range of meat and meat products, and, due to their toxicity, can cause adverse effects on the consumers. BAs are generally produced by microbial decarboxylation of amino acids in food products, and their formation is influenced by different factors associated with the raw material, microorganisms, processing, and conservation conditions. These factors do not act in isolation but rather have combined effects that dictate the final concentration of BAs in meat and products. Despite their strong influence on food quality, there is no specific regulation regarding BA content in meat and meat products, with the exception of histamine in fishery products. Therefore, the present paper reviews the main BAs in meat and products, effect of technological factors influencing biogenic amines content in meat and products and their use as markers of quality.

1. Introduction

The rapid increase in the production of meat (products) in the last decades and the desire to prolong its shelf-life cause the search for new methods of meat quality evaluation. As an alternative to sensory and microbiological analyses, it is increasingly often proposed to determine certain chemical indicators, such as biogenic amines (BAs), as an indicator for food safety and quality evaluation (*Chmiel et al.,* 2022). BAs are low molecular weight substances in food mainly produced by the decarboxylation of free amino acids under the influence of endogenous or bacterial enzymes or by amination or transamination of aldehydes and ketones (*Schirone et al.,* 2022). Spermine (SPM), spermidine (SPD), putrescine (PUT), cadaverine (CAD), tryptamine (TRP), phenylethylamine (PHE), histamine (HIS), and tyramine (TYR) commonly exist in meat and meat products and rapidly accumulate, especially during spoilage (*Jastrzębska et al.,* 2024). SPD and SPM are also naturally occurring BAs in fresh meat (*Schirone et al.,* 2022). Several factors associated with raw materials, such as chemical composition, pH, temperature, some handling and manufacturing operations (e.g., fermentation, ripening, curing), time of storage, and post contamination can influence BA presence in foods (*Dabadé et al.,* 2021). High concentrations of these compounds in food are associated with food poisoning, due to their toxicological effects, potentially causing adverse effects like nausea, headaches, gastric and intestinal problems, and

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pseudo-allergic responses (*Shashank et al.,* 2021). Despite their strong influence on both public health and food quality, specific legislation only covers histamine in fishery products, and no criteria have been established for other BAs or other food such as meat (*Schirone et al.,* 2022).

In the light of these considerations, the present study aimed to summarize the influence of the major environmental and process factors on the occurrence of BAs in raw meat and meat products produced by several manufacturing processes, taking into account both food quality and safety aspects of accumulation of BAs. This work intends to inspire establishment of more accurate permissible levels of BAs in meat and products.

2. Factors influencing biogenic amine formation

Meat and meat products are particularly subjected to BA production due to their high protein and amino acid content. Thus, the availability of BA precursors, free amino acids, and the presence of microorganisms able to biosynthesize enzymes catalyzing BA decarboxylation, are essential factors that affect the formation of BAs in meat and meat products (*Liu et al.,* 2024). Proteolytic activity can arise as a consequence of acidity increase, dehydration, temperature and the action of sodium chloride in some meat derived products, but also by microbial activity during fermentation and/or other food production processes, and during storage (*Dabadé et al.,* 2021). Many microorganisms which are present in meat and meat product, such as *Lactobacillus* spp., *Pseudomonas* spp., Enterobacteriaceae, and *Enterococcus* spp., exhibit decarboxylase activity (*Liu et al.,* 2024). A high content of some BAs in food can also be an indicator of poor microbiological quality (*Chmiel et al.,* 2022).

Since amino acid decarboxylase has higher activity under low pH conditions, to some extent, microbial amine production is a physiological reaction to resist an acidic environment (*Gardini et al.,* 2016). Also, low pH enhances the cathepsin activity in raw meat, resulting in the degradation of muscle proteins into peptides and free amino acids, substrates of amino acid decarboxylase (*Gökoğlu,* 2003).

Temperature is another factor influencing the formation of BAs in meat and meat products (*Liu et al.,* 2024). High temperature during storage or fermentation accelerates protein decomposition and the growth of microorganisms, increases the activity of amino acid decarboxylase and proteolytic enzymes and, hence, accelerates the formation of BAs in meat and meat products. Formation of BAs is also influenced by atmospheric conditions, especially the atmospheric conditions in the packaging. The proportion of oxygen in packaging mainly affects the type, quantity, and metabolism of microorganisms in meat and meat products, thus leading to the differences in the formation of BAs (*Gardini et al.,* 2016).

The intrinsic properties of meat (products), influence BA generation. Due to the unique protein composition and softer texture of chicken meat, its BA content is generally higher than in beef and pork, under the same storage conditions (*Liu et al.,* 2024). For meat products, salts are often added during fermentation, curing or storage. High salt concentrations contribute to less accumulation of BAs in meat products, due to reduction of the metabolic activities of decarboxylating microorganisms (*Gardini et al.,* 2016). Gram-negative bacteria are more inhibited by increasing salt concentrations than Gram-positive microbiota (*Gardini et al.,* 2016). The content of BAs in fermented meat products was, due to the specific manufacturing process, significantly higher than those in cured or other kinds of meat products (*Schirone et al.,* 2022). Fermented meat products have conditions conducive to the growth of microorganisms, thus facilitating the production of BAs (*Schirone et al.,* 2022). Smoking treatment also influences BA formation in meat products, because smoking reduces water content and increases salt diffusion, thus inhibiting aminopeptidase, release of free amino acids, and ultimately, generation of BAs (*Gardini et al.,* 2016).

3. Biogenic amines in raw meat

In raw meat, the presence of BAs depends on different factors, such as meat origin, the specific microbiota, storage conditions, and meat shelf-life (*Schirone et al.,* 2022). Hence, inappropriate hygiene and storage conditions can increase the incidence of BAs. As mentioned above, environmental factors such as temperature and pH are of crucial importance for BA formation. However, packaging solutions can control the increase of BA in raw meat and meat preparations (*Chmiel et al.,* 2022). *Chmiel et al.* (2022) indicated that storage conditions are very important, since product stability was higher in cold rooms and dry conditions, than in display cases exposed to light. Raw meat mostly contains the polyamines, PUT, SPD, and SPM, in particular the last

two (*Schirone et al.,* 2022). Many researchers proposed using the ratio of SPM to SPD for raw meat quality evaluation, because it is independent from microbiological activity and relatively stable over time, as well as the sum of CAD + PUT to evaluate raw meat decay independently from the animal species. HIS and TYR start to increase after some days of storage, presuming the initial microbial count is not high (*Schirone et al.,* 2022). Quantification of TRP, CAD, PHE, and PUT is considered useful for early determination of meat spoilage in poultry, pork, and beef, based on the study by *Woinowski et al.* (2019).

4. Biogenic amines in processed meat

BAs can be found in a wide range of meat products. Fermented meat products are a primary source of BAs, mainly due to bacterial and fungal activities. These microorganisms take advantage of the available nutrients, the favourable water activity (at the beginning of the process), and the total and partial oxygen absence ensured by the environment, the former promoting the specific production of BAs like HIS, while reducing the amounts of PUT and CAD (*Schirone et al.,* 2022). In fermented meat products, main bacterial groups responsible for BA production are Enterobacteriaceae and *Pseudomonas*, some strains belonging to the genera *Staphylococcus* and *Bacillus*, and the lactic acid bacteria (*Li et al.,* 2020). Fermented products like sausages and salamis can impair food safety in regard to the accumulation of TYR, HIS, PUT, and CAD. TYR was the most frequent and abundant BA found in dry fermented sausages in Spain's retail sector, while fermented sausages and salamis from Portugal and Serbia had low amounts of TYR, CAD and PHE, and HIS was present only in two samples (*Latorre-Moratalla et al.,* 2017; *Alves et al.,* 2017). Different smoking processes can reduce the level of BAs due to the reduction of free amino acids in dry cured hams (*Martuscelli et al.,* 2009). Also, the diffusive phenomena of salt and water influence amino acid decarboxylase and/or amino oxidase activities (*Dabadé et al.,* 2021). TYR is the most common amine in cured meat products (*Schirone et al.,* 2022). Heat treatments do not affect the BA concentration in meat products. Chicken meat did not show any polyamine loss after different heat treatments, while beef and pork meat after cooking showed only slight reduction of BAs (*Schirone et al.,* 2022). Low levels of BAs usually indicate high quality of the raw materials, good hygiene and handling procedures, and suitable drying/fermenting conditions.

5. Biogenic amine index and recommended limits

To control the processing and development of various food (meat, fish, etc.), and to signal degree of food freshness and/or deterioration, BAs have been frequently employed as quality indexes. However, establishing a biogenic amine index (BAI) that reliably predicts product quality is no simple matter. The BAI calculated from the sum of HIS, TYR, PUT, and CAD (mg/kg) was proposed for meat (*Wójcik et al.,* 2022). Good quality fresh meat should not have a BAI exceeding 5 mg/kg. Acceptable meat, but with signs of initial spoilage, has a BAI of between 5 and 20 mg/kg. Meat of low hygienic quality is classified in the range 20–50 mg/kg, and spoiled meat has a BAI above 50 mg/kg (*Ruiz-Capillas and Herrero,* 2019; *Hernández-Jover et al.,* 1996). The usefulness of BAI as a quality index depends on many factors, which include the nature of the product (fresh, canned, modified atmosphere, fermented, etc.). BAI levels have proven to be more satisfactory in fresh meat and meat products and heat-treated products than in fermented products, due to the latter having varying amounts of BAs over time, owing to the number of different factors involved in the processing (ripening, maturation, starter, additives, etc.) (*Ruiz-Capillas and Herrero,* 2019).

Since the consumption of foods containing high amounts of BAs have been associated with health hazards, restricted consumption of BA-rich products is recommended (*Wójcik et al.,* 2022). However, international permissible levels of biogenic amine consumption are lacking. Numerous consumer organizations and food safety agencies, which include the European Food Safety Authority (EFSA), the Food and Drug Administration (FDA), Food Safety Commission of Japan (FSCJ), and the World Health Organization (WHO), are dealing with this problem (*Ruiz-Capillas and Herrero*, 2019). One of the toxins targeted by the *FDA* (2025) and *EFSA* (2011) is HIS in fish and fish products. The maximum level of tolerated HIS in meat was determined as 100 mg/kg (*Feddern et al.,* 2019), whereas daily HIS consumption should not exceed 50 mg (*Rabie et al.,* 2014). Also, *Danchuk et al.* (2020) suggested a HIS level between 50 and 200 mg/kg could have a harmful effect on consumer health,

and levels above 200 mg/kg exhibit toxic properties. *Feddern et al.* (2019) reported the toxic effects of TYR content above 100 mg/kg. For healthy people who do not take drugs from the group of monoamine oxidase inhibitors (MAOI), *EFSA* (2011) recommended a daily limit of 600 mg of TYR, 50 mg for people taking third-generation MAOI drugs and 6 mg for people taking classic MAOI drugs. PHE levels above 30 mg/kg show toxic effects on consumer health status (*Feddern et al.,* 2019). *EFSA* (2011) also suggested that information on PUT and CAD have proven insufficient to draw any conclusions on limits.

6. Conclusion

One of the major challenges in modern times is to produce adequate amounts of safe food. Due to the impact of BAs on human health, it is of great importance to prevent the excessive accumulation of these compounds in food. Controlling these compounds implies a deep understanding of the formation and monitoring mechanisms, and an ability to reduce biogenic amines during the processing and storage of meat and products. Such control of BAs would benefit consumers, public authorities, and industry. Apart from being a food safety concern, BAs also serve as an indicator of quality. Obtaining deeper knowledge about levels that could be used as guidelines in the different meat and products that are available on the market is needed. Such an endeavour requires a commitment, not only from public institutions, but also from production sectors and commercial processors, and ultimately from consumers.

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Review Paper

Alpha-gal syndrome: allergy to red meat

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ABSTRACT

Meat allergies are increasingly present, both in children and in adults, and are a group of heterogeneous allergic reactions with different syndromes. Alpha-gal syndrome (AGS), also known as α-gal allergy or mammalian meat allergy (MMA) is a type of acquired allergy characterized by a delayed onset of symptoms (3–8 hours) after consumption of mammalian meat. It is a reaction to the carbohydrate, galactose-α-1,3-galactose (α-gal), where the body is overwhelmed with immunoglobulin E (IgE) antibodies when exposed to the carbohydrate. In most cases, it is the result of previous exposure to certain tick bites and was first reported in 2002. Allergy symptoms vary greatly from person to person and include rash, hives, nausea or vomiting, difficulty breathing, drop in blood pressure, dizziness or fainting, diarrhoea, severe stomach pain and possible anaphylaxis.

1. Introduction

Food allergens of animal origin are substances that the bodies of some consumers recognize as foreign and respond to by activating the immune system. The most common allergens from animal-based foods are proteins found in meat, milk, eggs, fish, shellfish, and other seafood. Meat very rarely causes allergic reactions and was first mentioned as a potential allergen about 25 years ago, at a time when increasing attention was being paid to food allergies in general (*Platts-Mills,* 2015).

It was once believed that only children with atopic dermatitis or those already allergic to cow's milk could develop an allergy to mammalian meat (*Werfel et al.,* 1997). There are no definitive estimates of the prevalence of such allergies, but what is notable are regional variations, primarily due to local dietary habits. Today, various IgE-mediated reactions and their cross-sensitizations are recognized to many types of meat species, including beef, pork, lamb, poultry, kangaroo, whale, and crocodile.

In recent years, there has been increasing mention of a new form of delayed anaphylaxis to red meat, triggered by the oligosaccharide Gal-α1,3Gal-β1,4GlcNAcR (α-gal), after which the allergy was named—α-gal syndrome (*Wilson et al.,* 2017). This type of allergy is also referred to as mammalian meat allergy (MMA) or tick bite-induced meat allergy (*Woodfolk et al.,* 2015).

1.1. Cross allergic reactions to red meat

Serum albumins are α-helical proteins with a molecular weight of approximately 70 kDa. They have the ability to pass through the capillary

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endothelium, which means they can also be found in epithelial tissues, animal fur, and bird feathers. Based on this fact, it can be concluded that individuals who are allergic to beef are often also allergic to milk, due to the presence of the serum albumin Bos d 6 (*Martelli et al.,* 2002). A similar example is the "bird-egg syndrome," where primary sensitization occurs through inhalation of bird serum albumin, but symptoms later also appear after consumption of chicken meat (*Szepfalusi et al.,* 1994). On the other hand, cross-reactivity between serum albumins of mammals and birds is rare, as it occurs only between phylogenetically similar species. The "pork-cat syndrome" involves primary sensitization to cat serum albumin (Fel d 2), followed by sensitization to pork serum albumin (Sus s 1). In some cases, due to epitope spreading of IgE, affected individuals can also become sensitized to bovine serum albumin (Bos d 6) (*Posthumus et al.,* 2013). One of the key characteristics of these proteins is that they are sensitive to heat and lyophilization (freeze-drying), so such processing methods can reduce the severity of allergic reactions (*Fiocchi et al.,* 1998).

1.2. Alpha gal (α-gal)

Alpha-gal is a sugar molecule (galactose-α-1,3-galactose) found in the cells of most mammals (except humans and primates). It is present in meat, internal organs, milk, gelatine, and other animal-derived products. It was first mentioned as early as 1925 (*Landsteiner et al.,* 1925) as a molecule structurally similar to the B blood group antigen. It is also notable for being a major target of IgM, IgG, and IgA antibodies in immunocompromised patients. The allergy to α-gal was first formally identified by Thomas Platts-Mills in 2002 in the United States, and later in 2007 by Sheryl van Nunen in Australia (*Velasquez-Manoff,* 2018). When Platts-Mills himself was bitten by a tick and developed an allergic reaction to α-gal, his team concluded that there was a link between tick bites and the development of the allergy (Figure 1).

This is the first food allergy associated with carbohydrates, rather than proteins. A defining characteristic of this type of allergic reaction is that the reaction occurs 3–6 hours after exposure to the allergen (*Commins et al.,* 2014). This delay is due to the time needed for the digestion, processing, and transport of these epitopes to the target tissue. Although most studies have focused on α-gal present in glycoproteins, it is also found in glycolipids. This supports the hypothesis that lean wild game meat is less likely to trigger a reaction in allergic individuals (*Galili et al*., 1987). Bites from some tick species—such as the Lone Star tick (*Amblyomma americanum*) and *Ixodes holocyclus*—are linked to the development of this allergic response. When a tick bites, it injects this carbohydrate into the human body via its saliva. The immune system then releases a surge of IgE antibodies to fight off this foreign sugar. After this sensitization, future consumption of red meat (beef, pork, lamb, etc.) containing the same α-gal can trigger an allergic reaction. Some individuals also develop allergic reactions to gelatine and certain dairy products derived from mammals (*CDC,* 2021). Traces of this carbohydrate can also be found in certain medications, including nonsteroidal anti-inflammatory drugs (NSAIDs), other analgesics, and pain relievers (*Vaz-Rodrigues et al.,* 2022). It is also present in the drug cetuximab, which is used in immunotherapy for metastatic colorectal cancer, specifically in the Fab fragment of this recombinant monoclonal antibody. Allergic reactions have also been reported in α-gal syndrome patients with blood type O who received plasma or platelets from

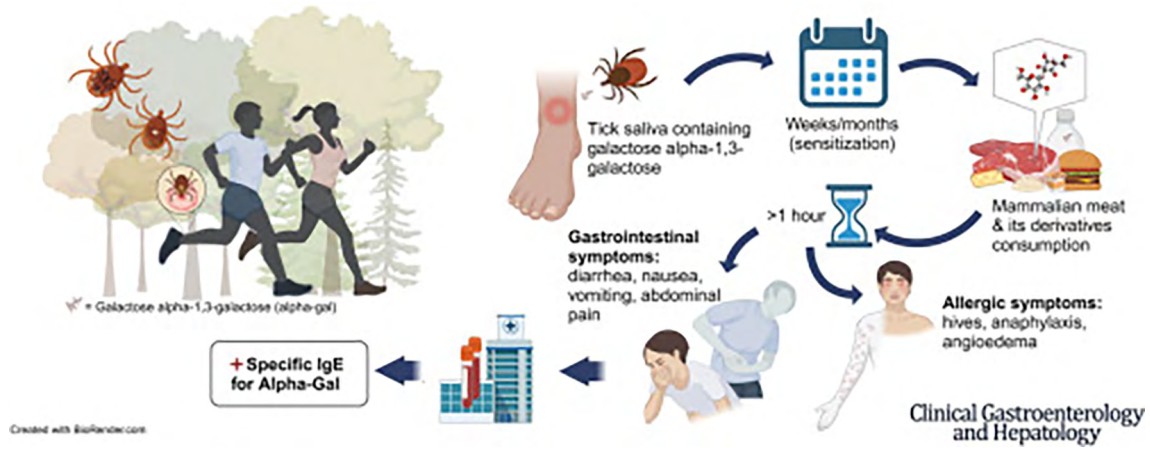

Figure 1. Formation of α-gal allergic reaction (*Lesmana et. al*, 2025)

donors with blood type B (*Miller et al.,* 2024), with the relevant moieties depicted in Figure 2.

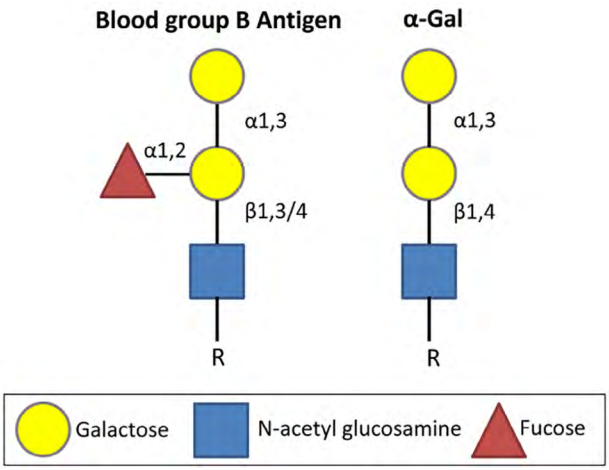

Figure 2. Comparison of the structures of α-Gal and blood group B antigen (*Lesmana et. al*, 2025)

After the delayed onset (3–6 hours), the allergic response is similar to most food allergies, particularly IgE-mediated allergies, and includes intense itching across the body, hives, angioedema, gastrointestinal symptoms, and the possibility of anaphylaxis (*Mabelane et al.,* 2019). Anaphylactic reactions occur in approximately 60% of affected individuals. There have been cases where gastrointestinal symptoms occurred without itching, hives, or other skin-related issues. In 70% of cases, the reaction is accompanied by respiratory problems, and it is particularly harmful to individuals with asthma. An increased risk has been found in people who do not have blood type B or AB, as the B blood group antigen is structurally similar to α-gal and may provide a certain level of natural protection (*Vaz-Rodrigues et al.,* 2022).

The severity of symptoms and the overall reaction to α-gal correlate with the amount of α-gal carbohydrate present in the consumed food or medication. It has been found that avoiding mammalian meat and preventing further tick bites leads to a decrease in serum IgE antibody levels (*Platts-Mills et al.,* 2020). In addition, treatment with antihistamines can be effective, while severe cases may require hospitalization and the administration of epinephrine (*Commins,* 2020). In 2020, the U.S. Food and Drug Administration (FDA) approved the genetic modification of pigs so they do not produce α-gal sugars. These pigs, developed under the trademarked name GalSafe, could be safely consumed by individuals with α-gal allergy, used in drug production, and their organs could be used for xenotransplantation (*Dolgin,* 2021).

Investigating a suspected case of meat allergy requires a multidisciplinary approach. Diagnosis of this allergic reaction begins with a clinical suspicion of α-gal syndrome, based on a detailed medical history and the individual's clinical symptoms. Diagnosis is typically challenging, and no single specific test is recommended over others. Because the onset of symptoms is often significantly delayed, it can be difficult to connect the reaction to the consumption of red meat several hours earlier. Skin prick testing with relevant animal proteins may be of limited use in this context, due to significant variability in food preparation and the lack of well-validated results. A commercially available test for α-gal includes bovine thyroglobulin and is accessible through certain manufacturers (*Apostolovic et al*., 2017).

The most commonly used test is a serological test, specifically the measurement of IgE antibodies against α-gal (>0.1 IU/mL), used in combination with the patient's clinical symptoms.

2. Conclusion

Although traditionally considered rare, meat allergy is increasingly being recognized in individuals of all ages. Raising awareness of α-gal allergy—both among healthcare professionals and the general public, especially in tick-endemic regions—is a crucial first step toward improving diagnosis and treatment. As with the management of any food allergy, treatment of this allergy is based on allergen avoidance. However, for α-gal patients, this principle of self-protection is complicated by the lack of proper labelling of mammalian-derived ingredients in food, medications, and vaccines. The approval of α-gal-free pork products would be an effective pathway for developing "α-gal-safe" foods, drugs, and implantable medical devices (*Commins, 2020*). The prevalence of α-gal allergy remains unknown and continues to pose a challenge with regard to its widespread integration into medical education and awareness efforts in the healthcare system.

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Review Paper

Cooked-emulsified rabbit meat sausages: a technological challenge or sustainable solution?

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ABSTRACT

Rabbit meat is characterized by a high content of biologically valuable proteins, low levels of fat, cholesterol, and sodium, appreciable quantities of n-6 and n-3 polyunsaturated fatty acids (PUFAs), and substantial amounts of B-complex vitamins, particularly vitamin B12. Given its favorable nutritional profile, rabbit meat is well-suited for the formulation of cooked-emulsified sausages, particularly those with added value aimed at health-conscious consumers. From a technological standpoint, rabbit meat possesses specific characteristics that can be an advantage or challenge in the production of cooked-emulsion sausages. Therefore, this paper highlights the technological characteristics of rabbit meat and considers its suitability for the production of cooked-emulsified sausages.

1. Introduction

Rabbit meat is a nutritionally valuable source of protein (18.6–22.4%) (*Dalle Zotte & Szendrő*, 2011), characterized by excellent digestibility and a notable content of essential amino acids, including lysine, threonine, leucine, valine, isoleucine, and phenylalanine (*Honrado et al.,* 2022; *Siudak & Palka*, 2022; *Deng et al*., 2024). It is classified as a lean meat, exhibiting a fat content that varies between 1.8% and 8.8%, depending on the anatomical part of the carcass (*Dalle Zotte & Cullere*, 2019). Importantly, rabbit meat contains appreciable quantities of both n-6 and n-3 polyunsaturated fatty acids (PUFAs), which are essential for human health. Among these PUFAs, linoleic acid (an n-6 fatty acid) and α-linolenic acid (an n-3 fatty acid) are predominant, besides long-chain n-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are known for their cardioprotective and anti-inflammatory properties (*Dalle Zotte & Szendrő*, 2011). In comparison to other meats, rabbit meat also has lower cholesterol content, averaging about 47 mg/100 g, with levels influenced by the animal's diet (*Hernàndez & Dalle Zotte*, 2010). As a white meat, it contains less iron (1.1–1.3 mg/100 g) and zinc (0.55 mg/100 g) than red meats like beef, lamb, or pork (*Lombardi-Boccia et al*., 2005). Another notable nutritional advantage of rabbit meat is its low sodium

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content (37–47 mg/100 g), which makes it suitable for inclusion in diets for individuals with hypertension (*Dalle Zotte & Szendrő*, 2011; *Dalle Zotte & Cullere*, 2019). Simultaneously, rabbit meat serves as a valuable source of essential minerals, including potassium (428–431 mg/100 g), phosphorus (222–234 mg/100 g), and selenium (9.3–15 µg/100 g), which are integral to numerous physiological processes (*Dalle Zotte & Szendrő*, 2011; *Dalle Zotte & Cullere*, 2019). In addition, rabbit meat is particularly rich in B-complex vitamins, which play a crucial role in metabolism, nervous system function, and the synthesis of red blood cells (*Dalle Zotte & Szendrő*, 2011).

Due to its high digestibility, nutritional profile, and low allergenic potential, rabbit meat is frequently recommended for nutritionally sensitive populations, such as children, pregnant women, the elderly, and individuals with health conditions including allergies, diabetes, obesity, and hypertension (*Składanowska-Baryza & Stanisz*, 2019; *Suvajdžić et al*., 2023). Despite its nutritional advantages, rabbit meat consumption remains low (*Cullere & Dalle Zotte*, 2018; *Szendrő et al*., 2020; *Siddiqui et al*., 2023). This limited acceptance is influenced by several factors, including socio-demographic characteristics, cultural background, the perception of rabbits as companion animals, concerns regarding animal welfare, as well as the meat's sensory characteristics and price (*Siddiqui et al*., 2023). Therefore, innovative strategies are required to increase the consumption of rabbit meat. One promising approach involves developing novel, health-oriented products that align with contemporary dietary preferences (*Suvajdžić et al*., 2023). Cooked-emulsified rabbit meat sausages are a viable option, as this type of product offer convenience and ease of preparation. Accordingly, this paper highlights the technological characteristics of rabbit meat and considers its suitability for the production of cooked-emulsified sausages.

2. Rabbit meat in the production of cooked-emulsified sausages

Cooked sausages are primarily composed of a meat batter or meat emulsion, which is subjected to heat treatment after being stuffed into casings (*Vuković*, 2020; *Vasilev et al*., 2023). The production of emulsion-type cooked sausages is a complex technological process in which the composition and interaction of the main filling components – meat, fat tissue, salt, and water – play a crucial role (*Vuković*, 2020). In the production of cooked sausages, meat with high water-binding capacity is preferred (*Vuković*, 2020). The water-binding capacity of meat depends on salt-soluble myofibrillar proteins, particularly myosin and actin (*Santhi et al*., 2017). During meat batter preparation, myofibrillar proteins are released through mincing and interact with added salts, becoming well hydrated and capable of binding added water (*Vuković,* 2020). In the presence of salt, approximately one-fifth of myofibrillar proteins become solubilized, and up to one-third do so when salt is combined with phosphates. The remaining myofibrillar proteins swell, retaining a significant portion of the water naturally present in the meat as well as the added water (ice) during batter preparation (*Puolanne*, 2010). On the other hand, the water-binding capacity of meat is positively correlated with the pH and is most pronounced before the onset of postmortem rigor, when the pH is the highest (*Vuković*, 2020). The production of cooked-emulsified sausages requires meat with pH between 5.8 and 6.2. Within this range, the protein structure is favorable for maximum water-binding capacity and exhibits good emulsifying and gel-forming properties, resulting in improved emulsion stability and a firmer product texture (*Santhi et al*., 2017). According to literature data, the ultimate pH of rabbit meat ranges from 5.7 to 6.0 (*Kozioł et al*., 2015; *Sampels & Skoglund*, 2021; *Frunză et al*., 2023), which is considered optimal for the preparation of cooked sausage batters. However, the ultimate pH of rabbit meat is influenced by a range of factors, including genetics, environmental conditions, diet, rearing system, as well as pre- and post-slaughter conditions (*Kumar et al*., 2023). A low ultimate pH leads to decreased water-binding capacity, resulting in both technological and economic losses (*de Oliveira Paula et al*., 2020). Conversely, rabbit meat with a high ultimate pH is generally avoided due to its negative impact on product shelf life (*Suvajdžić et al*., 2023).

The formation of an interfacial protein film around fat globules plays a crucial role in stabilizing the fat phase during the production of cooked-emulsified sausages (*Glorieux et al*., 2019). Primarily composed of myofibrillar proteins, interfacial protein film prevents fat coalescence and the release of liquefied fat as the temperature rises during heat treatment, such as pasteurization (*Barbut & Youssef*, 2016). Concurrently, heating induces the formation of a three-dimensional gel network by myofibrillar proteins, which physically entraps both fat and

water fat (*Vuković*, 2020). After that, cooling promotes additional stabilization of the protein network and initiates partial crystallization of the fat phase (*Glorieux et al*., 2019). Fat globules, coated with interfacial protein film and integrated within the protein matrix, enhance the gel rigidity and its structural integrity (*Glorieux et al*., 2019). In a comparison of rabbit, beef, and chicken meats for frankfurter production, *Whiting & Jenkins* (1981) concluded that protein solubility, water-holding capacity, emulsifying capacity, and binding strength were approximately equal among these meat types. Moreover, the low collagen content in rabbit meat (5.71 to 7.97 mg/g) enhances the stabilization of fat globules and promotes greater water retention (*Bueno et al*., 2023).

On the other hand, the production of cooked-emulsified rabbit meat sausages could be a technological challenge due to the low quantity of rabbit fatty tissue as a source of essential fats. Fatty tissue in rabbits is primarily located in the submandibular, scapular, and perirenal regions of the carcass. Obtaining sufficient quantities of rabbit fatty tissue can be particularly difficult, especially in the context of industrial-scale sausage production (*Dalle Zotte & Cullere*, 2019). As the most commonly used raw material in the production of cooked-emulsified sausages, pork back fat is a suitable solution since it contributes to stable emulsion formation, reduces losses during heat treatment, and enhances juiciness and texture (*Polak et al*., 2023). Nevertheless, replacing rabbit fatty tissue with pork back fat compromises both the concept of an authentic rabbit meat product and the expectations of modern health-conscious consumers. To replace animal fat, various hydrocolloid systems, such as alginate, carrageenan, xanthan gum, cellulose derivatives, starches, and pectins, have been investigated (*Petracci & Cavani*, 2013). Nevertheless, the use of other functional ingredients, such as vegetable fiber has shown greater advantages from both technological and nutritional aspects, owing to their favorable rheological properties and dietary benefits (*Petracci & Cavani*, 2013). Vegetable fiber can be used either alone or in combination with plant oils, which increase the content of polyunsaturated fatty acid in the product and make it prone to the oxidation process (*Vasilev et al*., 2023). Therefore, the replacement/reduction of fatty tissue in cooked-emulsified sausages presents a significant technological challenge, as it influences the aroma, color, and texture of these products (*Vasilev et al*., 2023). However, cooked-emulsified sausages with reduced fat content and with functional ingredients are labeled as added-value products, which are more appreciated by consumers, particularly health-conscious individuals.

The color of cooked-emulsified sausages is a very important sensory property, as it creates the consumer's first impression. Due to its low myoglobin content, rabbit meat is relatively light in color (high L^*), with low contributions to redness (a*) and yellowness (b*) (*Suvajdžić et al*., 2023). Therefore, the incorporation of rabbit meat into meat products significantly increases lightness and reduces redness (*Ignacio et al*., 2019). The low myoglobin content limits the development of nitrosyl-myoglobin after the addition of nitrites into meat batter, and consequently, limits the formation of product color (*Suvajdžić et al*., 2023). However, the addition of specific spices, such as paprika, can solve the problem and make a product color more acceptable for consumers (*Polak et al*., 2023). According to *Mićović et al*. (2021), paprika increases the redness and yellowness in frankfurter-type sausages, since it contains red and yellow pigments. Additionally, paprika decreased L^* in frankfurter-type sausages, making them darker than control samples (*Mićović et al*., 2021).

3. Conclusion

Given its nutritional profile, as well as its dietetic and technological properties, rabbit meat is a suitable raw material for the production of cooked-emulsified sausages. The protein solubility, water-holding capacity, emulsifying capacity, and binding strength in rabbit meat is optimal and approximately equal to that of beef and chicken meat. However, the limited amount of rabbit fatty tissue presents a challenge, which can be addressed by incorporating pork back fat, hydrocolloids, or vegetable fiber as functional ingredients. While the use of pork back fat in the production of cooked-emulsified rabbit meat sausages compromises the dietetic properties and authenticity of rabbit meat products, replacing rabbit fatty tissue with other functional ingredients offers a promising alternative.

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Review Paper

Incorporation of microalgae in the development of innovative dairy products

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ABSTRACT

Global food demand is projected to rise in the future, increasing pressure on food systems and intensifying the search for sustainable protein sources. Microalgae are increasingly recognized as a promising alternative due to their high protein content, favourable essential amino acid profile, polyunsaturated fatty acids, vitamins, minerals, and diverse bioactive compounds with antioxidant potential. Dairy products, as widely consumed nutrient-rich foods, represent an ideal platform for microalgae incorporation to improve nutritional quality and promote more sustainable diets. The aim of this manuscript was to provide a concise overview of dairy product formulations incorporating microalgae, with an emphasis on future prospects and associated challenges. The most thoroughly studied algal species are *Arthrospira platensis* and *Chlorella vulgaris*, and the possibility of their incorporation into fermented milks, particularly yogurt, cheese and ice creams. Documented benefits include increased protein content, improved fatty acid composition, enhanced antioxidant activity, and potential stimulation of starter and probiotic bacterial growth. Nevertheless, technological and sensory challenges remain, including modifications in texture, viscosity, and colour, as well as the development of bitter, herbaceous, or marine-like flavours that may reduce consumer acceptance. Future research should focus on identifying suitable microalgal species, refining processing technologies, and developing flavour-masking approaches to enable the production of microalgae-enriched dairy products that are both nutritionally enhanced and sensorially acceptable to a broad consumer base.

1. Introduction

Global food demand is projected to rise by 35–56% by 2050, and potentially by up to 62% when the impacts of climate change are considered (*Van Dijk et al*., 2021). The projected increase in the intake of animal-derived protein poses particular challenges for both food security and environmental sustainability. Consequently, the development and adoption of alternative protein sources for direct human consumption will be essential (*Henchion et al*., 2017).

Microalgae are regarded as one of the most promising alternative protein sources for future. The term microalgae are not based on phylogenetic classification, but it refers to a heterogenous group of unicellular photosynthetic microorganisms, both eukaryotic and prokaryotic (*Barros de Medeiros et al*., 2022). Microalgae are a sustainable alternative protein source and are also characterized by high

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contents of essential fatty acids and vitamins. Furthermore, due to their abundance of bioactive compounds, they are increasingly recognized as valuable ingredients in the formulation of functional foods (*Hernández et al*., 2022).

Dairy products are widely consumed across the globe and are an important source of essential nutrients. The incorporation of microalgae into dairy matrices has been proposed as a strategy to enhance their nutritional profile by providing additional proteins, essential fatty acids, vitamins, and antioxidants, thereby contributing to the development of more sustainable and health-promoting products (*Hernández et al*., 2022).

The aim of this manuscript is to provide a concise overview of dairy product formulations incorporating microalgae, with an emphasis on future prospects and associated challenges.

2. Microalgae composition

According to *AlgaeBase*, the leading global database of algal taxonomy, approximately 50,000 microalgal species have been described to date (*algaebase.org*). Nevertheless, only a limited number of these species are used in food production, with *Arthrospira* (*Spirulina*) *platensis* and *Chlorella vulgaris* being the predominant species applied in the dairy industry (*Hernández et al*., 2022).

Microalgae are a rich source of protein, which can be as high as 70% of dry biomass (*Lucakova et al*, 2022). The protein content of *Arthrospira platensis* is in the range 55-70% per dry matter and 42-55% in *Chlorella vulgaris* (*Barros de Medeiros et al*., 2022). Both species produce high-quality proteins containing all essential amino acids (*Lucakova et al*., 2022). Although they are deficient in sulphur-containing amino-acids, the amino acid profiles of these microalgae are comparable to the conventional protein sources, egg and soybean (*Becker*, 2007). The main obstacle for using microalgae as a protein source is the presence of a cellulosic cell wall, which is indigestible in the human gastrointestinal tract (*Fasolin et al*., 2019). This limitation can be addressed through various cell disruption techniques, including enzymatic hydrolysis, microwave treatment, pulsed electric fields, high-pressure processing, ultrasonication, osmotic shock, and chemical methods (*Barros de Medeiros et al*., 2022).

The lipid content in microalgae is usually in the range 20-50% per dry matter biomass, and is influenced by the metabolic status of the cells and cultivation conditions (*Barros de Medeiros et al*., 2022). Microalgae are a source of polyunsaturated fatty acids (PUFAs) ω-3 and ω-6 fatty acids, and sterols (*Randhir et al*., 2020). *C. vulgaris* is a source of α-linolenic acid (ALA, C18:3 n-3) and linoleic acid (LA, C18:2 n-6) (*Hernández et al*., 2022), whereas *A. platensis* is particularly rich in ω-6 fatty acids, with γ-linolenic acid (GLA, C18:3 n-6) being predominant (*Senila et al*., 2025). Other microalgal species, including *Nannochloropsis gaditana, Nannochloropsis oculata, Pavlova lutheri, Phaeodactylum tricornutum*, and *Tetradesmus pseudonana*, are valuable sources of eicosapentaenoic acid (EPA, C20:5 n-3), while *Pavlova lutheri* is recognized for its high content of docosahexaenoic acid (DHA, C22:6 n-3) (*Lucakova et al*., 2022). PUFAs produced by microalgae are of special interest, since they contain lower levels of contaminants (dioxins, polychlorinated biphenyls, methyl mercury) compared to fish oils (*Barros de Medeiros et al*., 2022).

Carbohydrates, which make up to 20% of the dry biomass, are usually accumulated in microalgae in the form of starch or other polysaccharides, such as glycogen, β-glucan etc, depending on the species. Exopolysaccharides are of special interest due to their beneficial health effects, such as antioxidant capacity and antitumor effects (*Barros de Medeiros et al*., 2022; Lucakova et al., 2022). Moreover, some of the carbohydrates produced by microalgae can serve as prebiotics (*Eltanahy et al*., 2021).

3. Microalgae in dairy products

Microalgae can be incorporated into dairy products, especially fermented milk, ice creams and cheeses in order to increase protein content and deliver bioactive compounds (*Behesthipour et al*., 2013). The incorporation into fermented dairy products can be done either before fermentation, directly into the milk or after fermentation into the finished product (*Hernández et al*., 2022). The approach where microalgae are added before fermentation process is preferred, since their interaction with lactic acid bacteria has a pivotal role in the product quality (*Albuquerque et al*., 2024). Yogurt was the most extensively studied fermented dairy product, in which microalgae incorporation has been shown to improve the nutritional profile, particularly by increasing the protein content (*Luwidharto et al*., 2025). *A. platensis* was most frequently used in the studies, typically at concentrations not exceeding 1% (*Barkallah et al*., 2017; *Bchir et al*., 2019;

Silva et al., 2019; *Atallah et al*., 2020; *Khaledabad et al*., 2020).

The addition of microalgae to yogurt can affect its functional and technological properties. Bioactive peptides formed during fermentation, together with carotenoids, chlorophylls, phycocyanin, and other pigments, as well as phenolic compounds originating from microalgae, contribute to enhanced antioxidant activity (*Albuquerque et al*., 2024). Multiple studies (*Barkallah et al*., 2017; *Atallah et al*., 2020; *Khaledabad et al.*, 2020; *Mesbah et al*., 2022; *Nazir et al.*, 2022) have reported a significant increase in antioxidant capacity when *A. platensis* is added to yogurt. However, antioxidants can change stability during processing and refrigerated storage, highlighting the need for optimized formulation strategies to preserve antioxidant potential throughout the product's shelf life (*Albuquerque et al*., 2024). The addition of microalgae to fermented dairy products can promote the growth and survival of starter cultures and probiotic bacteria, owing to the bioavailability of trace elements, vitamins, and growth-promoting bioactive compounds (*Caporgno and Mathys*, 2018). Studies by de *Mocanu et al.* (2013), *Khaledabad et al.* (2020), and *Luwidharto et al.* (2022) demonstrated that *A. platensis* addition leads to higher viable counts of lactic acid bacteria in fermented dairy products, potentially enhancing their functional properties.

However, the development of yogurt-based product with microalgae comes with the challenges, especially regarding textural and sensory properties. Interactions between lipids from microalgae and milk can affect emulsion creation and the structure of yogurt with regard to the texture and stability of the final products (*Albuquerque et al*., 2024). Interactions between proteins from microalgae and milk proteins should be also taken into consideration, since they can form protein complexes which affect yogurt viscosity. Microalgal fortification enhanced the viscosity of yogurt, which is attributed to protein and exopolysaccharide contents (*Bchir et al*., 2019; *Luwidharto et al*., 2022). However, the incorporation of microalgae into yogurt affects not only its nutritional value but also key sensory attributes, particularly flavour and colour, which strongly influence consumer acceptance. Unique aroma and taste notes could challenge acceptance in markets with a preference for traditional flavours (*Zarrin et al*., 2014; *Silva and Pandolfi*, 2020). As such, the impact that microalgae can have on sensory properties can pose both as an opportunity for innovation and obstacle to broader market appeal.

Cheese is a highly versatile category of dairy product, encompassing a wide variety of types that differ in technological processes, sensory characteristics, physicochemical properties, etc. Most studies exploring the incorporation of microalgae have focused on soft white-brined cheeses, quark, and cheese spreads (*Garofalo et al.,* 2022). The microalgal species most commonly investigated for incorporation into cheese are *A. platensis* and *C. vulgaris*. Regarding cheese analogue composition, the addition of algae has the most profound impact on protein and fat content (*Agustini et al*., 2016; *Darwish*, 2017; *Golkmani et al*., 2019; *Bosnea et al*., 2020). The antioxidant activity was also improved also by the addition of microalgae to cheese, mainly due to the higher levels of phenolic compounds, flavonoids, β-carotene, etc. (*Darwish*, 2017; *Ismail et al*., 2023; *Lousada Falcão et al*., 2023).

The addition of microalgae had effects on the starter culture and probiotic growth in cheese. Studies by *Mazinani et al.* (2016) and *Golkmani et al.* (2019) demonstrated that the inclusion of *A. platensis* can enhance bacterial growth and viability, even during prolonged storage. Microalgae addition can also markedly influence cheese texture, one of its most critical quality attributes. This effect is often linked to the water-binding capacity of microalgal proteins and exopolysaccharides (*Luwidharto et al*., 2025). Reported textural changes differ by cheese type. *Golkmani et al.* (2019) observed reduced hardness in Feta-type cheese, whereas *Darwish* (2017) found increased hardness, cohesiveness, gumminess, and chewiness in Karaish cheese. In the study by *Falcão et al.* (2023), cream cheese and quark with added *C. vulgaris* showed decreased firmness and softer texture than the control. Regarding the sensory attributes, a bitter taste is one of the biggest challenges to overcome when microalgae are added (*Golkmani et al*., 2019; *Bosnea et al*., 2020). It was reported that the bitter taste was more pronounced with the increasing level of microalgae and increased storage time (*Golkmani et al*., 2019).

Ice cream is a complex dairy product composed of milk, sweeteners, stabilizers, emulsifiers, flavours, and colourants (*Durmaz et al*., 2020). Microalgae, rich in natural pigments and bioactive compounds, present a promising alternative to food colourants, which are perceived as "unhealthy", and certain stabilizing agents in ice cream formulations (Hernández

et al., 2022). Among various microalgae species, *A. platensis* has been the most extensively studied for ice cream fortification (*Malik et al*., 2012; *Agustini et al*., 2016; *Balensiefer et al*., 2021). Fortified products have demonstrated compositional changes, including increased protein and fat content. Fortified ice creams also showed changes in other important parameters, such as prolonged melting time, and higher overrun due to enhanced air incorporation (*Malik et al*., 2012; *Agustini et al*., 2016). Rheological properties were also influenced; for instance, a reduction in viscosity was observed *(Agustini et al*., 2016), attributed to the lower water absorption capacity of *A. platensis* compared to conventional stabilizers (*Robertson*, 2014). The addition of microalgae had impact also on sensory properties, since excessive supplementation intensified bitterness (*Durmaz et al*., 2020).

4. Conclusion

The incorporation of microalgae into dairy products can significantly enhance their nutritional and functional properties, most notably through increased protein content, enrichment with essential fatty acids, vitamins, minerals, and a boost in antioxidant capacity. Additionally, microalgae contribute bioactive compounds, such as phycocyanin and carotenoids, which could offer health-promoting effects. However, their application also introduces technological and sensory challenges. Fortification often alters rheological characteristics and product stability, while sensory attributes can be negatively affected with the development of increased bitterness, herbaceous and marine-like aromas, which can limit consumer acceptance. These drawbacks can be mitigated through careful selection of microalgal species, optimization of cultivation and processing conditions, and adjustment of inclusion levels to balance functionality with sensory appeal. Future research should focus on species screening, microalgae pre-treatment (e.g., cell disruption, deodorization), and integration with flavour-masking strategies to develop dairy products that are both functionally enhanced and organoleptically acceptable to a broad consumer base.

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Original Scientific Paper

Circular economy approaches in the dairy industry: strategies for waste utilisation and sustainable production

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ABSTRACT

The dairy industry is a major part of the global agri-food system, associated with food production, but also with industrial waste and emissions. Significant amounts of whey, sludge, wastewater, and packaging are generated by the dairy chain. Effluents from dairy processing contain high loads of organic matter, suspended solids, fats, and nutrients. Circular economy principles offer the dairy sector an approach to transition from a linear to a regenerative model by transforming waste into value-added resources. Circular economy strategies applied in the dairy industry, including waste valorisation, energy recovery, nutrient recycling, and the use of biodegradable and recyclable packaging, can substantially improve the environmental performance of dairy systems, and contribute to sustainable development. The present work highlights the potential of circular economy access to transform dairy waste streams into valuable resources, contributing to environmental sustainability and economic resilience.

1. Introduction

The dairy sector plays an important role in ensuring food security by providing milk and dairy products. However, this comes with significant environmental challenges, such as greenhouse gas emissions, high water consumption, and large volumes of organic waste (*Milani et al.*, 2011; *Wang and Serventi*, 2019; *Olusegun*, 2024; *Mwirigi et al.*, 2025).

The circular economy is a regenerative model designed to keep materials in use for as long as possible, extract maximum value from resources, and reduce impact on the environment (*Geissdoerfer et al.*, 2017). In the context of the dairy industry, this involves optimising resource use, valorising waste and co-products, minimising waste, and designing processes that support closed-loop systems (*Adesra et al.*, 2021). Given the growing regulatory, market and consumer pressures, the shift to sustainable production in the dairy sector has become essential. In this context, applying circular economy principles offers a valuable approach for transforming dairy industry waste into useful resources.

2. Dairy industry waste: sources and types

Dairy processing produces diverse by-products and waste. The volume, concentration and composition of dairy wastes varies depending on the scale and nature of operations, and it can be classified into two types: wastewater, i.e., effluent and solid waste (*Adesra et al.*, 2021).

Wastewater in the dairy industry originates from different processing stages: from milk production on farm, milk receipt at the dairy, to processing

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and product packaging (*Watkins and Nash*, 2014). Approximately one to three litres of wastewater are produced for every litre of milk produced (*Kushwaha et al*., 2011). This effluent is often characterised by a high organic load. The organic load of the effluent is monitored by biological oxygen demand (BOD) and chemical oxygen demand (COD) in the range of 0.1-100 $kg.m^{-3}$ with an index of biodegradability (BOD5/COD) typically in the range 0.4-0.8. Organic matter content is mainly due to the presence of carbohydrates (lactose) and proteins derived from milk. Additionally, fat content (0.1-10.6 $kg.m^{-3}$) suspended solids (0.1-22 $kg.m^{-3}$) and nutrients (N and P) also contribute to the contamination levels. Hygiene requirements in milk processing facilities and frequent cleaning result in effluents containing detergents and other cleaning agents (*Prazeres et al*., 2012; *Slavov*, 2017). Without adequate treatment, the discharge of these effluents into natural water bodies can lead to eutrophication and degradation of aquatic ecosystems (*Prazeres et al*., 2012). As such, effective wastewater management is a cornerstone of sustainable dairy operations (*Chokshi et al*., 2016).

In addition to effluents, the dairy industry generates substantial amounts of solid waste, such as treatment sludge, residual solids from milk processing, and non-biodegradable packaging. Sludge is generally classified into two categories: chemical sludge and biological sludge. It contains both biodegradable organic matter and non-biodegradable solids (*Kwapinska et al*., 2019), and such, can be valuable but also potentially hazardous when improperly managed (*Frąc et al*., 2017). Sludge properties depend on the specific treatment technology applied (aerobic or anaerobic) and on the chemicals used in physicochemical processes. The quantity of sludge produced is directly proportional to wastewater volume (*Adesra et al*., 2021). Packaging waste, mainly plastics, contributes to marine and terrestrial pollution unless recycled (*Esposito et al*., 2020). Untreated disposal of dairy processing wastes through landfilling or incineration can lead to long-term environmental hazards, such as methane emissions and leachate pollution. Implementing proper segregation and recycling mechanisms, such as diverting organic waste, can significantly mitigate these impacts (*La Pera et al*., 2023; *Scharff et al*., 2023; *U.S. Environmental Protection Agency*, 2025).

3. By-products of dairy industry

The main by-products of the dairy industry, whey and buttermilk, were traditionally considered as waste, but are now being recognised for their rich nutritional composition and potential applications. Whey, by-product of cheese production, is rich in lactose, proteins, and minerals. For every kilogram of cheese produced, up to 9 litres of whey is generated (*Barukčić et al*., 2019; *Lappa et al*., 2019). Buttermilk is a by-product obtained during the churning of cream into butter, and it contains lactose, proteins, vitamins, and minerals, as well as bioactive compounds, such as probiotics and peptides. Due its rich nutritional content and functional properties, buttermilk is increasingly being valued not only as beverage but also as an ingredient in food formulations for improving texture, flavour, and shelf life (*Shah*, 2000; *Hameed et al*., 2023). These by-products offer opportunities for value addition when appropriately processed and integrated into food, feed, or other industrial applications (*Prazeres et al*., 2012).

4. Circular economy strategies in the dairy industry and waste valorisation pathways

Circular economy implementation in the dairy industry involves a range of strategies which aim to convert waste into valuable resources. Valorisation is a core principle of the circular economy, focusing on closing material loops, minimising environmental impact, and maximising resource efficiency (*Olusegun*, 2024; *Udourioh et al*., 2025).

Whey and buttermilk, once considered low-value by-products, have been successfully transformed into valuable ingredients in the dairy industry. Whey proteins, rich in essential amino acids and bioactive peptides, are isolated using membrane filtration technologies and incorporated into high-value products, such as sports nutrition supplements, infant formulas, and functional foods, due to their excellent nutritional and functional properties. This valorisation not only improves economic returns but also supports sustainability by reducing waste (*Smithers*, 2008). Buttermilk, containing milk fat globule membrane components, proteins, and bioactive lipids, has gained attention for its emulsifying properties and potential health benefits. It is increasingly used in beverages, bakery products, and nutritional formulations, contributing to product innovation and enhanced nutritional profiles. Together, these examples illustrate the dairy industry's shift

towards circular economy principles by converting traditional low-value streams into high-value functional ingredients (*Smithers*, 2008).

Dairy sludge can be treated through anaerobic digestion to produce biogas (typically CH_4 and CO_2). This renewable energy source can fulfil the facility's energy demand, while the nutrient-rich digestate can be used as an organic, nutrient-rich biofertiliser (*Scott and Blanchard*, 2021; *Duan et al.*, 2025). Anaerobic digestion contributes not only to waste reduction but also to lessening climate change stimulation (*Massé and Massé*, 2010).

Membrane-based separations, such as microfiltration, ultrafiltration, nanofiltration and reverse osmosis, are effective for selectively separating and concentrating valuable molecules while enabling the treatment and reuse of dairy process water. These techniques facilitate the recovery of components like proteins and lactose, contribute to reducing waste volumes, and enhance resource efficiency by closing production loops within the dairy industry (*Luo et al*., 2011; *Reig et al.*, 2021; *Ahmad et al.*, 2022).

An emerging valorisation pathway involves converting lactose-rich streams, such as those from cheese whey, into bioplastics. Through microbial fermentation processes, lactose-containing residues can produce polymers like polylactic acid (PLA) and polyhydroxyalkanoates (PHAs), which are biodegradable and suitable for use in food packaging. For example, *Bacillus megaterium* has been shown to produce polyhydroxybutyrate (PHB), a type of PHA, from whey (*Obruca et al*., 2011). Biorefinery approaches also propose the use of whey for PHA production, providing a more environmentally acceptable alternative to conventional plastics (*Asunis et al*., 2020). Microbial processes converting deproteinised dairy streams into biohydrogen and PHAs demonstrated high yields of biopolymer (~62%) illustrating both sustainability and circular economy potential (*Colombo et al*., 2019).

Biodegradable packaging films composed of proteins, polysaccharides and microbial cellulose represent a promising shift towards sustainable materials with decreased environmental impact. Whey protein-based films have excellent oxygen barrier and good mechanical properties, degrade naturally, and can be formulated to include active compounds, such as antimicrobial and antioxidant agents. Recent advancement includes multilayer structures integrating whey-based layers designed for strength and shelf life without compromising environmental performance (*Potrč et al*., 2020; *Reichert et al*., 2020; *Bugnicourt et al*., 2021; *Kandasamy et al*., 2021; *Singh et al*., 2025). Despite these benefits, biodegradable and compostable packaging materials face challenges, such as higher production costs than their fossil fuel-based counterparts, limited shelf-life stability, and consumer adaption (*Peelman et al*., 2013).

The application of circular economy principles in the dairy industry has unlocked new revenue streams by converting low-value or waste materials into marketable products. Whey valorisation, for instance, contributes to economic growth by enabling the recovery of functional ingredients and supporting sustainability within dairy operations (*Țița et al.*, 2024). Investments in renewable energy technologies, such as anaerobic digestion of whey, which generates biogas to provide energy and reduces carbon emissions, offer long-term cost savings and energy autonomy for dairy facilities (*Pires et al.,* 2021).

5. Conclusion

The Circular economy is a more sustainable pathway for dairy industry, aligning economic profitability with positive environmental impact. By implementing circular economy principles, dairy producers can reduce their ecological footprints, enhance resource sustainability, and build more resilient supply chains. Realising the full potential of these principles in the dairy sector will require investment in advanced technologies, as well as strong stakeholder engagement and policy support.

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Review Paper

Hygiene indicators in a milk processing plant – a review

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ABSTRACT

This review paper focuses on the current microbiological parameters employed to evaluate the hygiene of raw milk and dairy products within processing plants. Key indicators of raw milk hygiene and overall quality include the total bacterial count (TBC) and somatic cell count (SCC). As the primary raw material in dairies for producing drinking milk and various dairy products, milk offers an ideal environment for the proliferation and spread of numerous microorganisms. In the dairy industry worldwide, contamination after pasteurization is commonly detected using indicator groups such as coliform bacteria, enterobacteria, total gram-negative bacteria, *Pseudomonas*, and gram-positive spore-forming bacteria. Enterobacteriaceae serve as hygiene indicators because they encompass a broad range of bacteria, including harmful species like *Salmonella* and *Klebsiella pneumoniae*. However, the standard coliform test depends on lactose fermentation, and since not all harmful enterobacteria—such as *Salmonella*, *Shigella*, or *Yersinia*—can ferment lactose, they may go undetected. Contamination of final dairy products can cause outbreaks of foodborne illnesses and incur additional expenses due to product recalls. Therefore, it is advisable to monitor hygiene indicators throughout milk production and processing to evaluate the effectiveness of the production process and decrease the necessity for repeated testing of final products.

1. Introduction

Milk, being the primary raw material in dairy plants for producing drinking milk and various dairy products, provides an ideal environment for the growth and proliferation of diverse microorganisms. The multiplication of these microorganisms can lead to food contamination and subsequently cause foodborne illnesses in consumers (*Lakticova et al.,* 2020). Data from the United States Foodborne Disease Surveillance System indicate that, between 2009 and 2015, dairy products ranked as the second most frequent source of outbreaks, following fish (*Dewey-Mattia*, 2018). Prompt and systematic monitoring of food quality, particularly milk quality, is indispensable for ensuring hygiene, safety, and public health throughout the entire food supply chain. The microbiological data obtained through such surveillance plays a pivotal role in validating food safety management systems and mitigating the incidence of product recalls and associated economic losses (*Kadyan et al*., 2020; *Poghossian et al.,* 2019).

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Milk contains all the essential amino acids needed by the human body (*Ahmed et al.,* 2019), but in its raw form, it also carries a variety of microorganisms. Some of these can be harmful and cause foodborne illnesses, while others, though not dangerous, can lead to spoilage (*Souad et al.,* 2021; *Merwan Ahmedsham et al*., 2018). To make milk safer and extend its shelf life, it undergoes heat treatment—pasteurization. This process kills or deactivates 99.99% of both harmful and harmless microorganisms (*Golić et al.,* 2019), including all vegetative forms of bacteria, psychotropic microorganisms, yeasts, molds, and certain unwanted enzymes, all while maintaining the milk's nutritional quality. Despite pasteurization, milk can still become contaminated. This can happen due to contact with unclean processing equipment, improper handling by workers, contaminated packaging, or if the pasteurization process itself is not performed correctly (*Postoli and Shehu*, 2018).

Maintaining proper hygiene is essential in producing safe and high-quality milk and dairy products, as it helps reduce microbial contamination. One way to assess cleanliness and safety is through the use of indicator organisms. These are specific microbes whose presence signals the overall sanitary condition of the food or processing environment. They can reveal contamination that may have occurred after cleaning procedures, during handling, or during storage. Additionally, these indicators can suggest the possible presence of harmful pathogens that could pose a risk to public health. In the global dairy industry, common groups of indicator bacteria used to detect post-pasteurization contamination include coliforms, Enterobacteria, total Gram-negative bacteria, *Pseudomonas*, and Gram-positive spore-forming bacteria (*Hervert et al.,* 2016). These groups help monitor cleanliness and ensure that safety standards are being met.

This review will discuss the current microbiological parameters used to assess the hygiene of raw milk and dairy products in the processing plant.

2. Milk production hygiene

Advanced milking automation is increasingly replacing manual methods on leading dairy farms. Nonetheless, proper hygiene training remains essential for all personnel, as contamination primarily stems from milking equipment and human contact. The total bacterial count (TBC) and somatic cell count (SCC) are key indicators of raw milk hygiene and overall quality (*Rodrigues et al.,* 2017). These are directly influenced by factors such as barn cleanliness, milking techniques, udder hygiene before, during, and after milking, equipment sanitation, and prompt cooling of milk post-milking. High TBC and SCC levels—common in milk from many dairy farms in Serbia—signal breakdowns in hygiene practices before and during milking. Literature data indicate that enhancing corrective and preventive hygiene measures related to farm conditions, milking procedures, and immediate milk cooling after milking leads to significant and continuous improvements in milk quality. This is reflected in improved TBC and SCC results. At the beginning of a study in Serbia, only 19.7% of milk samples met first-class quality standards, whereas by the end, this number had increased to 50.0%, clearly demonstrating the effectiveness of the implemented measures (*Mihajlović et al.,* 2022). These tests (TBC and SCC) mostly reflect on-farm hygiene rather than the final quality of dairy products.

However, two main groups of bacteria in raw milk can affect product quality: psychrophilic and psychrotolerant bacteria (like *Pseudomonas*), spore-forming bacteria (like *Paenibacillus* and *Clostridium*). If raw milk is poorly refrigerated or stored too long, these bacteria can multiply and produce enzymes that survive pasteurization. These enzymes can continue breaking down milk components, causing off-flavors, aroma issues, and texture problems in dairy products. For example, high bacterial counts in raw milk can reduce cheese yield and cause defects during aging. In UHT products, leftover enzymes can lead to age gelation and spoilage during long-term storage, with signs like thickening, sediment, and bad odors (*Martin et al.*, 2023; *Murphy et al.,* 2016).

Spore-forming bacteria are another major group of microorganisms in raw milk that significantly impact the quality of finished dairy products. These bacteria, from the Bacillales and Clostridiales orders, form endospores that originate in the farm environment, make their way into raw milk, survive processing, and later grow, causing spoilage (*Gopal et al.,* 2015). Psychrotolerant spore-formers, which can grow at refrigeration temperatures, are a particular concern for liquid milk spoilage. Research shows they are responsible for 40–50% of the cases where milk in the U.S. reaches the 20,000 CFU/mL limit set by the Pasteurized Milk Ordinance during its shelf life. These bacteria are considered the main contributors to the limited shelf life of liquid milk (*Alles et al.,*2018; *Reichler et al.,* 2018).

Addressing these key microbial contaminants in raw milk is essential for improving the quality of dairy products. It is a critical focus for the entire dairy supply chain, because consumer dissatisfaction with product quality directly impacts purchasing behavior and long-term consumption.

3. Hygiene indicators in dairy products

In developing countries, over 20% of milk production is lost due to early spoilage and microbial contamination occurring at different points throughout the production process (*Fox et al.,* 2017). Some of this loss may result from dairy products spoiling too early, often because of poor sanitation or contamination after pasteurization during processing. Bacteria such as coliforms, Enterobacteriaceae, and other Gram-negative organisms are common indicators of these hygiene issues in dairy products around the world (*Hervert et al.,* 2016). The use of Enterobacteriaceae as an indicator of hygiene covers a wide range of bacteria, including harmful species, such as *Salmonella* and *Klebsiella pneumoniae* (*Hervert et al.,*2017). The detection of these bacteria in milk suggests that safety protocols may have been followed during processing, but that subsequent handling of the milk was likely poor. Common sources of contamination with these bacteria include human or animal feces, water, equipment, and poor personal hygiene (*Yilma and Faye,* 2006). When equipment and facilities are not properly designed for cleanliness, they can have hidden areas where bacteria like *Salmonella* can survive and thrive, even after cleaning. These bacteria do not always appear in predictable patterns—they can come from many different sources. They can also stick to surfaces and form protective layers (called biofilms), making them harder to remove and increasing the risk that the food will be contaminated (*Hervert et al.*, 2017). Additionally, pests like rodents and insects can carry *Salmonella* and spread it further.

The choice of which hygiene indicator to use depends on the objective. For routine monitoring during production, it is not very important whether Enterobacteriaceae or coliform counts are used. However, coliforms are easier to cultivate and identify, making them more practical when the goals are to determine which species of bacteria are present and to assess their potential risks to consumers. On the other hand, Enterobacteriaceae are more suitable for assessing overall food hygiene and safety (*Souad et al.,* 2021).

Dairy products, like yogurt, can carry Enterobacteriaceae. That is why it is essential to follow strict hygiene and manufacturing practices at every step of the production process. Just because common foodborne pathogens are not found in yogurt does not necessarily mean it is safe, as other harmful bacteria from the Enterobacteriaceae family could still be present. Monitoring for Enterobacteriaceae helps assess whether the preventive hygiene measures in place are effective. This kind of testing offers valuable information about the cleanliness, quality, and safety of yogurt products and helps ensure that sanitation procedures in the production facility are working properly (*Knezevic et al.,* 2021). That study found that 21.29% of yogurt samples contained more than 10 CFU/g of Enterobacteriaceae, indicating problems such as poor hygiene, processing problems, or post-production contamination (*Knezevic et al.,* 2021).

Coliforms are Gram-negative, rod-shaped bacteria, able to survive with or without oxygen. They can ferment lactose to produce gas and acid (*Feng et al.,* 2002). For a long time, coliform bacteria counts have been used to check the hygiene of dairy products. For example, studies in the U.S. from 2001 to 2010 found that 7.6–26.6% of liquid milk samples were contaminated with coliforms after production (*Martin et al.,* 2012). In a study conducted in Cameroon (2012–2013), most yogurt samples had high levels of coliforms—over 10^2 CFU/mL. From these samples, 21 different species of Enterobacteriaceae were found among the 72 bacterial isolates originally identified as coliforms. The standard test for coliform bacteria relies on lactose fermentation, but not all harmful Enterobacteriaceae—such as *Salmonellae, Shigella*, or *Yersinia*—can ferment lactose, so they would not be detected. Replacing lactose with glucose in the test would help detect a wider range of these bacteria, including harmful ones. Because Enterobacteriaceae are more resistant to harsh conditions than coliform bacteria, they can serve as more reliable indicators of hygiene and sanitation in food production (*Knezevic et al.,* 2021).

Research shows that raw milk typically contains low levels of coliform bacteria and/or *E. coli*. However, their presence in raw milk does not necessarily predict their levels in final cheese products. This is because the cheesemaking and ripening processes can greatly influence the growth and survival of these bacteria. Various factors—such as the type of starter cultures, salting methods, aging conditions, and ripening duration—affect the final composition, texture, and bacterial content of the cheese.

Since there are hundreds of different raw milk cheeses with differing characteristics, it is difficult to generalize the number of indicator bacteria present in all of them. Usually, an initial increase in bacterial numbers occurs during early ripening, followed by a decline as the cheese matures. This early increase may be due to bacteria multiplying or being trapped in the curd during production (*Metz et al.,* 2020). Research shows that *E. coli* and other indicator bacteria are commonly found in raw milk, typically at low levels—often below 100 CFU/g, and under 10 CFU/g in high-quality milk. During the early stages of cheese production, these bacteria can multiply, but their numbers generally decline significantly during aging and ripening, except in fresh, unripened cheeses. By the time the cheese is fully matured, indicator bacteria are usually either absent or present at very low levels—often below the limits (10–100 CFU/g) set by many countries. When high levels of indicator bacteria are found in cheese, the cause is usually poor-quality raw milk, unsanitary production conditions, or both. However, raw milk cheeses made from clean, high-quality milk and produced using good manufacturing practices typically do not have high levels of these bacteria (*Metz et al*., 2020).

4. Milk plant processing hygiene

Good hygiene practices (GHP) form the foundation of any effective food safety management system and ensure that the Hazard Analysis and Critical Control Points (HACCP) system operates effectively. GHP helps to prevent the introduction and spread of harmful microorganisms, minimize the risk of food contamination during processing, handling, and storage, ensure compliance with national and international food safety regulations, and maintain consumer trust by showing a commitment to producing safe and high-quality food (*Postoli and Shehu,* 2017).

Keeping food contact surfaces clean, a fundamental of GHP, is essential to prevent cross-contamination and maintain food safety. Inadequately cleaned surfaces can harbor microorganisms that reduce a product's shelf life, but also pathogens that pose serious health risks if transferred to food. The high incidence in Europe of outbreaks (around 39%) associated with cross-contamination highlights its importance as a food safety risk (*Whitehead and Verran,* 2006). To mitigate these risks, food safety management systems like HACCP, supported by prerequisite programs (PRPs), emphasize the necessity of effective cleaning and sanitation protocols.

However, the variability in cleaning outcomes across the food industry highlights the importance of systematically evaluating and adjusting cleaning schedules to ensure effectiveness. Manufacturers have a legal and ethical responsibility to maintain cleanrooms by designing and following targeted cleaning programs, especially for surfaces that come into direct contact with food. Microbiological tests, such as surface swabbing and agar contact plates, are commonly employed to detect bacterial contamination on food contact surfaces. Quantitative analyses determine the presence of indicator microorganisms on surfaces to assess overall hygiene levels, with high counts signaling inadequate cleaning or sanitation. These indicators, such as total bacterial counts and Enterobacteriaceae counts, help assess the potential risk of contamination during food production. Qualitative analyses, on the other hand, focus on detecting specific pathogens—such as *Salmonella* spp. or *Listeria monocytogenes*—and reveal whether these dangerous microorganisms are present or absent in the tested area, rather than how many are present. Together, these two data provide a comprehensive picture of both the overall hygiene status and specific safety threats in the production environment (*Postoli and Shehu*, 2017).

5. Conclusion

Relying solely on final product control to ensure food safety has its drawbacks. Contaminated food products can lead to outbreaks of foodborne illness and result in additional costs due to product recalls. Therefore, it is recommended to use hygiene indicators throughout the milk production and processing process, which help to assess how well the production process is functioning and reduce the need for repeated controls of finished products. By consistently applying and monitoring GHP, food businesses can reduce the risk of foodborne illnesses and product recalls, while also providing documented proof of their commitment to food safety and hygiene standards.

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Original Scientific Paper

Fatty acid composition of beef patties with replacement of beef fat by walnut oil and cake

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ABSTRACT

This study investigated the effects of replacing beef fat with walnut oil and walnut cake on the fatty acid composition and nutritional value of beef patties. Nine groups were prepared: four with differing levels of walnut oil, four with walnut cake, and one control without substitution. Fatty acid composition was determined by gas chromatography, and nutritional indices were calculated. Substitution with walnut derivatives reduced saturated fatty acids (SFA) and increased unsaturated fatty acids (UFA), including both monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA), with a marked increase in oleic acid (C18:1 n-9) and linoleic acid (C18:2 n-6). Beef patties composed with walnut oil showed a more favourable profile than those with cake, as reflected in lower atherogenic (AI) and thrombogenic indices (TI), and higher values of the hypocholesterolaemic/hypercholesterolaemic ratio (HH), nutritional value index (NVI), and health-promoting index (HPI). The best results were observed in beef patty BP4 (100% fat replacement with walnut oil), which had the highest UFA and PUFA/SFA ratio, and the lowest AI and TI values.

1. Introduction

Burger patties are semi-products made from minced and shaped meat, most commonly from beef and lamb, while pork is used less frequently (*Smajić,* 2014). According to the *Rulebook on minced meat, semi-products, and meat products (BiH,* 2013*)*, a burger patty is produced by shaping various types of minced fresh meat from domestic ruminants, with the possible addition of other ingredients, such as up to 10% minced onion. Together with ćevapi, pljeskavica is an essential part of local traditional cuisine in Bosnia and Herzegovina. Pljeskavica is considered an extension of the burger, as it contains more additions and is sometimes referred to as the "poor burger" (*Operta,* 2016).

Meat products such as patties and hamburgers have gained immense popularity and are considered symbols of the fast-food industry. When grilled, the patty develops a brown crust on the surface while retaining a juicy interior, which can be filled with ingredients such as cheese, ham and onions (*Stajić et al.,* 2024). These products typically contain significant amounts of animal fat, which is high in cholesterol and saturated fatty acids. A high intake of these components, along with a low ratio of polyunsaturated to saturated fatty acids (PUFA/SFA), is recognized

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as a risk factor for coronary heart disease (*Pleadin et al.,* 2016). Accordingly, producers increasingly seek to optimize the fatty acid profile of meat products to enhance their nutritional value and consumer acceptability (*Pleadin et al.,* 2015). One key strategy involves increasing the PUFA/SFA ratio, often by replacing animal fat with vegetable oils. Unlike animal fats, vegetable oils are cholesterol-free and naturally rich in unsaturated fatty acids. Numerous studies have investigated nutritional enhancement of meat products through the inclusion of various vegetable oils such as flaxseed, olive, sunflower, walnut, soybean, sesame, grape seed, avocado, and hemp oil. Improvements in the nutritional profile of beef patties have been reported through the replacement of animal fat with walnut oil (*Fadiloglu et al.,* 2023; *Botella-Martinez et al.,* 2021; *Lucas-Gonzalez et al.,* 2023), sesame oil (*Abdul Aali et al.,* 2021) and flaxseed oil (*Anwar et al.,* 2023). Walnut oil (*Juglans regia L.*) is especially valued for its health benefits and favourable fatty acid profile, being rich in linoleic, linolenic and oleic acids (*Saxena et al.,* 2009; *Hussain et al.,* 2021). Additionally, walnut oil is a source of carotenoids, fat-soluble vitamins, and essential minerals such as zinc, copper, iodine, calcium, magnesium, iron, phosphorus, and cobalt (*Popovici et al.,* 2014). It also contains secondary metabolites like tocopherols, flavones, and polyphenols, which have antioxidant, antidiabetic, anti-inflammatory, and anticancer effects (*Yang et al.,* 2022). Its consumption has been linked to improved lipoprotein profiles and a reduced risk of cardiovascular disease and certain cancers (*Rabrenović & Gajić-Krstajić,* 2010). The aim of this study was to evaluate the fatty acid composition of beef patties in which beef fat was partially or completely replaced by walnut oil and walnut cake.

2. Materials and methods

2.1 Production and sampling

Walnuts used for oil and cake production originated from the Gajzenhajm-139 variety grown in Bosnia and Herzegovina and were purchased locally. Walnut oil and cake were produced in the laboratory; after removing shells and impurities, the walnuts were pressed using a manual screw press (Piteba, Netherlands). The oil was filtered, and the cake was dried at room temperature. Beef muscle tissue (shoulder and neck) and adipose tissue were collected immediately after slaughter from the Malak farm (Kakanj, Bosnia and Herzegovina). Meat and fat were ground separately, both using a grinder attachment with a 3.8 mm hole diameter (Bosch, Germany). The ratio of meat to fat in the mixture was 80:20. Common food additives such as salt (2.0%), ground black pepper (0.10%), and baking soda (0.30%) were added, all sourced from a local store. Ground beef tissue was divided into nine parts corresponding to experimental groups with different levels of replacement of beef fat with walnut oil or cake. The sample codes and composition are as follows: BP CON – control with 100% beef fat; BP1, BP2, BP3 – 15%, 30% and 45% replacement with walnut oil; BP4 – 100% replacement with walnut oil; BP5, BP6, BP7 – 15%, 30% and 45% replacement with walnut cake; BP8 – 100% replacement with walnut cake. After adding the food additives, mixing and re-grinding to achieve homogeneity, the mixtures were cooled at 4°C for 24 hours. Subsequently, the appropriate walnut products were added according to the formula. Patties were formed into balls weighing 120-125 g and mechanically shaped (Maxima, Netherlands). Frying was performed on an induction cooktop (Easyline, Italy) with minimal use of sunflower oil until the internal temperature of the patties reached 78°C.

2.2 Methods

2.2.1 Determination of fatty acid composition

Methyl esters of fatty acids were prepared according to the standard method (*BAS EN ISO*, 2018), which involves dissolving glycerides in iso-octane and transesterification using a methanolic solution of potassium hydroxide. The fatty acid composition was determined by gas chromatography. The fat content in the analyzed samples of beef patties was determined using the reference method (*BAS ISO*, 2007), which involves fat extraction by the Soxhlet method using organic solvents. The extracted fat was then used for the determination of fatty acid composition. Approximately 60 mg of the extracted fat was weighed into a glass test tube with a glass stopper, followed by the addition of 4 mL of iso-octane. After the fat was dissolved, 200 μL of methanolic potassium hydroxide solution was added. The mixture was vigorously shaken twice for 30 seconds and then left to react at room temperature. Once the mixture clarified, a glycerol layer separated at the bottom of the test tube. To neutralize the mixture, 1 g of sodium hydrogen sulphate monohydrate was added. The solution was again shaken vigorously twice for 30 seconds. After the crystals settled, 500 μL of the

resulting solution was transferred into an injection vial, followed by the addition of 1 mL of iso-octane. The vial was then sealed and shaken. The fatty acid composition was determined using a Shimadzu QP2010 gas chromatograph with a mass spectrometer and a Supelco SP-2560 capillary column (100 m × 0.25 mm × 0.20 μm). Chromatographic conditions were as follows: injector and detector temperatures were 220°C, column temperature was 170°C, and the injection volume was 1 μL. Helium was used as the carrier gas at a flow rate of 0.9 mL/min. Individual fatty acids were identified by comparing their retention times with those of pure commercial standards. The content of fatty acids was expressed as a percentage of the total identified fatty acids.

2.2.2 Determination of nutritional indices

Based on the fatty acid composition, nutritional indices were calculated for the analyzed beef patties, including the atherogenic index (AI), thrombogenic index (TI), and the hypocholesterolaemic/hypercholesterolaemic index (HH). The nutritional indices were calculated according to *Guimarães et al., (2013)*.

2.2.3 Statistical data analysis

Statistical data analysis was performed using the software Past 3.15 (*Hammer et al., 2001*). Multivariate data analysis was conducted using principal component analysis (PCA). The principal components are intended to explain the variability of the data and visually present the relationships among the samples within the dataset.

3. Results and discussion

Fatty acid analysis (Table 1) showed that UFA predominated in all beef patties. The highest UFA level was found in BP4 (85.98%), where beef fat was fully replaced with walnut oil, consistent with its naturally high UFA content. The lowest UFA level was in BP CON (70.17%) due to the high SFA content in beef fat. Oil-based patties had more UFA than cake-based ones, reflecting the higher UFA concentration in pure oil compared to the cake, which also contains protein and fibre. Among the cake-based patties, BP8 had the highest UFA content (79.07%), and BP5 the lowest (71.70%), likely due to lower fat replacement. *Fadiloglu et al.,* (2023) reported 75.44% UFA in patties with 50% fat replacement using walnut oil, lower than all our oil-based patties (BP1–BP4), supporting the effectiveness of full replacement. Within UFA, MUFA were more prevalent than PUFA. BP4 had the highest MUFA content (59.95%), and BP CON the lowest (54.25%). Oil-based patties showed slightly higher MUFA than cake-based ones, indicating a stronger enrichment effect from direct oil addition. Oleic acid was the dominant MUFA, ranging from 56.90% (BP4) to 51.97% (BP CON), in line with previous studies. *Fadiloglu et al.,* (2023) reported much lower MUFA content (29.83% and 33.26%), further confirming the success of our fat replacement. The rise in oleic acid mirrored the proportion of walnut oil, which is naturally rich in this fatty acid. The PUFA level ranged from 15.92% (BP CON) to 26.03% (BP4), with linoleic acid as the most abundant. Oil-based patties had higher PUFA and linoleic acid levels than cake-based ones, due to greater availability of polyunsaturated acids in oil. Among cake-based patties, BP8 (100% replacement) had the highest linoleic acid (15.42%), and BP5 the lowest (13.34%), matching the fat replacement level. These trends align with earlier findings showing PUFA increases with higher plant-based ingredient proportions. *Fadiloglu et al.,* (2023) reported linoleic acid levels of 39.27% and 41.46% in their patties, likely higher than in ours due to differences in formulation and raw materials.

The SFA content was highest in BP CON (29.82%) and lowest in BP4 (13.96%), reflecting the fat type, with animal fat being naturally richer in SFA than vegetable oil. Cake-based patties (BP5-BP8) had intermediate SFA levels, lower than BP CON but higher than oil-based ones, due to residual oil and other matrix components in the cake. These results confirm the SFA-reducing effect of both walnut oil and cake, with oil showing a stronger impact. *Fadiloglu et al.,* (2023) reported SFA values of 24.56% and 31.05% in their patties, supporting the trend of reduced SFA with fat substitution. Palmitic acid was the main SFA, highest in BP CON (14.17%) and lowest in BP4 (7.87%), decreasing with greater walnut oil inclusion, consistent with previous findings on vegetable oils. Stearic acid followed the same trend, from 11.47% (BP CON) to 4.83% (BP4). These reductions align with studies showing lower SFA with increased plant-based fat. *Fadiloglu et al*., (2023) found comparable stearic acid levels (11.68%) at 50% walnut oil replacement in patties, further supporting our results. In our patties, PUFA/SFA ratios ranged from 0.53 to 1.86, all exceeding the recommended minimum of 0.40

(*UK Department of Health,* 1994; *Wood et al.,* 2004, 2008), indicating good nutritional quality. The highest ratio was in BP4 (100% walnut oil), supporting prior evidence of improved fatty acid profiles with plant-based fat substitution. Patties with walnut cake had lower PUFA/SFA ratios than oil-based ones, but still met nutritional standards. A similar pattern was seen in the ω-6/ω-3 ratio, which declined as walnut oil or cake increased. The lowest ratio was in BP4 (5.02), a significant drop from the control (22.76). This supports the nutritional benefit of plant-based fats, as recommended ratios range from 1:1 to 4:1 (*Simopoulos,* 2002, 2004; *Wood et al.,* 2004, 2008). Cardiovascular indices also improved with fat replacement. BP4 had the lowest AI (0.10) and TI (0.24). *Fadiloglu et al.,* (2023) reported similar improvements using walnut oil. Though cake-based patties had slightly higher AI and TI than oil-based

Table 1. Fatty acid composition of beef patties

FATTY ACIDS (%)	BP CON	BP 1	BP 2	BP 3	BP 4	BP 5	BP 6	BP 7	BP 8
C14:0	1.08	1.02	0.82	0.65	0.25	0.94	0.80	0.66	0.39
C15:0	0.67	0.58	0.41	0.39	0.25	0.54	0.53	0.40	0.26
C16:0	14.17	11.60	10.20	9.14	7.87	13.48	13.07	12.60	10.98
C17:0	1.35	1.02	0.82	0.65	0.51	1.35	1.07	0.66	0.39
C18:0	11.47	7.25	6.53	5.87	4.83	11.05	10.67	9.95	8.63
C20:0	1.08	0.73	0.54	0.39	0.25	0.94	0.80	0.66	0.26
C14:1	0.13	0.15	0.14	0.26	0.25	0.27	0.40	0.53	0.65
C16:1	1.21	0.58	0.82	1.04	1.14	1.21	1.33	1.59	1.96
C17:1	0.67	0.58	0.54	0.78	1.02	0.81	1.07	1.13	1.31
C18:1 n-9	51.97	56.84	54.67	55.48	56.90	52.43	52.67	53.32	55.03
C22:1 n-9	0.27	0.15	0.27	0.52	0.64	0.40	0.53	0.66	0.78
C18:2 n-6	13.23	15.95	18.36	18.41	19.30	13.34	13.47	13.93	15.42
C18:3 n-3	0.67	2.18	3.81	4.05	4.32	1.08	1.20	1.33	1.44
C20:4 n-6	2.02	1.45	2.04	2.35	2.41	2.16	2.40	2.52	2.48
SFA	29.82	22.20	19.32	17.09	13.96	28.30	26.94	24.93	20.91
UFA	70.17	77.88	80.65	82.69	85.98	71.70	73.07	75.07	79.07
MUFA	54.25	58.30	56.44	57.88	59.95	55.12	56.00	57.29	59.73
PUFA	15.92	19.58	24.21	24.81	26.03	16.58	17.07	17.78	19.34
ω-6	15.25	17.40	20.40	20.76	21.71	15.50	15.87	16.45	17.90
ω-3	0.67	2.18	3.81	4.05	4.32	1.08	1.20	1.33	1.44
PUFA/SFA	0.53	0.88	1.25	1.45	1.86	0.58	0.63	0.71	0.92
MUFA/SFA	1.82	2.63	2.92	3.39	4.29	1.95	2.08	2.30	2.86
ω-6/ω-3	22.76	7.98	5.35	5.13	5.02	14.35	13.22	12.37	12.43
AI	0.26	0.20	0.17	0.14	0.10	0.24	0.22	0.20	0.16
TI	0.73	0.45	0.35	0.30	0.24	0.66	0.62	0.57	0.46
HH	4.45	6.06	7.16	8.20	10.21	4.79	5.01	5.36	6.54
NVI	4.48	5.53	6.00	6.71	7.84	4.71	4.85	5.02	5.80
HPI	3.79	4.97	5.98	7.05	9.69	4.16	4.49	4.93	6.30

SFA – saturated fatty acids; UFA – unsaturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; AI – atherogenic index; TI – thrombogenic index; HH – hypocholesterol/hypercholesterol index; NVI – nutritional value index; HPI – health promoting index; BP CON – beef patty with 100% animal fat; BP1 – beef patty with 15% of animal fat replaced by walnut oil; BP2 – beef patty with 30% of animal fat replaced by walnut oil; BP3 – beef patty with 45% of animal fat replaced by walnut oil; BP4 – beef patty with 100% of animal fat replaced by walnut oil; BP5 – beef patty with 15% of animal fat replaced by walnut press cake; BP6 – beef patty with 30% of animal fat replaced by walnut press cake; BP7 – beef patty with 45% of animal fat replaced by walnut press cake; BP8 – beef patty with 100% of animal fat replaced by walnut press cake.

ones, they still outperformed the control, confirming benefits even with partial replacement. The HH ratio (4.45–10.21) and NVI (4.48–7.84) rose with increasing walnut oil or cake, with BP4 again showing the best values. This is consistent with literature on plant oil enrichment. The HPI, a key fat quality index, was also highest in BP4 (9.69) and lowest in BP CON (3.79), confirming a significantly improved lipid profile. Overall, BP4 showed the most favourable nutritional profile across all indices (AI, TI, HH, NVI, HPI), due to full replacement of animal fat with walnut oil, rich in unsaturated fatty acids, particularly ω-3 and ω-6. As a result, BP4 had the lowest atherogenic and thrombogenic potential and the highest values for health-promoting indices.

3.1 Principal component analysis (PCA) of the fatty acid composition of beef patties

Principal component analysis (PCA) was conducted based on a correlation matrix including 28 parameters for 9 groups of beef patties in which beef fat was partially or completely replaced with walnut oil and cake.

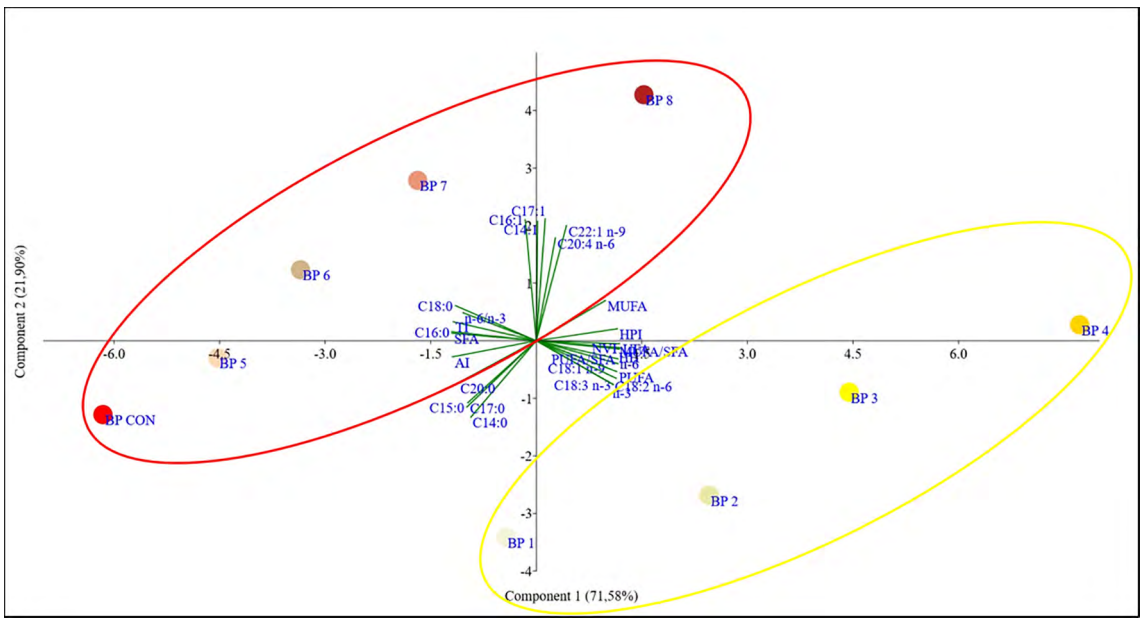

Figure 1. Distribution of acrylamide content category and potato cultivar in 2024

Figure 1 shows that PCA grouped the beef patties according to the type and level of beef fat replacement-walnut oil (yellow ellipsoid) or walnut cake (red ellipsoid). The control group (100% beef fat) was positioned on the left side of the plot and showed the highest levels of SFA, ω-6/ω-3 ratio, and of the nutritional indices, AI and TI, indicating the least favourable nutritional profile. Beef patties with increasing levels of walnut oil or cake shifted rightwards, associated with higher UFA, MUFA, and PUFA levels, and improved nutritional indices (HPI, NVI, HH). Based on the nutritional indices HPI, NVI, and HH, as well as the PUFA/SFA ratio, the patties with complete replacement of beef fat by walnut oil (BP4) were the most nutritionally acceptable among all examined beef patties. PCA clearly separated the least (BP CON) and most (BP4) favourable beef patties along the first principal component.

4. Conclusion

Fatty acid analysis of beef patties showed that UFA were dominant in all of them, with the highest proportion observed in BP4 (85.98%), where 100% of beef fat was replaced with walnut oil, while the control patties (BP CON) contained 70.17% UFAs. Fat replacement led to a decrease in SFA content, including C16:0 and C18:0, and a simultaneous increase in MUFA (including C18:1 n-9) and PUFA (including C18:2 n-6), with these changes being more pronounced in beef patties constituted with oil compared to those with cake. Consequently, significant improvements in nutritional indices were recorded in the BP4 beef patties, with an increase in the PUFA/SFA ratio, HH, NVI and HPI, and a decrease in AI and TI compared with other patty types. Beef patties containing walnut cake also showed positive changes, although to a lesser extent.

These results are preliminary data on the fatty acid composition and nutritional value of beef patties enriched with walnut oil and cake, contributing to the development of healthier meat products and the valorization of food industry by-products, thereby supporting sustainability in the food sector.

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Review Paper

Valorization of plant-based agro-industrial residues for recovery of bioactive peptide-rich fractions and their application in muscle foods

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ABSTRACT

Plant-derived protein hydrolysates are gaining increasing attention as sustainable sources of bioactive peptides with functional and health-promoting properties. This review highlights the potential of utilizing agro-industrial by-products as substrates for producing bioactive peptide-rich hydrolysates and explores their applicability in muscle-based food formulations. Enzymatic hydrolysis is the most commonly used method for obtaining these fractions, offering a green and efficient strategy for valorizing plant-based waste. Various studies have demonstrated that such hydrolysates can enhance bioactivities, improve techno-functional characteristics, and contribute to the overall quality and shelf life of meat products. Specifically, protein-rich residues from plant processing, such as oil cakes, brans, and other wastes, offer the dual benefit of supporting clean-label food formulations while reducing environmental impact through waste valorization. Overall, protein hydrolysates of these sources represent a promising class of natural ingredients for the reformulation of healthier and more sustainable meat products.

1. Introduction

Bioactive peptides (BPs) are defined as food components encoded within the primary structure of proteins, which, upon release, exert effects on biological processes and substrates, thereby providing beneficial impacts on body functions and overall health (*Bhat et al*., 2015; *Singh et al*., 2022). Bioactive peptides have been reported to exhibit a wide range of physiological effects, including antithrombotic, antihypertensive, antioxidant, antibacterial, immunomodulatory, anticancer, and cholesterol/ blood pressure-lowering properties.They have been shown to impact not only human health but also the sensory and functional quality of food products (*Toldrá et al*., 2020; *Du and Li*, 2022; *Singh et al*., 2022; *Karami and Akbari-Adergani*, 2019). Due to these positive impacts and their origin from highly biocompatible sources, BPs are considered promising compounds with potential applications in food, medicine, pharmaceuticals, cosmetics, and various other fields (*Akbarian et al*., 2022). There is a growing global interest in BPs derived from food proteins, with the market value estimated at USD 48.6 billion in 2020 and projected to reach USD 95.7 billion by 2028 (*Du and Li*, 2022). It is well established that various plant- and animal-based foods contain signif-

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icant amounts of BPs. In addition to meat- and milk-derived BPs, plant-based sources such as legumes, cereals, fruits, and seeds have also been reported as important reservoirs (*Singh et al*., 2022). Beyond their beneficial effects on health, plant-derived biopeptides are emphasized as more accessible, sustainable, and cost-effective sources compared to those of animal origin (*Akbarian et al*., 2022; *Singh et al*., 2022). Therefore, investigating the isolation of plant-based BPs from alternative sources and their application in food formulations represents a highly relevant and valuable research area.

The agri-food industry generates large volumes of by-products and waste at both on-farm and off-farm levels. Accounting for nearly 50% of total agricultural output, these wastes pose challenges in safe disposal and contribute significantly to environmental pollution (*Ben-Othman et al*., 2020). According to the United Nations Environment Programme (UNEP) Food Waste Index Report 2024, 19% of food available to consumers is wasted globally, totaling 1.05 billion tons per year across retail, food service, and households. More than half of it originates from households- equivalent to over one billion meals discarded each day worldwide. Another striking finding from the report is that approximately 60% of all food waste consists of plant-based food products, highlighting the critical need for valorizing plant-derived residues in sustainable food systems (*UNEP*, 2024). This context underscores the importance of developing value-added strategies for the utilization of plant-based food residues, particularly through the recovery of functional components such as protein hydrolysates.

Based on these insights, this review aims to highlight the potential use of plant-derived protein hydrolysates in muscle food formulations, with a specific emphasis on the by-products as sustainable sources of bioactive peptides.

2. Obtaining biopeptide-rich fractions from plant-sourced proteins

BPs can be derived from various plant sources such as legumes, seeds, nuts, cereals, and fruits through processes like enzymatic hydrolysis, fermentation, and other extraction techniques. Among these methods, enzymatic hydrolysis is the most commonly applied approach, as it enhances the nutritional and functional quality of protein sources. It is considered as safe and environmentally-friendly green technique due to the absence of residual organic solvents or toxic chemicals during the reaction process (*Angulo and Márquez*, 2023). In common applications of enzymatic hydrolysis, one or more proteases are employed to break down proteins into smaller peptides and free amino acids. The resulting fraction, known as the "hydrolysate", primarily contains dipeptides and tripeptides, which often exhibit enhanced bioactivities and functional properties compared to the parent protein chain (*Eckert et al*., 2019; *Zinina et al*., 2022). Figure 1 presents the main steps of the production of plant protein hydrolysates and bioactive peptides through enzymatic hydrolysis. The steps mainly cover the application of pre-treatments in the raw material, enzymatic hydrolysis application, protein separation and purification, assessment of bioactivity, identification of peptides in the sub-fractions, and verification of bioactivity after chemical peptide synthesis (*Ozturk-Kerimoglu et al.*, 2025). One major challenge in working with plant protein hydrolysates is the presence of impurities such as fibers and non-protein compounds, which may compromise peptide quality and safety; thus, effective protein purification is essential. Several techniques such as membrane filtration, gel permeation chromatography, ion-exchange chromatography, and reverse-phase high-performance liquid chromatography (HPLC)

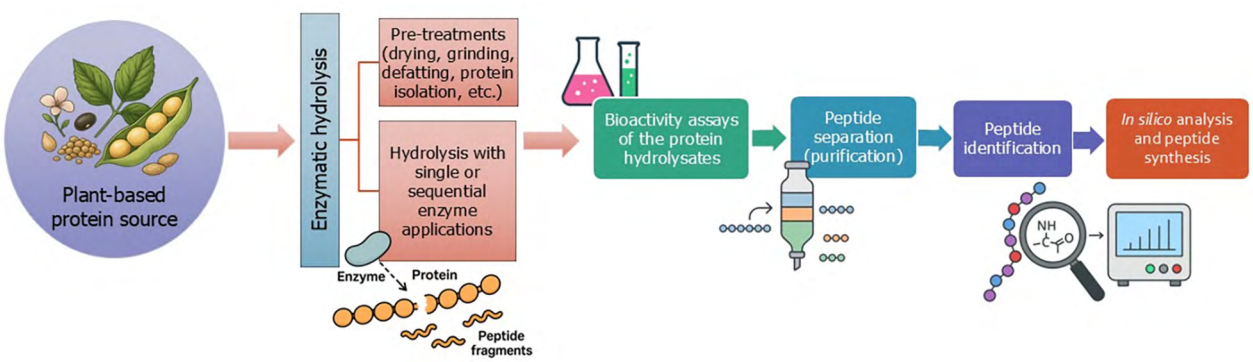

Figure 1. Key steps in obtaining plant-based protein hydrolysates and bioactive peptides through enzymatic hydrolysis

can be employed for the purification process, each offering distinct advantages depending on the properties of the hydrolysate and the specific goals of the process (*Kadam et al*., 2024).

2.1. Plant-derived by-products as potential sources of functional protein hydrolysates

As mentioned above, the processing of food raw materials such as fruits, vegetables, seeds, and nuts generates a substantial amount of valuable residual fractions. Valorizing these residues, particularly those with high protein potential, through various approaches and converting them into novel food ingredients is of critical importance for sustainability. In this context, several studies have focused on the utilization of certain plant-based production wastes by transforming them into protein hydrolysates. For example, *Zaky et al*. (2019) reported that the hydrolysate obtained from the protein concentrate of rice bran (a valuable by-product of rice) digested with Flavourzyme contains bioactive components which can act as natural antioxidants in food applications. *Ospina-Quiroga et al.* (2022) investigated the physical and oxidative stabilities of simple oil-in-water emulsions containing protein hydrolysates from olive seed, sunflower, rapeseed, and lupin meals. They found out that all the emulsions were physically stable, and the hydrolysates could be successful candidates to retard oxidation in food emulsions as natural antioxidants. Another study was carried out by *Ahmed et al*. (2023) on protein hydrolysates derived from orange peel using *Aspergillus niger* WA 2017 protease, where it was reported that exhibited strong antioxidant and antitumor activities, with optimized conditions significantly enhancing DPPH radical scavenging capacity and reducing Ehrlich Ascites Carcinoma cell viability by over 60% *in vitro*, while also increasing the life span of tumor-bearing mice *in vivo*. *Zeng et al*. (2025) applied a bacterial-enzymatic co-fermentation to palm kernel cake, and concluded that this application significantly improved the nutritional quality by reducing fiber, increasing protein solubility, and releasing sugars. This pretreatment also enhanced *in vitro* intestinal digestibility and beneficially modulated gut microbiota, highlighting its potential for broader application of this by-product in animal feed production. Collectively, these findings demonstrate the growing potential of plant-based by-products as valuable sources of protein hydrolysates with diverse functional and biological activities, supporting their integration into both food and feed systems as part of sustainable and health-oriented formulation strategies.

3. Applications of hydrolysates obtained from plant-derived proteins and/or by-products in meat product formulations

The utilization of bioactive peptide-rich protein hydrolysates in various food systems is a relatively new and actively evolving research area. Due to their high biological activity, low toxicity, and ease of metabolism in the human body, there is a growing interest in food-derived bioactive peptides for the development of functional food formulations (*Singh et al*., 2022). This approach is particularly prominent in muscle-based foods. In the production of processed meat products, a variety of ingredients and additives are used for different purposes; however, in recent years, synthetic additives have increasingly been replaced with more natural alternatives in response to shifting consumer expectations. When evaluating strategies for designing healthier meat formulations, one key approach is the enhancement of product quality through the incorporation of natural and functional additives (*Ayuso et al*., 2025). In this context, the use of plant-based protein hydrolysates in various meat products has emerged as a promising strategy.

Studies involving the use of various plant-based protein hydrolysates in meat product formulations and their key findings are presented in Table 1. As seen in the table, various plant protein sources such as chickpea, mung bean, soybean, red lentil, broad bean, pea, pumpkin seed, rapeseed, and rice berry have been investigated for their potential use in meat products or animal cell culture systems. These types of hydrolysates have been shown to enhance bioactivities -particularly antioxidant activity- as well as techno-functional properties. Evaluating the applications of plant-based hydrolysates obtained from by-products, it is observed that some valuable residues, such as canola meal (*Mirzapour et al*., 2022) and tea waste (*Zhao et al*., 2014), have been utilized in meat systems as antioxidant and emulsifying agents, demonstrating their broad application potential. Moreover, it has become evident that plant-derived food industry residues are being explored for even more innovative purposes in meat systems. For example, *Flaibam et al.* (2024) reported that protein-rich extracts and hydrolysates derived from plant-

based agro-industrial waste, particularly soybean and peanut meals, represent a promising and cost-effective alternative in cultured meat production. Overall, these findings highlight the growing potential of plant-based protein hydrolysates, specifically those derived from agro-industrial by-products, as multifunctional ingredients in both conventional and cell-based meat systems, aligning with current demands for health-oriented, sustainable, and clean-label food solutions.

Table 1. Applications of plant-based protein hydrolysates in meat systems

Hydrolysate material	The applied enzyme(s)	Meat system	Key findings	Reference
Soy protein	Microbial-derived extracellular protease	Pork and fish model meat system	• It was reported that hydrolysates slowed down the formation of secondary oxidation products, suggesting their potential use as natural antioxidant sources for controlling lipid oxidation.	*Oliveira et al.* (2014)
Tea dreg	Alcalase®, Neutrase®, Protamex®	Chicken patties	• Hydrolysates produced using Protamex® have been shown to exhibit high in vitro antioxidant activity and to exert antioxidant effects in liposome–meat model systems. The importance of identifying specific peptides at this stage has been emphasized. • Formulation of chicken patties with hydrolysates resulted in reduced peroxide and TBARS values.	*Zhao et al.* (2014)
Canola meal	Alcalase® and Flavourzyme®	Chicken nugget	• Alcalase-produced canola protein hydrolysate (CPH) showed higher antioxidant activity and protein recovery. • Composite coating plus CPH reduced oil uptake and increased moisture and frying efficiency. • CPH-treated nuggets had longer shelf life and delayed oxidation.	*Mirzapour et al.* (2022)
Chickpea, mung bean, soybean, red lentil, broad bean, pea, pumpkin seed, rapeseed	Trypsin and carboxypeptidase B	Animal cell culture medium	• Specifically, chickpea protein hydrolysates were developed as novel microcarriers for cultured meat applications. • The microcarriers demonstrated enhanced cytoaffinity, supporting cell attachment, proliferation, and differentiation into muscle and fat cells. • Improved cell compatibility was attributed to the exposure of lysine and arginine residues, offering a scalable and cost-effective substrate for cell-based meat production.	*Kong et al.* (2023)
Soybean meal and peanut meal	Alcalase™ 2.4 L	Animal cell culture medium	• Soybean and peanut bran were processed to obtain protein-rich hydrolysates as plant-based alternatives to fetal bovine serum (FBS). • The hydrolysates showed comparable protein profiles to FBS and contained high levels of leucine and proline, supporting *in vitro* cell growth and viability. • This approach offers a cost-effective strategy to reduce dependence on animal-derived components in cultured meat production.	*Flaibam et al.* (2024)
Rice berry and mung bean	Bromelain and Protease FP51®	Pork meat batter	• Rice berry and mung bean hydrolysates bound to protein particles at the microstructural level. • These plant proteins had limited impact on texture but may influence color when combined with bromelain. • Meat batters with 1.0% κ-carrageenan and plant proteins showed improved cooking loss and sliceability.	*Saengsuk et al.* (2024)

4. Conclusion

The focus on plant-derived, biopeptide-rich protein hydrolysates underscores a broader industry trend toward incorporating sustainable and functionally active ingredients in food systems. Particularly when derived from agro-industrial by-products, these hydrolysates offer a valuable opportunity to address both environmental and health-related challenges. Their demonstrated bioactivities and techno-functional contributions in meat systems support their integration into clean-label product development. These hydrolysates also show great promise in the field of cultured meat production, offering a sustainable and bioactive alternative for supporting cell growth and functionality. As research continues to uncover new bioactivities and optimize hydrolysate production methods, plant-derived protein hydrolysates are expected to play an increasingly important role in the formulation of next-generation, health-oriented muscle foods.

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Original Scientific Paper

Amino acid profiling of commercial dog and cat foods: comparative analysis and principal component evaluation

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ABSTRACT

This study evaluated the amino acid composition of commercial dog and cat foods and examined differences between them. Fifteen samples were analyzed using ion-exchange chromatography, followed by principal component analysis (PCA). Cat food contained higher levels of most amino acids, especially taurine, and had greater total amino acid (protein) content than dog food. PCA showed clear separation between the two groups, driven mainly by taurine and overall amino acid concentration. Glycine, histidine, and methionine contributed modestly to group differentiation. Dog food samples had more uniform amino acid profiles, while cat food samples showed greater variability, particularly in glycine, glutamic acid, leucine, and alanine content. These findings reflect species-specific nutritional requirements and indicate that cat foods, due to stricter amino acid demands, show higher variability between formulations, while dog foods are formulated in a more uniform way. Further research with a larger and more diverse sample set is needed to confirm these results and assess broader formulation trends in the pet food industry.

1. Introduction

Amino acids are essential nutrients that play a fundamental role in the growth, maintenance, metabolism, and overall health of pets. As the building blocks of proteins, amino acids play a crucial role in enzyme activity, immune system function, and energy metabolism. Dogs (*Canis familiaris*) and cats (*Felis catus*), although both classified within the order *Carnivora*, differ significantly in their physiological requirements for amino acids due to their distinct evolutionary paths, digestive capacities, and metabolic adaptations (*Li & Wu*, 2023).

Although anatomically classified as carnivores, dogs exhibit several metabolic characteristics of omnivores. They are capable of synthesizing certain nutrients endogenously, such as converting β-carotene into vitamin A, tryptophan into niacin, cysteine into taurine, and linoleic acid into arachidonic acid (*NRC*, 2006). This metabolic adaptability enables dogs to tolerate a broader range of dietary ingredients, including plant-based sources, and to thrive on well-balanced vegetarian diets when appropriately supplemented with nutrients that are otherwise absent or insufficient in plant materials (*Oberbauer & Larsen*, 2021). In contrast, cats are

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obligate carnivores with limited ability to regulate amino acid catabolism and maintain consistently high activity of nitrogen-degrading enzymes, regardless of protein intake (*Che et al.*, 2021). As a result, their protein requirements are high and cannot be met through plant-based diets. Furthermore, cats are particularly sensitive to deficiencies in certain amino acids, especially arginine, where even a single deficient meal can cause life-threatening hyperammonaemia, and taurine, which they cannot synthesize in sufficient amounts and must obtain directly from animal tissues (*Legrand-Defretin*, 1994).

To ensure dietary adequacy, organizations such as the National Research Council (NCR), the European Pet Food Industry Federation (FEDIAF) and the Association of American Feed Control Officials (AAFCO) have published species-specific nutritional recommendations. These guidelines define minimum and maximum levels of essential amino acids in pet food, adapted to life stage and physiological state. Notably, most essential amino acids are required in higher amounts in cat food compared to dog food, reflecting the cat's obligate carnivorous nature and its reduced biosynthetic capacity for several key compounds.

In both Serbia and the European Union, the use of amino acids in pet food is regulated as part of the broader framework governing nutritional additives. While their inclusion is permitted, neither system sets specific minimum or maximum levels for amino acids in finished products. In Serbia, the Rulebook on the Quality of Animal Feed (*Official Gazzete*, 2017) defines general quality standards but leaves the formulation of amino acid content to manufacturers, based on the nutritional requirements of the target species. Similarly, in the EU, Regulation (EC) No 1831/2003 (*European Union*, 2003) authorizes the use of amino acids as feed additives, with recommended levels guided by non-binding industry standards, such as those developed by FEDIAF or other organizations.

Since no regulation at the national, European, or international level defines the total amino acid content required in finished pet food, manufacturers are responsible for formulating products in accordance with recommended nutrient profiles and may supplement specific amino acids as additives to optimize the overall amino acid composition.

Given the wide range of formulations on the pet food market and the growing use of alternative ingredients, the amino acid profile of commercial diets may vary significantly. This study aimed to analyze and compare the amino acid composition of selected dog and cat foods, using principal component analysis (PCA) to explore whether systematic differences exist between formulations intended for each species, and how these differences align with established nutritional expectations.

2. Materials and methods

2.1. Sample collection

A total of 15 pet food samples, 7 dog foods (DF1-7) and 8 cat foods (CF1-8), were collected and analyzed during 2025. All samples were submitted by regular clients of the Institute of Meat Hygiene and Technology in Belgrade, with the objective of the full amino acid profiling of their products. Analyses were conducted at the Laboratory for Instrumental Chemistry within the Institute. The products represented various manufacturers and formulations and were analyzed as received, without further processing. Each sample was stored in its original packaging under ambient conditions until laboratory analysis.

2.2. Determination of amino acid composition

Amino acid content in the pet food samples was quantified using a Biochrom 30+ amino acid analyzer (Biochrom, Cambridge, UK), based on the ion-exchange chromatography method outlined in the standard method (*ISO*, 2005). The analytical process relied on strong cation-exchange chromatography coupled with post-column derivatization using ninhydrin, enabling accurate detection of amino acids. Absorbance was measured at 570 nm, except in the case of proline, which was detected at 440 nm due to its unique properties. Prior to chromatographic separation, samples were hydrolyzed in 6M hydrochloric acid (Merck, Germany) at 110 °C for a duration of 24 hours. After hydrolysis, the solutions were cooled to ambient temperature (20 °C), diluted to a final volume of 200 mL with a pH 2.2 buffer (Biochrom, Cambridge, UK), and filtered through a 0.22 μm PTFE membrane. The resulting filtrates were refrigerated until analysis. Individual amino acids were identified by matching their retention times with those in a reference standard mixture containing 21 amino acids (Sigma-Aldrich, St. Louis, MI, USA), and concentrations were determined using norleucine as the internal standard.

2.3. Statistical analysis

Statistical processing was performed using JMP® 11 software (SAS Institute Inc., Cary, NC, USA). Principal component analysis (PCA) was applied to explore patterns and differences in the amino acid composition of pet food samples. The data set included quantified values for all detected amino acids, and PCA was conducted using standardized variables to allow for meaningful comparison across samples.

3. Results and discussion

The amino acid composition of 15 commercial pet food samples was determined and is presented in Table 1, which shows the individual amino acid concentrations and total amino acid content (ΣAA) expressed in g/100 g of product. Cat food samples generally exhibited higher levels of most amino acids compared to dog food samples, particularly for taurine, arginine, lysine, and leucine. This difference is consistent with established nutritional knowledge and previous reports, as domestic cats have inherently higher dietary requirements for protein and several essential amino acids than dogs, due to their limited ability to down-regulate amino acid catabolism and to synthesize certain amino acids, such as taurine and arginine (*NRC*, 2006, *Kanakubo et al*., 2015).

To further explore differences in amino acid profiles between the two product types, principal component analysis (PCA) was applied. The PCA results are shown in Figure 1, with the first two principal components accounting for 94.82% of the total variance (PC1 = 90.7%, PC2 = 4.12%). The PCA plot revealed a clear separation between cat and dog food samples along the PC1 axis, suggesting that systematic differences exist in their amino acid profiles.

Among the individual amino acids, taurine was identified as the most discriminating variable, with markedly higher values in cat food. This finding is in line with the obligate carnivorous nature of cats and their dietary dependence on preformed taurine. In addition, glycine, histidine, and to a lesser extent

Table 1. Amino acid composition and total amino acid content (g/100 g sample) of analyzed pet food products.

	CF1	CF2	CF3	CF4	CF5	CF6	CF7	CF8	DF1	DF2	DF3	DF4	DF5	DF6	DF7
Ala	2.04	3.53	2.88	2.12	3.08	3.76	2.79	2.00	2.19	1.44	1.47	1.90	1.36	1.87	1.08
Arg	2.28	3.74	2.81	2.10	3.04	3.80	2.77	2.13	2.14	1.44	1.48	2.21	1.35	2.05	1.02
Asp	2.82	4.67	3.58	2.63	3.77	4.48	3.75	2.85	2.73	1.84	1.77	2.74	1.71	2.78	1.3
Cys	0.32	0.82	0.56	0.63	0.67	0.71	0.48	0.36	0.45	0.45	0.42	0.32	0.18	0.34	0.24
Phe	1.32	2.22	1.65	1.4	1.81	2.13	1.61	1.27	1.29	1.04	1.02	1.29	0.87	1.28	0.71
Gly	3.08	5.29	4.53	3.02	4.83	6.55	3.83	2.95	3.17	1.86	2.05	2.90	1.78	2.15	1.46
Glu	4.66	7.75	5.89	5.23	6.30	7.54	5.86	4.84	4.55	4.12	3.89	4.41	3.65	4.44	2.77
His	0.84	1.22	0.69	0.59	1.07	1.37	0.87	0.68	0.76	0.57	0.58	0.80	0.40	0.60	0.36
Ile	1.05	1.85	1.33	1.24	1.55	1.63	1.46	1.05	1.07	0.83	0.81	0.93	0.70	1.16	0.54
Leu	2.20	3.80	2.83	2.39	3.12	3.62	3.14	2.11	2.20	1.81	1.84	2.16	1.54	2.20	1.28
Lys	2.07	3.60	2.70	2.00	2.86	3.30	3.14	2.17	2.09	1.26	1.14	1.86	1.17	2.26	0.9
Met	1.10	1.55	1.24	1.13	1.30	1.61	1.54	1.24	0.98	0.65	0.74	0.88	0.42	1.04	0.59
Pro	1.61	2.53	2.85	2.39	3.12	3.02	2.34	1.26	2.33	1.54	1.11	1.27	1.32	1.54	1.00
Ser	1.45	2.49	1.92	1.55	2.03	2.49	1.94	1.50	1.46	1.22	1.16	1.48	1.02	1.33	0.81
Tau	0.40	0.90	0.61	0.69	0.58	0.24	0.79	0.70	0.33	0.32	0.22	0.28	0.29	0.39	0.21
Tyr	0.95	1.59	1.15	0.95	1.25	1.44	1.11	0.84	0.88	0.75	0.74	0.89	0.54	0.83	0.45
Thr	1.26	2.18	1.61	1.28	1.72	2.03	1.66	1.27	1.23	0.91	0.88	1.22	0.81	1.21	0.63
Trp	0.40	0.66	0.41	0.30	0.50	0.54	0.49	0.37	0.38	0.27	0.27	0.37	0.25	0.35	0.28
Val	1.28	2.33	1.77	1.60	2.04	2.19	1.81	1.27	1.43	1.06	1.07	1.16	0.91	1.42	0.71
ΣAA	31.14	54.31	41.03	33.27	44.67	52.46	41.4	30.87	31.69	23.41	22.67	29.08	20.28	29.26	16.35

CF – cat food; DF – dog food.

methionine, contributed modestly to group separation. However, no single amino acid, aside from taurine, was sufficient to account for the divergence between the two groups. Rather, the dominant factor driving the separation was the total amino acid content, which closely reflects the overall protein level in the product. These results emphasize that while certain amino acids, like taurine, are species-specific dietary requirements, the broader distinction between dog and cat foods lies in their cumulative amino acid concentrations. PCA thus proved to be an effective analytical tool for identifying these formulation patterns and nutritional strategies tailored to species-specific needs.

In addition to group separation, PCA results also revealed differences in the degree of data dispersion between the two product types. Dog food samples displayed a more uniform amino acid composition, clustering more tightly in the PCA plot and showing smaller variability across most individual amino acids. In contrast, cat food samples were more dispersed, indicating greater formulation variability. Quantitatively, the total amino acid content (ΣAA) in cat food varied from 30.87 to 54.31 g/100 g, with a range of 23.44 g/100 g, whereas in dog food, it ranged from 16.35 to 31.69 g/100 g, yielding a smaller range of 15.34 g/100 g. Among individual amino acids, glycine, glutamic acid, leucine, and alanine showed notably higher variation in cat food samples. For instance, glycine ranged from 3.02 to 6.62 g/100 g in cat food (range: 3.60 g/100 g), compared to 1.44 to 3.15 g/100 g in dog food (range: 1.71 g/100 g). Similar patterns were observed for glutamic acid (range: 3.09 g/100 g vs. 1.78 g/100 g in cat and dog food, respectively), leucine (1.69 g/100 g vs. 0.92 g/100 g in cat and dog food, respectively), and alanine (1.76 g/100 g vs. 1.11 g/100 g in cat and dog food, respectively). The observed ranges of amino acid concentrations, as well as the ratios between cat and dog food samples, are consistent with the results of previous studies conducted in other countries (*Leiva et al.*, 2019, *Choi et al.*, 2024).

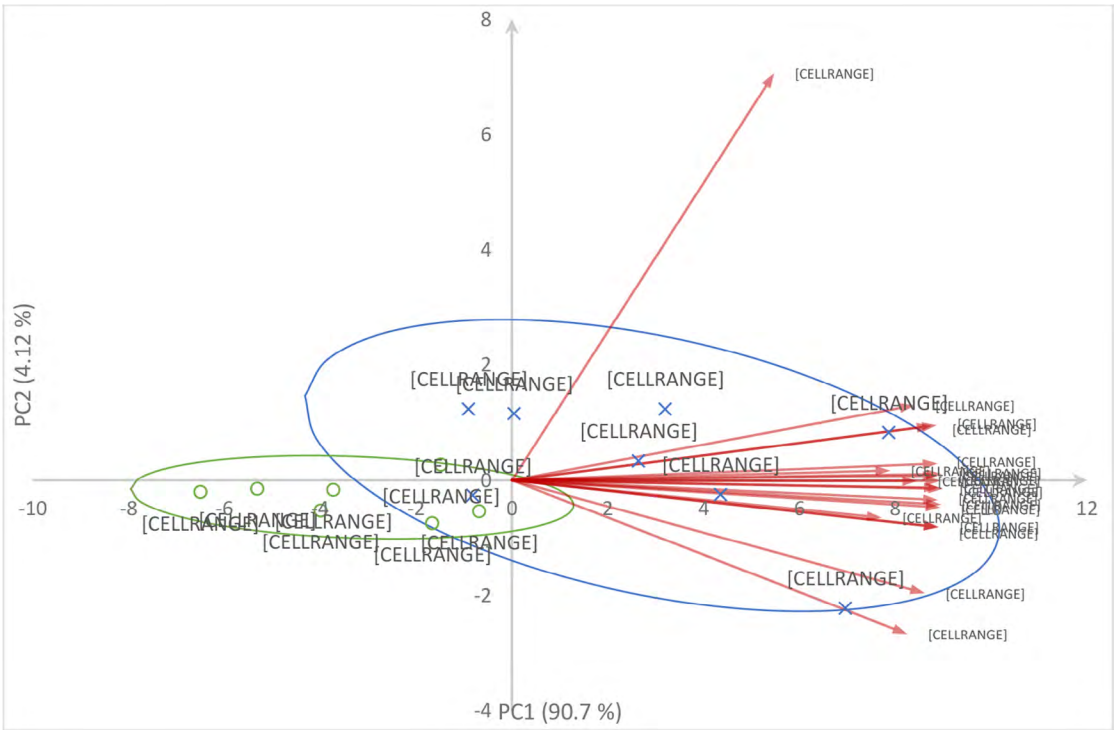

Figure 1. Principal component analysis (PCA) of amino acid composition in dog and cat food samples.

4. Conclusion

This study demonstrated clear differences in the amino acid composition of commercial dog and cat foods. Cat food samples contained higher levels of most amino acids, particularly taurine, and had significantly greater total amino acid (protein) content. PCA analysis confirmed a distinct separation between the two product types, driven primarily by taurine and cumulative amino acid levels.

Dog food formulations were generally more uniform, which may reflect a consistent industry

approach to meeting the nutritional needs of omnivorous species, whereas cat foods, designed to satisfy stricter amino acid requirements, showed greater variability between formulations. In contrast, the greater variability observed in cat food may reflect manufacturer-specific strategies to meet the stricter amino acid requirements of obligate carnivores. This reinforces the conclusion that, aside from taurine, the most defining feature of feline food formulations is their consistently higher and more variable total amino acid content.

Further research involving a larger and more diverse sample set is needed to confirm these findings and to better understand formulation trends and nutritional consistency across the pet food industry.

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Original Scientific Paper

Application of protein concentrate-based gel emulsions for animal fat reduction in meat emulsions: proximate composition, technological, and textural properties

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ABSTRACT

The present study investigated the effects, on the quality of model system meat emulsions, of reducing beef fat by 50% using a gelled emulsion that contained sunflower oil plus different proteins. Meat emulsions were prepared in four different formulations as follows: 20% beef fat (control; C), 10% beef fat combined with 10% gelled emulsion containing almond protein concentrate (A50), 10% beef fat combined with 10% gelled emulsion containing hazelnut protein concentrate (H50), and 10% beef fat combined with 10% gelled emulsion containing sodium caseinate (SC50). Chemical composition, pH, water-holding capacity (WHC), and textural properties were evaluated. The highest moisture content was recorded in the H50 group ($P<0.05$). A50 and H50 meat emulsions contained similar protein contents as SC50, but no significant differences were recorded in the total fat content of the groups. Compared to the control, all treatments containing gelled emulsions exhibited significantly higher WHC ($P<0.05$), with the highest values observed in the plant protein-based meat emulsions (A50 and H50), which even surpassed the SC50 treatment ($P<0.05$). Texture profile analysis showed that hazelnut-based meat emulsions (H50) were of greater hardness, while almond-based meat emulsions (A50) had lower hardness ($P<0.05$). However, both treatments led to lower cohesiveness, springiness, and chewiness compared to the control ($P<0.05$). Overall, incorporating gelled emulsions into emulsified meat systems yielded beef fat substitution and supported a healthier product profile without diminishing chemical composition or functional quality.

1. Introduction

In recent years, there has been an increase in research on fat replacement strategies due to the negative health impacts of animal-derived fats present in meat and meat products. In particular, the elevated saturated fat content in these products contributes to increased low-density lipoprotein (LDL) levels, thereby heightening the risk of cardiovascular disease and posing significant concerns for human health (*Ozen et al*., 2022). According to the World Health Organization (WHO), cardiovascular diseases remain the leading cause of death globally; in 2019, approximately 17.9 million people died from these diseases, accounting for 32% of all global deaths (*WHO*, 2021). Therefore, new strategies are being developed to reduce animal fat consumption in favor of oils rich in unsaturated fatty acids (*Badar et al*., 2021).

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Incorporating gelled emulsions is a practical and effective method in meat product reformulation, helping to improve health attributes without diminishing overall quality. A gelled emulsion forms a gel-like structure and demonstrates mechanical behavior similar to that of a viscoelastic solid (*Öztürk-Kerimoğlu et al*., 2021). In the present study, considering the emulsifying properties of proteins, different plant-based protein concentrates (almond and hazelnut) were used in the formation of gelled emulsion systems, while a standard formulation containing sodium caseinate, a milk-derived protein, was also prepared. The preference for plant-based protein sources is significant not only from a nutritional perspective, but also in terms of sustainable food production strategies. A review of the literature suggests that protein concentrates are commonly used in the development of plant-based meat analogs (*Jia et al*., 2021; *dos Santos et al*., 2022; *Ramos-Diaz et al.*, 2022). However, to the best of our knowledge, no study has been conducted on substituting beef fat in meat emulsions with gelled emulsions containing almond and hazelnut protein concentrates. In this context, in the current research, we targeted a reduction in the animal fat content of meat emulsion systems through the incorporation of gelled emulsions formulated with plant-sourced proteins. This approach is expected to decrease the reliance on animal-derived fats and, consequently, mitigate the associated environmental impact.

2. Materials and methods

2.1 Preparation of plant protein concentrates, gelled emulsions, and model meat emulsions

Sodium caseinate and microbial transglutaminase (mTG) were purchased from A&D Chemicals Inc. (İstanbul, Türkiye) and SternEnzym Inc. (Ahrensburg, Germany), respectively. Raw almonds and hazelnuts, fresh and pre-packaged, were procured from the local market of İzmir, Türkiye, and ground into a fine powder using a grinder (Mioji, Türkiye). Subsequently, protein concentrates were obtained by applying the alkaline solubilization and acid precipitation method, with minor modifications, as described by *Daliri et al.* (2021). For the production of meat systems, post-mortem beef muscles (i.e., boneless round) and beef backfat were supplied from a local butcher located in İzmir, Türkiye, and transported to the laboratory under cold chain conditions. Refined sunflower oil was purchased from Migros Ticaret Inc. (İzmir, Türkiye), while the other ingredients (curing agents, etc.) were supplied by Kimbiotek Chemicals Inc. (İstanbul, Türkiye).

The oil-in-water (O/W) gelled emulsion system was prepared with some modifications of the method previously described by *Öztürk-Kerimoğlu et al.* (2021). Firstly, the water phase was prepared as follows: non-meat proteins (2.5%) were homogenized with cold water (22.5%) at 6000 rpm for 40 seconds using an Ultraturrax (IKA, Germany). Separately, mTG (0.35%) and cold water (24.65%) were mixed at the same speed and for the same duration. The two phases were then mixed at 5800 rpm for 20 seconds. Subsequently, the oil phase (50%) was added to the system in a controlled manner while mixing at 1125 rpm for 5 min. Thereafter, the emulsion was subjected to an additional homogenization at 5800 rpm for 3 min and then stored at 4°C for 12 h to enable gelation through cold setting.

Table 1 presents the formulations of model system meat emulsions. For the production of meat systems, beef and beef backfat were separately minced using a meat grinder (Arnica W2000 Grande, Türkiye) equipped with a 3 mm diameter plate. Then, the

Table 1. The formulations of model system meat emulsions

	Ingredients (g)						
Treatments*	Beef	Beef Fat	Gelled emulsion	Water (ice)	Salt (NaCl)	Sodium tripolyphosphate	Sodium nitrite
C	500	100	0.0	50	7.5	2.5	0.075
A50	500	50	50	50	7.5	2.5	0.075
H50	500	50	50	50	7.5	2.5	0.075
SC50	500	50	50	50	7.5	2.5	0.075

Legend: *C: Control meat system formulated with 20% beef fat, A50: Reduced-fat meat system formulated with 10% beef fat and 10% gelled emulsion containing almond protein concentrate, H50: Reduced-fat meat system formulated with 10% beef fat and 10% gelled emulsion containing hazelnut protein concentrate, SC50: 10% beef fat and 10% gelled emulsion containing sodium caseinate.

minced meat was pre-mixed at 500 rpm for 1 min in a food processor (Thermomix TM5, Vorwerk, Germany). Next, the other ingredients were added, and the mixture was homogenized under the same conditions for 2 min. After partially adding the ice and the lipid phase (fat or gelled emulsion), the mixture was further mixed at 1100 rpm for 3 min. After adding the remaining ice, an additional mixing step was performed at the same speed. Finally, the emulsification was completed by mixing at 2000 rpm for 1 min to obtain the final model meat emulsion.

2.2 Analyses

Total protein content was determined using the Kjeldahl method, as described by *Moore* (2010), by multiplying the nitrogen content by the nitrogen-to-protein conversion factor (6.25). Moisture and ash content were measured according to *AOAC* (2012). pH value was measured using a WTW pH 3110 Set 2 pH meter (WTW, Germany). Water-holding capacity (WHC) was measured after heat treatment and centrifugation of the samples, based on the method described by *Hughes et al.* (1997) with minor modifications. Texture profile analysis (TPA) of the model meat emulsions, presenting hardness, cohesiveness, springiness, gumminess, and chewiness, was carried out using a texture analyzer (TA-XT2, Stable Micro Systems, Haslemere, UK) by compressing suitable samples to 50% of their original height using a 30 kg load cell, at a crosshead speed of 1 mm/s. The pre-test and post-test speeds were set at 1 mm/s and 5 mm/s, respectively. Statistical analysis was performed by analysis of variance (ANOVA) followed by Duncan's post-hoc tests in the SPSS software (v.26, IBM, USA) within a confidence interval of 95%. All the analyses were performed at least in triplicate. Mean values are presented and discussed below.

3. Results and discussion

3.1 Chemical composition and pH

Proximate composition and pH values of the model meat systems are presented in Table 2. Moisture content is a key quality indicator that determines the WHC and emulsification properties of meat products; hence, high moisture content plays a key role in preserving the functional properties of the product (*Qiao et al*., 2001). Total moisture contents of the model meat emulsions were between 63.49-66.47%, and the lowest moisture content was found in the control (C) group, while the highest mean moisture content was recorded in the H50 meat emulsion with hazelnut protein concentrate ($P<0.05$). The findings indicated that gelled emulsions containing hazelnut protein have the potential to enhance water retention in meat systems and entrap water within the emulsified meat matrix. Formerly, *dos Santos et al.* (2022) mentioned that the moisture content of the final product could be improved by increasing WHC and emulsion stability through the use of concentrations.

The protein contents of the treatments ranged between 19.65-21.03%, and indeed, almond and hazelnut meat emulsions had similar protein contents to that of the sodium caseinate meat emulsion. Although the model meat emulsions with plant-sourced proteins had lower protein contents than the control ($P<0.05$), the plant-derived components were mentioned as having the potential to improve gel formation and emulsion stability (*Toldrá*, 2006). Thus, the functional properties of proteins should also be considered as quality-determining factors alongside total protein content (*Toldrá*, 2006).

The fat content of the treatments ranged between 9.47%-11.76%, and no statistically significant differences were observed between the groups (Table 2). In a study conducted by *Ghribi et al.* (2018), sausages were formulated using different concentrations of chickpea protein concentrate, with fat contents ranging from 14.27% to 19.04%. The absence of differences in total fat content among the groups in our study is attributed to the fixed total lipid concentration used in the formulation (20%). Under these conditions, the similar fat levels in the model meat emulsions suggest that the gelled emulsion treatments exhibited a fat retention capacity comparable to the control, indicating a technological advantage. However, to ensure lipid modification, further analysis of the fatty acid composition and unsaturated fatty acid levels is required.

The ash contents of the model meat emulsions were between 3.41-4.70%. The ash contents of the gelled emulsion groups, in which animal fat was reduced, were similar to that of the control.

The pH of the model meat emulsions ranged between 5.85-6.10. The lowest pH belonged to the control meat emulsion group ($P<0.05$), indicating that utilization of gelled emulsions with different protein concentrates effected a rise in pH in each of the other groups. The pH values measured in our model meat emulsions were consistent with those

Table 2. Chemical composition and pH value of model system meat emulsions

Treatments*	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	pH
C	63.49^{c}±0.30	11.76±0.96	21.03^{a}±0.65	3.71^{ab}±0.0	5.85^{b}±0.13
A50	65.29^{b}±0.05	10.0±0.62	19.90^{b}±0.19	4.54^{ab}±0.38	6.07^{a}±0.05
H50	66.47^{a}±0.004	10.96±1.26	19.65^{b}±0.12	3.41^{b}±0.70	6.13^{a}±0.17
SC50	65.62^{b}±0.26	9.47±0.48	20.25^{ab}±0.30	4.70^{a}±0.25	6.10^{a}±0.01

Legend: *C: Control meat system formulated with 20% beef fat, A50: Reduced-fat meat system formulated with 10% beef fat and 10% gelled emulsion containing almond protein concentrate, H50: Reduced-fat meat system formulated with 10% beef fat and 10% gelled emulsion containing hazelnut protein concentrate, SC50: 10% beef fat and 10% gelled emulsion containing sodium caseinate.

reported in a study that used soy, pea and sunflower protein concentrates in hybrid meat emulsions (*dos Santos et al*., 2022). The pH level in meat products is a critical factor that directly affects basic quality parameters such as color, WHC, and tenderness, thereby determining both the functional and sensory characteristics of the final product.

3.2 Water-holding capacity (WHC)

The WHC of the control, A50, H50, and SC50 model meat emulsions was determined to be 68.42%, 80.85%, 81.97%, and 76.06%, respectively (Figure 1). Compared to the control group, significantly higher WHCs were observed in all treatments with gelled emulsions (P<0.05). The highest WHC was recorded in model meat emulsions with plant-proteins (A50 and H50), and these treatments even presented higher WHCs than the SC50 meat emulsion containing sodium caseinate (P<0.05). Accordingly, it was shown that gelled emulsions prepared with either of the plant protein concentrates have good potential to raise the WHC of the emulsified model meat systems, thereby improving technological quality and product yield. The high WHC can provide significant technological and economic advantages in meat emulsions. Formerly, *Nacak et al.* (2021) reported that increasing the added level of gelled emulsions in sausage formulations considerably increased the WHC of the emulsions, and the main reason for this was attributed to the water-binding properties of the protein-based ingredients in the gelled emulsion, such as gelatin and egg white powder. WHC is a fundamental technological feature that improves the textural quality of meat products and reduces weight loss during cooking, thereby determining both production efficiency and consumer satisfaction (*Huff-Lonergan and Lonergan*, 2005).

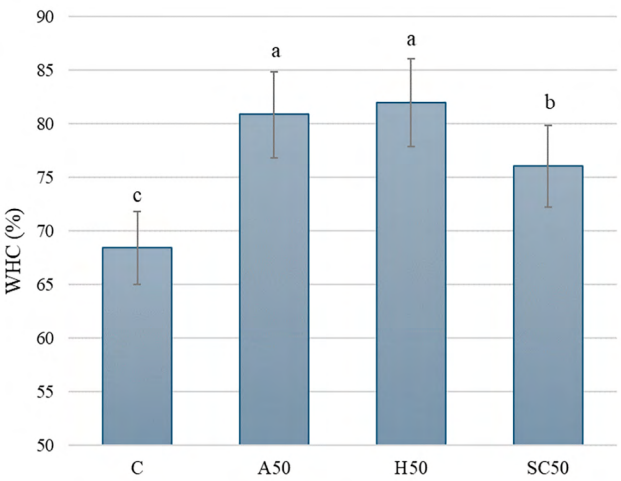

Figure 1. WHC of model system meat emulsions (%)

Legend: *C: Control meat system formulated with 20% beef fat, A50: Reduced-fat meat system formulated with 10% beef fat and 10% gelled emulsion containing almond protein concentrate, H50: Reduced-fat meat system formulated with 10% beef fat and 10% gelled emulsion containing hazelnut protein concentrate, SC50: 10% beef fat and 10% gelled emulsion containing sodium caseinate.

3.3. Texture

As shown in Table 3, the model meat emulsion systems exhibited significant differences in hardness, cohesiveness, springiness, gumminess, and chewiness (P< 0.05). Hardness results revealed that the highest value among treatments was found in H50 model meat emulsions, while the lowest values were observed in control and A50 meat emulsions (P<0.05). It was reported that the hardness of meat emulsions increases with higher protein content (*Santhi et al.*, 2017). However, in our study, the protein content of the harder H50 group was not higher when compared with the other groups. In this situation, it is suggested that the fibrous structure formed a complex network in this group due to its ability to retain water, thereby resulting in greater hardness.

A50 and H50 meat emulsions had similar cohesiveness values, which were both lower than the control (P<0.05). In contrast, *Yang et al*. (2025) reported that replacing pork fat with almond protein isolate-based Pickering emulsions in pork sausage formulations significantly increased cohesiveness. The differing results observed in our study are likely attributable to variations in the behavior of pork versus beef fat, as well as differences in the fat replacer emulsion systems employed.

Springiness was the highest in the C group among treatments (P<0.05), so the extent of recovery of the sample height was the highest in this treatment (*Fan et al*., 2020). This outcome was likely due to the rigid structure of animal fat compared to the pastry-like structure of the gelled emulsions.

The A50 group exhibited the lowest gumminess among treatments (P<0.05), which pointed to a lower energy requirement for mastication due to a softer and less dense texture. Chewiness values of A50 and H50 meat emulsions were significantly lower than those of the C and SC50 model meat emulsions ($P < 0.05$). These findings were in line with the softness of the meat emulsion containing gelled emulsions with almond protein (A50). However, although H50 meat emulsions with hazelnut protein had higher hardness, the lower chewiness of this group was due to the lower cohesiveness and springiness (compared with other treatments). Chewiness can be affected by the moisture-to-protein ratio of the product, with higher ratios typically resulting in less chewiness (*Nacak et al*., 2021). This trend was also reflected in the results of the current study. TPA is widely used to characterize the textural attributes of meat emulsions and serves as a reliable indicator of their overall quality (*Lee et al*., 2025).

4. Conclusion

The findings of the present study indicated that utilization of gelled emulsions formulated with plant-based protein sources is an effective way to reduce animal fat in emulsified meat systems. The studied formulations containing almond and hazelnut protein concentrates provided promising results in terms of technological functionality and product yield of the model meat emulsions. Although textural differences were evident among treatments, the incorporation of plant protein-based emulsions successfully supported fat reduction without compromising the functional qualities measured, so this approach would thereby contribute to healthier meat product formulations. Future research should focus on optimizing the fatty acid composition and exploring both sensory attributes and oxidative quality in meat products formulated with gelled emulsion systems containing different plant proteins.

Table 3. Textural parameters of model system meat emulsions

Treatments*	Hardness (N)	Cohesiveness	Springiness (mm)	Gumminess (N)	Chewiness (N x mm)
C	11.26^{c}±0.48	0.22^{a}±0.04	0.24^{a}±0.03	2.54^{a}±0.51	0.62^{a}±0.20
A50	9.33^{c}±1.17	0.15^{b}±0.05	0.13^{c}±0.02	1.41^{b}±0.64	0.17^{c}±0.04
H50	18.07^{a}±1.87	0.15^{b}±0.02	0.13^{c}±0.02	2.80^{a}±0.54	0.38^{bc}±0.12
SC50	13.95^{b}±0.39	0.19^{ab}±0.02	0.18^{b}±0.02	2.61^{a}±0.23	0.48^{ab}±0.07

Legend: *C: Control meat system formulated with 20% beef fat, A50: Reduced-fat meat system formulated with 10% beef fat and 10% gelled emulsion containing almond protein concentrate, H50: Reduced-fat meat system formulated with 10% beef fat and 10% gelled emulsion containing hazelnut protein concentrate, SC50: 10% beef fat and 10% gelled emulsion containing sodium caseinate.

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Original Scientific Paper

Chia mucilage-based nanoemulsion coatings with sage essential oil for improving the oxidative stability and shelf life of fresh turkey breast

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ABSTRACT

This study evaluated chia mucilage (CM)-based edible coatings enriched with sage essential oil (SEO) nanoemulsions for improving oxidative stability, microbial safety, and quality of turkey breast strips during refrigerated storage. Coating solutions (C, CS1: CM + 0.5% SEO, CS2: CM + 1% SEO, CS3: CM + 1.5% SEO) were analyzed for total phenolic content (TPC), total antioxidant activity (TAA), and antimicrobial activity. TPC and TAA values increased with SEO concentration, with CMS3 reaching 139.13 mg GAE/g and 55.53% scavenging activity. Although disk diffusion assays showed limited direct antimicrobial activity, the controlled release of SEO during storage provided effective microbial inhibition. In turkey meat samples, the evaluated parameters included coating yield, color parameters (L*, a*, b*), lipid oxidation (TBARS), total mesophilic aerobic bacteria (TMAB) counts, and sensory attributes. CMS2 and CMS3, compared with the other coating solutions, exhibited higher coating yield (96.24% and 94.68%), better color stability, and lower lipid oxidation. After 12 days at 1 °C under MAP, CMS3 reduced TBARS by ~60% (0.41 vs. 1.03 mg MDA/kg) and TMAB counts by 1 log unit, supporting its potential as a clean-label preservation strategy.

1. Introduction

The limited shelf life of poultry products, particularly turkey meat, is primarily attributed to its high moisture and protein content, which accelerates microbial spoilage and oxidative deterioration (*Baltic et al*., 2019). As a valuable source of high-quality dietary protein with low fat content, essential amino acids, and beneficial long-chain polyunsaturated fatty acids, turkey meat is increasingly favored by health-conscious consumers (*Marangoni et al*., 2015; *Keykhosravy et al*., 2020). However, due to regulatory restrictions, the use of synthetic preservatives is not permitted in fresh meat products, which renders them more vulnerable to spoilage and underscores the need for the development of effective and natural preservation approaches (*Shaltout*, 2024).

In recent years, edible coatings have gained attention as an innovative approach to improving the quality, safety, and shelf life of perishable foods. These coatings form a thin, protective layer on the surface of food, which can modulate gas exchange, reduce moisture loss, and serve as a carrier for bioactive compounds such as antioxidants and antimicrobials (*Valencia-Chamorro et al.*, 2011; *Chen et*

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al., 2021). Among natural bioactives, essential oils have been widely studied for their potent antimicrobial and antioxidant properties; however, their direct incorporation into foods is limited due to volatility, hydrophobicity, and strong sensory attributes (*Prakash et al*., 2018; *Sharma et al*., 2022).

To address these challenges, essential oils are increasingly being encapsulated in nanoemulsion-based coatings, which improve their stability, dispersion, and controlled release (*Choi & McClements*, 2020; *Thakur et al*., 2024). Such nanoemulsion coatings not only preserve the transparency and appearance of foods, but also effectively slow down oxidative rancidity and microbial growth during storage (*Shokri et al*., 2020; *Mushtaq et al*., 2023). Moreover, plant-derived hydrocolloids like chia mucilage have demonstrated excellent water-holding capacity and matrix-forming ability, further enhancing the structural and functional properties of these coatings (*Baraketi & Khwaldia*, 2024).

In this context, the present study investigates the application of chia mucilage-based edible coatings enriched with varying concentrations of sage essential oil (SEO) nanoemulsions, applied via the dipping method, on turkey breast meat. The study evaluates their effects on oxidative stability, microbial quality, and sensory attributes during refrigerated storage, offering valuable insights for the development of sustainable, natural, and clean-label preservation strategies for poultry products.

2. Materials and methods

Materials: Fresh turkey breast fillets were purchased from a local butcher shop in İzmir, Türkiye, on the day of processing. Sage essential oil (SEO) was obtained from Doğal Yağlar A.Ş. (İstanbul, Türkiye). Chia seeds (*Salvia hispanica* L.) were sourced from Organik Tarım Ürünleri Ltd. (İzmir, Türkiye) for mucilage extraction. Tween 80 (HLB 15) and all analytical grade chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Preparation of Chia Mucilage and Nanoemulsions: Chia mucilage (CM) was prepared following the method of *Akhavan et al.* (2022) with minor modifications. Chia seeds were hydrated in distilled water (1:10, w/v) at 45 °C under constant stirring for 15 min, followed by centrifugation (4100 rpm, 15 min). The viscous mucilage layer was collected, filtered, and stored at 4 °C until use. Nanoemulsions containing SEO (0.5%, 1%, or 1.5%, w/w) were produced by blending SEO with Tween 80 (1%, w/w) and distilled water, pre-homogenizing (Ultra-Turrax, 12,000 rpm, 2 min), and then applying high-pressure homogenization (120 MPa, 3 passes) to achieve submicron droplet sizes (*Ghosh et al*., 2013). The nanoemulsions were subsequently mixed with 10% (w/w) CM and stored at 4 °C for 12 h before coating.

Coating Application: Turkey breast strips (1 cm thick) were trimmed of visible fat and connective tissue and immersed in the coating solutions (1:3 sample-to-solution ratio) for 10 min at 4 ± 1 °C to ensure that the cold chain was maintained throughout the process. Excess solution was allowed to drain on stainless-steel racks for 30 min (chilled condition). Control samples (CONT) were immersed in distilled water. All samples were packaged using modified atmosphere packaging (MAP; 50% CO_2, 50% N_2) with a Henkelman Boxer42 system and stored at 1 °C for 12 days. Five experimental groups were prepared: CONT (control, distilled water), CM (10% chia mucilage), CMS1 (10% CM + 0.5% SEO), CMS2 (10% CM + 1% SEO), and CMS3 (10% CM + 1.5% SEO).

Analyses: Coating solutions were evaluated for total phenolic content (TPC) using the Folin-Ciocalteu method, expressed as mg gallic acid equivalent (GAE)/g (*Singleton et al*., 1999), total antioxidant activity (TAA) using the DPPH radical scavenging assay (*Brand-Williams et al*., 1995), and minimum inhibitory concentration (MIC) against *Escherichia coli* and *Staphylococcus aureus* using the broth microdilution method (*CLSI*, 2018). Meat samples were assessed for coating yield (*Shokri et al*., 2020), color parameters (L*, a*, b*) using a colorimeter (*Hunt et al.*, 1991), lipid oxidation by thiobarbituric acid reactive substances (TBARS) expressed as mg MDA/kg (*Tarladgis et al*., 1960), and total mesophilic aerobic bacteria (TMAB) counts on Plate Count Agar incubated at 30 °C for 48 h (*ISO*, 2013). Sensory evaluation was performed by a trained panel (n=10) to assess appearance, color, texture, juiciness, and overall acceptability on a 9-point hedonic scale. All measurements were performed in triplicate, and data were analyzed by ANOVA and Tukey's test at a significance of $p < 0.05$.

3. Results and discussion

Coating solution analyses: The total phenolic content (TPC) and total antioxidant activity of the coating solutions increased significantly with the rising concentration of sage essential oil (Table 1).

Due to its high content of phenolic compounds and terpenoids, SEO markedly enhanced the antioxidant potential of the coating matrix. Particularly, the coatings containing 1% and 1.5% SEO exhibited significantly higher TPC values compared to the CM (chia mucilage only) formulation ($p < 0.05$). Similarly, DPPH radical scavenging activity (TAA) showed a concentration-dependent increase, indicating that lipid-soluble phenolic constituents and volatile components in the nanoemulsions improved the radical-quenching ability of the coatings. The antioxidant properties of the coatings are linked bioactive compounds such as borneol, monoterpenes (α- and β-thujenes), eucalyptol, α-caryophyllene (humulene), and β-caryophyllene (*Ghorbani & Esmaeilizadeh*, 2017).

The disk diffusion assay revealed that the coating solutions exhibited limited or no direct antimicrobial effect (Figure 1). This can be attributed to the low diffusion capacity of hydrophobic compounds in the agar medium and the encapsulation of SEO within the nanoemulsion matrix, which restricts its immediate release. Therefore, the antimicrobial efficiency of the coatings is expected to be more pronounced on the surface of meat products through a controlled release mechanism during storage.

Table 1. Total Antioxidant Activity and Total Phenolic Content (TPC) of Coating Solutions

Coating Solution	DPPH(%)	Total phenolic compounds(mg GAE/g)
C	50.20± 0.61^{c}	3.54±0.32^{d}
CS1	50.94±0.57^{c}	23.8±0.23^{c}
CS2	53.4±0.23^{b}	77.8±0.17^{b}
CS3	55.53±0.19^{a}	139.13±0.5^{a}

C: Chia mucilage only; CS1: 10% CM + 0.5% sage essential oil (SEO); CS2: 10% CM + 1% SEO; CS3: 10% CM + 1.5% SEO. Different letters (a–d) within the same column indicate statistically significant differences ($p < 0.05$).

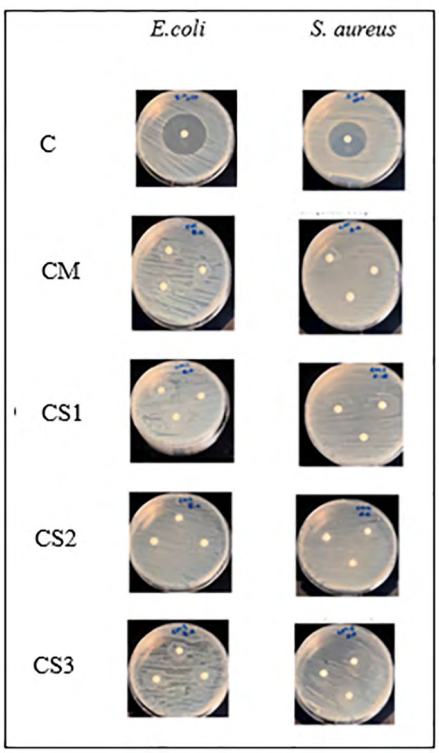

Figure 1. Antimicrobial activity of coating solutions against *Escherichia coli* and *Staphylococcus aureus*. C: Chia mucilage only; CS1: 10% CM + 0.5% sage essential oil (SEO); CS2: 10% CM + 1% SEO; CS3: 10% CM + 1.5% SEO.

Turkey breast analyses: The coating yield ranged from 93.60% (K) to 96.24% (CMS2). The highest yield was recorded in CMS2 (10% chia mucilage + 1% SEO), which was significantly higher than the control and CM groups ($p < 0.05$). The presence of chia mucilage and SEO nanoemulsions likely enhanced the adhesion of the coating film onto the meat surface due to increased viscosity and emulsifying capacity. Conversely, the control group had the lowest yield, as it lacked a polysaccharide matrix to retain the coating solution. On day 0, L* values (lightness) ranged from 44.58 (CMS3) to 48.56 (CM) (Table 2). The inclusion of SEO, particularly at 1.5%, slightly reduced lightness (lower L*), indicating a mild darkening effect likely due to the inherent color of the essential oil. The a* (redness) values showed a slight decrease in all coated groups compared to the CONT, possibly due to the coating acting as a barrier to oxygen, thereby slowing the formation of oxymyoglobin. These findings are consistent with previous reports, where oregano essential oil coatings (*Zheng et al.*, 2023) and cinnamon essential oil nanoemulsions (*Wang et al.*, 2021) also maintained color stability in chicken fillets. The b* (yellowness) values decreased with higher SEO levels, with CMS3 showing the lowest b* value (2.02), indicating that SEO incorporation can subtly shift surface color, but not to an unacceptable degree.

Table 2. Coating yield and color parameters of turkey breast meat coated with chia mucilage based nanoemulsions

Sample	Coating yield (%)	L*	a*	b*
cont	93.60 ± 0.20^{c}	45.85±0.36^{b}	4.14±0.13^{a}	4.01±0.93^{a}
CM	94.38 ± 0.48 ^{bc}	48.56±0.63^{a}	3.54±0.37^{b}	3.12±0.22^{ab}
CMS1	94.82 ± 1.02 ^{bc}	48.50±0.50^{a}	3.64±0.09^{b}	3.40±0.80^{b}
CMS2	96.24 ± 0.89 ^{a}	47.31±0.45^{ab}	3.42±0.59^{b}	2.34±0.44-^{c}
CMS3	94.68 ± 0.58 ^{bc}	44.58±0.85^{c}	3.62±0.75^{b}	2.02±0.35^{c}

Legend: **CONT:** Turkey breast strips coated with water, **CM:** Turkey breast strips coated with 10% chia mucilage, **CMS1:** Turkey breast strips coated with 10% chia mucilage + 0.5% sage essential oil, **CMS2:** Turkey breast strips coated with 10% chia mucilage + 1% sage essential oil, **CMS3:** Turkey breast strips coated with 10% chia mucilage + 1.5% sage essential oil. Different letters (a–e) within the same column indicate statistically significant differences ($p < 0.05$).

TBARS values, reflecting lipid oxidation via malondialdehyde (MDA) formation, increased during refrigerated storage for all groups (Figure 2). On day 0, values were 0.22–0.24 mg MDA/kg with no significant differences ($p > 0.05$). By day 12, the control group reached 1.03 mg MDA/kg, about 2.5 times higher than its initial value, indicating advanced oxidation. In contrast, SEO-coated samples (CMS1–CMS3) showed strong inhibition, with CMS3 (1.5% SEO) achieving the lowest value (0.41 mg MDA/kg), a ~60% reduction compared to the control. This effect results from the antioxidant phenolics in SEO and the oxygen-barrier properties of chia mucilage. While storage time significantly affected TBARS ($p < 0.05$), coated groups, especially CMS2 and CMS3, displayed much slower oxidation, confirming the synergistic protection offered by CM-SEO coatings. In line with our results, other researchers have also reported lower TBARS or MDA values when essential oils and edible coating or active packaging applications were used on meat products (*Gautam et al.*, 2023; *Zimoch-Korzycka & Jarmoluk*, 2017; *Amiri et al.*, 2019; *Fiore et al.*, 2021). Throughout the 12-day storage period, TMAB counts gradually increased in all groups, reflecting normal microbial growth in chilled poultry. The control reached 5.70 log CFU/g by day 12, nearing the spoilage limit, whereas coatings with SEO significantly suppressed microbial proliferation. CMS3 (1.5% SEO) achieved the lowest TMAB count (4.60 log CFU/g), about 1 log lower than the control (Figure 2). The antimicrobial effect of sage essential oil compounds (*Speranza et al.*, 2013), aided by their slow release from the nanoemulsion and the protective chia mucilage matrix (*Barazandegan*, 2022) kept microbial levels in CMS2 and CMS3 below 5 log CFU/g during storage. Sensory evaluation results indicated that all parameters' scores remained similar across all treatments (Figure 2).

4. Conclusion

The application of chia mucilage-based coatings enriched with SEO nanoemulsions significantly improved the oxidative and microbial stability

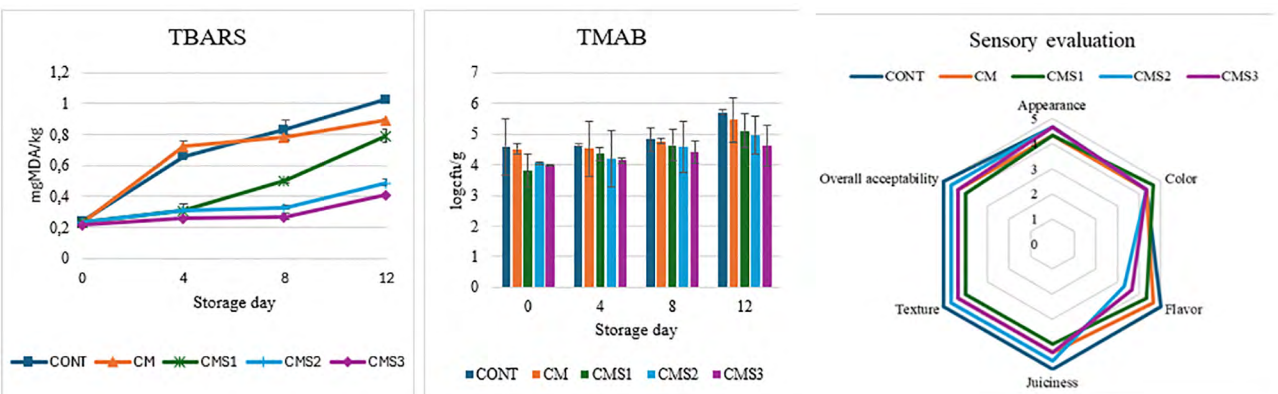

Figure 2. Changes in lipid oxidation (thiobarbituric acid reactive substances (TBARS), mg MDA/kg) and total mesophilic aerobic bacteria (TMAB, log CFU/g) and sensory evaluation of turkey breast strips coated with chia mucilage based nanoemulsions

of turkey breast meat during storage. CMS3 (1.5% SEO) exhibited the most effective performance, achieving the lowest TBARS values and a notable reduction in TMAB counts while preserving surface color and coating yield. These results indicate that the synergistic effects of chia mucilage and SEO provide strong antioxidant and antimicrobial protection, delaying quality deterioration. Therefore, such coatings hold potential as sustainable, clean-label solutions for extending the shelf life of poultry products. Further research should explore sensory evaluations and industrial-scale applications.

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Original Scientific Paper

Evaluation of greener solvent options for polycyclic aromatic hydrocarbon extraction

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants with known carcinogenic and mutagenic effects. In order to monitor their presence in complex food and environmental matrices, liquid-liquid extraction (LLE) was employed as a reliable sample preparation technique. To minimize environmental impact, green solvents are used as alternatives to more hazardous traditional solvents. Quantification and confirmation of 16 target PAHs were performed using gas chromatography coupled with tandem mass spectrometry (GC-MS/MS), ensuring high sensitivity and selectivity. This study compared toluene and ethyl acetate as PAH extraction solvents in water samples at lower and higher concentrations (10 µg/L and 100 µg/L). Both solvents extracted similar amounts of the 16 target PAHs from water, although the AGREE tool favours ethyl acetate over toluene, due to the former's environmental and safety benefits.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of persistent organic compounds composed of two and more fused aromatic rings. One of the main ways they are created is through the combustion of coal, wood and waste. As such, they can be found in soil, water and air, both through human activity and natural processes (*Agency for Toxic Substances and Disease Registry - ATSDR*, 1995; *Iko Afé et al.,* 2021). Due to their mutagenic and carcinogenic properties, these compounds pose a significant risk to human health. First, several PAHs are classified by IARC as known or potential carcinogens. Second, their metabolites can cause mutations in DNA molecules (*Wilson and Jones,* 1993; *Agency for Toxic Substances and Disease Registry - ATSDR*, 1995; *Liu et al.*, 2015).

Because of their demonstrated toxicity and high abundance, regular analysis of PAHs is very important, primarily for human health and environmental safety. This is accomplished through regular monitoring of water, air, and soil. Similarly, food safety is ensured by routinely checking the levels of PAHs in, e.g., smoked and grilled products (*Kumar et al.*, 2021; *Imrigha et al.*, 2022).

Modern and routine analysis and quantification of these compounds is mainly performed using gas chromatography with mass detection or high-performance liquid chromatography with fluorescence detection (*Zhang et al.*, 2020). However, it is also essential to use mass detection, especially as

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detection limits and regulatory requirements continue to become stricter over time.

Alongside the requirements for increasingly lower detection limits, there is a rising demand for the implementation of environmentally friendly solvents in the extraction of these compounds from diverse matrices. Commonly used organic solvents for the extraction of PAHs, e.g., toluene, dichloromethane, chloroform and hexane, can present very serious health risks, primarily due to their toxicity, volatility and high carcinogenic potential (*Jinadasa et al*., 2020).

In order to reduce health and environmental risks, there is an increasing need to use greener solvents, i.e., chemicals that are less toxic, more biodegradable and can be produced from renewable sources (*U.S. Environmental Protection Agency - EPA*, 2005). Green chemistry aims at sustainable development, reduction of pollution and better use of resources. Green analytical chemistry focuses on reducing the harmful impact of analytical methods on human health and the environment, while on the other hand, ensuring the high quality of the results is not impaired. Some of the principles of green analytical chemistry are reduction of the use of dangerous chemicals, replacement of toxic solvents, miniaturization and automation of processes and analysis of multiple analytes with one method (*Capello et al.*, 2007; *Kaya et al*., 2022).

Ethyl acetate is one of the more acceptable solvents for the environment, being a safer solution compared to commonly used solvents, because of its lower toxicity and higher biodegradability. Its volatility prevents persistence and bioaccumulation in the environment. Additionally, it can be sustainably produced from bioethanol and acetic acid (*Armenta et al*., 2008).

Liquid-liquid extraction (LLE) is a one of a widely used technique for isolating PAHs prior to chromatographic analysis. Liquid-liquid extraction is a separation technique based on the differential solubility of compounds in two immiscible liquids, mainly between the organic and aqueous phases. The analytes of interest, such as PAHs, partition preferentially into the organic phase due to their non-polar characteristics. Some of the parameters that influence extraction efficiency (EE) are choice of solvent, pH adjustment and sample volume (*Manoli and Samara*, 1999; *Peña-Pereira et al*., 2009; *Rezaee et al*., 2006).

The aim of this study was to compare toluene and ethyl acetate as extraction solvents for PAHs in water samples at two concentration levels, lower and higher (10 µg/L and 100 µg/L). A non-polar aromatic solvent (toluene), is traditionally favoured for its high affinity for hydrophobic PAHs. Ethyl acetate, a moderately polar solvent, is a greener alternative due to its lower toxicity and better biodegradability.

In order to compare the analytical performance of the two solvents and their impacts on the environment, the EE of the solvents was monitored, while the analytical greenness (AGREE), proposed by *Pena-Pereira et al*. (2020), was calculated. AGREE is a quantitative measure of method greenness comparing 12 principles of green analytical chemistry.

2. Materials and methods

The CRM mixture of 16 US EPA PAHs, i.e., Naphthalene (Naph), Acenaphthylene (Acy), Acenaphthene (Ace), Fluorene (Flu), Phenanthrene (Phen), Anthracene (Ant), Fluoranthene (Flt), Pyrene (Pyr), Benzo(a)anthracene (BaA), Chrysene (Chry), Benzo(b)fluoranthene (BbF), Benzo(k)fluoranthene (BkF), Benzo(a)pyrene (BaP), Dibenzo(a,h) anthracene (DahA), Benzo(g,h,i)perylene (BghiP) and Indeno(1,2,3-cd)pyrene (IcdP), was purchased from Supelco (Bellefonte, PA, USA). Deuterated Benzo(a)pyrene Internal Standard (IS) was purchased from HPC Standard GmbH (Cunnersdorf, Germany). Stock standard solutions were prepared by dissolving the reference standard in acetonitrile and stored in a freezer at -20°C. Ethyl acetate and toluene were purchased from Honeywell Specialty Chemicals (Seelze, Germany), water was purchased from VWR International (Leuven, Belgium). Solvents used were HPLC grade.

The simplified Small Volume Liquid-Liquid Extraction (SV-LLE) method was used for sample preparation (*ISO*, 2005). A standard solution of PAHs was prepared in acetonitrile (ACN). Serial dilutions were made to achieve concentrations of 1, 10, 20, 50, and 100 µg/L and were prepared in two solvents, ethyl acetate and toluene. In order to test the EE, two PAH concentration levels were prepared, 10 and 100 µg/L in water. For each extration, aqueous PAH solution (250 µL) was dispensed into a 2 mL screw top vial and extracted two times with 1 mL of extraction solvent. The vials prepared in this way were vortexed for 1 min, after which organic phase was removed and another portion of extraction solvent was added. After 1 min of vortexing, the second organic phase was added to first one and

then evaporated under a current of N_2. After evaporation, 250 µL of ethyl acetate or toluene was added, as appropriate.

All extracts were analysed using GC-MS/MS. All data were acquired using a Shimadzu GCMS-TQ8050NX gas chromatograph with triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan), with the sampling unit consisting of an autoinjector (AOC-20i) and autosampler (AOC-20s) from the same manufacturer. A fused silica SH-I-5ms capillary column (Length 30 m × I.D. 0.25 mm × film thickness 0.25 µm, Shimadzu, USA) was used for GC separation. The injector operated in spitless mode, injecting 1 µL at 280°C. The oven temperature program was: 40°C (hold for 1.0 min), ramped at 25°C/min to 120°C (no hold), ramped at 10°C/min to 160°C (hold for 10.0 min), ramped at 15°C/min to 220°C (hold for 5.0 min), and ramped at 5°C/min to 325°C (hold for 7.0 min). Helium was used as the carrier gas with flow of 2 mL/min in a linear velocity mode of 51.0 cm/sec. Acquisition mode was MRM. The temperature of transfer line was set to 300°C, ion source temperature was set at 240°C. MRM transitions and collision energies (CE) of analysed compound were set according to *U.S. Environmental Protection Agency - EPA* (2007) and *Wang et al*. (2019).

All measurements were carried out in triplicate. EE was used as a quantitative measure to evaluate the effectiveness of an extraction process in isolating PAHs from water. EE was calculated as the ratio of the concentration of the analyte in the extract to its initial concentration in the sample, multiplied by 100 to express the result as a percentage (*European Commission Directorate-General for Health and Food Safety*, 2023).

$$Extraction\ Efficiency = \frac{C_{ex}}{C_{ini}}\ x\ 100$$

where C_{ex} is concentration of PAHs in the extracted organic phase and C_{ini} is initial concentration of PAHs in the water sample before extraction.

Statistically significant differences between the EE of the two solvents were tested by two-way ANOVA with factors (solvent and concentration) using Minitab 17.

The AGREE metric was used to evaluate the methods greenness, and it was calculated by AGREE software (*Pena-Pereira et al*., 2020). This green chemistry assessment tool based on the 12 principles of green analytical chemistry (sample treatment, sample amount, device positioning, sample preparation procedure, degree of automation, derivatization, amount of waste, number of analytes determined, energy consumption, source of reagents, toxicity, operator's safety). A standardized approach was used to compare the ecological impact of different extraction procedures quantitatively. It is visually represented as circular clock-like diagram, where each hour position (1 to 12) around the edge corresponds to one of the 12 principles of green analytical chemistry. AGREE scores from 0 to 1 are, respectively, not green at all and fully green (*Pena-Pereira et al*., 2020).

3. Results and discussion

In this study, toluene and ethyl acetate were evaluated as extraction solvents for PAHs in water samples at two concentration levels (10 µg/L and 100 µg/L). The results of PAH EE with two different extraction solvents at two concentration levels are shown in Table 1. Two-way ANOVA was performed for each PAH to test the effects of solvent, concentration, and their interaction on extraction recovery. Table 1 summarizes the p-values for all tested PAHs. There was no statistically significant difference in recovery between toluene and ethyl acetate, between low and high concentration levels at a confidence level of 95%, using the previously described method of extraction and in a water matrix (i.e., in samples that were not loaded with matrix compounds). When the interaction term was not statistically significant ($p > 0.05$), there was no indication that the solvent's influence fluctuated with concentration. This means that variations among solvents were uniform across both concentration levels, while variations among concentrations were uniform across both solvents. Under these circumstances, the primary impacts of solvent and concentration were analysed directly.

The EE was higher than 70% for all analytes and in both solvents, except for naphthalene, probably due to its high volatility. The lowest EE was observed for naphthalene at the lower concentration level when using ethyl acetate as the extraction solvent. The highest EE at the lower spiking level was recorded for benzo(b)fluoranthene with ethyl acetate, while for toluene, the highest EE was 86.9% for both benzo(a)pyrene and benzo(a)anthracene. At the higher spiking level, the lowest EE was again observed for naphthalene, with a value of 65.6% and 70.6, in ethyl acetate and toluene, respectively. The highest EE at this level was obtained for pyrene when using toluene, and for chrysene when using ethyl acetate,

reaching 94.1%. For the lower PAHs, compounds with 2-3 rings in their structure, we detected lower EEs than in higher PAHs (four and more rings), probably due to the number of rings and its correlation to the higher volatility of those compounds (*Satouh et al*., 2021).

In this study, we decided to readjusts weights (Figure1) for criteria number 10 (source of reagents, is it possible to be renewable) and number 12 (operator's safety), so the weights of these criteria were set to four due to their importance and ability to differentiate chromatographic methods. Based on the AGREE evaluation, ethyl acetate with a score of 0.56 demonstrated a more favorable environmental and analytical greenness profile than toluene, scoring 0.37. While neither solvent achieved optimal green chemistry standards (score closer to 1.0), the higher-scoring solvent aligns better with the principles of green analytical chemistry, particularly in terms of lower toxicity, biodegradability and safety.

Table 1. Extraction efficiency of poly aromatic hydrocarbons (%) dissolved in water using two different solvents at two different levels

No.	Compound	$Spike_t$ 10	$Spike_{EA}$ 10	$Spike_t$ 100	$Spike_{EA}$ 100	$p\text{-value}_S$	$p\text{-value}_c$	$p\text{-value}_{cxs}$
1	Naph	67.4 ± 15.0	63.5 ± 4.4	70.6 ± 9.9	65.6 ± 6.5	0.122	0.246	0.520
2	Acy	73.6 ± 9.1	72.9 ± 8.8	72.2 ± 6.0	74.5 ± 4.8	0.872	0.753	0.993
3	Ace	75.3 ± 12.5	73.7 ± 7.8	76.1 ± 5.9	75.4 ± 3.2	0.743	0.737	0.896
4	Flu	77.9 ± 4.3	78.2 ± 9.4	70.3 ± 12.4	79.1 ± 3.4	0.102	0.464	0.120
5	Phen	74.3 ± 4.8	77.8 ± 7.4	72.1 ± 7.4	81.2 ± 4.1	0.071	0.847	0.377
6	Ant	75.3 ± 5.8	81.2 ± 4.8	82.0 ± 3.6	83.7 ± 8.2	0.233	0.153	0.492
7	Flt	80.9 ± 6.1	80.1 ± 9.7	79.6 ± 6.2	89.2 ± 4.0	0.149	0.192	0.092
8	Pyr	81.0 ± 7.4	87.3 ± 6.8	94.4 ± 4.9	89.4 ± 5.5	0.835	0.052	0.096
9	BaA	86.9 ± 9.5	85.5 ± 6.7	83.8 ± 10.5	80.9 ± 9.0	0.519	0.267	0.824
10	Chry	84.4 ± 2.5	90.8 ± 6.3	83.3 ± 5.9	94.1 ± 8.4	0.088	0.819	0.635
11	BbF	81.6 ± 9.9	95.1 ± 8.3	87.1 ± 4.7	89.2 ± 4.1	0.786	0.112	0.786
12	BkF	81.1 ± 7.7	89.4 ± 8.5	83.6 ± 7.4	88.6 ± 4.2	0.094	0.815	0.660
13	BaP	86.9 ± 3.2	84.0 ± 6.7	88.5 ± 8.6	84.6 ± 9.5	0.376	0.781	0.896
14	DahA	80.1 ± 10.1	81.9 ± 9.3	86.2 ± 10.6	86.2 ± 6.2	0.838	0.283	0.843
15	IcdP	82.4 ± 9.7	80.9 ± 8.1	85.5 ± 8.1	85.9 ± 6.0	0.103	0.345	0.537
16	BghiP	83.7 ± 6.7	80.5 ± 7.7	81.5 ± 7.3	81.8 ± 4.2	0.074	0.200	0.363

Legend: T – Toluene; EA – Ethyl acetate; ± Standard deviation; s - solvent, c - concentration, cxs – concentration × solvent. The PAH compounds are listed in Materials and Methods; p-values from two-way ANOVA regarding the effects of solvent type (*Solvent*), concentration level (*Concentration*), and their interaction *Solvent* × *Concentration* on extraction recovery for each PAH. *Solvent* evaluates the overall disparity between toluene and ethyl acetate, averaged across various concentrations. *Concentration* evaluates the total disparity between low (10 µg L^{-1}) and high (100 µg L^{-1}) values, averaged over tested solvents. The *Solvent* × *Concentration* analysis examines if the solvent impact varies with different concentration levels.

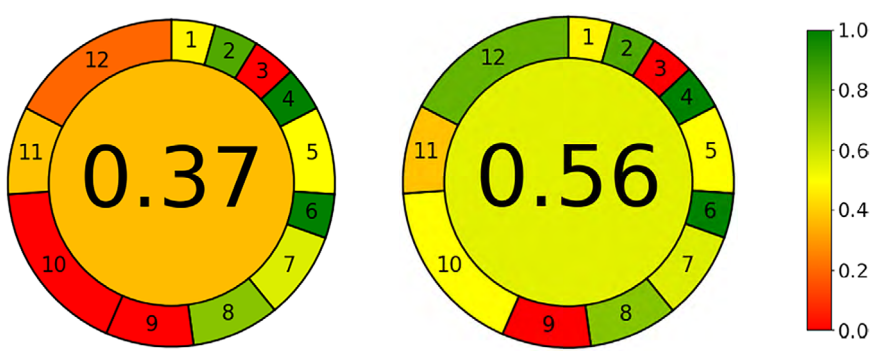

Figure 1. Evaluation of the solvents' greenness using the AGREE method

Therefore, from a sustainability perspective, ethyl acetate, the solvent with the 0.56 AGREE score, is the preferred choice, provided it maintains acceptable analytical performance.

4. Conclusion

PAHs are persistent, bioaccumulative, and toxic pollutants that pose serious risks to human health and ecosystems. Their testing and monitoring are essential for assessing exposure risks, guiding regulatory policies, and implementing effective environmental and public health protections. Continued research and improved analytical methods are key to managing and mitigating the impact of PAHs in our environment.

Quantitatively, both solvents achieve similar extraction efficiencies. However, the AGREE tool clearly favours ethyl acetate due to its better environmental and safety profile. Thus, from a green chemistry standpoint, ethyl acetate is the preferred solvent for sustainable and responsible analytical workflows. Additional investigations are necessary to thoroughly evaluate the EE of green solvents, especially for more complex matrices—such as food products, feed and environmental samples—where matrix effects and co-extractives may considerably affect performance.

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Original Scientific Paper

Utilization of pumpkin seed protein isolate as a phosphate replacer in model meat emulsions: effects on chemical and techno-functional properties

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ABSTRACT

This study aimed to evaluate the potential of pumpkin seed protein isolate (PSPI) as a natural alternative to phosphates in model meat emulsions. PSPI was extracted from cold-pressed pumpkin seed oilcake using an alkaline solubilization and acid precipitation method. Model meat emulsions were formulated by replacing sodium tripolyphosphate (STPP) with PSPI at three levels (1%, 1.5%, and 2%) and compared with a phosphate-containing control group. Chemical composition (moisture, fat, protein, ash) and techno-functional properties, i.e., pH, water-holding capacity (WHC), total expressible fluid, expressible fat (EFAT), jelly-fat separation, and cooking yield were evaluated. Results showed that the addition of PSPI significantly reduced fat content and increased protein content in comparison to the control. The control sample exhibited the highest WHC and the lowest fat and fluid separation. However, increasing levels of PSPI improved WHC and reduced EFAT values, with the 2% PSPI group exhibiting emulsion stability comparable to that of the control group. In line with the stability analyses, the highest cooking yield was observed in the control group, while the lowest value belonged to the P1 group. Although the functional performance of PSPI was slightly lower than that of STPP in some parameters, PSPI demonstrated notable potential in maintaining key quality attributes, such as moisture retention and emulsion stability. In conclusion, PSPI can serve as a functional and natural alternative to phosphates in meat emulsions, contributing to the development of healthier and clean-label meat products.

1. Introduction

Phosphates, commonly used in meat products as sodium and potassium salts of phosphoric acid, serve to enhance water-holding capacity, stabilize pH, control lipid oxidation, and improve texture and flavor in processed meats (*Thangavelu et al*., 2022). Nevertheless, in recent years, changing dietary habits and the increasing awareness of consumers have led to a growing recognition of the relationship between food and health, resulting in an increased demand for "clean label" products. Consequently, phosphates used in meat products are being replaced with various natural alternatives, such as potato starch and rice starch (*Resconi et al*., 2016), winter mushroom (*Choe et al*., 2018), *Jerusalem artichoke* powder and sodium carbonate (Öztürk & *Serdaroğlu*, 2018), eggshell calcium powder and low methoxyl pectin (*Kavuşan et al*., 2021), and seaweed powder dietary

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fiber (*Yuan et al*., 2023), in different kinds of meat products.

Plant-based proteins offer considerable health benefits due to their high nutritional value, low fat absorption, and rich content of branched-chain amino acids (*Xiao et al.*, 2023). They are widely employed as gelling agents to improve the texture of meat products, while their surface-active properties facilitate emulsification and stabilization of fats in processed meats, such as sausages and meatballs (*Ma et al*., 2022). The incorporation of plant-derived proteins, such as pea, flaxseed, pumpkin seed, and sunflower seed proteins, into food products has steadily increased (*Kumar et al.*, 2022; *Langyan et al*., 2022).

Pumpkin seed meal, obtained as a by-product from the extraction of pumpkin seed oil, has attracted attention due to its high protein content of approximately 66.54% *(Gao et al*., 2022a). Pumpkin seed protein is effective as natural functional ingredient in emulsified meat products to improve emulsification, texture, cooking yield, and oxidative stability (*Gao et al*., 2022b; *Tomczyńska-Mleko et al*., 2023; *Rong et al*., 2025). Despite these benefits, studies on the use of pumpkin seed protein isolate (PSPI) in meat products remain limited (*Gao et al*., 2022b; *Baig et al*., 2023). The use of plant protein isolates, such as soybean in chicken sausages (*Muguruma et al*., 2003), pea in beef sausages (*Gomezulu et al*., 2022) and in hybrid meat products (*Wang et al*., 2025), and chickpea in pork meat batters (*Broucke et al*., 2025), as phosphate replacers has been investigated. There is no research available on the use of PSPI for this purpose. Accordingly, this study aims to address the existing gap in the literature by investigating the potential of PSPI as a phosphate replacer. For this purpose, the effects of incorporating PSPI at different levels (1%, 1.5%, and 2%) are based on the equivalent amount of sodium tripolyphosphate (STPP) on the chemical and techno-functional properties of model system meat emulsions.

2. Materials and methods

2.1. Materials

Pumpkin seed (*Cucurbita pepo*) oilcake obtained by cold pressing was kindly donated by Mecitefendi Herbal Products Food Industry and Trade Limited Company (Izmir, Turkey). Beef (72.21% moisture, 22.1% protein, 4.5% fat, 1.19% ash, pH: 5.6) and beef fat were purchased from Dincer Meat & Steakhouse (Tekirdag, Turkey). All the chemicals were of analytical grade (Sigma-Aldrich Chemie GmbH, Germany) and used without purification.

2.2. Production of pumpkin seed protein isolate

PSPI was prepared with modifications based on the method of *Boye et al*. (2010). Pumpkin seed oilcake was milled (60 mesh) and defatted with hexane (1:5, w/v). The defatted flour was dispersed in water (1:20, w/v), homogenized (UltraTurrax, 12000 rpm, 5 min), and the pH was adjusted to 8.5 using 1 M NaOH. After stirring (25 °C, 200 rpm, 65 min), the suspension was centrifuged (4000 rpm, 15 min, 25 °C). The supernatant was acidified to pH 4.5 (1 M HCl) and centrifuged again. The resulting protein precipitate was washed twice (distilled water, 1:2, w/v), freeze-dried (-55 °C, 0.250 mBar), and stored at -20 °C until use. The chemical composition of PSPI was 7.47% moisture, 85.45% protein, 5.72% fat, and 1.36% ash.

2.3. Production of model system meat emulsion and experimental design

For the model meat emulsions, the control (C) sample was prepared using 500 ppm sodium tripolyphosphate (STTP), while the other treatment groups were formulated with 1% (P1), 1.5% (P2), and 2% (P3) PSPI as a replacement for STTP (Table 1). Model meat emulsions were produced according to *Yüncü-Boyacı et al*. (2024). Firstly, beef and beef fat were minced separately using a 3 mm plate grinder (Arnica, Turkey). Minced meat was then homogenized in a Thermomix (TM5, Vorwerk, Germany) at 500 rpm for 1 min. Curing agents (salt, sodium nitrite) were added and mixed at 500 rpm for 2 min. Fat, PSPI, and/or STPP, and half of the ice were added and emulsified at 1100 rpm for 3 min, followed by the addition of the remaining ice and further emulsification for another 3 min. The final batter was homogenized at 2000 rpm for 1 min, maintaining the temperature below 10 °C throughout. Portions (30 g) were filled into 50 mL polypropylene tubes and centrifuged (Nüve NF 400, Turkey) at 2500 g for 15 min to eliminate air bubbles. Model emulsions were cooked in a water bath at 70 °C for 30 min, then immediately cooled. All analyses were conducted within 72 h of production.

Table 1. Formulation of model meat emulsions

Ingredients (%)	Treatments*			
	C	P1	P2	P3
Beef	68	68	68	68
Beef fat	20	20	20	20
Ice	10	10	10	10
Salt	1.5	1.5	1.5	1.5
Sodium ripolyphosphate (STPP)	0.5	-	-	-
Pumpkin seed protein isolate (PSPI)	-	1	1.5	2
Sodium nitrite	0.015	0.015	0.015	0.015

*C: Model meat emulsions containing sodium tripolyphosphate (500 ppm). P1: Model meat emulsions produced using 1% PSPI. P2: Model meat emulsions produced using 1.5% PSPI. P3: Model meat emulsions produced using 2% PSPI.

2.4. Analyses

Moisture and ash content were measured according to the *AOAC* (2012). Fat content was determined by the chloroform-methanol extraction according to *Flynn and Bramblett* (1975). Crude protein was determined using the Kjeldahl method by multiplying 6.25 (nitrogen-to-protein conversion factor) (*Morr*, 1981). Triplicate measurements of pH values were conducted using a WTW pH 3110 set 2 pH meter from Germany. Water holding capacity (WHC), total expressible fluid (TEF), and expressible fat (EFAT) were determined in triplicate according to *Hughes et al.* (1997) with some modifications. The amounts of separated gel and fat in the meat emulsions were determined using the method of *Bloukas and Honikel* (1992). The cooking yield (CY) was calculated based on the methodology established by *Murphy et al.* (1975). The impact of PSPI on chemical composition and techno-functional properties of model meat emulsions was assessed using analysis of variance (ANOVA) followed by Duncan's post-hoc tests in the SPSS software.

3. Results and discussion

3.1. Chemical composition

Table 2 presents the moisture, fat, protein, and ash contents of the meat emulsion systems. Moisture values indicated that the C, P1, and P3 groups had the highest moisture content, while the P2 group showed a comparatively lower moisture content (62.28%) ($P<0.05$). This situation is explained by the high protein content in this group. The fat content of treatments decreased with the addition of PSPI regardless of the utilization ratio ($P<0.05$). This finding is consistent with the results reported by *Wang et al.* (2025), who studied the reduction of phosphate levels using PSPI. Besides, no statistical differences were observed between P2 and P3 ($P>0.05$). In contrast to the fat contents, the addition of PSPI to the formulation led to an increase in protein content ($P<0.05$); however, no statistical difference was observed between the P2 and P3 ($P>0.05$). This finding can be attributed to the higher protein content of the PSPI (ort protein içeriği). The protein content reported by *Gao et al.* (2022a), in a study utilizing PSPI, was 15.22%, which is lower than the values observed in the current study. Similar to our results, it was determined that the fat content of low-fat duck meat sausages with added soy protein isolate decreased compared to the control group (*Moirangthem et al*., 2022). The C group exhibited the highest ash content (2.25%), while the P3 group (1.81% ash) showed the second highest value among all treatments ($P<0.05$). On the contrary, some researchers have reported that the addition of pea protein isolate or rice protein to beef patties, and of soy protein isolate to low-fat duck meat sausages, did not result in a significant difference in ash content compared to the control groups (*Baugreet et al.,* 2016; *Moirangthem et al*., 2022). The pH values, which have a significant impact on the technological properties of meat products, are presented in Table 2. The pH value of the treatments decreased with the addition of PSPI to the meat emulsions, and the highest pH value was observed in group C containing STPP ($P<0.05$). On the other hand, no statistical differences were observed between the P2 and P3 groups ($P>0.05$). In a study by *Öztürk and Turhan* (2020), pumpkin seed kernel flour was used as a fat replacer in beef meatballs, and the pH values ranged from 5.98 to 6.22. These values are consistent with those obtained in the present study.

Table 2. Chemical composition and pH value of model meat emulsion batters

Treatments*	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	pH
C	64.14±0.72^{a}	20.39±0.99^{a}	12.56±1.31^{c}	2.25±0.22^{a}	6.20±0.01^{a}
P1	65.61±0.95^{a}	16.22±0.20^{b}	16.86±0.66^{b}	1.31±0.48^{b}	5.90±0.01^{b}
P2	62.28±0.14^{b}	14.85±0.37^{c}	21.42±0.35^{a}	1.45±0.11^{b}	5.88±0.01^{c}
P3	64.11±1.08^{a}	14.18±0.70^{c}	19.90±0.96^{a}	1.81±0.01^{ab}	5.88±0.01^{c}

*C: Model system meat emulsion containing sodium tripolyphosphate (500 ppm), P1: Model system meat emulsion formulated with 1% pumpkin seed protein isolate, P2: Model system meat emulsion formulated with 1.5% pumpkin seed protein isolate, P3: Model system meat emulsion formulated with 2% pumpkin seed protein isolate. $^{a-d}$ Different letters in the same row indicate a significant difference ($P < 0.05$).

3.2. Techno-functional properties

Water-holding capacity (WHC) is a vital quality parameter in the meat industry, as it directly influences tenderness and juiciness—two essential attributes that determine consumer acceptance of meat products (*Yüncü-Boyacı et al*., 2024). The highest WHC was observed in the C group (81.55%), which contains STPP ($P<0.05$). In the other groups, a significant increase in the proportion of PSPI incorporated into the formulation resulted in a progressive enhancement of the WHC values of the meat emulsions ($P<0.05$). In a study conducted by *Helikh and Yongfeng* (2025), the WHC was reported as 74.2%, which closely aligns with the findings of the present study that utilized PSPI. TEF values ranged between 15.40% (C) and 29.92% (P1). These results indicate that the TEF value of treatments increased with the addition of PSPI ($P<0.05$). Similarly, in a study by *Wang et al.* (2025), where phosphate was replaced with modified chickpea protein in pork emulsions, the total released fluid ranged from 13.29% to 21.79%, and consistent with the present findings, the control group containing phosphate showed the lowest fluid release. Similar to the TEF value, the highest EFAT value (6.24%) among the experimental meat models was observed in our P1 group, which contained 1% PSPI ($P<0.05$). On the other hand, the lowest values (ranging from 2.73% to 3.23%) were recorded in the C and P3 groups ($P<0.05$). Therefore, it can be concluded that incorporating PSPI at levels above 2% improves emulsion stability, exhibiting an effect comparable to that of STPP. Jelly and fat separation of meat emulsions were significantly affected by the replacement of STPP with PSPI (Table 3). While the lowest value was observed in the C group, reformulated samples had higher values ($P<0.05$). Besides that, no statistical differences were observed between the reformulated samples ($P>0.05$). This result was consistent with the WHC, TEF, and EFAT values. Compared to the study conducted by *Helikh and Yongfeng* (2025), which involved the addition of PSPI, the fat holding capacity (73.8%) reported in their work was higher than that observed in the present study. In terms of cooking yield, the highest value was observed in our control group with 85.28% ($P<0.05$). In the groups formulated with PSPI, cooking yields decreased, with the lowest cooking yield detected in the P1 group at 63.09% (Table 3). This indicates that increasing PSPI levels led to a partial improvement in cooking yield.

Table 3. Techno-functional properties of model system meat emulsions

Treatments*	WHC (%)	TEF (%)	EFAT (%)	Jelly and Fat Separation (%)	Cooking Yield (%)
C	81.55±0.92^{a}	15.40±0.63^{c}	2.73±0.14^{c}	9.50±1.00^{b}	85.28±1.48^{a}
P1	72.45±1.01^{d}	29.92±1.47^{a}	6.24±0.57^{a}	21.25±0.25^{a}	63.09±0.87^{c}
P2	74.34±0.92^{c}	26.23±0.29^{b}	4.88±0.48^{b}	20.83±1.26^{a}	68.50±0.90^{b}
P3	75.99±0.38^{b}	25.01±0.56^{b}	3.23±1.11^{c}	22.04±0.71^{a}	68.10±0.69^{b}

Legend: *C: Model system meat emulsion containing sodium tripolyphosphate (500 ppm), P1: Model system meat emulsion formulated with 1% pumpkin seed protein isolate, P2: Model system meat emulsion formulated with 1.5% pumpkin seed protein isolate, P3: Model system meat emulsion formulated with 2% pumpkin seed protein isolate. WHC: Water holding capacity; TEF: Total expressible fat, EFAT: Expressible fat. $^{a-d}$ Different letters in the same row indicate a significant difference ($P < 0.05$).

4. Conclusion

The findings of this study demonstrate the potential of PSPI as a promising phosphate replacer in meat emulsions. Incorporating PSPI at increasing levels led to significant reductions in fat content and increases in protein content. However, replacing sodium tripolyphosphate (STPP) with PSPI resulted in decreased WHC, TEF, cooking yield, and jelly and fat separation values in the model meat emulsions. Although the reformulated samples showed slightly lower performance than the phosphate-containing control group in some functional parameters, the use of 2% PSPI exhibited comparable effects to STPP, particularly in terms of TEF. These results highlight the potential of PSPI to serve as a clean-label alternative to phosphates in meat products. Further studies may focus on optimizing PSPI functionality through protein modification techniques and evaluating its effects on sensory attributes and shelf-life characteristics in real product formulations.

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Original Scientific Paper

Survey on Serbian consumer acceptance of edible insects as alternative source of animal proteins

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ABSTRACT

This study aimed to evaluate the state of public perception of edible insects as an alternative source of animal proteins as part of the human diet in Serbia. Data were gathered via an online survey involving 1,845 participants who completed a Google Forms questionnaire shared via social media channels. The findings indicate that 37.8% of participants expressed a willingness to consume edible insects. In comparison, only 8.5% of respondents reported having previously consumed edible insects in some form (whole, processed as flour, or in dishes). Logistic regression analysis revealed that women were more open to the possibility of consuming edible insects as food compared to men. Additionally, 57.1% of participants selected grasshoppers as the most suitable edible insect for consumption. The majority of respondents preferred processed insects or dishes made from insects as the most favourable way to consume them, rather than whole insects. Regarding the age of the participants and willingness to consume edible insects, no statistical significance was determined between different age class types. In conclusion, this study revealed that the population in Serbia shows a low readiness to consume edible insects as an alternative source of animal proteins. Furthermore, this study demonstrated a higher acceptability of processed insects as food, which could be very useful for promoting specific market strategies in the future.

1. Introduction

Edible insects are a highly promising and sustainable alternative source of animal protein (*Zhou et al.,* 2022). The *Coleoptera* order represents the most widely consumed group of insects worldwide, accounting for 31% of total insect consumption. Additionally, the *Lepidoptera* order contributes significantly, representing 18% of global consumption (*Tuhumury et al.,* 2021). Entomophagy, the practice of consuming insects, is primarily associated with cultures in Asia, Africa, and Latin America (*Sikora and Rzymski,* 2025). In recent years, this trend can also be observed in European Union (EU) countries (*Čobanović and Pajičić*, 2024). According to several authors (*Bbosa et al.,* 2019; *Mancini et al.,* 2022*; Tuhumury et al.,* 2021; *Zhou et al.,* 2022), edible insects and their products are nutritionally valuable foods containing high levels of proteins, essential fatty acids, fats, polyunsaturated fatty acids, vitamins, and minerals (*Čobanović and Pajičić*, 2024). These nutrients offer various beneficial properties for the human body compared to conventional meat types (*Zhou et al.,* 2022). Compared to traditional livestock farming, insect farming offers several benefits: (i) lower greenhouse gas and ammonia emis-

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sions, (ii) less land use, (iii) minimal water requirements, and (iv) efficient, cost-effective nutrition (*Čobanović and Pajičić*, 2024; *Mancini et al.,* 2022; *Zhou et al.,* 2022).

The first studies to assess willingness to buy and eat edible insects were based on data gathered from Dutch (*Lensvelt and Steenbekkers*, 2014; *Pascucci and de Magistris*, 2013) and Belgian (*Capparos Megido et al*., 2014) consumers. In Serbia, there are no registered producers of edible insects, and the sector lacks specific legislation regulating it. Current legal acts, such as the Law on Food Safety (*Official Gazette*, 2019a) and the Law on Veterinary Matters (*Official Gazette*, 2019b), do not include reared insects within the category of farmed animals, nor do they recognize edible insects as acceptable food for consumers. However, as Serbia works towards EU membership, it will have to align its laws with those of the EU; thus, there will be a need to regulate the edible insect market and insect-based food. Therefore, this study aimed to assess the public perception of edible insects as an alternative source of animal protein in the human diet in Serbia.

2. Materials and methods

2.1 Data collection, survey and sample

Data for this study was collected via an online survey form. The survey was conducted between 2024 and 2025 via the Google Forms platform. Permission was obtained from the Ethics Committee (01-05/2024) for conducting the survey. The unique URL for the survey was shared across various social media channels, including Instagram, Facebook, WhatsApp, Reddit, and Discord. Participation in the survey was voluntary, with no financial compensation offered for completion. The survey was completed only by individuals older than 18 years of age who hold Serbian citizenship or who declared themselves as Serbian. The first section of the survey included questions designed to gather general information about respondents, such as their age and gender. The second section investigated opinions regarding the consumption of edible insects, intending to determine whether respondents would consume them and, if so, which edible insects they would prefer. In the third section, respondents could write their opinion in open form on whether edible insects can be a good alternative source of animal proteins. One thousand eight hundred forty-five individuals filled out the survey. Respondents who answered "No" to the question "Would you consume insects?" did not fill in the rest of the questions. Instead, they were directed to the end section of the survey.

2.2 Data analysis

Participant responses to the survey, collected via Google Forms, were saved in Google Sheets. Afterward, the data were exported and analyzed using IBM SPSS Statistics Version 27 (Armonk, New York, USA). The participants' answers to the questions in the first section of the survey are presented in the form of frequencies. A logistic regression analysis was conducted to determine whether willingness to consume edible insects was influenced by factors such as age and gender. Statistical significance was established at $P < 0.05$.

3. Results

General information about the participants, including their gender and age, is presented in Table 1. The study had a higher proportion of female participants and a younger population (<40 years of age). When asked about their lifetime consumption of edible insects, 156 participants (8.5%) reported having consumed them, whereas 1,689 participants (91.5%) reported no such experience. When asked about their knowledge of entomophagy, 860 participants (47.0%) responded affirmatively, while 971 participants (53.0%) reported being unfamiliar with the term.

Regarding their willingness to consume edible insects, 697 respondents (37.8%) indicated that they

Table 1. General information about participants.

Gender	
Male	780 (42.3%)
Female	1065 (57.7%)
Total	1845 (100%)
Age	
<30	828 (44.9%)
31 – 40	715 (38.8%)
41 – 50	167 (9.1%)
51 – 60	76 (4.1%)
>60	59 (3.2%)
Total	1845 (100%)

would consider doing so (Table 2). Among these, 398 participants (57.1%) selected grasshoppers as their preferred choice. Furthermore, they were also asked what they preferred more: products (processed insects) or whole insects, and 64.4% of participants chose products as their preferred choice. The results of the logistic regression analysis revealed a statistically significant difference concerning gender and the willingness to consume edible insects (Table 3). Male participants were 53% less likely to consume edible insects in comparison to female participants. However, no statistically significant differences were observed regarding age ($P > 0.05$).

In Figure 1, the positive and negative attitudes of survey participants towards edible insects are summarized. In most cases, the main negative attitudes towards edible insects originated from religion or tradition, while positive attitudes resulted from their high nutritive value and reduced environmental pollution.

Table 2. Participants' answers to the questions "Have you ever consumed insects?" and "Would you consume insects?".

Have you ever consumed insects?			Would you consume insects		
Gender	*Yes*	*No*	***Gender***	*Yes*	*No*
Male	91 (11.7%)	689 (88.3%)	**Male**	375 (48.1%)	405 (51.9%)
Female	65 (6.1%)	1000 (93.9%)	**Female**	322 (30.2%)	743 (69.8%)
Total	156 (8.5%)	1689 (91.5%)	**Total**	697 (37.8%)	1148 (62.2%)

Table 3. Influence of gender and age on willingness to consume edible insects.

Variable	**B**	**S.E.**	**Wald**	**OR**	**95% C.I. for EXP(B)**		**Sig.**
					Lower	*Upper*	
Gender							
Female	0.000	0.000	0.000	1.000	-	-	< 0.001
Male	-0.759	.098	60.117	0.468	0.386	0.567	< 0.001
Age							
<30	0.000	0.000	0.000	1.000	-	-	0.447
31 – 40	-0.101	0.105	0.921	0.904	0.735	1.111	0.337
41 – 50	-0.264	0.172	2.338	0.768	0.548	1.077	0.126
51 – 60	-0.193	0.244	0.622	0.825	0.511	1.331	0.430
>60	0.179	0.288	0.387	1.196	0.680	2.103	0.534

4. Discussion

The findings of this research indicate that the population in Serbia is reluctant to accept entomophagy, as 62.2% of surveyed participants declared an unwillingness to consume edible insects. These observations align with the results of other studies conducted in European countries (*Mandolesi et al*., 2022; *Ribeiro et al*., 2022) and previous studies in Serbia regarding the willingness to purchase edible insects (*Andric et al*., 2023). In the present study, age was not a significant factor in determining willingness to consume edible insects, which was contrary to the findings reported by other authors (*Andric et al*., 2023; *Cicatiello et al*., 2016; *Hartmann et al*., 2015; *Lammers et al*., 2019).

However, in contrast to other research studies (*Cicatiello et al*., 2016; *Hartmann et al*., 2015; *Lauerati et al*., 2016; *Tan et al*., 2016; *Verbeke*, 2015; *Videbaek and Grunert*, 2020), female participants in this study showed a greater willingness to consume edible insects. This outcome could be attributed to more traditional attitudes among men in Serbia, including social norms and acceptability, as well as the larger sample size of surveyed male consumers. In agreement with earlier investigations regarding insects as food (*Andric et al*., 2023; *Bartkowicz and Babicz-Zielińska*, 2020; *Grasso et al*., 2019; *Orsi et al*., 2019; *Van Thielen et al*., 2018; *Tuccillo et al*., 2020; *Wilkinson et al*., 2018), the majority of respondents willing to consume edible insects preferred processed insect products over whole insects,

likely due to the reduced visual impact, making them more acceptable from a sensory perspective. This discovery is consistent with the predominantly negative consumer perceptions of edible insects, which are frequently influenced by cultural or religious beliefs. Notably, the novelty and exoticism of edible insects significantly shape consumer attitudes, acting as both a catalyst for acceptance and a barrier to adoption. Although this study provides valuable insights into consumer perceptions of edible insects as an alternative source of animal protein and novel food, its limitations should also be acknowledged. Namely, this study was based on an online survey, which does not exclude volunteer bias, and it cannot be confirmed that declarations made about consuming edible insects will correlate with the actual choices of consumers in everyday life.

5. Conclusion

This study demonstrates that Serbian consumers exhibit a low willingness to accept edible insects as a part of their diet, with religion, culture, and concerns over safety and sensory properties as the primary barriers. Informing consumers about the advantages of edible insects as a food source could increase overall acceptability in Serbia. To further foster acceptance, it is crucial to promote insect-based foods, such as protein powder and flour, which mitigate disgust associated with whole insects as food while offering versatility in the application of edible insects as additives in regular food. Marketing strategies should focus on countering misconceptions while further emphasizing the nutritional and environmental benefits of edible insects and slowly normalizing them as part of a daily diet.

Figure 1. Consumer attitudes towards edible insects as food.

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Original Scientific Paper

Determination of perfluorinated substances (PFAS) in muscle tissue from wild boar from three districts of Serbia – Zlatibor, Pčinj and Raška

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ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are recognized as significant environmental pollutants due to their widespread industrial use and persistence. Wild boars are known bioindicators of environmental contamination with PFAS, as they accumulate these compounds through their omnivorous diet and foraging behaviour. In this study, muscle tissue samples (n = 15) from 15 respective wild boars were collected during the 2024–2025 hunting season as part of the Serbian National Residue Monitoring Program. Samples were obtained from three Serbian districts—Zlatibor, Pčinj, and Raška—and analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). PFAS compounds were detected in 9 of the 15 wild boars, with total PFAS concentrations ranging from <0.170 to 3.179 µg/kg and an average concentration of 1.030 µg/kg. The results suggest regional differences in PFAS exposure and confirm the suitability of wild boar as a bioindicator species for PFAS contamination in Serbia.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are anthropogenic synthetic chemicals, that number over 4,000, and they are use extensively throughout the world in many industrial sectors. They are resistant to degradation at high temperatures, and are water, oil and dirt repellent (*Buck et al., 2011*). Also, they resist biodegradation, photooxidation, and hydrolysis due to the strength of the carbon-fluorine bond (*Sznajder-Katarzyńska et al.,* 2019).

In the latest scientific opinion of the European Food Safety Agency (EFSA), four PFAS compounds were assessed as toxic—perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorohexanesulfonic acid (PFHxS) and perfluorooctanesulfonic acid (PFOS). This requires countries to eliminate or reduce the release of these chemicals into the environment. (*European Food Safety Authority,* 2020).

Because wild boars are omnivores and search for food by burrowing into the ground, it is assumed that wild boar meat is most likely contaminated with PFAS compounds (*Food and Agriculture Organization of the United Nations,* 2018). Over the past twenty years, various studies have shown high lev-

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els of PFAS compounds in the tissues and organs of wild boars across Europe, especially in the regions of Central Europe (*Kowalczyk et al.,* 2018; *Stahl, et al.,* 2012; *Felder, et al.,* 2023; *Schröder et al.,* 2024; *Arioli et al.,* 2019; *Death et al.,* 2021). In Hesse, Germany, 506 muscle tissue samples from wild boars were tested for the presence of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). PFOA concentrations ≤ 7.4 µg/kg and PFOS concentrations ≤ 28.6 µg/kg were detected in muscle tissue. The concentration of PFOA was lower than the LOQ of 1 µg/kg in 456 samples (90.1%), a majority of all of the samples examined. PFOS concentrations lower than the LOQ were found in 329 of the 506 (65%) wild boar muscle samples. In 2019, PFOS was detected in 25% of muscle samples from wild boar collected in North West Italy at concentrations lower than those reported from Germany (*Arioli et al.,* 2019).

In Regulation of the European Union Commission from December 7 2022 (*European Commission,* 2022), the maximum permissible amounts for four PFAS compounds (PFOS, PFOA, PFNA, PFHxS) in meat of wild animals (except bear meat) were prescribed individually as well as the aggregate value, and they are an integral part of the regulations of the Republic of Serbia (*Official Gazette of the Republic of Serbia,* 2024). The maximum levels (applies to the sum of linear and branched stereoisomers, whether they are chromatographically separated or not) in meat of game animals are: PFOS – 5.0 µg/kg, PFOA – 3.5 µg/kg, PFNA – 1.5 µg/kg, PFHxS – 0.6 µg/kg and sum of all four PFAS – 9.0 µg/kg. For the sum of PFOS, PFOA, PFNA and PFHxS, lower bound concentrations are calculated on the assumption that all the values below the limit of quantification are zero.

The aim of this study was to determine the concentration of four PFAS compound (PFOS, PFOA, PFNA and PFHxS) in tissue (leg muscle) of wild boars in three districts of Serbia, Zlatibor, Pčinj and Raška, and compare the results. Zlatibor and Raška districts border each other, while the Pčinj district is about 300 km away from Zlatibor. This is the first study in Serbia to examine the concentration of PFAS in game meat.

2. Materials and methods

2.1. Sampling

PFAS levels were measured in tissue samples from wild boars in the period of one hunting season, within the framework of the Serbian National Residue Monitoring Program (autumn-winter 2024 -2025). The total number of wild boars analysed was 15, with one tissue sample per animal. Wild boar tissue samples were stored at -18°C. Frozen samples were thawed at 4°C one day before the analysis and subsequently homogenized.

2.2. Reagents and standards

All solvents used were of HPLC grade—acetonitrile (Honeywell, Germany), methanol (Honeywell, Germany), water (Honeywell, Germany), ammonium acetate (Sigma Aldrich, Germany) and a QuEChERS kit (Phenomenex, USA). PFAS standards were of analytical purity manufactured by Wellington Laboratories Inc., Canada. Native Perfluorinated Compound Solution/Mixture was used as a calibration standard and Mass-Labelled PFAS Extraction Standards Solution was used as an internal standard. As a control, FAPAS QC Material /Fish/T0687QC and FAPAS QC Material /Dried Egg/T06142QC (FAPAS, UK), were used.

2.3. Sample preparation and measurement

During the development of the method and sample extraction plan, the guidelines stated in the works that dealt with the topic of PFAS compound analysis in samples of animal origin were taken into account (*EURL for Halogenated POPs in Feed and Food,* 2022; *Kowalczyk et al.,* 2018). Homogenized sample was weighed into a polypropylene centrifuge tube. Acetonitrile and internal working standard were added. The tube was centrifuged and the entire supernatant was transferred to a polypropylene tube. QuEChERS were added, and the tube was shaken vigorously. The tube was again centrifuged and the supernatant was transferred to a glass cuvette, and evaporated to dryness under a stream of nitrogen at 50 °C. The dry residue was dissolved in acetonitrile and vortexed. The sample was transferred to a polypropylene cuvette and centrifuged. The supernatant was transferred to an HPLC vial.

2.4. Instrumental analysis

Boar tissue samples, which were previously determined to be free of PFAS compounds, were used as blank samples, calibration and spiking. Four blank samples of muscle tissue were spiked with PFAS calibration working standard, within the cali-

bration range of 0.1 to 5 µg/L. One blank sample was spiked with PFAS calibration working standard corresponding to concentrations of 2 µg/L (QC spike).

Analysis was performed by LC-MS/MS with a mass detector, using an HPLC device and a mass detector LCMS-8050 (Shimadzu, Japan). LC-MS/MS system equipped with RP Kinetex C18 column 100 mm × 2.1 mm, particle size 2.6 µm, and PFAS Delay Column Restek 50 × 2.1 mm, particle size 5 µm. The chromatographic separation was performed on a gradient with 2mM ammonium acetate in water/acetonitrile, 95%:5%, v/v, and 10% of acetonitrile/methanol, 60:40, v/v, as mobile phases.

2.5. Quality control

The results for QC materials were within the range of certified values, and the results for QC spike were within the range of 80-120%. The limit of quantification (LOQ) obtained in the validation process was 0.1 µg/kg.

3. Results and discussion

Tissue samples from Zlatibor District (numbers 1 to 6) and Pčinj District (numbers 7 to 12) came from sows about 4-5 years old and that weighed between 70 and 80 kg. Tissue samples from Raška District (numbers 13 to 15) came from young wild boars, about 2 years old and that weighed around 40 kg. The results are presented in Table 1.

In samples 1, 2, 3 and 6 from Zlatibor District, only PFOS was detected out of the four PFAS compounds. Accordingly, concentrations of PFOS in these samples was equal to the concentrations of total PFAS, and they ranged from 0.170 µg/kg to 0.267 µg/kg. In samples 4 and 5, no PFAS compounds were detected.

In the samples from Pčinj District, PFOS was detected in samples 7, 8, 9, 11 and 12, PFOA in samples 7, 8, 9 and 11, and PFNA in samples 7, 9 and 11. PFHxS was not detected in any wild boar from Pčinj District. The concentrations of total PFAS in tissue samples from Pčinj District were higher than in those from Zlatibor District, due to the higher concentration of PFOS that ranged from 0.216 µg/kg to 0.675 µg/kg, but also because of presence of PFOA and PFNA that were not detected in the wild boar from Zlatibor District (Figure 1). In wild boar from Pčinj District, the concentration of PFOA was the highest of all the PFAS compounds, as it ranged from 0.355 µg/kg to 1.909 µg/kg, and it had the greatest impact

Table 1. PFAS concentrations in tissue of wild boars from three districts in Serbia. Perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorohexanesulfonic acid (PFHxS) and perfluorooctanesulfonic acid (PFOS). ND = not detected.

District	Wild boar tissue sample number	Concentration in µg/kg				
		PFOS	PFOA	PFNA	PFHxS	Total PFAS
Zlatibor	**1**	0.170	ND	ND	ND	0.170
	2	0.183	ND	ND	ND	0.183
	3	0.267	ND	ND	ND	0.267
	4	ND	ND	ND	ND	ND
	5	ND	ND	ND	ND	ND
	6	0.207	ND	ND	ND	0.207
Pčinj	**7**	0.586	1.706	0.662	ND	2.954
	8	0.216	0.355	ND	ND	0.571
	9	0.234	0.450	0.160	ND	1.415
	10	ND	ND	ND	ND	ND
	11	0.675	1.909	0.595	ND	3.179
	12	0.321	ND	ND	ND	0.321
Raška	**13**	ND	ND	ND	ND	ND
	14	ND	ND	ND	ND	ND
	15	ND	ND	ND	ND	ND

of the four compounds on the high concentration of total PFAS in these game animals. The concentration of PFNA ranged from 0.160 µg/kg to 0.662 µg/kg. In samples 7, 9 and 11 (from Pčinj District), all three PFAS compounds were detected. The concentration trend was PFOA > PFOS > PFNA, except for sample 7, in which the concentration of PFNA was higher than that of PFOS and accordingly, for this wild boar tissue, the concentration trend was PFOA > PFNA > PFOS.

In wild boar tissue from Raška District, no PFAS compounds were detected.

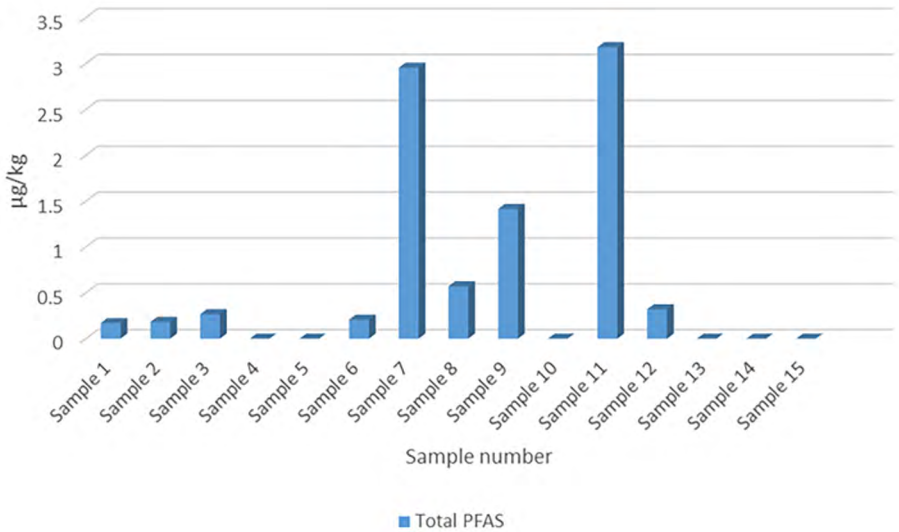

Figure 1. Total PFAS concentrations in wild boar from three districts in Serbia

4. Conclusion

The present study on wild boar leg tissue suggests that wild boars living in Serbia are exposed to PFAS. All obtained concentrations of PFAS compounds were within the limits prescribed by European and Serbian regulatory authorities. Comparing the districts, the highest concentration of PFAS compounds in the boar tissue was found in Pčinj District. Among these samples from Pčinj District, PFOA had the highest concentration of the four studied PFAS compounds. The higher concentration of PFAS compounds in Pčinj District compared to in Zlatibor District can be attributed to greater environmental pollution, due to a higher concentration of industrial plants and larger settlements in the former location.

The absence of PFAS compounds in wild boar from Raška District can be explained by the fact that the wild boars from which the samples were taken were younger than those from Zlatibor and Pčinj Districts, rather than by the absence of environmental pollution.

This study shows that the wild boar can be used as bioindicators of environmental pollution with PFAS in Serbia in future research. The results so far indicate the need for continued monitoring of PFAS compound concentrations in the game meat.

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Review Paper

Review of antimicrobial-resistant ESKAPEE pathogens in food sources and their relevance to the One Health approach

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ABSTRACT

Antimicrobial resistance (AMR) is a growing concern globally, particularly with ESKAPEE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp., and *Escherichia coli*) which have developed multidrug resistance. While traditionally associated with healthcare settings, these pathogens are increasingly being found in food sources, particularly meat products, and agricultural environments. This review explores the mechanisms by which ESKAPEE pathogens acquire antimicrobial resistance, their occurrence in meat and other food products, and the implications for food safety. It also discusses the environmental reservoirs, including water sources, and the challenges posed by the spread of these pathogens within the meat production and processing chains. Surveillance programs monitoring the prevalence of AMR in the food industry, especially in relation to meat, are outlined, highlighting the need for improved detection and control measures. The review emphasizes the importance of a One Health approach, recognizing the interconnectedness of human, animal, and environmental health. Addressing AMR in meat and food production requires coordinated efforts across sectors, including public awareness campaigns and enhanced food safety protocols, to limit the spread of resistant ESKAPEE strains.

1. Introduction

Antimicrobial resistance (AMR) is a natural evolutionary process by which microorganisms adapt to antimicrobial agents (*Fair & Tor*, 2014), but it has become a major global health threat, often referred to as a silent pandemic, with over 700,000 deaths annually (*O'Neill*, 2016). In 2019, AMR was directly responsible for approximately 1.27 million deaths and contributed to nearly 4.95 million additional deaths (*WHO*, 2018). Limited treatment options for infections caused by resistant bacteria further exacerbate the issue (*Magiorakos et al*., 2012). The World Health Organization (WHO) has identified priority pathogens, particularly multidrug-resistant (MDR) Gram-negative bacteria from the ESKAPEE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp., and *Escherichia coli*), as critical threats (*Tacconelli*, 2020; *WHO*, 2023). While multi-drug resistant (MDR) infections

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are commonly associated with hospitals, resistant bacteria are increasingly found in animals, food, and aquatic environments (*Lepuschitz et al*., 2019; *Wyres & Holt*, 2018). Antibiotic use in agriculture promotes resistance development, which can be transmitted to humans through direct contact, contaminated food, or the environment (*Argudín et al*., 2017). Resistance often spreads via horizontal gene transfer through mobile genetic elements (*Gajic*, 2023). Extended-spectrum β-lactamase (ESBL)- and carbapenemase-producing bacteria, once limited to hospitals, are now also detected in animals (*Dahms et al*., 2015; *Michael et al*., 2015), and the emergence of colistin-resistant *Enterobacteriaceae* carrying *mcr* genes raises further concerns (*Liu et al*., 2016).

2. Understanding antimicrobial resistance pathways in ESKAPEE bacteria

Antimicrobial resistance genes can be located on bacterial transposons, plasmids, or the chromosome (*Giedraitienė et al*., 2011). These genes, especially when carried on mobile genetic elements (MGEs), can be acquired by bacteria through horizontal gene transfer or arise through genetic mutations, enabling pathogens, particularly those from the ESKAPEE group, to develop various mechanisms of resistance. ESKAPEE pathogens have developed multiple mechanisms of antibiotic resistance. These mechanisms include: drug inactivation, modification of antibiotic binding sites, active drug efflux via efflux pumps, reduced drug uptake and biofilm production (*Oyenuga et al*., 2024).

2.1 Drug inactivation

Numerous bacterial enzymes are capable of permanently altering or neutralizing the effects of antibiotics. These include aminoglycoside-modifying enzymes, β-lactamases and chloramphenicol acetyltransferases. Among them, β-lactamases are particularly well-studied and widespread. They function by hydrolysing the β-lactam ring, a core structural component of all β-lactam antibiotics, thereby rendering carbapenems, cephalosporins, penicillins and monobactams ineffective (*Reygaert*, 2018).

2.2 Modification of antibiotic binding sites

Many resistant bacteria evade antimicrobials by modifying their target sites. In multi-drug resistant *S. aureus* (MRSA), mutations in *mecA* or *mecC* lead to production of altered penicillin-binding proteins (PBPs), such as PBP2a, which have low affinity for β-lactam antibiotics, allowing cell wall synthesis to continue despite treatment (*Tang et al*., 2014; *Oniciuc et al*., 2017; *Abebe & Birhanu*, 2023; *Santajit & Indrawattana*, 2016). Similarly, *E. faecium* and *Enterococcus faecalis* resist glycopeptides like vancomycin and teicoplanin by substituting D-Ala-D-Ala with D-Ala-D-Lac or D-Ala-D-Ser in peptidoglycan precursors, a change mediated by resistance genes such as *VanA*, *VanB*, and others, which prevent antibiotic binding (*Giedraitienė et al*., 2011).

2.3 Active drug efflux via efflux pumps

Efflux pumps are membrane proteins that help bacteria expel antimicrobial agents, lowering intracellular antibiotic levels and reducing drug efficacy (*Santajit & Indrawattana*, 2016). These pumps play a key role in multidrug resistance by exporting a wide range of antibiotics. Five major efflux pump superfamilies are recognized: ABC (ATP-dependent), and four proton sodium gradient-driven families, SMR, MFS, RND, and MATE. A sixth group, the PACE family, has also been identified (*Du et al*., 2018). In *P. aeruginosa*, systems like MexAB-OprM and MexCD-OprJ contribute to resistance against fluoroquinolones, aminoglycosides, and carbapenems (*Jamal et al*., 2023).

2.4 Reduced drug uptake

Bacterial antibiotic susceptibility depends on the balance between drug entry and efflux. One resistance mechanism involves reduced membrane permeability, limiting drug uptake (*Uddin et al*., 2021; *Santajit & Indrawattana*, 2016). In Gram-negative ESKAPEE bacteria, porins facilitate antibiotic entry, but mutations or loss of porins, such as in *K. pneumoniae*, can block drug entry and confer resistance to β-lactams (*Santajit & Indrawattana*, 2016).

2.5 Biofilm production

Biofilm formation involves three main stages: initial bacterial adhesion to a surface, followed by growth and secretion of extracellular polymeric substances (EPS) that form a protective matrix, and finally, detachment. Detachment can occur actively

via quorum sensing and enzymatic matrix degradation, or passively due to physical forces like shear stress or human handling (*Laverty et al*., 2014).

3. Presence of ESKAPEE pathogens in the food chain

Holman et al. (2021) reported that *E. faecalis* and *E. faecium* were isolated from swabs and ground beef in a beef processing facility, with *E. faecalis* being more prevalent and present at all sampling points. Most *E. faecalis* isolates exhibited high resistance to lincomycin (97.4%) and quinupristin-dalfopristin (93.2%), along with varying levels of resistance to tetracycline, erythromycin, tylosin, and ciprofloxacin, while *E. faecium* isolates showed similar resistance profiles, except for ciprofloxacin, to which they remained susceptible. Notably, only one *Enterococcus* isolate showed full susceptibility to all 16 tested antimicrobials, with no resistance recorded to linezolid, penicillin, or vancomycin in any isolate. Complementary findings were reported in retail food studies across Europe. *Pesavento et al.* (2014) detected *E. faecium* in 35.5% of raw meat (beef, poultry, pork) and 44.9% of ready-to-eat products (cheese, salads, ham) in Italy, with resistance most commonly found to erythromycin (22.1%), tetracyclines (16.4%), gentamicin (13.6%), and ciprofloxacin (10.7%). In Turkey, *Sanlibaba et al.* (2018) found *E. faecium* in 61.9% of pre-packaged chicken meat samples, with dominant resistance to rifampicin (81.7%) and significant rates for ampicillin (73.3%), erythromycin (45%), and ciprofloxacin (31.7%). Similarly, a Polish study revealed that 31.6% of fermented milk products were contaminated with *E. faecium*, showing resistance to streptomycin (29.7%), erythromycin (14.9%), and tetracyclines (10.9%) (*Chajęcka-Wierzchowska et al*., 2020). Together, these findings underscore the widespread presence of multidrug-resistant *Enterococcus* spp. across diverse food sources in Europe.

Contamination of meat and meat products with *S. aureus* is widespread. *Wu et al.* (2018) found it in 35% of 1850 retail meat samples, with high resistance rates to ampicillin (85.4%), penicillin (84.6%), erythromycin (52.7%), and tetracycline (49.3%). Similarly, raw chicken meat contained *S. aureus* strains fully resistant to cefpodoxime and cloxacillin (100%), with notable resistance to ceftazidime and piperacillin/tazobactam (92.5%), clindamycin (72.5%), and methicillin (57.5%) (*Herve & Kumar*, 2017). *K. pneumoniae* was detected in 46.7% of raw calf and chicken meat samples in Turkey, with all isolates resistant to ampicillin and amoxicillin, and 29% resistant to aztreonam (*Gundogan et al.,* 2011). In another study, fresh raw poultry showed the highest contamination (13.8%), and isolates displayed resistance to ampicillin (92.3%), tetracycline (31.3%), and trimethoprim-sulfamethoxazole (18.2%) (*Liu et al.,* 2016). *A. baumannii* was found in 20% of raw meat samples, exhibiting high resistance to gentamicin (87.2%), tetracycline (79.5%), erythromycin (74.4%), and ciprofloxacin (59%) (*Askari et al*., 2020). *P. aeruginosa* was isolated from 29 out of 370 samples of raw, frozen, and processed beef products, with most isolates resistant to ampicillin (89.6%), penicillin (86.2%), and tetracycline (82.7%) (*Rezaloo et al*., 2022). In Egypt, 139 *Enterobacterales* strains isolated from RTE foods including meats showed general susceptibility, though resistance to ampicillin, third-generation cephalosporins, and carbapenems was noted. *Edris et al*., (2023) reported *Enterobacter* spp. in 274 meat-related products, with *E. cloacae*, *E. hormaechei*, and *E. kobei* resistant to β-lactams, cephalosporins, and, in some cases, glycopeptides; several strains were MDR or even extensively drug resistant (XDR). *Menck-Costa et al*. (2023) found ESBL-producing *E. coli* in 37% of 450 meat samples, especially in chicken (109/150), with additional resistance to tetracycline (51%), ciprofloxacin (46%), and fosfomycin (38%). Notably, 45% of these *E. coli* strains were MDR, though all were susceptible to imipenem. Another study detected *E. coli* in 33.8% of milk and dairy products, with 88.1% of tested strains resistant to at least three antimicrobials (*Sarba et al.,* 2023). An *E. coli* O157 strain was also identified in milk (0.2% of *E. coli* isolates) (*Sarba et al.,* 2023). Although not meat-related, *E. coli* from vegetables also showed resistance to penicillin (100%) and other antibiotics (*Datta et al*., 2024).

4. The role of the One Health concept in combating AMR

The complexity of AMR at the human-animal-environment interface requires an integrated, multidisciplinary approach. The One Health concept, recognizing the interconnectedness of human, animal, and environmental health, is key to addressing antibiotic resistance gene spread. These genes move through hospitals, farms, and water sources, acting as “reactors” for resistance (*Gajić*, 2023). However, the One Health approach has not been widely imple-

mented. Strengthening the understanding of genetic links among MDR organisms can enhance national strategies. While clinical resistance is well-documented, data on environmental resistance is lacking. National and international strategies emphasize prudent antibiotic use, infection prevention, surveillance, and scientific knowledge. WHO's GLASS initiative recommends tools like whole-genome sequencing (WGS) for understanding resistance patterns and transmission routes (*WHO*, 2022). "One Health genomics," using metagenomics and advanced sequencing, helps map AMR, identify resistance hotspots, and clarify genetic relationships between MDR bacteria, guiding targeted interventions.

4.1 AMR: a public health issue in Serbia

AMR poses a significant public health issue in Serbia. In 2019, approximately 1,800 deaths were directly linked to AMR, with 7,100 deaths associated with resistant infections, surpassing mortality from diseases like diabetes and chronic respiratory conditions. Serbia ranks 68th globally in AMR-associated mortality per 100,000 people. The main pathogens contributing to the AMR burden are *E. coli* (1,800 deaths), *S. aureus* (1,200), *K. pneumoniae* (986), *P. aeruginosa* (617), and *Streptococcus pneumoniae* (493), which cause bloodstream, intra-abdominal, respiratory, and urinary tract infections (*Institute for Health Metrics and Evaluation*, 2019).

5. Conclusion

Effectively addressing AMR requires full implementation of the One Health approach, which promotes global collaboration, innovation, long-term investment, and strong governance. The rise of multidrug-resistant ESKAPEE pathogens, including those with resistance to last-resort antibiotics, poses a serious clinical challenge. Overuse of antibiotics in healthcare and food production worsens the problem, increasing pressure on health systems. Reducing AMR depends on restricting antibiotics to treatment, avoiding their use for prophylaxis or growth promotion, and enforcing strict regulation, monitoring, and reporting to limit environmental contamination. A coordinated, cross-sectoral effort is essential to combat AMR.

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Original Scientific Paper

Process hygiene of cattle carcasses in one slaughterhouse in Serbia during 36 months

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ABSTRACT

Process hygiene in slaughterhouses plays a key role in ensuring product safety and protecting the health of both workers and final consumers. A three-year monitoring was conducted in one slaughterhouse in Serbia, using non-destructive sampling and testing according to microbiological criteria for total viable count (TVC), *Enterobacteriaceae* (EC), and the presence of *Salmonella* spp. The results showed that all TVC and EC values were below the prescribed m and M limits, while *Salmonella* spp. was detected in two samples. These findings highlight the need for continuous monitoring. Only consistent implementation of self-control measures, application of the HACCP system, and ongoing staff training can ensure meat safety and protect public health. This study emphasizes that properly applied hygiene measures can significantly reduce the risk of microbiological contamination of meat.

1. Introduction

The demand for beef as a protein source is increasing globally, although in most countries it represents less than half of total meat consumption, while offering a high sensory quality in both developed and, increasingly, developing countries (*Smith et al.*, 2018). A major global challenge in maintaining the hygiene and safety of meat products is the steady growth of the population, which puts extra pressure on production systems and control measures. It is generally accepted that the primary sources of microorganisms on processed carcasses are contaminated hides and possible rupture of the gastrointestinal tract, with additional contamination arising from unclean tools, as well as workers' hands and clothing (*Diyantoro and Wardhana*, 2019). The level of animal cleanliness at the time of entry into the slaughterhouse plays a crucial role. Animals in inadequate hygienic condition are a significant source of microbial contamination in slaughterhouses. Microorganisms present on their skin, hair, and hooves can easily be transferred to equipment, working surfaces, and the meat itself during slaughter operations (*Hong et. al.*, 2024). These facts show how important it is to keep track of everything from the farm to the slaughterhouse, to ensure hygiene and safety are really being followed all the way through.

In most countries, including Serbia, a scientifically-based preventive food safety system is implemented, founded on the principles of hazard analysis and critical control points (HACCP). The implementation of HACCP is not feasible without the

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application of good manufacturing practice (GMP) and good hygiene practice (GHP). These control systems are often supported by microbiological food criteria, which include safety parameters focused on foodborne pathogens and hygiene indicators (*Serbian Regulation*, 2019). The number of total viable counts (TVC) and *Enterobacteriaceae* (EC) and the presence or absence of *Salmonella* spp. are the process hygiene criteria for cattle carcasses, as specified in the Serbian Regulation on General and Specific Conditions of Food Hygiene and Microbiological Criteria for Food (*Republic of Serbia*, 2024). As the *World Health Organization* (2024) states, bacterial pathogens are among the most significant causes of foodborne diseases. Each year, they cause illnesses that range from mild gastrointestinal disorders to severe and fatal outcomes. Contaminated food threatens both public health and nutrition security. It also generates major socioeconomic consequences. These challenges highlight the need for governments to prioritize food safety. Evidence-based policies and effective control systems are essential to safeguard health. In 2023, *Salmonella* spp. remain one of the most important bacterial zoonotic agents transmitted through food in Europe, with 77,486 reported human cases (*EFSA and ECDC*, 2024).

The highest levels of *Salmonella* spp. contamination on cattle hides are typically found on the brisket and distal leg areas, likely due to prolonged contact with floors contaminated with feces while the animals are standing or lying down during the pre-slaughter phase, including time spent in the stunbox. This contamination pattern is reflected on the carcass, with higher microbial loads at skin-opening sites (brisket, distal leg) and hide-meat contact areas (rump, flank) (*Buncic and Sofos*, 2012). The detection on carcasses of *Enterobacteriaceae* (EC), a bacterial family widely distributed in the environment, may indicate fecal contamination. Total viable count (TVC) clearly reflects the general hygiene level in slaughterhouses (equipment, tools, etc.) (*Mrdović et al*., 2017). This highlights the importance of microbiological monitoring of hygiene indicators in the assessment of hygiene levels in slaughterhouses and the control of carcass contamination to protect the health of both working staff and final consumers.

2. Materials and methods

Slaughterhouses are required to have a self-control plan and establish a food safety assurance system at all stages, in accordance with the principles of good manufacturing and hygiene practice and hazard analysis and critical control points (HACCP), according to the local food safety law (*Serbian Regulation*, 2019). Sample collection was undertaken from January 2022 to December 2024 (Table 1). During this period, 180 samples were collected from one slaughterhouse in Serbia and the number of microorganisms on the carcasses were determined using standard methods (*ISO*, 2015). At each visit to the slaughterhouse five carcasses were randomly selected and sampled at step of the slaughtering process – after the final washout before chilling. The non-destructive swabs were taken at four sites on each carcass (rump, flank, brisket and neck; Figure 1). In this study a non-destructive sampling method was applied in order to avoid damaging the musculature. This approach followed the recommendations of the Guide for the Application of Microbiological Criteria for Food (*Ministry of Agriculture, Trade, Forestry and Water Management of the Republic of Serbia*, 2011) and, therefore, required a stricter evaluation of the results than would have been the case

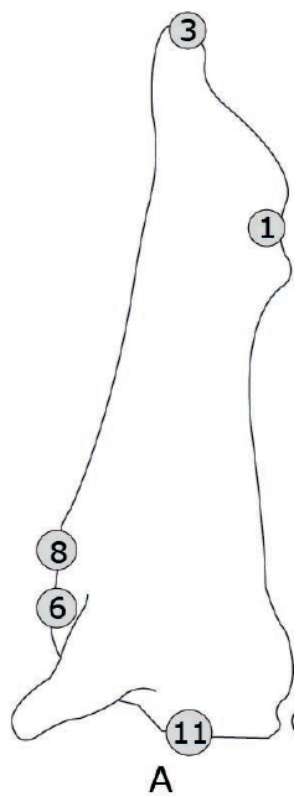

Figure 1. Microbiology of the food chain — Carcass sampling for microbiological analysis according to the ISO 17604:2015 standard (*ISO*, 2015). The circle indicate the sampling sites on the cattle carcass surface, and the numbers correspond to defined sampling positions: (1) Rump, (3) Flank, (6 & 8) Brisket, (11) Neck.

if the limit prescribed for the destructive method by Serbian regulation (*Serbian Regulation*, 2024) had been used. Process hygiene was monitored and evaluated in accordance with the local law (*Serbian Regulation*, 2024). The samples collected were conditionally stored in a hand refrigerator and transported to the Institute of Meat Hygiene and Technology in Belgrade. Process hygiene criteria for cattle were: TVC, EC count and presence/absence of *Salmonella* spp. In addition, the evaluation of the presence of *Salmonella* spp. on carcasses was conducted using the rolling window sampling approach. This method ensures systematic and continuous monitoring, with results assessed through overlapping "windows" of data that shift forward as sampling progresses. By applying this principle, contamination patterns can be more precisely detected and the effectiveness of hygienic measures evaluated. Sampling for *Salmonella* spp. was carried out with the abrasive sponge method, covering a minimum surface area of 400 cm², and the number and locations for sampling for the presence of *Salmonella* spp. were the same as those previously specified. According to the Regulations, results are evaluated in series of 50 samples (10 consecutive series with 5 samples each). The acceptable process hygiene level is defined as a maximum of 2 positive samples out of 50 per series, in accordance with the domestic legislation (*Serbian Regulation*, 2024) and the Guide for the Application of Microbiological Criteria for Food (*Ministry of Agriculture, Trade, Forestry and Water Management of the Republic of Serbia*, 2011). In Serbia, the regulation prescribes limits for process hygiene test results for cattle carcasses (Table 1).

Table 1. Process hygiene criteria for cattle – non destructive method.

Microorganisms	**Sampling plan**		**Limits**		**Analytical reference method**	**Stage where criterion is applied**	**Action in the case of unsatisfactory results**
	n	c	m	M			Improvement of slaughter hygiene and re-evaluation of the process
Total Viable Count			2.8 log CFU/cm²	4.0 log CFU/cm²	SRPS ISO 4833-1: 2014	Carcasses after final washing, before chilling	
Enterobacteriaceae			1,2 log CFU/cm²	2 log CFU/cm²	SRPS ISO 21528-2:2009		
Salmonella	50	2 (2)*	Absence in the area tested per carcass.		SRPS ISO 6579:2008		Improvement of slaughter hygiene, re-evaluation of process control, determination of animal origin, and implementation of biosecurity measures on farms

2.1 Statistical analysis

Statistical analysis of the results was performed using Microsoft Office Excel 2016. The average logarithmic TVCs and ECs for each carcass were calculated, based on the previously transformed log values from four corresponding sampling sites per carcass. Subsequently, the average daily logarithmic values were computed. The average daily logarithmic count for *Salmonella* spp. cannot be calculated due to regulatory requirements, which specify only the presence or absence of *Salmonella* spp. Data on *Salmonella* presence were analyzed using Microsoft Excel. The prevalence was calculated as the proportion of positive samples relative to the total number

of tested carcasses, expressed as a percentage. This calculation involved dividing the number of positive samples by the total number of samples and multiplying by 100.

3. Results

3.1 Total viable count numbers and trends

The mean monthly TVCs on cattle carcasses during the three-year monitoring period ranged from undetected for September and November 2022 to 2.7 log CFU/cm² for October 2024 (Table 2). In most months, the TVCs remained below the reference value *m* (2.8 log CFU/cm²), and none exceeded the maximum permitted value *M* (4.0 log CFU/cm²). Over the three-year period, none of the samples exceeded the *m* or *M* values, indicating that 100% complied with the satisfactory criterion.

3.2 Enterobacteriaceae numbers and trends

During the monitoring period, the average monthly ECs on cattle carcasses ranged from undetected in January, May, July, September, November, December 2022 and January and February 2023 to 1.2 log CFU/cm² in August 2023 (Table 3). All monthly values were well below the reference value *m* (1.2 log CFU/cm²) and did not exceed the maximum permitted value *M* (2.0 log CFU/cm²). Throughout the study period, no sample surpassed the *m* or *M* thresholds, demonstrating 100% compliance with the satisfactory process hygiene criterion for EC.

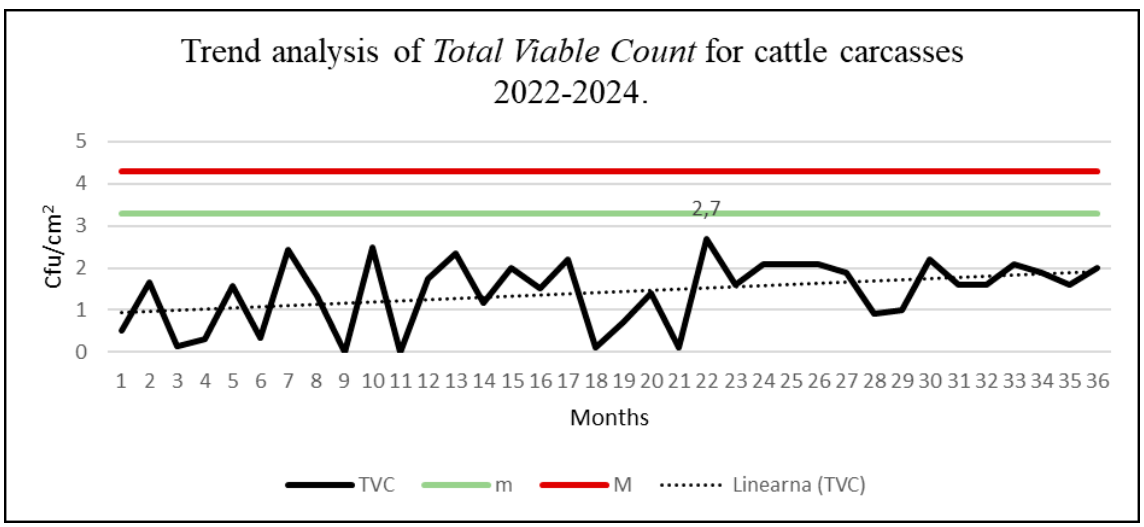

Figure 2. Trend analysis of total viable counts (TVCs) on cattle carcasses, 2022-2024.

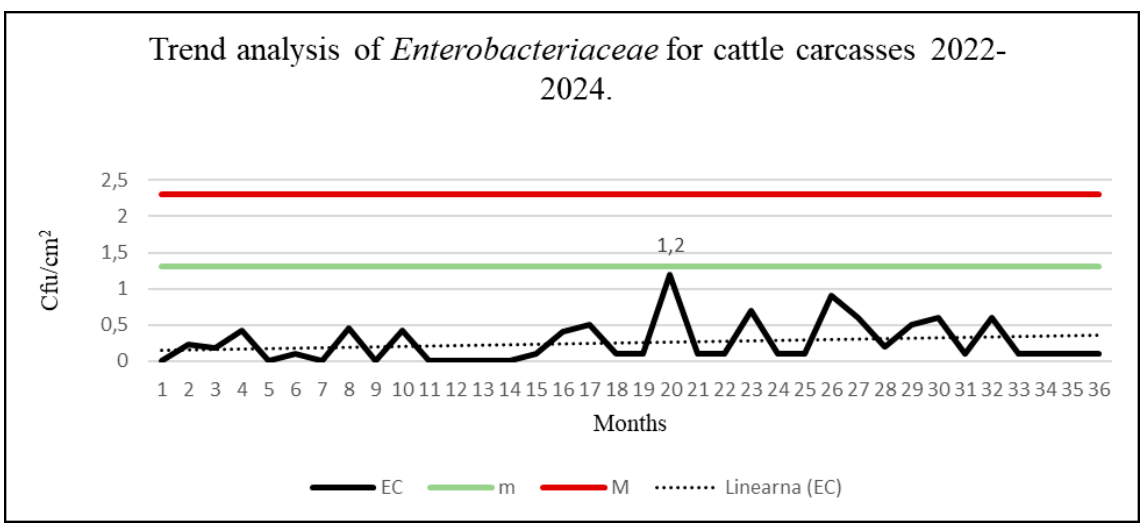

Figure 3. Trend analysis of *Enterobacteriaceae* counts (EC) on cattle carcasses, 2022-2024.

3.3 Salmonella spp. presence/absence

The presence of *Salmonella* spp. was detected on 2 out of 180 cattle carcasses. The detection limit for *Salmonella* spp. is set at two occurrences per 50 carcasses. Since *Salmonella* spp. were detected in the slaughterhouse, it was necessary to verify the origin of the animals and the biosecurity measures on the farms of origin, review process controls, and improve slaughter hygiene.

4. Discussion

Overall, all results for process hygiene indicators on cattle carcasses stayed below the threshold values set by *m* and *M*, confirming full compliance with the satisfactory hygiene criteria. In many months, neither TVC nor *Enterobacteriaceae* were detected, and when they were present, their levels were still below the reference limit. *Salmonella* spp. was detected on only 2 of the 180 carcasses tested (1.11%), and satisfactory results were obtained using the rolling window method, confirming the effectiveness of the applied hygiene measures. By contrast, *Mrdović et al.* (2017) reported that *Salmonella* spp. were not detected on any of the 610 cattle carcasses examined in either a large-scale or a small-scale slaughterhouse in Serbia during a five-year monitoring period. In their study, TVC and EC were consistently within the satisfactory or acceptable regulatory ranges, confirming good slaughter hygiene practices. On the other hand, *Gutema et al.* (2021) observed a considerably higher prevalence of *Salmonella* spp. (8.6%) on cattle carcasses in two small-scale slaughterhouses in Ethiopia. Their study also detected *Salmonella* in rectal content (7.1%) and hide samples (7.1%). Interestingly, genetic analysis revealed that direct transfer from feces or hides to carcasses occurred only in a small number of cases. The majority of contamination originated from cross-contamination during the slaughter process, largely linked to poor hygiene practices, such as insufficient cleaning of equipment and limited training of slaughterhouse workers. Similarly, *Nakamura et al*. (2023) demonstrated that TVC levels were highest immediately after skinning, particularly on the outer carcass surface, but decreased significantly after washing, reaching low levels. *Enterobacteriaceae* were mainly detected on outer surfaces, especially around the anus and splitting site, while inner surfaces showed consistently low levels (*Nakamura et al*., 2023). These observations are consistent with our findings, where TVC and EC levels remained below regulatory thresholds throughout the monitoring period. Overall, our results correspond well with those of *Mrdović et al.* (2017) in Serbia and *Nakamura et al.* (2023) in Japan, both demonstrating low TVC and EC levels under adequate hygiene practices, whereas the higher prevalence of *Salmonella* spp. reported by *Gutema et al.* (2021) in Ethiopia suggests that differences in slaughterhouse infrastructure, hygiene management, and biosecurity measures may contribute to variation in carcass contamination rates. These findings show that the hygiene measures in place were effective in keeping microbial contamination to a minimum, although the occasional detection of *Salmonella* underlines the importance of ongoing monitoring, checks on biosecurity measures, and consistent application of hygiene procedures to ensure food safety and meet legal requirements.

5. Conclusion

Adhering to the HACCP system, along with the prerequisite GMP and GHP, makes it possible to maintain satisfactory microbiological criteria for cattle carcasses at critical points in production. Findings from this three-year study show that the slaughterhouse consistently upheld a high level of process hygiene, with TVC, EC, and *Salmonella* spp. results all remaining within the satisfactory limits set by regulations. Continuous monitoring, together with regular staff training and education, is essential to ensure the ongoing safety of beef and to maintain these high hygiene standards.

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Review Paper

Food safety, consumer trust and emerging trends

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ABSTRACT

The complexity of today's global food industry demands robust food safety systems that go beyond technical solutions. Sustainable production, advanced technologies, and circular economy principles are essential to meet the needs of a growing population. Despite regulatory controls, incidents of contamination persist, impacting consumer trust as a key foundation of food safety. Modern food safety systems must address both accidental and intentional risks, including food fraud, through transparent communication and proactive management. Establishing a strong food safety culture, with leadership and risk awareness, is crucial for transitioning from reactive to proactive systems. Ultimately, integrated approaches and stakeholder engagement are vital for building resilient, trustworthy, and sustainable food systems.

1. Introduction

The growing food industry and its complexity have intensified its significance due to the need for a robust and trusted global food system and food safety mechanisms. These mechanisms have to extend beyond the technical solutions in the area of food production, processing, distribution, and consumption (*Vagsholm et al.*, 2020). As the world population grows, feeding the global population requires a sustainable approach, with reducing food loss, new and sophisticated technologies, introducing circular economy, with improved effectiveness and efficiency, and taking care of the environment (*Singh*, 2025; *Kilibarda et al.*, 2023). Food production depends on numerous factors, including weather conditions, geopolitical situations, as well as supply and demand on the market (*FAO*, 2025). Food security requires proactive decision-making with improved food safety and innovation in production and distribution to achieve a secure system (*Chandimali et al.*, 2025; *Karabasil et al.*, 2023).

This article explores recent advances in food safety and food safety culture assessment, and the evolution of consumer trust.

2. Food systems and safety

Food used to be produced and sold locally. The journey from the producer to the consumer was short, and raw materials for production were used from the local area. Today, the situation is different. Food systems are complex, raw materials are procured from different geographical latitudes and distances, and

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the product is often a complex mixture of them with appropriate technological processing (*Aybar Espinoza et al*., 2026). Nowadays, it is quite rare to sell food from the doorstep, but distributors and wholesale and retail networks are involved in the process. This situation further complicates the food production chain, and therefore, a proactive approach to risk control must be taken. The risks are numerous, and there is practically no point in the production chain where possible contamination cannot occur (*Zahir et al*., 2025; *Yeak et al*., 2025). Today, more and more food is placed and sold on the international market, and therefore, the number of links that make up the chain of production, distribution, and consumption is becoming longer and more complex (*Grabs & Carodenuto*, 2021). In general, consumers have a greater preference for local or domestic products compared to products from the global market. This consumer attitude is more of a habit than an element consistent with assessed risk or a record of proven risk (*Polleau & Biermann*, 2021). There are several reasons that affect market stability and consumer confidence, including foodborne disease agents, increasingly recorded chemical food contaminants, transboundary diseases, intentional food contamination, and food fraud. Although regulatory and process control requirements are constantly being improved, there are still incidents related to risks in the food and food production chain, such as Salmonella, residues of antimicrobial drugs, pesticides, metal fragments, etc (*Aybar Espinoza et al*., 2026; *Karabasil et al*., 2025; *Karabasil et al*., 2021).

Nowadays, the demands of the consumers are getting more prominent and are realized through different levels. Entities in the food business want to cope with it and keep the trust of consumers, as a foundation of food safety. The consumer will forgive an error and incidents that occurs during the process of production, storage, and distribution, but only in case they received clear and transparent information regarding the product and the further procedure (*Gannon et al*., 2022). In order to fulfil the requirements of the legislator and the requirements of the market and ensure a sufficient amount of safe and nutritional food for the population's diet, producers must follow and introduce in the system new and sophisticated technologies that require additional resources in the competitive market (*Chandimali et al*., 2025; *Harnessing*, 2025).

3. Food control

If there is no safe food, we cannot have secure food. Food safety is based on several levels of control: application of legal requirements, application of the rules of good hygiene practice and good production practice, principles of HACCP, verification, and official control (*Radu et al*., 2023). We are aware that the food sector is a sector that operates with risks. The risks are numerous, and according to the risk assessment and analysis by the regulatory body and food business operators, they must be eliminated or kept under control with application of adequate measures. However, we witness that incidents occur, and control fails to prevent microbiological, chemical, or physical contamination (*Zahir et al*., 2025; *Yeak et al*., 2025). These situations undermine consumer confidence, which is the foundation of food safety. In the 21st century, in addition to food safety, which primarily deals with the prevention of accidental contamination in the food chain, food systems must take care of the prevention of intentional contamination and food defence, as well as food fraud (*Neuberger et al*., 2025; *Marin et al.*, 2025; *Davidson et al.*, 2017). In order to keep all these challenges under control, regulatory authorities and food systems must develop and implement management systems based on constant monitoring and control of critical parameters along the entire chain of production, distribution, and consumption (Manning, 2020). Control systems have to use modern technology, which implies the use of information technologies, including artificial intelligence (*Harnessing*, 2025).

In order to enable sustainable production, distribution, consumption and improve awareness of food safety principles, nowadays we are talking about a food safety culture that contains collective values and rules in one system (*Tongyu Wu & Wallace*, 2025). Key elements of a food safety culture are leadership, communication, risk, and environmental awareness. Leadership and adequate communication in a system that operates with risk, such as the food industry, are key proactive components that drive the system and create such an internal environment where employees, through compliance with established procedures, ensure the integrity of the process from a reactive to a proactive system (*Manning*, 2020).

4. Consumer trast

The history of consumer trust in food production systems includes global connectivity and a complex network that has an impact on consumer expectations and includes a demand for transparent and traceable information that has an impact on consumer attitudes and product placement (*Anastasiadis et al*., 2022). The integrity of the chain of food production, distribution and consumption is often affected by incidents as a result of failure, change of food properties, evident finding of biological, chemical, or physical risk. Although not all recorded incidents represent a direct health hazard, each recorded incident affects consumer confidence and the integrity of the food chain (*Vainio et al*., 2020). Therefore, the responsibility of the food chain lies in the corresponding transparency and traceability. The improvement of this aspect includes numerous parameters such as sustainability of the agricultural production chain, origin of raw materials and products, composition and properties of products, processing and used technology, supply chain, regulatory requirements, awareness, and responsibility of consumers. The fact is that the expected growth trend of the world's population by 2050 is expected to reach 10 billion people, which creates enormous pressure on food systems to place a sufficient amount of safe food on the market (*FAO*, 2025; *Roy*, 2024). In order to achieve this, food production must be significantly increased, which can represent a significant obstacle for the food system, if it does not transition in the coming years to sustainable production systems with the best possible utilization of raw materials with minimal waste while preserving the environment. Modern consumers' expectations are very closely related with the sustainable and responsible production with care for our planet and keeping resources for next generations (*Karabasil et al*., 2023; *Jiang et al.,* 2021).

5. Conclusion

Food safety is multidimensional and complex, and influences both organisational effectiveness and the confidence of consumers. Technological innovations offer powerful tools for risk mitigations, but their success depends on stakeholder engagement and as well as other sectors. Only an integrated, holistic approach will enable food systems to achieve a compact and resilient state with an important element of consumer trust.

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Review Paper

Food irradiation – perspectives, challenges and questions

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ABSTRACT

Application of the irradiation process in the food technology is recognized as a significant factor for achievement of the food safety in the chains of the distribution and general consumption. This position is based on the numerous scientific and research studies which confirmed benefits and uniqueness of the treatment with ionizing radiation, as well as the limits of usage. Last decade and post corona period are characterized with increased global demands for safe food in all segments of the industry due to higher awareness of the consumers regarding quality and safety of the food on the market. Technological innovations such as electron beam and X-Ray sources improved the ways for the facilities to fulfil obligatory safety standards and reduce their operational costs. Irradiation services is now more accessible to the food producers easing its inclusion in the food technology process. Beside that, needs for the establishing sustainable agricultural development and reducing quantity of the waste containing non consumed food, indicate some new applications and perspectives of the irradiation process.

1. Introduction

Food irradiation is the technological process which use a strictly controlled dose of ionizing radiation, to protect foodstuffs from deterioration caused by microorganisms, insects, and metabolic activity to prolong their shelf life without leaving any residual effect on processed foods, unlike preservatives and chemical pesticides (*Pavlov et al*., 2020). This activity provide pasteurization and sterilization of the food products without raising their temperature (cold process) and does not induce alteration of food attributes (taste, structure, visual appearance, nutrient content) when the recommended doses are used. Foodstuffs are processed by ionizing radiation after being packed into final consumer packaging which is a unique feature of food irradiation technology (*Molins*, 2001) and provide food safety in all segments of the production and distribution to the markets. Beside irradiation with ionizing radiation, some other types of the radiation such as UV is commonly used for food treatment (*Bisht at al*., 2021), as well as ozone treatment, and all these technologies can be characterized with different benefits and drawbacks. Process of the irradiation of the foodstuff with ionizing radiation will be subject of this review paper contributing to the presentation of the benefits, limitations and disadvantages of the food irradiation process as well as perspectives of this technological process.

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2. Ionizing radiation

Ionizing radiation is characterized with extremely short wavelengths and high intensity inducing changes in atom structure by expelling an electron from the orbit thus forming a reactive ion specie. This process cannot make changes in the atom nuclei so irradiated atoms are not radioactive after treatment and material quickly loses this absorbed energy. Effects of the irradiation can be distinguished as direct and indirect ones. In the direct effect, cell components such as carbohydrates, DNA, and lipids are directly damaged by ionizing radiation whereas in indirect effect, free radicals and reactive species from water radiolysis react with cells or food components. This free radicals has very short live and they are cannot be identified in the water component of the foodstuff after irradiation. Since the main ingredient of fresh products is water so indirect effect is the principal mechanism for ionizing irradiation treatment (*Fan at al*., 2020). On the other side, after irradiation of the dry products, longer live transient free radicals are formed which can be used for determination of the irradiated products. The mentioned effects of ionizing radiation treatment destroying different micro-organisms, bacteria, pests or insects, preventing sprouting and slowing down the ripening and aging of the treated fruit and vegetables. Induced radiolytic byproducts are not unique just to irradiation process since similar or identical products can be found in food that has been cooked or pasteurised which significantly complicate post detection of the irradiation process. Ionizing radiations are also well-known useful for inactivating different pathogens in various food products (Jeong et al., 2015). Gamma and X-rays can penetrate food to a depth of about 1m whereas an electron beam can only penetrate foods to a depth of about 3cm similar as soft X-rays which is useful characteristic used for specific purposes such as irradiation of the surface of the eggs. (*Kataoka at al*., 2021)

Sources of the gamma radiation are mostly consisted from radioactive isotope Cobalt-60 or in some cases Cesium-137. Gamma rays provide good penetration depths enabling efficient process of the irradiation which can be easily monitored and controlled. Due to the complicate protection issues to prevent radiation exposures gamma sources are now replacing with electron beam and X-rays sources as more accepted alternative.

Electron beams can be described as a focused stream of high-energy electrons produced in electron accelerator with energies up to 10 MeV and moderate penetrating depth due to reaction with the irradiated material. Accelerators are characterized with smaller health hazard and environmental impact since radiation is generated only when needed contrary to the gamma sources but the equipment used are more complex and difficult for maintenance.

X-rays radiation is focused electromagnetic radiation generated in the high energy X-Ray tubes with energies up to 5 MeV or 7.5 MeV, characterized with high penetrating power similar to gamma radiation and minimal environmental impact such electron beam sources.

According to the IAEA Database of Industrial Irradaition facilities DIIF, today there are 294 active facilities worldwide, from which is 184 categorized as gamma irradiators while rest of 110 employs electron beam or X-ray technology. Distribution of the facilities can be connected to the level of the implementation of the irradiation technology not just for the food processing so almost 20% of the facilities are located in the USA.

The choice of irradiation method and the dose applied will depend on the material treated and the intended purpose for treatment and the effectiveness of process depends on proper delivery of dose and its reliable measurement. It is important that the techniques used for dose determination are standardized and carried out accurately and that the process is monitored and controlled. During the irradiation process foodstuff are not in direct contact with the radiation source and their integrity is fully preserved.

3. Food irradiation process

Food products can be divided in two categories, food products with higher moisture content (fresh products) and products with lower moisture content. The irradiation process is determined by type of the product as well as purpose of the treatment and is characterized by applied dose of the irradiation. Ionizing radiation process is very effective for sterilization whether is used for medical consumables or foodstuff since induced effects on the living cells while minimum effects on the non-living material are detected.

The SI unit of ionizing radiation for absorbed dose is a Gray (Gy) and corresponds to the absorption of one joule of energy in a mass of one kilogram (1Gy = 1J/kg). Duration of the time that material is exposed to the ionizing radiation and the strength of the source (energy of the electrons or X-rays or

activity of the gamma source) determine the irradiation absorbed dose.

According to the applied doses, process of the irrradiation can be divided in 3 catogeries: (*Mshelia at al.*, 2023)

- **Radurization** is low dose irradiation process (0,75-2,5 kGy) equivalent to the food and beverage pasteurization and is commonly used as tool for the phytosanitary irradiation for the sprout inhibition and ripening delay as well as insect and parasite elimination extending shelf life and minimizing microbial burden (*Golding at al.*, 2023).
- **Radicidation** is middle dose irradiation process (2,5-10 kGy) are useful for the reduction of the number of the various non-spore forming pathogens such as disease-causing bacteria like *Salmonella, E. coli* and *Listeria* in fresh products (meat, poultry, seafood etc.) and inactivation of the enzymes which can significantly extend shelf life (*Gautam at al.*, 2023).
- **Radappertization** is high-dose irradiation (more than 10 kGy), particularly important to produce meat, poultry, and fishery products which are shelf-stable at ambient temperature. (Plaček et al., 2004). Today, application of high-dose irradiation of food products has been expanded to the sterilization of ready-to-eat (RTE) food, hospital diets (including food for immuno-compromised patients), and space food (*Feliciano et al.*, 2014; *Song et al.*, 2012)

Although all international standards define 10 kGy as maximum limit, use of higher doses is allowed if necessary for a legitimate technological purposes which include examples such as microbial decontamination of the dried herbs and spices (European Union 15 kGy, Australia 30 kGy) and sterilization e.g. space food. (USA 42 kGy for food for astronauts). (*Feng at al.*, 2017, *Feliciano et al.*, 2018). Some scientific studies noted occurrence of undesirable specific sensory characteristics like off-odour, decrease viscosity, discoloration/darkening, lipid oxidation, and decrease in its textural properties. However, these problems can be solved by combining irradiation with other treatment technologies (*Odueke et al.*, 2017, *Chen et al.*, 2016).

The first ever application of an electron accelerator to food irradation is processing of the spices for the sausage production 1958 in Stuttgart, Germany. This facility became obsolete almost the same year since Germany introduced a general ban on food irradiation due to lack of scientific and confident evidences for the safety of the irradiated food. (*Ehlermann*, 2016).

The first legislation act which is addressed to irradiation process is *Food Additive Amendment* enacted by US Congress in 1958. where all sources of the ionization are recognized as food additives. This act enable introduction of the food irradiation in the legislation regarding food safety. From that period use of the irradiation facilities is increased although general acceptance is not achieved due to public fear of the nuclear issues, especially during *Cold war* and nuclear threats as well as nuclear accidents such as Chernobyl and Fukushima. Nevertheless, in 2019, it was reported that irradiation of agricultural products is carried out in more than 60 countries of the world and more than 200,000 tons of irradiated products are produced annually in Europe (*Obodovskiy*, 2019).

4. Safety of the irradiated foodstuffs and labeling

After first implementation of the irradiation to foodstuff many research groups start investigation of the effects of the irradiation process and safety of the treated food regarding formed radiolytic byproducts and sensory attributes. International groups of experts evaluated periodically the results of wholesomeness testing and in 1980. decision is issued as "…the irradiation of any food commodity to an overall average dose of 10 kGy presents no toxicological hazard, hence, toxicological testing of food so treated no longer required…", and "…irradiation of foods up to an overall average dose of 10 kGy introduces no specific nutritional or microbiological problems…" (*WHO*, 1981). In 1997, an FAO/IAEA/WHO Study Group on High Dose Irradiation examined the results of safety studies carried out on foods irradiated in the dose range 25–60 kGy to achieve the intended technological objective of radappertization. The conclusion was that such foods are both safe to consume and nutritionally adequate (*WHO*, 1999). In 2011, the European Food Safety Authority (EFSA) reviewed the evidence and reasserted the opinion that food irradiation is safe (*EFSA*, 2011) with conclusions that there are no microbiological risks for the consumer linked to the use of food irradiation and its consequences on the food microflora and that most of the chemical substances formed during irradiation were also formed in food that has been subjected to other processing treatments and

that the quantities in which they occur in irradiated food were not significantly higher than those being formed in heat treatments.

When food is irradiated, water molecules may split and form free radicals which can react with food components, potentially forming new compounds called radiolytic products such as 2-alkylcyclobutanones (2-ACBs), furans and other volatile compounds as well as free radical-derived products from the reaction of free radicals with proteins, carbohydrates and lipids. Scientific research indicates that the chemical changes induced by food irradiation are qualitatively similar to those produced by conventional cooking methods but are generally less extensive. Some vitamins, particularly B1 and A, E, and K, can be somewhat sensitive to irradiation, the losses are comparable to or less than those that occur during conventional food processing methods like canning or heat pasteurization. (Ravindran at al, 2019) With proper dose control and processing conditions, nutritional losses in irradiated foods can be minimized, maintaining the food's nutritional quality. Since the materials used for packaging are exposed to radiation during the treatment, these materials must be radiation resistance and no mitigation of the toxic substances into food is allowed nor production of the additional sensory attributes or affect on the present ones. (*Verde et al*., 2013).

Mandatory specific labeling of the irradiated foods and foodstuff establish need for the development of the reliable and validated analytical techniques for the detection of the irradiated food, both defined in Codex Alimentarius. All irradiated food products must be labeled with "Radura" sign which represent plant-like image placed in the circle with solid lower half and dashed upper half indicating penetration of the radiation. In most cases this symbol is green colored indicating ecological nature of the process. (FDA, 2008). Aim of the labeling is to ensure that consumers are fully informed whether foods, or ingredients within them, have been irradiated since irradiation is not fully accepted in all countries while some countries strictly ban irradiation of the foodstuff (*Barkai-Golan at al*, 2017).

As each technological process, food treatment with ionizing radiation can be characterized with benefits, limits and disadvantages which are usually confirmed with scientific research. While benefits are mostly already stated in the text, as major limitations must be noted:

- Irradiation has no effect on the viruses and prions
- Some spore-forming bacteria such as Bacillus and Clostridium species are radiation resistant so radiation treatment with doses below 10 kGy can only reduce the number of spores, but in conjunction with freezing can prevent the growth of spores (*Markland at al*., 2016).
- Irradiation is not suitable for some fruits and vegetables such as tomatoes, grapes and cucumber due to reduction in some vitamins and possible loss of color and texture (Hwang et al. 2015; Mohamed, 2016).
- Irradiation of lipid products such as egg and dairies using high doses in an oxygen rich environment can lead to hydrogen peroxide formation, resulting to unpleasant odour and flavour (*Feng et al.*, 2017; *Ham et al.*, 2017)
- Although nutritional values of the irradiated foodstuff is not significantly decreased, slight reduction in vitamin content such as vitamin-C and vitamin-E.

5. Perspectives of the food irradiation

The acceptance of the food irradiation process is balancing between two opposite poles, major scientific approval and confirmation and consumer doubts and skepticism based on the lack of the information and false presumptions regarding radioactivity. Food irradiation is often linked with radioactive contamination of the food and environment generally which commonly induce rejection of the irradiated food. The European Union has strict food safety regulations so advanced irradiation technologies must be regulated and confirmed. But diverse regulatory standards make difficult task to fully implement challenging requirements including mandatory labeling practices and extensive authorization procedures which create barriers to worldwide trade in the situation where organic and natural food is fully promoted. Additional public interest include waste reduction and sustainable development where food irradiation can play significant role since it can minimize food waste and food post-harvest losses which are major reasons for the global lack of safe food. The changes in the consumer habits and increased need for the production ready-to-eat and packaged food will open some perspectives for the ionizing irradiation industry which can be summarized as:

- The increasing global need for safe food for all inhabitants with proven safety and increased longevity will induce higher demands for the irradiation services.

- Trade and transportation routes from the distance countries require additional time and initiate need for the extended shelf life of the packaged foodstuff.
- Reducing quantities of the food waste will generate new value and improve environmental status enabling sustainable development and minimizing global malnutrition across the world-wide.
- Increasing number of the irradiation facilities in the world with more efficient process should reduce commercial irradiation cost thus expanding range of the clients which can afford this service based on the cost/benefit analysis.

6. Challenges in the food irradiation process

Food irradiation presents growing market since nowadays there is increase of society's need for safe food and waste reduction agenda. The food supply chains benefit from irradiation technologies which enables safety of the packaged foodstuff and prolong shelf life which are necessary for the efficient worldwide distribution. The rise of consumption of the clean-label and organic foods directs food manufacturers to use irradiation technology since it provides a substitution for artificial chemical preservatives. So, role players in this industry will meet severe challenges such as:

- Public perception of the term radiation is major reason for lower acceptance since irradiation is commonly mixed with term radioactive which has dangerous connotation,
- Legislation approval is based on concise scientific confirmation of the irradiation process so additional research of the application on the other types of the foodstuff and new packaging materials through analysis of the radiolytic byproducts and their effects on the food and human health must be conducted as well as properly funded,
- Distribution of the irradiation facilities is not uniform in the world thus limiting producers in some parts of the world to provide safe food for domestic consumption and export,
- Growing demands for the irradiation services due to global increase in the food production and longer transportation routes with provided food safeness must be followed with proper capacities of the irradiation services,
- Structural technological changes from gamma radiation facilities to E-beam and X-Ray facilities due to improving radiation protection and reducing impact on the environment must be realized without losses in the installed capacities,
- Food irradiation is not fully adopted in all countries which can complicate trade agreements. International trade agreements must properly address to this issue,
- Competitive processes such as ozone treatment or cold plasma can decelerate the development and research in field of the irradiation due to lesser safety and security issues.

7. Conclusion

Ionizing radiation can be applied on a number of agricultural products and foodstuff in order to provide safe food. This activities include sprout inhibition, disinfestation, reduction or elimination of food borne pathogens and delayed ripening of fruits. The process involves application of the strictly determined radiation dose which is clearly confirmed through proper scientific research. The quality and safety of irradiated food at the defined and approved dose level for commercial use can be properly described and confirmed if it is compared with food preserved using other methods of preservation which are already accepted. Scientific community should actively participate in the promotion of the irradiation treatments in all segments of the human activities including foodstuff, medical consumables, waste etc. Increased knowledge regarding food irradiation can change public perception regarding application of the ionizing radiation in the human activities and improve public acceptance of the process itself.

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Review Paper

Risk ranking and prioritization of Aflatoxin M_1 in milk: a strategic tool for strengthening the national food safety system in Serbia

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ABSTRACT

In Serbia, aflatoxin M1 (AFM1), a carcinogenic metabolite of aflatoxin B1 (AFB1), is a common contaminant in milk and poses a significant food safety risk. Due to climate change, aflatoxin contamination remains a recurring and serious food safety issue, particularly for vulnerable groups, such as children. In this context, key priorities include AFM1 surveillance, public health risk quantification, regulatory assessment, and exposure modelling. These efforts are essential to support science-based decision-making and evidence-informed policy design. This paper proposes a structured, integrated methodology for risk ranking and prioritization of AFM1 in milk, with the aim of strengthening the national food safety system in Serbia. The approach enables systematic identification of high-risk regions, critical contamination periods, and vulnerable population groups. It also facilitates evidence-based monitoring and more efficient allocation of resources. In addition, the proposed methodology supports regulatory alignment with European Union (EU) food safety standards, enhances risk governance, and increases consumer confidence. Ultimately, risk ranking and prioritization are presented as essential tools for improving food safety outcomes, reducing human exposure to chemical hazards, and protecting public health in the dairy sector.

1. Introduction

Aflatoxins are secondary metabolites produced by *Aspergillus* fungi, mainly *A. flavus* and *A. parasiticus*. Among them, aflatoxin B_1 (AFB_1) is the most toxic and is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (*IARC*, 2012). When ingested by dairy cows *via* contaminated feed, AFB_1 is metabolized into aflatoxin M_1 (AFM_1), which is excreted in milk. AFM_1 retains genotoxic and hepatotoxic properties, posing a significant health risk, particularly to vulnerable groups, such as children and pregnant women.

In Serbia, aflatoxin contamination in milk has been a recurring issue since 2012, closely linked to climate change effects, such as rising temperatures and droughts, which promote fungal growth in corn—the primary cattle feed (*Milićević et al.*, 2020). Studies confirm a correlation between climate variability and increased AFB_1 contamination, leading to

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elevated AFM_1 levels in milk, especially during dry seasons (*Milićević et al.*, 2019). Exposure assessments conducted in Serbia (*Milićević et al.*, 2021) indicate that children are particularly at risk from chronic AFM_1 intake. Although concentrations generally remain within national limits, Serbia's maximum residue level (0.25 µg/kg) is significantly higher than the EU standard (0.05 µg/kg), highlighting the need for stricter controls and harmonized food safety policy. Given the way AFM_1 enters the food chain—combined with its thermal resistance and carcinogenicity—it requires a more structured, science-based risk management approach, which is still lacking in Serbia despite prior monitoring efforts.

Risk ranking is a key component of risk assessment, involving scientific evaluation and comparison of hazards based on potential health impact and exposure likelihood. Risk assessors analyze toxicological and exposure data, while risk prioritization, led by risk managers, determines how to act, where to intervene, and how to allocate limited resources effectively (*FAO,* 2020).

This paper presents a science-based methodology for the risk ranking and prioritization of AFM_1 in milk, tailored to the national context of Serbia and aligned with international food safety principles. The proposed framework supports transparent, evidence-driven risk management in dairy production, particularly in response to climate-related contamination challenges. It aims to improve milk safety for all population groups, with a focus on protecting infants, children, and pregnant women. This initiative is grounded in ongoing interdisciplinary research led by national food safety and public health experts and provides a foundation for future decision-support tools in Serbia's dairy sector.

2. Methodology: risk ranking and prioritization of chemical hazards in food

2.1 Risk ranking: what to monitor

Risk ranking is a structured, evidence-based method used to identify which chemical hazards in food present the greatest public health concern (*FAO,* 2020; *Van der Fels-Klerx et al.*, 2015) (Figure 1). It answers the question: "What should we monitor?" by evaluating the severity of health effects (e.g., genotoxicity, carcinogenicity) as well as the probability of exposure, which is primarily influenced by contamination levels, dietary habits, and population vulnerability. This step is especially critical in systems with limited monitoring capacity, as it enables scientific prioritization of hazards for surveillance and control. International bodies, such as the Food and Agriculture Organization (FAO) and the European Food Safety Authority (EFSA), emphasize that ranking should be transparent, reproducible, and rooted in data to support credible decision-making.

2.2 Risk prioritization—how and where to act

Risk prioritization builds on ranking to address the next essential question: "How and where should action be taken?" It translates scientific insights into management strategies, integrating additional practical considerations, primarily public and political sensitivity, economic and trade impact, and feasibility of implementation (infrastructure, technology, logistics) (*FAO,* 2020; *Van der Fels-Klerx et al.*, 2015). The result is a ranked list of actionable interventions, adapted to national circumstances. While based on scientific risk assessments, prioritization also reflects stakeholder values, regulatory feasibility, and capacity for mitigation, making it the bridge between risk assessment and practical food safety management.

2.3 Methodological approaches: top-down vs bottom-up

2.3.1 Top-down—based on public health outcomes

The top-down approach uses population-level health metrics to prioritize risks (*FAO,* 2020). These metrics include disease burden indicators (Disability-Adjusted Life Years (DALYs) and Quality-Adjusted Life Years (QALY), epidemiological data on incidence, mortality, and disease attribution, and global frameworks, such as WHO's Foodborne Disease Burden Epidemiology Reference Group (FERG) methodology. This approach is typically applied to well-characterized hazards and is commonly employed by public health agencies to inform high-level policy decisions, for instance, assessing the attributable risk of AFM_1 in hepatocellular carcinoma incidence.

2.3.2 Bottom-up—based on hazard and exposure

When health outcome data are limited, as is commonly observed in developing countries, the bottom-up approach provides an alternative by focusing on:

- Exposure Context, e.g., consumption patterns and contaminated food items (such as milk and maize-based feed), measured levels of AFM_1, and exposure routes and vulnerable groups (children, infants).
- Hazard Characteristics, e.g., identity and toxicological profile of AFM_1 (Group 1 carcinogen), and threshold values, such as the lower bound of the benchmark dose ($BMDL_{10}$) and the margin of exposure (MOE) for risk estimation.
- Contextual Considerations, e.g., socio-economic capacity, technical infrastructure, and feasibility of intervention (*FAO,* 2020).

This method is especially relevant for AFM_1 in Serbia, where public health data may be limited, but contamination monitoring and exposure modelling can inform effective, evidence-based interventions (*Milićević et al.*, 2021; *Udovicki et al.*, 2023).

2.3.3 Methodological tools used in bottom-up risk ranking

The bottom-up approach combines scientific data with context-driven analysis using quantitative, semi-quantitative, and qualitative tools (*Van der Fels-Klerx et al.*, 2018). Quantitative tools like the MOE and hazard quotient (HQ) assess health risks by comparing estimated human exposure to toxicological benchmarks. For genotoxic carcinogens such as AFM_1, MOE below 10,000 or HQ above 1 indicate a public health concern. When data availability is limited, semi-quantitative tools are commonly used. Although they retain certain quantitative aspects, their evaluations are less robust than those produced by qualitative tools. Among others, these include scoring method, in which numerical weights are assigned to factors, such as prevalence, severity or exposure; risk matrices which map severity against likelihood; and Multi-Criteria Decision Analysis (MCDA) which integrates scientific, social, and economic dimensions to support balanced, transparent decision-making.

Qualitative tools are generally used when both resources and available data are severely limited, making it the most basic assessment approach. These tools include drafting decision trees and flowcharts designed to classify hazards into risk categories. The advancement of risk assessment methodologies can be enhanced through integration with EFSA-developed databases and tools. These include OpenFoodTox, which provides toxicological profiles of food and feed; DietEx tool, which estimates dietary exposure; or RACE, a tool that provides an assessment of acute and chronic exposure in different populations.

2.4 Risk ranking and prioritization: focus on the dairy chain

When determining the risk from aflatoxin exposure, multiple factors specific to the dairy chain have to be considered. Firstly, AFB_1 has a rapid biotransfer and carry-over, meaning that AFM_1 will appear in milk within 24 hours of AFB_1 ingestion from contaminated feed. Secondly, AFM_1 is heat-stable, meaning it persists through pasteurization and other processing methods, remaining in final dairy products. Furthermore, special attention should be paid to exposure in infants and children, as they consume more milk relative to their body size.

2.4.1 Risk ranking: key inputs and indicators

In order to effectively perform risk ranking of AFM_1, it is essential to integrate several sources of data. Toxicological data must be considered first, notably the classification of AFM_1 as an IARC Group 1 carcinogen and EFSA's $BDML_{10}$ of 4 µg/kg bw/day. Next, the carry-over rate (COR) from feed to milk requires evaluation of multiple variables, including AFB_1 concentration in feedstuffs, the cows' diet composition, lactation stage, and herd productivity metrics. Lastly, exposure assessment in humans should incorporate both milk consumption patterns and monitoring data on AFM_1 levels. Analytically, this translates to applying MOE and HQ methodologies, supplemented by scoring methods that weigh hazard severity against exposure prevalence and population vulnerability.

2.4.2 Prioritization of risk management actions

After hazards are ranked, prioritization focuses on where interventions will have the greatest impact. For AFM_1 in milk, priority actions could include: targeting regions prone to drought and maize contamination; focusing on periods of elevated risk (late summer–winter); identifying high-risk farm profiles (e.g., heavy silage use, poor hygiene); and enhancing monitoring of milk intended for infants or export. Based on the determined focus of priority, recommended measures could include: implementing risk-based seasonal monitoring; training farmers in feed management and mycotoxin control; gradually aligning Serbian MRLs

with EU standards to reduce public health risks and enhance trade opportunities.

2.4.3 Case study: Serbia

Following the severe AFM_1 milk contamination crisis in 2013, triggered by extreme weather and widespread maize contamination, several scientific studies have established a rationale for the development of a structured risk ranking and prioritization framework in the Serbian dairy sector. The findings of these studies point to AFM_1 in milk and dairy products as a priority hazard, with confirmed presence not only in fluid milk but also in traditional cheeses from regional markets. This observed high threat highlights the need for broader monitoring (*Milićević et al.*, 2020; *Torović,* 2015; *Torović et al.,* 2021; *Udovicki et al.,* 2018).

Several studies (*Milićević et al.,* 2021; *Torović,* 2015; *Torović et al.,* 2021) emphasize that population-specific co-factors must be incorporated into risk assessments to accurately evaluate the risk of developing diseases. A critical example is the synergistic effect between AFM_1 exposure and Hepatitis B, which significantly exacerbates the risk of hepatocellular carcinoma. Furthermore, some populations, primarily children, must be recognized as vulnerable groups, as they consistently exhibit the highest exposure due to their higher milk intake relative to body weight. This exposure usually exceeds safety thresholds during peak contamination periods, especially in school milk programs (*Milićević et al.,* 2021; *Udovicki et al.,* 2023). Climate change has been identified as a significant factor in crop contamination by mycotoxins. Extreme weather conditions, such as floods and droughts, which have occurred in Serbia in recent years, have led to the promotion of fungal growth and aflatoxin accumulation in feed, further increasing the scientific attention, especially from the perspective of risk analysis (*Milićević et al.,* 2019). These findings provide the scientific foundation for a data-driven, risk-based management system for AFM_1 in Serbia's dairy chain, aligned with international food safety standards.

Figure 1. Conceptual flowchart for managing AFM_1 risk in milk supply chain

3. Conclusion

Risk ranking and prioritization of AFM_1 in milk are essential for enhancing food safety in Serbia's dairy sector. The direct transfer of AFB_1 from contaminated feed to milk, combined with AFM_1's thermal stability and its proven genotoxic and hepatocarcinogenic effects, necessitate a preventive, evidence-based strategy. National data confirm the value of exposure-based models in identifying high-risk groups—especially children—and in guiding timely, targeted interventions. Implementing structured risk assessment frameworks will enable more effective monitoring, optimized resource allocation, and better alignment with EU food safety standards. This, in turn, will contribute to improved public health protection, greater consumer confidence, and enhanced regulatory and export readiness. Rather than functioning as standalone tools, risk ranking and prioritization should be embedded within a broader, integrated strategy to build a resilient and future-oriented national food safety system.

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Original Scientific Paper

Detection of peanut allergens in retail food items in Serbia (2021-2024)

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ABSTRACT

Food allergies are a significant and growing public health concern globally, impacting millions of individuals and placing immense pressure on healthcare systems and personal well-being. Peanuts are one of the 14 fundamental food allergens, making them potentially harmful. As a result, the goal of this mini review is to assess the prevalence of peanuts in commercial food products in Serbia between 2021 and 2024. The current study summarizes the qualitative detection of specific DNA sequences using real-time PCR techniques on 212 commercial food goods, including frozen desserts, biscuits and related products, cream products, snacks, and sausages. Undeclared peanut allergen DNA occurred across different food categories, with positive results observed in frozen desserts (n=3), biscuits (n=4), and snacks (n=2). Peanut allergen DNA was detected in six samples with a precautionary allergen label (PAL) statement, while in the case of three samples in which peanut allergen DNA was detected, the presence of the allergen was not stated at all.

1. Introduction

Food Allergy is a growing global public health concern. The presence of undeclared allergenic ingredients or the presence of traces of allergens due to contamination during food processing poses a great health risk to sensitised individuals (*Janković et al*., 2024).

These disorders are identified by unfavourable immune responses to specific food proteins and can cause a wide range of symptoms, from slight irritation of the skin and intestinal problems to severe, life-threatening anaphylactic reactions. The growing frequency of food allergies, particularly in industrialized nations, highlights the crucial need for effective prevention, diagnostic, and management techniques (*Sicherer & Sampson*, 2018).

Peanut allergy is a significant public health concern, affecting a substantial portion of the population worldwide, with prevalence rates varying across different regions. It is characterized by an adverse immune response to proteins found in peanuts, which can manifest in a range of symptoms from mild reactions like hives and itching to severe, life-threatening anaphylaxis. Unlike some other food allergies, peanut allergy often persists throughout life, necessitating strict avoidance and careful management (*Food Allergy Research & Education*, 2024).

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The allergic reaction is primarily mediated by immunoglobulin E (IgE) antibodies, which recognize specific peanut proteins, known as allergens. These allergens, such as Ara h 1, Ara h 2, and Ara h 3, are encoded by specific genes within the peanut genome (*Palladino and Breiteneder*, 2018). Upon exposure, IgE binds to these proteins, triggering a cascade of immune responses that lead to the symptoms of an allergic reaction.

Peanut allergy prevalence varies considerably across Europe. Studies have shown rates ranging from 0.2% to 7.7% in different European populations (*Spolidoro et al.*, 2023). Data on peanut allergy prevalence in Serbia is more limited, but available research suggests it is within the range observed in other European countries (*Davidovic et al.*, 2022).

2. Materials and methods

This study investigated the qualitative presence of peanut (*Arachis hypogaea*) DNA in a range of commercial food products available in Belgrade, Serbia, between 2021 and 2024. A total of 212 food samples were analysed, encompassing five categories: frozen desserts (n=44), biscuits and related products (n=73), cream products (n=25), snacks (n=63), and sausages (n=7). These samples were randomly selected from various retail outlets, including supermarkets and local markets, within the Belgrade area.

2.1 Sample collection and preparation

Commercial food goods belonging to the specified categories were purchased between January 2021 and December 2024. Upon collection, samples were labelled and stored according to the manufacturer's instructions until processing.

2.2 DNA extraction

All selected products were analysed using validated methods for food allergen peanut. Analysis was carried out according to the SRPS EN ISO 15634-4:2023. The DNA from product samples was extracted using protocol with cetyltrimethylammonium bromide – CTAB. The DNA was quantified by spectrophotometry using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The applied CTAB extraction method resulted in DNA preparations with an OD260 nm/OD280 nm ratio of 1.8 to 2.0 in all samples, indicating the high quality of the extracted DNA.

2.3 Real-Time PCR for qualitative detection of peanut DNA

The pure DNA preparations were used as templates for amplifying, identifying, and qualitative detection using real-time PCR. Real-time PCR assays were performed with Aria MX (Agilent Technologies). The program used an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and primer annealing and elongation at 60°C for 1 min. The qualitative detection of peanut-specific DNA sequences was performed using real-time PCR, Aria MX. Real-time PCR was performed to detect a specific DNA sequence from the Ara h 2 gene of peanut.

3. Discussion

The findings of this study, which involved the qualitative screening of 212 commercial food products from Belgrade, Serbia, between 2021 and 2024, revealed the presence of peanut DNA in nine products across the tested categories. Notably, the detection of peanut DNA in these nine products suggest the presence of undeclared peanut material, as the study focused on identifying its presence irrespective of labelling information. Undeclared peanut allergen DNA across different food categories was detected in frozen desserts (n=3), biscuits (n=4), and snacks (n=2). Peanut allergen DNA was detected in six products with a precautionary allergen label (PAL) statement, while in the case of three products in which this peanut DNA was detected, the potential presence of the allergen was not stated at all.

Comparing our findings to other studies investigating undeclared allergens in food products is crucial for contextualization. While the prevalence of undeclared peanut DNA in our sample set (9 out of 212, approximately 4.2%) appears relatively low, it still represents a tangible risk to allergic consumers in Belgrade. Studies from other regions have reported varying rates of undeclared allergens, influenced by factors such as food regulations, manufacturing practices, and the specific food categories analysed (*Allen et al*., 2014, *Sprikkelman et al*., 2016, *EFSA*, 2014; *Sampath et al*., 2021). In Europe, the prevalence of undeclared allergens has been a concern, with studies highlighting issues in various food products, including peanuts (*Vadas & Perelman,* 2003, *Martínez-*

Pineda & Yagüe-Ruiz, 2022). For Serbia, while comprehensive data may be less extensive, some studies indicate the presence of undeclared allergens is also a concern, reflecting the need for stringent control measures and robust analytical methods like the one described here (*Davidović et al*., 2022).

4. Conclusion

The presence of undeclared peanut allergens in food is a major global health hazard that requires ongoing attention and proactive measures. The discovery of undeclared peanut allergen DNA in Belgrade, Serbia, exposes the risk of peanut allergen exposure from the local food supply and emphasizes the importance of food businesses implementing strong allergy management practices. The issue of undeclared peanut allergens is further complicated by the inconsistent and often unreliable use of PAL. Our study, which detected peanut DNA in products not labelled with PAL, supports concerns about the inadequacy of relying on PAL alone to protect allergic consumers.

The Voluntary Incidental Trace Allergen Labeling (VITAL) program takes a formal, risk-assessment-based approach to PAL, with the goal of providing more consistent information to both businesses and consumers. Our findings show that, even with systems like VITAL, a comprehensive plan is necessary. This strategy must involve strong allergen management methods by food producers, stricter regulatory oversight, better labelling, comprehensive consumer education, and continuing monitoring for undeclared allergens. Finally, limiting or eliminating allergen contamination throughout the food supply chain is critical for preserving the safety and well-being of people with peanut allergies.

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Review Paper

Aflatoxin M1 in donkey milk from indigenous breeds in the Zasavica natural reserve

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ABSTRACT

Donkey milk is considered highly valued for its hypoallergenic properties and distinctive nutritional benefits. However, there is limited information on its potential contaminants, such as aflatoxin M1 (AFM1). This study aimed to thoroughly assess the safety of milk from Balkan and Banat donkey breeds raised under extensive organic (free-range) conditions, focusing on a significant concern - AFM1 concentrations across different seasons. Monthly samples were collected over the course of a year, resulting in a total of thirty-six pooled samples. AFM1 levels were quantified using a validated enzyme-linked immunosorbent assay (ELISA), with results showing concentration of AFM1 in donkey milk remained below the detection limit throughout the year.". This study underscores the need for additional research on both the nutritional and safety aspects of donkey milk, particularly in relation to seasonal variations, potential presence of other environmental toxins, and differences among donkey breeds.

1. Introduction

Donkey milk is highly valued for its unique nutritional profile (*Živkov Baloš et al*., 2023; 2024), especially its similarity to human milk and hypoallergenic properties, making it a suitable alternative for individuals with cow's milk allergies, including infants (*Martini et al.*, 2021). The nutritional composition of donkey milk, rich in proteins, amino acids, fatty acids, lactose, and essential minerals, supports its potential as a beneficial food source (*Živkov Baloš et al*., 2023). Given its growing use in high-sensitive population., concerns about contaminants, such as aflatoxin M1 (AFM1), warrant further investigation into its safety for human consumption.

Aflatoxins, primarily produced by *Aspergillus flavus* and *Aspergillus parasiticus*, are mycotoxins with carcinogenic, mutagenic, and immunosuppressive properties (*Gizachew et al*., 2019). AFM1, a metabolite of aflatoxin B1 (AFB1) found in contaminated feed, poses significant health risks, particularly to vulnerable populations like infants. Classified as a human carcinogen by the International Agency for Research on Cancer (*IARC,* 2012), AFM1 can also impair growth in children and cause immune-related issues (*Tesfamariam et al*., 2020). While AFM1 contamination in commonly consumed milk, such as cow and goat milk, has been well-documented (*Ljubojević Pelić et al*., 2024), limited studies focus on its presence in donkey milk, despite growing

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interest in the latter due to its nutritional and hypoallergenic qualities.

The Balkan and Banat donkey breeds, known for their adaptability and high milk quality, are reared in the Zasavica Natural Reserve, providing an ideal setting for studying both the nutritional value and safety of their milk. Although research on donkey milk contamination is limited compared to other milk types, the growing demand especially among children underscores the importance of continuous monitoring to ensure it remains safe and free from contaminants. Understanding how environmental pollution may affect contamination levels is crucial. The present study aims to assess AFM1 concentrations in milk from Balkan and Banat donkeys over different seasons, addressing the need for further research on potential contaminants and their impact on milk safety and quality. This study is the first to examine AFM1 levels in the Balkan and Banat donkey breeds, contributing significantly to the knowledge base and the conservation of indigenous breeds.

2. Materials and methods

The study was conducted at the Zasavica Nature Reserve, which houses approximately 300 milking donkeys, making it the largest such farm in Southeastern Europe. The study area and the conditions for keeping donkeys are detailed in our previous researches (*Živkov Baloš et al*., 2024; *Ljubojević Pelić et al*., 2025). Briefly, the reserve is home to donkeys, horses, Serbian Podolian cattle, Mangalitsa pigs, and various bird species. Animals were raised in an extensive production system, grazing for nine months a year and drinking water from springs within the reserve. The pastures consist of meadow grass and are not fertilized due to the high number of animals. During winter, donkeys are kept indoors and fed a grain-based diet, mainly corn and triticale.

Milk samples were collected monthly from July 2023 to June 2024, with three pooled samples taken each month (nine per season) from the milk of all donkeys milked. Only milk from clinically healthy donkeys was sampled. Samples were aseptically collected in food-safe containers and transported to the laboratory in refrigerated containers (4°C). Aflatoxin M1 levels were determined using the enzyme-linked immunosorbent assay (ELISA) method, following the manufacturer's instructions (Neogen). Limit of detection of the test was 0.005 µg/kg.

3. Results and discussion

The results of the analysis of pooled donkey milk samples as well as the results of previous studies are presented in Table 1. The results of the examination of aflatoxin M1 presence in donkey milk showed that throughout the entire study period, across all four seasons, its concentration was < 0.005 µg/kg (Table 1).

Donkey milk is highly valued for its hypoallergenic properties and nutritional benefits, making it essential to investigate potential contamination with aflatoxin AFM1. AFM1 is a metabolite of AFB1 that is excreted into milk when lactating animals consume AFB1-contaminated feed (*Zentai et al*., 2023). The liver metabolizes AFB1 into AFM1, which represents the primary contamination pathway. While donkeys, like other mammals, can consume contaminated feed, their metabolic conversion of AFB1 to AFM1 and the transfer rate of aflatoxins into milk may differ from that of ruminants (*Tozzi et al*., 2016). This may be due to lower transfer rate, as suggested in limited data.

Research on AFM1 in donkey milk is limited, with most studies showing low or undetectable levels, consistent with our findings and studies in Greece (*Malissiova and Manouras*, 2017), Germany (*Gross et al.*, 2019), Italy (*Cammilleri et al*., 2019), Greece and Cyprus (*Malissiova et al*., 2016), and China (*Xiong et al*., 2022). This low presence of AFM1 may be linked to the specific feed used in donkey farming, particularly the primary diet of grass and hay (*Altafini et al*., 2020), which reduces AFM1 detection compared to milk from other species. In our study, donkeys grazed for nine months with no additional feed supplements, and only corn and triticale were provided during winter. Additionally, the low casein content in donkey milk compared to cow's milk (*Živkov Baloš et al*., 2023) may contribute to the reduced AFM1 presence, as aflatoxins have a high affinity for casein (Indyk et al., 2021). In animals exposed to mycotoxins, there is often a correlation between AFM1 levels and casein percentage, which may be another reason for the lower AFM1 levels in donkey milk (*Ljubojević Pelić et al*., 2024).

This study highlights the importance of monitoring AFM1 in donkey milk, especially with the increasing demand for this milk due to its health benefits. Further research is needed to explore seasonal and environmental factors affecting AFM1 levels and to investigate the specific physiological mechanisms in donkeys to ensure the safety and quality of donkey milk.

Table 1. Aflatoxin M1 (AFM1) in donkey milk samples

Donkey breed	Country	Analytical method	Number of positive samples/ number of samples	Concentration/ range	Reference
The Balkan and Banat	Serbia	ELISA	0/36	< 0.005 µg/kg	
	Serbia	ELISA	3/5 (60%)	Mean: 0.02 ± 0.02/ range: 0.005–0.035 µg/kg	Kos et al., 2014
	China (Xinjiang)	ELISA	0/70	< 0.005 µg/kg	Xiong et al., 2022
Indigenous Greek Donkey and Cypriot Donkey	Greece and Cyprus	ELISA	0/90 (79 from 6 Greek farms and 11 from a Cypriot farm)		Malissiova et al., 2016
	Greece	ELISA	5 /36 (13.9 %)	0.006–0.03 µg/kg	Malissiova and Manouras, 2017
Martina Franca, Amiata, Sant' Andrea, San Domenico, Argentato di Sologno, Sardo, Ragusano, Poitou	Italy	HPLC-FLD	1/63	1 sample between LOD - and LOQ (0.004 µg/kg)	Altafini et al., 2020
	Italy (Sicily)	ELISA	0/84	< 0.007 µg/kg	Cammilleri et al., 2019
	Croatia	ELISA	14/14	Mean: 0.005 ± 0.001/ range: 0.003–0.01 µg/kg	Bilandžić et al., 2014
Baudet du Poitou	Germany	EIA	0/6	< 0.005 µg/kg	Gross et al., 2019

4. Conclusion

The global consumption of donkey milk is expected to rise, particularly in Europe, due to the increasing prevalence of cow's milk allergies. Our study found that milk from Balkan and Banat breed donkeys is safe in terms of AFM1 contamination, with levels consistently below the detection limit. However, the potential presence of AFM1 and other toxic substances in donkey milk poses a food safety risk, highlighting the need for comprehensive monitoring and control measures.

Future research should focus on larger-scale studies, risk assessment, and developing preventive measures to ensure the safety and quality of donkey milk as it becomes an increasingly popular alternative milk source.

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Review Paper

Occurrence of nitrates in milk products-review

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ABSTRACT

This review explores the role of nitrates, primarily sodium nitrate and potassium nitrate, in cheese production, particularly for semi-hard and hard cheeses. Nitrates are added as food additives to control microbial growth and prevent defects caused by gas-producing bacteria. The initial spike of nitrate levels during cheese production decreases over time, with trace levels remaining in the final product. Various regulations around the world dictate nitrate levels in cheese, with notable differences between the EU and the US. Nitrates can also originate from natural and environmental sources, influenced by cows' diets and agricultural practices. Furthermore, nitrates act as antimicrobial agents during cheese ripening, supporting product quality by inhibiting spoilage bacteria. There are several techniques used to analyse nitrates (NO3-), including spectrophotometers, Raman spectrometry, infrared and per fluorometrically-interferon- irradiated (IR and FTIR) spectroscopy, AAS, fluorophotography, chemical luminance, mass spectrometry, MECA, EPR, and NMR, Gas-Liquid Chromatography (GLC) with Electron-Capture Detector (ECD), High-Performance Liquid Chromatography (HPLC) with UV detection.

1. Introduction

Nitrates, primarily in the form of sodium nitrate ($NaNO_3$) or potassium nitrate (KNO_3), are used in cheese production, particularly for certain semi-hard and hard cheeses like Emmental, Gouda, Swiss type and Edam. These compounds are added as food additives to control microbial growth, specifically to prevent the "late-blowing" defect caused by gas-producing bacteria such as *Clostridium tyrobutyricum* and *Clostridium butyricum*. These bacteria can produce carbon dioxide during cheese ripening, leading to undesirable cracks, slits, or holes in the cheese (*Zamrik,* 2013). The addition of nitrate salts causes an initial spike in nitrate levels in the cheese; however, during ripening, the concentration continues to decrease to trace levels. Good manufacturing practices must be applied by law to avoid big natural content in milk. Plant-based ingredients, such as truffles, clover, herbs, peppers, seeds, are naturally high in sodium nitrate, and could increase the amount of nitrates and nitrites, when added to certain cheeses, especially cheese spreads or processed cheese (*Genualdi et al*, 2018). The use of nitrates in cheese production is controversial due to potential health risks, primarily related to the formation of N-nitrosamines, which are genotoxic and carcinogenic compounds. Nitrates can be reduced to nitrites in cheese, and nitrites can react

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with secondary amines (present in proteins or other compounds) to form N-nitrosamines, some of which (e.g., N-nitrosodiethylamine) are classified as carcinogenic by the International Agency for Research on Cancer (IARC) (https://www.foodtimes.eu/food-system-en/nitrites-nitrates-and-nitrosamines-efsa-revises-risk-analysis/), (*Grey et al*, 1979).

2. Nitrates as cheese additives

Nitrates are typically added to cheese milk at concentrations ranging from 10 to 30 g per 100 kg of milk in some regions, though regulations vary. In the European Union, up to 150 mg/kg of nitrates can be added to cheese milk, while the CODEX General Standard for Food Additives sets a maximum residual limit of 50 mg/kg in the final cheese product. In contrast, the United States does not approve nitrates as food additives in cheese production. (*Zamrik*, 2013). Raw cow's milk may contain between 1 and 5 mg/L of nitrate and less than 0.1 mg/L of nitrite (*Indyk and Woolard*, 2010). Nitrates can also be present in cheese from endogenous sources, such as milk contaminated with nitrates from water, forage, or fertilizers, or from plant-based ingredients in processed cheeses. Beet root is natural source of nitrates (*Blom-Zandstra*, 1989) and it is used as livestock feed. Studies have found nitrate levels in cheese ranging from below detection limits to 26 mg/kg, with most samples (93%) containing less than 10 mg/kg, indicating primarily endogenous sources (*Genualdi et al*, 2018). The internal level of nitrites in milk depends strongly on the quality of nutrients provided to the cows (*Topçu et al,* 2009; *Cristea,* 2008). Diets rich in nitrate but poor in fermentable energy reduce the capacity of rumen microbes to fully convert nitrate into ammonia, resulting in nitrite accumulation. These elevated nitrite concentrations can enter the bloodstream and be secreted into milk, where they affect both the technological properties of cheese and the survival of nitrite-tolerant pathogens. Nitrates and nitrites in milk products can originate from cow diet, ingestion of nitrates from feed or water (*Taghawi et al.,* 2025; *Genualdi et al*, 2020; *Branova et al*, 1993). Environmental contamination originates from fertilizers, agricultural runoff, or industrial waste increasing nitrate in water supplies

3. Nitrate's mechanism of action

Nitrates act as antimicrobial agents by being reduced to nitrites by nitrate-reducing bacteria during cheese ripening. Nitrites then inhibit the growth of spoilage bacteria, ensuring product quality (*Topçu et al*, 2006). Ingested nitrate (NO_3^-) is eliminated through the kidneys in around 60 % of cases, whereas notable 25 % undergoes active concentration within the salivary glands. Saliva that contains nitrate (NO_3^-) is released into the oral cavity, where facultative anaerobic bacteria use nitrate (NO_3^-) reductase enzymes to transform nitrate (NO_3^-) into nitrite (NO_2^-) (*Tiso and Schechter*, 2015). Following ingestion, nitrite-rich saliva is further metabolized non-enzymatically in the stomach's acid to produce nitric oxide (NO) followed by numerous biological effects of NO. Nitrates prevent spoilage in cheese in two main ways: Inhibition of microbial growth and control of gas-producing bacteria. Inhibition of microbial growth occurs when nitrates (NO_3^-) are reduced to nitrites (NO_2^-) by enzymes (e.g., nitrate reductase) produced by certain bacteria naturally present in cheese or starter cultures. Nitrites are the active antimicrobial agents. Nitrites disrupt the metabolism of spoilage-causing and pathogenic bacteria by interfering with their respiratory processes, enzyme activity, or cell membrane integrity. This creates an unfavorable environment for microbial growth. Nitrites may also react to form nitric oxide (NO), which further enhances antimicrobial effects by damaging bacterial DNA or proteins. Nitrates are particularly effective at preventing late blowing, a spoilage defect in cheese caused by gas production (e.g., carbon dioxide or hydrogen). Late blowing results in undesirable cracks, fissures, or off-flavors in cheese. By inhibiting gas-forming bacteria, as mentioned earlier *Clostridium spp*, also *Streptococcus bovis*, nitrates help maintain the structural integrity and sensory quality of cheese during ripening. Nitrates work in conjunction with other cheese preservation factors, such as low water activity, high salt content, low pH (due to lactic acid production by starter cultures), and competitive exclusion by beneficial bacteria. This creates a hurdle effect, making it difficult for spoilage organisms to thrive.

Microorganisms targeted by nitrates belong to Clostridium species, coliforms and other pathogenic bacteria. *Clostridium tyrobutyricum* anaerobic, spore-forming bacterium is a primary cause of late blowing in cheese. It ferments lactic acid into butyric acid, carbon dioxide, and hydrogen, leading to off-flavors (rancid or fermented smells) and textural defects. Nitrates effectively inhibit its growth, preventing these issues. Other *Clostridium spp*. *C. sporogenes* or *C. butyricum* can also contribute to spoilage, and

nitrates suppress their activity. Coliform bacteria, such as *Escherichia coli* or Enterobacter species, are associated with early gas production and off-flavors in cheese, often due to poor hygiene during milk collection or cheese production. Nitrites derived from nitrates inhibit their proliferation. Other pathogenic bacteria particularly *Listeria monocytogenes* can survive in cheese, mostly in soft or semi-soft varieties. Nitrites have been shown to reduce its growth, enhancing cheese safety. *Staphylococcus aureus* are successfully limited by nitrates can and this pathogen is a concern in cheeses made from raw milk or under improper hygienic conditions. Nitrates may also have some effect against *Salmonella spp*. and certain strains of *Bacillus spp*., although their primary role is against anaerobes like Clostridium. Scientific studies and reviews, such as those published in journals like International Dairy Journal and Journal of *Masoud et al,* 2011 demonstrated that nitrates effectively controlled *Clostridium tyrobutyricum* in Gouda cheese, reducing gas formation during ripening. A review of *Van Horde et al*, 2010 highlighted the role of nitrates in preventing late blowing and their synergistic effect with starter cultures in semi-hard cheeses. Research also indicates that the efficacy of nitrates depends on factors like cheese pH, salt content, and ripening conditions. For example, nitrates are more effective in cheeses with a pH below 6.0, where nitrite formation is enhanced. The use of nitrates in cheese is regulated due to concerns about nitrosamine formation (potentially carcinogenic compounds). For example, the European Union permits nitrate use in specific cheeses (e.g., up to 150 mg/kg in Gouda or Edam), but levels are tightly controlled. Some cheese producers use lysozyme (an enzyme) or specific starter cultures to control Clostridium and other spoilage organisms as alternatives or complements to nitrates.

Nitrates prevent cheese spoilage by being converted to nitrites, which inhibit the growth of spoilage and some pathogenic microorganisms, particularly *Clostridium tyrobutyricum* (responsible for late blowing), but coliforms, and pathogens like *Listeria monocytogenes* and *Staphylococcus aureus* can survive in presence of nitrites. Their antimicrobial action disrupts bacterial metabolism and prevents gas production, preserving cheese quality. Nitrite-tolerant pathogens (*L.monocytogenes, E. coli, Salmonella spp.*) are food safety concern, regardless to nitrites because nitrite in cheese does not guarantee inhibition. Scientific studies confirm their efficacy, especially in hard and semi-hard cheeses, when used within regulatory limits.

4. Benefits of nitrates

Nitrates (NO_3^-) enhance cardiovascular health by lowering blood pressure. Nitric Oxide (NO) relaxes and widens arteries and veins, resulting in enhanced blood circulation and tissue oxygen supply throughout the body (*Bentley et al.*, 2017). They are also used in the form of dietary supplements, particularly in the context of sports nutrition. Over the past few years, the role of nitrates (NO_3^-) in promoting human physical condition and preventing diseases has received increasing attention from researchers and health professionals (*Affourtit et al.,* 2015). Dietary nitrate (NO_3^-) influences its effects by producing nitric oxide (NO) in the physiological system. Numerous physiological and pathological processes, such as cardiac contraction, immunomodulation, prevention of platelet aggregation, vasodilation, and neuronal transmission, depend on the intra and intercellular signaling molecule nitric oxide (NO) (*Kanno et al*, 2004).

5. Main risks associated with nitrates in cheese production

The use of nitrates in cheese production is controversial due to potential health risks, primarily related to the formation of N-nitrosamines, which are genotoxic and carcinogenic compounds. Nitrates can be reduced to nitrites in cheese, and nitrites can react with secondary amines (present in proteins or other compounds) to form N-nitrosamines, some of which (e.g., N-nitrosodiethylamine) are classified as carcinogenic by the International Agency for Research on Cancer (IARC) (https://www.foodtimes.eu/food-system-en/nitrites-nitrates-and-nitrosamines-efsa-revises-risk-analysis/), (*Grey et al,* 1979). Studies indicate that while N-nitrosamine formation is well-documented in cured meats due to high protein content and cooking at high temperatures, there is less data on their presence in cheese. Potential of nitrosamines exists, particularly in cheeses with added nitrates, as the protein-rich environment can facilitate nitrosamine formation under certain conditions (e.g., specific pH or microbial activity (*Grey et al,* 1979). Cheeses often contain lower levels of nitrosatable precursors and may have protective compounds (e.g., antioxidants like vitamin C), which reduce nitrosamine formation. Modern cheese-making practices, such as improved milk quality and pasteurization, have reduced the need for nitrate addition, further lowering this risk

(Johnson, 2017). Cured meats are nevertheless high in nitrite (NO_2^-) and nitrate (NO_3^-) contents and could be the reason for developing colo-rectal cancer (*Shakil et al,* 2022). Because of risk posing to nitrosamines from nitrates, The European Union (EU), and the World Health Organization's (WHO) Joint FAO/Food and Drug Administration (FDA) Expert committee on Food additives (JECFA), have established an ADI (Acceptable Daily Intake) of nitrates (NO_3^-) for a person with body weight (body-weight-kg-1) of 0–3.7 parts per million (b.w.), equivalent to 222 parts per million (ppm) Nitrate (NO_3^-) daily for an adult 60 kg and ADI of 0–0.07 mg kg-1 b.w. for nitrites (NO_2^-) (WHO/JECFA, 2015). While cheese contributes only a small fraction of dietary nitrate intake (0.2% of ADI in some studies), high consumption of nitrate-rich foods (e.g., vegetables, processed meats, and cheese) could lead to exceeding the ADI, particularly in vulnerable populations like infants. (*Karwowska M. and Kononiuk A.,* 2020; *Roila et al,* 2018). A study in Belgium found that cheese and meat products contributed 0.2% and 6% of the nitrate ADI, respectively, with vegetables being the primary source (50%). In Syrian white cheese, nitrate levels ranged up to 26 mg/kg, but nitrite levels were generally below detection limits, suggesting minimal risk from cheese alone (*Zamrik,* 2013). However, cumulative exposure from multiple sources remains a concern, especially if nitrate levels in cheese are not strictly controlled. (*Roila et al.,* 2018). Methemoglobinaemia is a condition where nitrate is reduced to nitrite in the body, oxidizing hemoglobin to methemoglobin, which impairs oxygen transport. This is particularly concerning for infants, who are more susceptible due to their immature enzymatic systems. Acute toxicity occurs at nitrate levels of 2–9 g (2000–9000 mg), which is unlikely from cheese consumption alone but could be a risk from contaminated water or other sources. (*Zamrik*, 2013) Studies suggest that the low nitrate levels in cheese (typically <10 mg/kg) do not pose a significant risk for methaemoglobinaemia. A U.S. study found no nitrite concentrations above 0.1 mg/kg in 64 cheese samples, indicating minimal risk from nitrites in cheese (*Genualdi et al,* 2018). Nitrate levels in raw milk are generally low, typically ranging from less than 1 to 12 mg/kg. Nitrite is often practically absent or at trace levels (*Zbikowski et al,* 2000). An old study found that nitrate content in milk from cows given drinking water with 0–180 mg/L nitrate remained very low, suggesting minimal impact from water contamination (*Kamemerer et al,* 1992). Another study reported a rapid increase in nitrate content (up to 5.6 mg/L in one cow and 3.2 mg/L in another) after administering sodium nitrate, but levels returned to a normal range of ~1 mg/L within 24 hours (*Geissler et al,* 1991). After administering potassium nitrate (KNO_3) to dairy cows, residual nitrate in milk reached 34.6 mg/L two hours after a 150 g dose, with elevated levels persisting up to 38 hours (*Baranová et al,* 1993). In a study from Shijiazhuang, China, liquid milk had an average nitrate content of 10.05 mg/kg (ion chromatography) and 9.95 mg/kg (cadmium column reduction method), with about 14% of samples exceeding regulatory limits. (*Ping et al.,* 2013). In Taiwan, nitrate concentrations in milk ranged from 0.3 to 417.7 mg/kg, with an average of 92.7 mg/kg. Nitrite was below the detection limit 0.07mg/kg in all samples. (*Yeh et al.,* 2013). In the United States, milk powders showed average nitrate concentrations of 17 ± 12 mg/kg, with nitrite detected above 2 mg/kg in 5 out of 39 brands, including both dairy and plant-based (soy and coconut) powders (*Genualdi et al,* 2020). In Taiwan, milk powder had an average nitrate content of 25.74 mg/kg (ion chromatography) and 25.66 mg/kg (cadmium column reduction method) (*Ping et al,* 2013). In a survey of commercial dry milk samples, nitrate levels were slightly higher (1–3 ppm more nitrate-nitrogen) in products from direct-fired (gas) dryers compared to indirect-heated (steam) systems (*Manning et al,* 1968). In Iran, infant formulas contained nitrate levels ranging from 0.221 to 1.347 mg/kg (mean 0.645 mg/kg) and nitrite from 0.045 to 0.263 mg/kg (mean 0.151 mg/kg). Baby foods had nitrate levels from 0.24 to 1.93 mg/kg (mean 0.99 mg/kg) and nitrite from 0.04 to 1.45 mg/kg (mean 0.36 mg/kg). All were below WHO acceptable daily intake limits (*Taghawi et al.,* 2025). Human breast milk nitrate levels vary by production stage: Colostrum (days 1–3 postpartum): 0.19 mg/100 mL nitrate, 0.08 mg/100 mL nitrite. Transition milk (days 3–7): 0.52 mg/100 mL nitrate, 0.001 mg/100 mL nitrite. Mature milk (days >7): 0.31 mg/100 mL nitrate, 0.001 mg/100 mL nitrite (*Hord et al,* 2011).

Nitrates in cheese can originate from contaminated water, forage, or fertilizers used in dairy farming, reflecting poor hygienic conditions or environmental pollution. Adding nitrates to mask poor hygiene during cheese production is a concern, as it may compensate for inadequate sanitation practices (*Zamrik,* 2013; *Topçu et al,* 2006). Research on Turkish and Syrian cheeses noted that nitrate

levels could be elevated due to external contamination (e.g., nitrate fertilizers or water). For example, Turkish white cheese samples showed nitrate levels up to 17.19 mg/kg, attributed to both added nitrates and environmental sources. Stricter controls on milk quality and production hygiene are recommended to minimize reliance on nitrate additives (*Topçu et al,* 2006). *Koréneková et al.* (2000), studied nitrate dynamics in Emmental cheese production, finding that nitrate levels decreased significantly from 81.2 mg/kg in milk to 3.3 mg/kg in the final product, with most nitrates passing into whey. This suggests that residual nitrates in cheese are minimal under controlled conditions. *Genualdi et al.* (2018) developed a method to measure nitrates and nitrites in U.S. cheeses, finding low levels up to 26 mg/kg for nitrates, none for nitrites above 0.1 mg/kg, indicating that endogenous nitrates dominate and intentional addition is rare in the *U.S. EFSA* (2022) updated risk analysis on N-nitrosamines in food, confirming their genotoxic and carcinogenic potential and emphasizing the need to monitor nitrate use in cheese and other products. *Zamrik* (2013) noted that nitrate use in Syrian white cheese (10–30 g/100 kg milk) could pose health risks if doses exceed recommended levels, particularly due to potential carcinogenicity. *Topçu et al.* (2022) found nitrate levels in Turkish cheeses ranging from 0.68–17.19 mg/kg, with higher levels in sheep's milk cheeses, highlighting the need for stricter controls to avoid masking poor hygiene. The risk of N-nitrosamine formation in cheese is lower than in cured meats due to lower protein content and the presence of protective antioxidants, but it remains a concern requiring further research (*Grey et al,* 1979). Regulatory limits and improved hygiene practices have reduced nitrate use, but environmental contamination and cumulative dietary exposure remain challenges. (*Zamrik,* 2013; *Topçu et al.,* 2022).

6. Methods for nitrates detection

There are several techniques used to analyze nitrates (NO_3^-), including spectrophotometers, Raman spectrometry, infrared and per fluorometrically-interferon- irradiated (IR and FTIR) spectroscopy, AAS, fluorophotography, chemical luminance, mass spectrometry, MECA, EPR, and NMR. Every method has its fundamental tenets, detection range, detection limits, sample throughput percentage, benefits, and drawbacks (*Lin et al,* 2019). Methods for determining nitrates in cheese typically involve dissolving the cheese in water, followed by reducing nitrate to nitrite and quantifying the nitrite content using a spectrophotometer. The International Dairy Federation standard method is a common approach. Several methods exist for detecting nitrate in cheese. These include spectrophotometry, ion chromatography, and cadmium reduction followed by spectrophotometry (*Tudor et al.* 2007). Ion chromatography separates the ions in a sample (including nitrate and nitrite) based on their affinity for a stationary phase. The separated ions are then detected, providing a means to quantify their concentrations (*Genualdi et al,* 2018). Cadmium Reduction involves reducing nitrate to nitrite using a cadmium column resulting nitrite is then measured using spectrophotometry by ISO14673-1;2004. Gas-Liquid Chromatography (GLC) with Electron-Capture Detector (ECD) involves nitration of a compound, extraction, and analysis by GLC-ECD (Tanaka et al, 1982). High-Performance Liquid Chromatography (HPLC) with UV detection uses HPLC to separate compounds and UV detection for nitrate, while UV/vis spectrophotometry is used for nitrite (*Srivichien et al.,* 2015). The choice of method depends on the specific needs and capabilities of the laboratory. Some methods, like cadmium reduction, may involve the use of toxic substances. Other methods, like ion chromatography, can provide more accurate and sensitive results for detecting low levels of nitrates and nitrites.

7. Conclusion

Good manufacturing practices must be applied by law to avoid big natural content in milk. Plant-based ingredients, such as truffles, clover, herbs, peppers, seeds, are naturally high in sodium nitrate, and could increase the amount of nitrates and nitrites, when added to certain cheeses, especially cheese spreads or processed cheese. Risk of nitrates exposure from cheese and milk products is rather low, comparing to cured meat and other protein rich products. Methods for detection of nitrates are various and they should be adjusted to needs of the laboratory sample throughput as well as to expected content of nitrates in samples.

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Original Scientific Paper

Effect of pH and water activity (a_w) on survival of Yersinia enterocolitica in fermented sausages: challenges and risk control

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ABSTRACT

Fermented sausages are a popular product in many cultures, but their production can pose a risk due to the presence of pathogenic microorganisms, such as *Yersinia enterocolitica*. This bacterium is known for its ability to survive in meat products, and its survival in fermented sausages is highly dependent on parameters such as pH and water activity (a_w). In this paper, the factors influencing the survival of *Y. enterocolitica* in fermented sausages are investigated, with special emphasis on the control of pH and a_w during production. Also, possible strategies for reducing the risk of contamination in the production process are considered.

1. Introduction

Yersinia enterocolitica is a bacterium that causes zoonotic disease in humans and is associated with consumption of contaminated meat. In fermented sausages, where the meat batter is subjected to the fermentation process, the survival and growth of microorganisms are influenced by various factors, among them pH and water activity (a_w) (*Baltić et al*., 2011, *Baltić et al*., 2009). The basic and most important strategy for meat preservation, including of fermented sausages, has been based for centuries on salt and its importance in controlling the growth of spoilage bacteria (especially pathogens), achieving the desired texture and enabling slicing (*Montanari et al*., 2022). These factors play a key role in the development or suppression of pathogens (*Savić & Savić*, 2002). Fermented sausages are considered shelf-stable if their a_w is around 0.92 (https://www.meatsandsausages.com/sausage-types/fermented-sausage), and pH is around 6.0 (*Olivares et al.*, 2010).

2. Materials and methods

For the purposes of the study, category I and II pork meat were used, as well as a certain proportion of pork fat. The meat was ground in a meat grinder to the desired degree of fineness. It was left to cool at a temperature of 0 to 5 °C. After 24 hours, a spice mixture (containing glucose, table salt, spices, and spice extracts) and 2.3% nitrite salt were mixed

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in, and then the minced meat was divided into four groups. A prepared culture of the reference strain *Y. enterocolitica* ATCC 9610 (8-9 log10 CFU/ml) was added to the stuffing of half the sausages. The starter culture was composed of *Lactobacillus sakei*, *Staphylococcus carnosus* and *Staphylococcus xylosus*. The starter culture was added in amount of 20 g per 200 kg of ground meat. The prepared stuffing was filled into casings to prepare narrower (34 mm) and wider (55 mm) diameter sausages as follows: the first sausage group (OI) consisted of narrower diameter sausages without a starter culture; the OII group consisted of wider diameter sausages without a starter culture; the OIII group consisted of narrower diameter sausages with a starter culture, and; the OIV group consisted of wider diameter sausages with a starter culture. The sausages were labeled by groups and placed on racks in the smokehouse (smoked at 20-23 °C, dried at 17 °C and a relative air humidity of 75%). The production process lasted 18 days for sausages of narrower diameter and 35 days for sausages of wider diameter.

Microbiological analyses were conducted by counting *Y. enterocolitica* bacteria according to *ISO* (2017). Chemical and physico-chemical analyses conducted were investigation of pH and a_w. Determination of pH was conducted by the direct method with a pH meter 150 (Testo, Germany) and was performed according to the manufacturer's instructions. The determination of a_w was conducted by using a method described previously (*Mitrović et al*., 2021). The obtained results were compared through statistical analysis using Microsoft Excel 2010 and GraphPad Prism software, version 8.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). All parameters were described by means and standard deviation (SD).

3. Results

At the beginning of the study, the mean number of *Y. enterocolitica* in the stuffing of the experimental (contaminated) groups of narrow and wide diameter sausages was 6.17±0.04 log CFU/g. In the narrow diameter sausages, i.e., group OI (group to which no starter culture was added), and group OIII (group to which starter culture was added), the average number of *Y. enterocolitica* on day 3 was 4.84±0.10 log CFU/g and 4.49±0.04 log CFU/g, respectively, and on day 7 was 5.30±0.01 log CFU/g and 4.99±0.04 log CFU/g, respectively. The presence of *Y. enterocolitica* was not detected in sausages with a narrower diameter on days 12 or 18 of the study (end of the production process) or in sausages with a wider diameter on day 25 of ripening.

At the end of ripening (day 18), the mean pH of the OI group sausages (without the added starter culture, narrower diameter) was 5.40±0.06, and of the OIII group (with added starter culture, narrower diameter) was 5.24±0.08. On day 35 (also the end of ripening for wider diameter sausages), in the wider sausage diameter OII group (without the addition of starter culture), the pH at the end of ripening was 5.24±0.03, and in the wider sausage group, OIV (with the addition of starter culture), the mean pH was 5.07±0.05.

It was found that the average a_w of the sausage stuffing on day 0 of the study was 0.9695±0.0007. At the end of ripening (day 18), the a_w of the narrower diameter OI group sausages were 0.9200±0.0007. At the end of ripening, the a_w of the narrower diameter sausages of the OIII group was 0.9235±0.0004. Also, at the end of ripening (day 35), the a_w of the wider diameter OII group was 0.9359±0.0006, while the a_w of the wider diameter of the OIV group sausages was 0.9379±0.0005.

4. Discussion

Food products are often treated with methods to prolong their shelf-life and to contribute to their safety, such as drying that reduces a_w, adding salt or sugar, or pH regulation with natural or added acids (*EFSA*, 2012). Studies show that using starter cultures, such as *Lactobacillus* spp., can significantly reduce *Y. enterocolitica* levels in fermented sausages. These added bacteria produce lactic acid, bacteriocins, and hydrogen peroxide, which inhibit pathogenic bacteria. Research indicates that proper drying and maturation periods enhance safety.

Lowering pH and reducing a_w through controlled fermentation and drying processes can prevent bacterial survival, growth of pathogens, and also extend the shelf life of the product (*Prpich et al*., 2021). *Y. enterocolitica* can lead to gastroenteritis, manifesting as diarrhea, abdominal pain, and vomiting. This pathogen is most commonly spread by consuming raw or undercooked meat, unpasteurized milk and contaminated water. *Y. enterocolitica* can survive and even reproduce at low temperatures, meaning refrigerated food can still be a source of infection. In rare cases, the bacterium can lead to severe complications such as septicemia, arthritis, or erythema nodosum (*Ivanović et al*., 2016).

Y. enterocolitica is known for its ability to survive in a wide range of pH values, but an acidic environment significantly reduces its viability (*Leroy et al*., 2015). The combination of low a_w and acidic pH can be an effective strategy to control this bacterium in food products (*Barbieri et al.*, 2021).

Y. enterocolitica and other microorganisms require a certain availability of water for growth and survival. However, reducing the a_w can limit the growth of bacteria, molds and yeasts (*Mitrovic*, 2016**).** For example, bacteria usually cannot survive when the a_w is below 0.9, while molds stop growing below a_w 0.7 (*Laranjo et al*., 2019).

5. Conclusion

During the ripening of the fermented sausages, there was a decrease in the a_w and number of *Y. enterocolitica*. The number of *Y. enterocolitica* bacteria was lower on all test days in the sausages to which the starter culture was added. The presence of *Y. enterocolitica* was not detected in the sausages with a narrower diameter on day 12, or in sausages with a wider diameter on day 25 of ripening.

The pH of all examined groups of fermented sausages decreased during the ripening process, and at the end of ripening it was lower in sausages with a wider diameter and in sausages in which starter cultures were used.

During the ripening process, the a_w of the control and experimental groups of fermented sausages of narrower and wider diameter decreased, but was higher in the sausages in which starter cultures were used.

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Original Scientific Paper

Dietary fibre intake in bread, cake and fermented vegetables

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ABSTRACT

White flour is sourced from the major storage tissue of the grain, which constitutes about 80% of its composition. Consuming pickled vegetables offers health benefits, including anti-cancer, anti-diabetic, and immune-regulatory effects. Our analysis examined 60 samples of bread, cake, and pickled vegetables using the total dietary fibre (TDF) method according to the AOAC standards. The confidence intervals for bread, desserts, and pickled vegetables were found to be relatively narrow: bread has a mean value of 3.02% (CI: 2.04-3.44), desserts have a mean value of 1.96% (CI: 1.39-2.52), and pickled vegetables have a mean value of 1.88% (CI: 1.68-2.08). However, despite containing significant amounts of total dietary fibre, bread, desserts, and pickled vegetables cannot be labelled as sources of fibre because they contain less than 1.5 grams per 100 kcal. Our results showed that bread has the highest dietary fibre content (3.02%). Pickled vegetables are also a source of fibre (1.88%), a finding confirmed in other studies. Dessert cakes vary greatly in fibre content (1.96%) depending on whether the cake is made of chocolate or another ingredient.

1. Introduction

Whole grain cereals, pulses, fruit and vegetables and potatoes are the main sources of dietary fibre. Nuts and seeds also contain high concentrations of fibre. Cellulose, along with hemicelluloses, is found in cereals (*Arzami et al.*, 2022). The lignified outer layers of whole grain products are the main source of fibre. Oats and barley are rich in a water-soluble, viscous type of polysaccharide known as β-glucan (*Gajdošová et al.*, 2007; *Lante et al.*, 2023). Pectins, another type of dietary fibre, are predominantly found in fruits and vegetables and share similar properties. (*Dietary Guidelines for Americans*, 2020). While dietary fibre is not an essential component of the diet, it plays a significant role in bowel function and alleviating gastrointestinal issues such as constipation (*EFSA*, 2010). White flour is derived from the major storage tissue of the grain, which makes up about 80% of the grain's composition. The grain typically consists of 10-15% protein, 80% starch, around 4% dietary fibre, soluble sugar, and minor amounts of minerals and vitamins, particularly B vitamins (*Shewry et al.*, 2023). Pickled vegetables are preserved and flavoured by being submerged in a solution of vinegar, salt, and often sugar. This pickling process allows the vegetables to ferment, giving them their distinct tangy flavour and extending their shelf life. Common types of pickled vegetables include cucumbers, carrots, beets, onions, cabbage, and peppers (*Featherstone*, 2016; *Tan et al.*, 2024). Consuming pickled vegetables

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benefits human health, such as anti-cancer effects, anti-diabetic effects and immune regulation (*Tan et al*., 2024). A study by *Galena et al*. (2022) suggests that positive changes in the abundance of certain bacterial species may be associated with consuming fermented vegetables. Future adequately powered human trials are needed to explore the relationship between fermented vegetables, health-related outcomes, and the microbial composition of the gut. In addition, dietary fibre intake is inversely associated with the risk of dementia *(Yamagishi et al*., 2022). Previously, published results for vegetables and meat products were reported (*Bajčić et al.*, 2019, *Trbović et al*., 2023). However, a higher intake of total dietary fibre within the typical American diet is unlikely to significantly reduce the risk of colorectal cancer (He et al., 2019). This study aims to provide new insights into dietary fibre in bread, desserts, and pickled vegetables.

2. Materials and methods

2.1 Samples

We analysed 60 samples of bread, cake and pickled vegetables using a high-quality laboratory blender (Robot Coupe Blixer 2 of 2.9 L, France). Following the manufacturer's guidelines ensured reliable and trustworthy results. Samples were taken from the following pickled vegetables: cauliflower, pickled cucumbers, mixed salad, garlic peppers and sauerkraut. For dessert, the following cake samples were collected: Mađarica (layered chocolate cake), cream puffs (vanilla cake), Kotor krempita (vanilla cake), baklava (honey, sugar and vanilla cake), and Rigo Janchi (chocolate cake).

2.2 Methods

The total dietary fibre (TDF) method includes correcting residue for undigested protein and mineral contamination, following the AOAC method. Dietary fibre is expressed in g/100 kcal, calculated based on the estimated energy value of the tested samples.

2.3 Statistical analysis

Statistical analyses including mean, standard deviation, minimum and maximum, median, confidence interval and dietary fibre in g/100 kcal were conducted using Microsoft Office 2010 Excel software and its Data Analysis Tools.

Table 1. Dietary fibre and basic statistical parameters in food items (bread, dessert cake and pickled vegetables)

Samples	Dietary fibre, % Mean ± SD (min-max)	Median, %	Confidence interval 95%, CI	Dietary fibre g/100kcal
Bread (n=37)	3.02±1.20 (1.28-5.41)	2.98	2.04-3.44	1.14
Dessert cake (n=7)	1.96±0.61 (1.27-2.85)	1.7	1.39-2.52	0.59
Pickled vegetables (n=16)	1.88±0.37 (1.39-2.43)	1.81	1.68-2.08	0.71

2. Results and discussion

The median is used when a dataset includes a value that significantly deviates from the rest of the series. This outlier can greatly influence the mean, but it does not affect the median at all. The median accurately represents the true values for bread, dessert, and vegetable products. In frequentist statistics, a confidence interval (CI) is defined as an interval that is expected to contain the estimated parameter. Typically, a confidence level of γ, such as 95% or 99%, is used. The confidence intervals for bread, dessert, and pickled vegetables are relatively narrow: bread has a mean value of 3.02%, which is close to the median value of 2.98% and CI 95% (2.04-3.44), dessert has a mean value of 1.96%, which is close to median value of 1.7% and CI 95% (1.39-2.52), and pickled vegetables have a mean value of 1.88%,

which is close to median value of 1.81% and CI 95% (1.68-2.08). According to the *FAO/WHO* (1998), the average energy factor for dietary fibre is 2 kcal per gram. As per the Regulation of EC (2006), food can be labelled as a source of fibre if it contains at least 1.5 grams of fibre per 100 kcal. However, bread, dessert, and pickled vegetables cannot be labelled as a source of fibre because they contain less than 1.5 grams per 100 kcal, despite having a significant amount of total dietary fibre, as noted in wheat by *Shewry et al*. (2023) (15.1% on dry basis), *Thebaudin et al.* (1997) (60% on dry basis), *Portugal et al*. (2013) (2.84%) and *Mongeau & Brassard* (white bread 2.10%). Our results showed that bread has the highest dietary fibre content (3.02%). Pickled vegetables are also a source of fibre, which has been confirmed in a study of *Knez et al*. (2023), pickled vegetables in fermented beetroot (0.8%), and red cabbage pickled (1.7%). Dessert cakes vary greatly in fibre content from 2.4-3.2% (*Care Omnia*, 2025) to 1.3% (My Food Data, 2025), depending on whether the cake is made of chocolate or another ingredient.

3. Conclusion

White flour is sourced from the major storage tissue of the grain, which constitutes about 80% of its composition. Consuming pickled vegetables offers health benefits, including anti-cancer, anti-diabetic, and immune-regulatory effects. In our analysis, we examined 60 samples of bread, cake, and pickled vegetables using the total dietary fibre (TDF) method, which includes adjustments for undigested protein and mineral contamination, according to the AOAC standards. This study aims to provide new insights into dietary fibre in bread, desserts, and pickled vegetables. Our results showed that bread has the highest dietary fibre content.

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Review Paper

The presence of virulence-related genes among *Listeria Monocytogenes* strains and their correlation with pathogenic potential

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ABSTRACT

Listeria monocytogenes remains a significant public health concern due to its high mortality rate in susceptible individuals. Contaminated food, particularly ready-to-eat products, are the primary route of transmission to humans. Previous studies have shown that *L. monocytogenes* exhibits heterogeneous virulence, with strains ranging from hypervirulent to hypovirulent. *L. monocytogenes* has been found to have pathogenicity islands and other virulence factors scattered across the bacterial genome (e.g. *inlAB* locus). Further, the *inlA-inlB* locus and LIPI-1 are conserved in almost all *L. monocytogenes* isolates, emphasizing their important role for pathogenicity. Literature data showed that using genetic virulence profiles to predict virulence potential offers useful information for risk assessment in the food sector, although it also has drawbacks. Also, the use of whole-genome sequencing as the gold standard approach, enhances quantitative microbiological risk assessment and improve listeriosis control.

1. Introduction

It is well known that the intracellular pathogen *Listeria monocytogenes* is the cause of listeriosis in humans and animals such as sheep, goats, cattle, pigs and fowl (*Borovic et al.*, 2014; *Headley et al.*, 2014; *OIE,* 2014; *Nilsson & Karlsson*, 1959; *Biester & Schwarte*, 1940; *Ramos et al.*, 1988). In ruminants, several predisposing factors contribute to the occurrence of listeriosis (*Burgess & Lohmann,* 2006; *Amene & Firesbhat,* 2016) including immune status, seasonal variations, production-related stress and management practices such as inadequate housing, overcrowding and insanitary conditions. This bacterium is widespread in farm environments, food processing areas and food products (*Lakicevic et al.*, 2022). Although *L. monocytogenes* evolves slowly, it exhibits a high degree of diversity (*Ragon et al.*, 2008; *Orsi et al.*, 2011). It is a member of a genus that currently includes 28 known species, 22 of which have been reported since 2010 (*Orsi et al.*, 2024). As noted by *Dreyer et al.* (2016), ruminants are exposed to a wide range of genetically diverse strains during their time on farms. Nonetheless, a particular hypervirulent genotype, ST1, is more frequently associated with rhombencephalitis than with other illnesses. Also, *L. ivanovii* has pathogenic potential to infect ruminants, causing abortions, stillbirth and sepsis. These are the two (*L. monocytogenes* and *L. ivanovii*)

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species that share many virulence factors, contributing to their ability to cause disease (*Orsi & Wiedmann,* 2016).

L. monocytogenes infection depends on various virulence factors involved in pathogenesis, the effectiveness of the host's immune system and the number of ingested bacteria (FAO/WHO, 2004). Since variations in virulence among strains of *L. monocytogenes* can influence infection and clinical outcome, assessing the virulence potential of isolates is crucial for public health (*Smith et al.*, 2019). Important virulence factors are encoded by the PrfA- regulated *inlAB* locus (involvement in adhesion) and the pathogenicity islands LIPI-1, LIPI-3, and LIPI-4 (*Gelbíčová et al.*, 2015; *Maury et al*., 2016; *Quereda et al.*, 2018). Significantly, all *L. monocytogenes* isolates have a conserved *inlAB* locus and the LIPI-1 island underlying their critical role in pathogenicity.

2. Internalins

Internalin A (*inlA*), an 80 kDa surface-invasion protein, is one of *L. monocytogenes*' primary virulence factors and was first reported by *Gaillard et al*. (1991). In the initial stage of intestinal barrier invasion, *inlA* mediates the pathogen's adherence and internalization into enterocytes via its specific receptor, E-cadherin (*Drolia & Bhunia,* 2019). Of the four divergent lineages, only hypovirulent lineage II strains harbor a truncated form of *inlA*, which could explain the low occurrence of these strains in human outbreaks (*Maury et al.*, 2016; *Van Stelten et al.*, 2010). There have been fewer documented listeriosis outbreaks in Japan compared to other countries, which may suggest that circulating strains of *L. monocytogenes* in Japan possess more mutations in *inlA* or in other virulence factors, potentially reducing their ability to invade cells (*Yamazaki et al.,* 2025). Internalin B (*inlB*), another a surface protein, also plays a pivotal role in initiating infection via its cellular receptor, c-Met, but unlike *inlA*, it does not possess the LPXTG motif (*Buchrieser*, 2007). *Quereda et al.* (2019), using the lineage I strain F2365, showed that *inlB* increases *L. monocytogenes* infection of the liver and spleen. Besides *inlA* and *inlB*, the literature also describes other members of the *inl* family, with *inlC*, *inlF* and *inlP* being extensively characterized (*Bierne et al.,* 2007; *Disson & Lecuit*, 2013; *Faralla et al.,* 2018; *Ghosh et al.*, 2018).

3. Listeriolysin

Listeriolysin (LLO), a pore-forming cytolysin encoded by *hly*A, enables bacteria to escape the phagosome and enter the cytosol of the infected cell where they multiply (*Birmingham et al.*, 2008; *Nguyen et al.*, 2019). To prevent lysis of the host plasma membrane, the activity of LLO must be regulated through multiple regulatory mechanisms. Recently, *Agbavor et al.* (2024) demonstrated that chaperone PrsA2 controls the secretion, stability, and folding of LLO throughout the bacterial infection. According to *Wang et al.* (2015), the flavonoid fisetin is an effective antagonist of LLO-mediated hemolysis. Furthermore, *Li et al.* (2020) concluded that morin, an edible flavonoid, prevents the oligomerization of LLO and effectively reduces the inflammation caused by *Listeria* infection. *Wang et al.* (2022) demonstrated that kaempferol reduces *L. monocytogenes* infection by inhibiting LLO pore formation and the inflammatory response.

4. Listeria pathogenicity island (LIPI-1)

The highly conserved *Listeria* pathogenicity island (LIPI-1) harbors the most significant virulence-associated genes of *L. monocytogenes*, including the previously mentioned *hly*, *actA*, *plcA, plcB* and *mlp* genes, which are positively regulated by transcriptional regulator PrfA (*Wiktorczyk-Kapischke et al., 2023*; *Sibanda & Buys, 2022*; *Osek & Wieczorek*, 2022; *Quereda et al.*, 2021*).* LIPI-1 is present in both pathogenic *L. monocytogenes* and *L. ivanovii* but it is absent in the avirulent *L. innocua*. However, LIPI-1 was discovered in atypical hemolytic *L. innocua* strains (*Johnson et al.,* 2004), which exhibited virulence potential in both the mouse model (*Bolger et al.*, 2014) and zebrafish larvae model (*Kaszoni-Rückerl et al.*, 2020) although these strains were less virulent compared to *L. monocytogenes.* The study of *Gradovska et al.* (2023) highlighted that *L. innocua* possesses diverse virulence potentials for cattle, underscoring its significance in the dairy production chain and cattle breeding. The authors also emphasize that unrecognized *L. monocytogenes* contamination and outbreak events could be indicated by the presence of *L. innocua*. Also, some isolates of *L. seeligeri* exhibit hemolytic activity and carry LIPI-1 (*Müller et al.,* 2010; *den Bakker et al.,* 2010). A previously healthy adult presenting with acute purulent meningitis is one of the rare

human cases of *L. seeligeri* that have been reported (*Rocourt et al.*, 1986).

L. ivanovii harbors a similar chromosomal island called LIPI-2 (22 kbp), which can cause listeriosis in ruminants, particularly sheep (*Vázquez-Boland et al.*, 2001). This region includes the genes *i-inlB2*, *i-inlL*, *i-inlK*, *i-inlB1*, *i-inlJ*, *i-inlI*, *i-inlH*, *i-inlG*, *smcL*, *i-inlF*, *i-inlE* and *surF3, which mainly code for listerial proteins of the internalin family (Sergeant et al.*, 1991; *Guillet et al.,* 2010).

4. Listeria pathogenicity island (LIPI-3)

Listeria pathogenicity island (LIPI-3), primarily found in lineage I isolates and typically absent from lineage II isolates, encodes listeriolysin S (LLS), a bacteriocin that alter the host intestinal microbiota (*Quereda et al.,* 2017). Oxidative stress induces the expression of LLS, which enhances its ability to evade phagosomes and increases pathogenicity. Although it is representative of clinical isolates, LIPI-3 is also found in food strains, emphasizing their possible risk to humans (*de Melo Tavares et al.,* 2020).

4. Listeria pathogenicity island (LIPI-4)

Listeria pathogenicity island (LIPI-4), a six virulence gene cluster (6 kbp), is linked to central nervous system infections and to maternal neonatal infections (*Maury et al.*, 2016). It was reported that LIPI-4 is most prevalent in hypervirulent CC4 and CC87 strains (*Kim et al.*, 2018). However, literature data also show that some *L. innocua* strains carry LIPI-4 (*Disson et al.*, 2021; *Moura et al.*, 2019). *Lee et al.* (2023) reported that LIPI-4 was highly conserved across examined strains of *L. innocua* and strains that had been previously studied (*Moura et al*., 2019). Nevertheless, LIPI-4 of *L. innocua* and its counterpart in *L. monocytogenes* have been shown to be much less similar, with sequence identities ranging from 83.7% to 84.0%, indicating that these two species possess distinct variants of LIPI-4 (*Lee et al.*, 2023).

5. Conclusion

Understanding the intraspecies variability of virulence genes in *L. monocytogenes* is essential for risk assessment, outbreak investigation and the development of targeted prevention and control strategies. Whole genome sequencing supports risk assessment and surveillance, helping to identify potentially high-risk strains in food production and processing environments. Despite extensive understanding of intraspecies pathogenicity, all strains are still classified as pathogenic for regulatory purposes.

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Review Paper

Phytonutrients in meat products: plant extraction and formulation of new functional products

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ABSTRACT

Enriching meat products with plant extracts has become an innovative strategy to improve product quality, extend shelf life and meet the growing demand for natural, functional foods. Plant extracts, especially those rich in polyphenols, flavonoids and terpenes, have strong antioxidant, antimicrobial and health properties. This article deals with the application of innovative extraction techniques in the isolation of biologically and pharmacologically active molecules from different plant species and that could be used as functional additives in meat products. Enrichment with plant extracts is a sustainable approach for the production of meat products with a clean label and added value. Therefore, this manuscript aims to present potent plant species, but also innovative extraction techniques, through the application of which, biologically valuable molecules are isolated, ensuring safety, quality and longer shelf life of meat products.

1. Introduction

Since the beginning of human civilisation, meat has been an essential part of the human diet. It is highly valued for its richness in high-quality protein, essential fatty acids, vitamin B_{12} and important minerals, such as iron and selenium, nutrients that are essential for the proper functioning of the human body (*Leroy et al*., 2023). The importance of meat in the human diet is also reflected in the growth of the global meat products market, which reached a value of $US 1,730.57 billion in 2024 and is expected to rise to around $US 2,710.39 billion by 2034, at a compound annual growth rate (CAGR) of 4.59% between 2025 and 2034 (*Precedence Research*, 2025).

Due to their high moisture and fat content, minimally processed meat products, especially those that are fresh or cooked with water or steam, are particularly susceptible to oxidative degradation and microbial spoilage (*FoodData Central*, 2019). In addition, meat processing itself can also contribute to contamination risks (*Mediani et al*., 2022). Inadequate implementation of preservation methods during processing can increase susceptibility to microbial contamination by bacteria, yeasts and moulds, which can lead to foodborne illness. At the same time, growing public concern over the health risks associated with synthetic additives, such as nitrates, has prompted the food industry to look for safer, natural alternatives, such as plant extracts (*Sabzal et al*., 2025).

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One promising approach is the fortification of meat products with plant extracts from medicinal plants. Thanks to advances in modern extraction technologies, various groups of bioactive phytonutrients, such as phenolic acids, flavonoids, terpenes, tannins and saponins, can now be efficiently isolated from these plants (*Terzić et al*., 2023). These compounds have strong antioxidant and antimicrobial effects by scavenging free radicals, chelating metal ions and destroying microbial membranes. As a result, they improve the stability, safety and shelf life of meat products. The production of high-quality plant extracts depends crucially on the effective extraction of biologically and pharmacologically active metabolites (*Olvera-Aguirre et al*., 2023).

In addition to medicinal plant extracts, essential oils, which are products of secondary metabolism in plants, also offer considerable potential for improving the quality and safety of meat products (*da Silva et al*., 2021). These volatile compounds have strong antimicrobial and antioxidant properties, making them effective natural preservatives. In recent years, innovative extraction methods have attracted considerable attention in the food industry due to their improved efficiency, lower energy consumption and higher extraction yields. These advanced techniques are particularly beneficial for maintaining the integrity of thermolabile compounds and promoting more sustainable small-scale production. The use of modern extraction technologies represents a pioneering approach in the development and formulation of new, safe and functional foods tailored to different consumer groups (*da Silva et al*., 2021).

For all these reasons, this article aims to emphasise the importance of using extracts of medicinal plants and essential oils, obtained with modern and environmentally friendly extraction technologies, as natural preservatives. Their use can significantly improve the quality, safety and shelf life of meat and meat products.

2. Plants as natural preservatives for meat products

The use of medicinal plants is closely linked to the global "clean label" movement, which reflects a growing consumer demand for products without synthetic preservatives and additives (*Beya et al*., 2021). This section introduces the various parts of medicinal plants, such as leaves, roots, seeds and bark of various species, that are used in specific concentrations to improve the quality and extend the shelf life of meat and meat-based products

Fruit-derived extracts

- lemon essential oil: applied at 1–4% in packaging films for fresh pork meat, it improved color, odour, and overall acceptance over 21 days at 4 °C (*Munekata et al*., 2023);
- pomegranate peel extract: used at 0.5–1% in films for fresh beef and 1–1.5% as powder in minced beef, it enhanced sensory attributes and reduced lipid oxidation (*Bellucci et al*., 2022);
- apple peel powder: incorporated at 3% in coating solutions for raw beef patties, it decreased lipid oxidation and *Salmonella* counts over 10 days at 4 °C (*Munekata et al*., 2023).

2.1 Seed and leaf extracts

- guarana seed extract: used at 250–500 mg/kg in raw lamb burgers, it improved color and reduced lipid and protein oxidation over 18 days at 2 °C (*Carvalho*, 2020);
- grape seed extract: employed at 100–600 mg/kg in beef sausages and 1000 mg/kg in pork liver pâté, it enhanced oxidative stability over extended storage periods (*El-Kholie et al*., 2014);
- broccoli leaf extract: applied at 0.1–0.5% w/w in ground beef patties, it significantly reduced lipid oxidation over 12 days at 4 °C (*Şimşek & Kılıç*, 2024);
- cranberry concentrate: used at 10% w/w in ground beef, it effectively suppressed various pathogens over seven days at both 21 °C and 7 °C (*Qiu & Wu*, 2007).

2.2 Herbal extracts and essential oils

- rosemary extract (E392): permitted at maximum levels ranging from 100 mg/kg in dried sausages to 5000 mg/kg in breakfast sausages and processed fishery products (*Plaskova & Mlcek*, 2023);
- cinnamon bark oil: included at 0.1–2% in natural preservative formulations for processed meats, it contributed to extended shelf life and microbial inhibition (*Hussain et al*., 2021);
- celery seed oil: used at 1–2% in combination with other essential oils for natural meat preservation, it enhanced antimicrobial properties (*Ghoname et al*., 2023).

To improve the oxidative stability, microbiological safety and overall quality of meat products, numerous plant-based additives were tested for their natural preservative potential. In addition to the plant species already studied, thyme (*Thymus vulgaris*), clove (*Syzygium aromaticum*), garlic (*Allium sativum*), green tea (*Camellia sinensis*) and sage (*Salvia officinalis*) have shown remarkable antioxidant and antimicrobial properties, making them promising candidates for extending the shelf life of fresh and minimally processed meat (*Shah et al*., 2014).

3. Extraction methods for plant-based meat preservatives

Ultrasound-assisted extraction (UAE) is based on the application of ultrasonic waves, typically in the frequency range of 20 kHz to 100 kHz, to a plant material–solvent mixture. The basic principle of UAE is acoustic cavitation, in which microscopic bubbles form, grow and collapse implosively in the solvent as the ultrasonic waves generate alternating high- and low-pressure cycles. This mechanical effect breaks up the cell walls, increases the penetration of the solvent and improves the mass transfer of target compounds from the material into the solvent. The ultrasonic energy breaks the cell walls and makes intracellular compounds more accessible. Cavitation enables deeper and faster penetration of the solvent into the plant matrix, and reduces extraction time and solvent consumption. The most important advantages are: preservation of thermolabile (heat sensitive) compounds, operation at relatively low temperatures, being environmentally friendly (often uses lower amounts or green solvents), scalable and adaptable for different matrices (*Singla & Sit*, 2021).

Microwave-assisted extraction (MAE) is based on the principle of dielectric heating, which occurs when polar molecules (e.g. water or polar solvents) align with an alternating electromagnetic field generated by microwaves (typically at 2.45 GHz). As these polar molecules are rapidly realigned by the alternating field, heat is generated by internal friction, resulting in rapid and uniform heating of the plant material-solvent mixture. Increased temperatures during the process improve the solubility and diffusion of the target compounds from the material into the solvent. Compared to conventional methods, MAE is faster, uses less solvent, is efficient for polar and moderately polar compounds, suitable for thermally stable bioactives and environmentally friendly when combined with green solvents (*Gonzaga et al*., 2025).

Supercritical fluid extraction (SFE) is a separation technique in which a supercritical fluid, i.e., a substance at a temperature and pressure above its critical point, is used to extract components from solid or liquid matrices. The most commonly used supercritical fluid is CO_2, due to its moderate critical conditions (critical temperature ~31.1 °C and pressure ~73.8 bar), its non-toxicity, its non-flammability and its easy removal from the final extract. The supercritical CO_2 acts as a selective solvent that dissolves specific bioactive compounds based on their solubility under controlled temperature and pressure. By changing the pressure and temperature, the dissolving power of CO_2 can be customised to specific desired compounds. Sometimes co-solvents (e. g. ethanol) are added to improve the extraction of polar substances. This extraction method, when based on solvent-free extracts (CO_2 evaporates without residue), works at low temperatures, is ideal for thermolabile compounds, is environmentally friendly and safe, with high selectivity and adjustable extraction parameters, and is suitable for applications in the food, pharmaceutical, cosmetic and food industries. Some of the limitations of SFE are the high initial cost, low effectiveness with very polar compounds unless modified with co-solvents, and precise control of temperature and pressure (*Sairam et al*., 2012).

Subcritical water extraction (SWE) — also known as hot water extraction under pressure — is an environmentally friendly extraction technique in which water is used at high temperatures (100 to 374 °C) and high pressure (to keep it in a liquid state) to extract bioactive compounds from various matrices. The dielectric constant of water decreases with increasing temperature, making it behave more like an organic solvent (e.g., ethanol or methanol). The viscosity and surface tension of the water are also reduced, which improves its ability to penetrate the plant material and dissolve both polar and moderately non-polar compounds. The advantages of this extraction process are: no toxic solvents (only water is used, making it environmentally friendly); efficient extraction of polar and semi-polar compounds; suitable for thermally stable compounds; potential for hydrolysis of glycosides, proteins and polysaccharides. Some of the limitations are: requires precise control of temperature and pressure; not ideal for thermolabile compounds that degrade at high temperatures; can cause undesirable chemical transformations if not properly optimised (*Nastić et al*., 2018).

Table 1. Extraction techniques of plant bioactive for improving meat product quality

Extraction method	Secondary metabolites	Application in meat products
Ultrasound-Assisted (UAE)	Polyphenols, flavonoids	Antioxidant/antimicrobial marinades, coatings
Microwave-Assisted (MAE)	Heat-sensitive compounds	Lipid oxidation prevention
Supercritical (SFE)	Essential oils, terpenes	Clean-label antimicrobial agents
Accelerated Solvent (ASE)	Phenolic-rich extracts	Processed meat shelf-life extension
Subcritical Water (SWE)	Polar compounds (e.g., tannins)	Green technology for antioxidant delivery

Accelerated solvent extraction (ASE) — also known as pressurised liquid extraction (PLE) — is a modern technique that increases the efficiency of solid–liquid extraction by accelerating the extraction process using conventional solvents (e.g., ethanol, methanol, hexane) with high temperatures (typically 50–200 °C) and high pressure (10–20 MPa or 1500–3000 psi). The high pressure keeps the solvent in a liquid state even above its boiling point, allowing it to better penetrate the plant material and dissolve the target compounds. The main mechanisms of this extraction process are elevated temperature, high pressure and a closed system that minimises solvent loss and environmental impact. The advantages of ASE are: faster extraction than conventional methods (e.g., Soxhlet); low solvent consumption; good recovery of thermally stable compounds; automatable and reproducible; suitable for high throughput laboratories; compatible with a wide range of sample types and solvents. Some of the limitations of ASE extraction are: not ideal for thermolabile compounds unless temperature is carefully optimised; initial equipment costs are relatively high; solvent must be removed after extraction (*Priego-Capote & Delgado de la Torre*, 2013).

Table 1. provides an overview of modern extraction techniques, the phytonutrients extracted through their application to plant species and their roles in meat products.

4. Conclusion

The integration of plant phytonutrients into meat products is a promising strategy to improve food quality, safety and functionality in response to growing consumer demand for environmentally friendly and natural products. Modern extraction techniques, including UAE, MAE, SFE, ASE and SWE, offer significant advantages in the efficient and sustainable isolation of bioactive compounds while preserving their antioxidant, antimicrobial and health-promoting properties. Bioactive compounds from medicinal plants, especially from extracts and essential oils, not only extend the shelf life and prevent spoilage of meat products, but also contribute to the development of functional foods with added value and potential health benefits. In addition, the use of environmentally friendly extraction technologies and unused plant resources supports sustainable food production and is in line with the principles of the circular economy. To fully exploit the potential of phytonutrients in the meat industry, continuous interdisciplinary research and innovation in plant extraction and formulation is essential.

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Original Scientific Paper

Hardness of dry-cured ham as a factor of consumer choice and its relationship to sensory acceptability

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ABSTRACT

This study investigated consumer attitudes towards Slovenian dry-cured ham, Kraški pršut with protected geographical indication (PGI), having different levels of hardness (soft, semi-hard and hard). A total of 626 participants took part in a two-step evaluation: a choice simulation of dry-cured ham pieces followed by a sensory evaluation using the check-all-that-apply (CATA) method on sliced samples and overall liking assessment. Instrumental measurements of texture profile confirmed the tactile-based classification of dry-cured hams (performed by the processor) into soft, semi-hard, and hard categories. Consumer choices of dry-cured ham pieces were relatively evenly distributed (32%, 29%, 38% for soft, semi-hard, and hard categories, respectively), but with a statistically significant preference for harder hams. Visual appearance was more often a reason for choice than tactile sensation (44% and 23%, respectively). Although choice simulation indicated some preference for hard hams, tasting of dry cured hams revealed that soft hams were more frequently perceived as 'juicy' and less frequently as 'very salty', albeit less frequently noted as 'mature'. These findings highlight the importance of both visual and tasting attributes in determining consumer preference for dry-cured ham.

1. Introduction

Texture is a critical quality attribute of dry-cured ham for consumer acceptability (*Morales et al.*, 2013). When buying dry-cured ham sold in pieces, consumers rely on its visual appearance and the tactile sensation of hardness. The hardness of dry-cured ham is influenced by the processing method, associated processing losses, and its final water content. Research indicates a negative non-linear relationship between water content and ham hardness. This relationship is further affected by factors such as pH, salt content, and the extent of proteolysis (*Petrova et al.*, 2015). A soft and pasty texture of dry-cured hams has been associated with negative consumer attitudes (*Morales et al.* 2013; *Virgili et al.*, 1995). To quantify mechanical texture properties, objective measurement methods, such as texture profile analysis (TPA) and stress relaxation (SR), are commonly used (*Fulladosa et al.*, 2021). However, these instrumental techniques cannot fully capture consumers' subjective perception of the texture of dry-cured ham. Therefore, sensory evaluation by consumers is essential for understanding their texture preferences, allowing the meat industry to tailor product characteristics and thereby enhance consumer satisfaction and market competitiveness. A positive consumer attitude towards a product means a greater preference for it (*Guerrero et al.*, 1998).

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Although numerous studies have explored consumer preferences for dry-cured hams, limited information is available for Kraški pršut (*EC,* 2012). The Slovenian processing industry seeks a deeper understanding of the relationship between visually based consumer choices and the sensory appreciation of the product. This study was, therefore, designed to investigate this relationship.

2. Materials and methods

The study was conducted with visitors during the main Slovenian agricultural fair. Participants (n = 626) were adults (>18 years old) and provided informed consent prior to the test. The material for this study was prepared by the biggest producer of Kraški pršut. Dry-cured hams (n=59) of similar maturation length were classified into hardness categories (soft, semi-hard, hard) based on tactile assessment of the exposed surface. Nine hams (three from each hardness category) were sliced to prepare 153 packages for sensory testing, while 50 ham quarters were prepared for the choice test. Care was taken that the samples (slices and pieces/quarters) included the key muscles: *semimembranosus* (SM), *biceps femoris* (BF), and *semitendinosus* (ST). Consumers were invited to select a preferred piece/quarter of dry-cured ham (as presented on the market) from randomly presented triplets (soft, semi-hard, hard). After making their choice, they were asked to articulate the reasons behind their selection. Their explanations were then categorized as relating either to visual appearance or to texture/tactile sensation. Afterwards they were offered nine slices (three from each ham group) for tasting and asked to evaluate the samples by checking the descriptors, i.e., using the check-all-that-apply method (CATA) to mark those descriptors they perceived during tasting and to provide an overall liking score (on a 1-5 Likert scale; see below).

2.1 Texture analysis of dry-cured ham muscles

Fifty-nine samples of dry-cured hams were used for instrumental texture analysis of SM, BF, and ST muscles of dry-cured ham according to *Škrlep et al.* (2012). The tests were conducted with a TPA analyzer (Ametek Lloyd Instruments, Ltd., Bognor Regis, UK) using a 50 kg load cell and a 50 mm diameter compression plate. For the SR test, samples were compressed to 25% of their initial height at 1 mm/s. For the TPA test, samples underwent two compressions to 50% at 1 mm/s. The following parameters were calculated: hardness, cohesiveness, gumminess, springiness, chewiness, adhesiveness and stress relaxation test (Y_{90}).

2.2. Consumer testing

2.2.1. Consumer choice of dry-cured ham based on visual and tactile appraisal

An initial choice test was conducted in which consumers were asked to select their preferred piece from random triplets of dry-cured ham samples that had been previously categorized into soft, semi-hard, and hard textures (this information was kept from consumers). The first question was: “Which piece of dry-cured ham would you choose?”. An optional follow-up question was: “Why did you choose this particular piece?”.

2.2.2. CATA analysis and overall liking score

The tasting was conducted as a blind test, during which participants were asked to select all sensory impressions they perceived from the provided CATA descriptors. The provided CATA descriptors were: very soft, soluble, very dry, raw, mature, tough, juicy, tasty, off flavour, aromatic, very fatty, sweet, very salty, other (with an option to specify), which were pre-selected in collaboration with the experts from the dry-cured ham industry. At the end of tasting, consumers rated their overall satisfaction using a 5-point scale, defined as 1 = I do not like it at all and have never bought this product; 2 = I do not like it and probably will not buy this product; 3 = I am not sure (neutral opinion); 4 = I like it and will buy it; 5 = I really like it and will definitely buy it.

2.3. Statistical analysis

Statistical analysis was conducted using SPSS 23.0. To verify if hardness groups could be separated based on texture traits a canonical discriminant analysis was performed. Fisher’s exact test was used to analyze CATA responses.

3. Results and discussion

3.1. Canonical discriminant analysis

Discriminant analysis revealed a clear separation of ham hardness groups based on instrumental texture measurements (Figure 1), confirming the accuracy of

the producer's tactile classification. Texture characterization showed that soft hams were less hard, gummy, and chewy, but more adhesive than hard hams, with semi-hard hams displaying intermediate properties (data not shown). Compared with hard hams, soft hams also contained higher moisture levels, greater water activity (a_w), and a greater PI, along with lower salt content (data not shown), consistent with findings reported in the literature (*Pérez-Alvarez et al*., 1999; *Ruiz-Ramírez et al*., 2006; *Ruiz-Ramírez et al*., 2005; *Serra et al*., 2005).

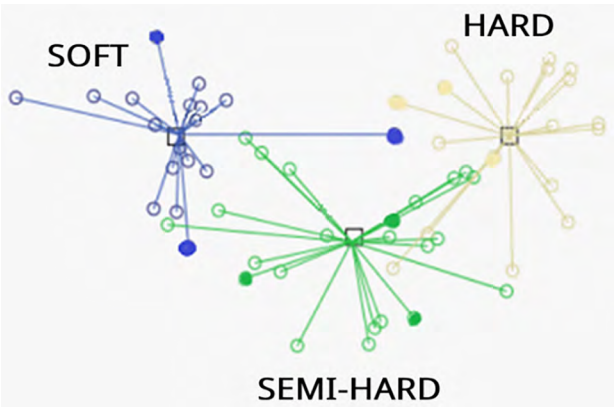

Figure 1. Canonical discriminant analysis based on texture traits of SM, BF, ST

3.2. Consumer testing of dry-cured ham

3.2.1. Choice simulation

Consumers selected dry-cured ham pieces from the different hardness categories in similar proportions with a slight preference for the hard ones (32%, 29%, and 38% for soft, semi-hard, and hard, respectively). Although the processor anticipated a clear preference for hard pieces, the difference was moderate. When justifying their choice, consumers more often attributed their selections to visual attributes (e.g. nice color, too fatty, meat to fat ratio) than to tactile sensations (data not shown). This can be attributed to the fact that appearance, especially colour and visual fat, is the most influential intrinsic attribute in shaping consumers' expectations (*Font-i-Furnols* & *Guerrero*, 2014). Furthermore, no differences in hardness group choice were found between genders or age groups.

3.2.1. Consumer preferences towards dry-cured ham

Despite important differences in the texture of dry-cured hams between the hardness groups, only small differences in consumer sensory perceptions were found (Figure 2). Namely, semi-hard hams were more frequently checked as 'mature' than soft hams, and soft hams were more frequently checked as 'juicy' and less frequently as 'very salty'. The perception of semi-hard hams as more mature than soft hams suggests that consumers associate maturity with hardness. This trend was also reflected in comparisons between soft and hard hams, although the difference was not statistically significant. No sensory differences (based on CATA) were detected between semi-hard and hard hams.

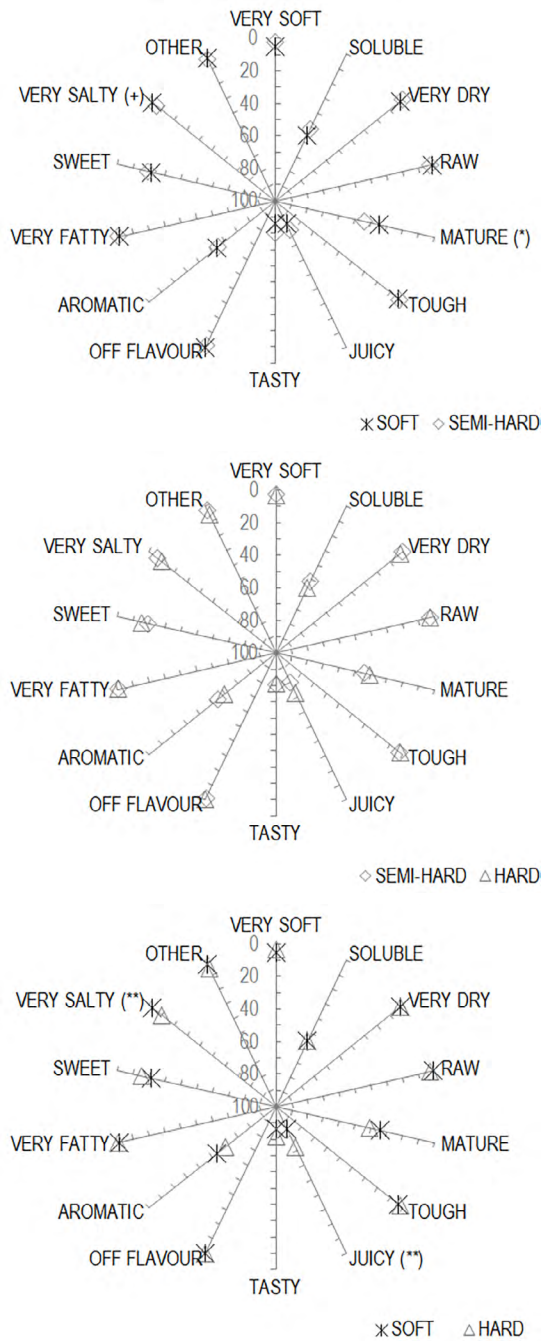

Figure 2. Results of CATA analysis with pair-wise comparisons of hardness groups

The higher frequency of perception of soft hams, compared with the other ham categories, as 'juicy' and less frequently as 'very salty' aligns with observed physicochemical traits (data not shown) and is supported by the existing literature. Studies have demonstrated that hams with higher moisture content and lower salt levels are perceived as juicier and less salty (*Savić et al*., 2024; *Schivazappa & Virgili*, 2020). The overall liking scores, which were high and statistically not different across all hardness categories (soft: 4.47, semi-hard: 4.42, hard: 4.53), indicate general consumer satisfaction with products regardless of hardness group and explain the small differences observed in the CATA responses. Once more, the result did not align with the processor's expectation that consumers would favour harder hams; in fact, there was a slight preference for softer hams, reflected in more frequent selection of 'juicy' and less frequent selection of 'salty' as sensory descriptors.

4. Conclusion

Discriminant analysis based on instrumental texture measurements validated the processor's classification of hams into three hardness categories. In the consumer choice simulation, selection relied more on visual than tactile perception. Although hard hams were slightly preferred in the choice step, soft hams were more frequently characterized as juicy and less frequently as salty.

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Original Scientific Paper

The influence of modified atmosphere and vacuum packaging on selected chemical freshness parameters of perch (*Stizostedion lucioperca*) and silver carp (*Hypophthalmichthys molitrix*) fillets

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ABSTRACT

The purpose of packing food in modified atmospheric conditions is to extend its sustainability by preventing both biochemical processes and growth of spoilage bacteria. The aim of our research was to examine the influence of packaging in modified atmosphere and vacuum on the total volatile basic nitrogen (TVB-N) content in muscle of perch (*Stizostedion lucioperca*) and silver carp (*Hypophthalmichthys molitrix*). Three groups of perch and silver carp fillets were investigated. The first two groups were packaged in modified atmosphere with different gas ratios: 70%CO_2+30%N_2 (group I) and 50%CO_2+50%N_2 (group II), whereas the third, control group, (group III) was vacuum packaged. During the trial, the packaged fish was stored at 3°C. Determination of TVB-N was performed on days 1, 4, 7, 9 and 14 of storage. The lowest increase in TVB-N was established in perch and silver carp fillets from group I, whereas the highest increase was established in group III. Statistically significant differences ($p < 0{,}001$) between the mean TVB-N in perch (group I: 17.00±0.94; group II: 25.00±0.91 and group III: 36.18±2.65 mg N/100 g) and silver carp fillets (group I: 20.64±1.45; group II: 26.74±0.31 and group III: 34.10±1.75 mg N/100 g) was established on day 14. Based on the obtained results, it can be concluded that, in terms of TVB-N content, the gas mixture consisting of 70% CO_2 and 30% N_2 was the most suitable of the two gas mixtures for packaging fresh perch and silver carp fillets.

1. Introduction

The fact that fresh fish is a highly perishable food item (pH > 6.0; water activity > 0.98) has influenced producers to focus on finding the optimal method of fish preservation. However, in recent years, consumers around the world have increasingly demanded that fresh fish be available at all times, as it has the most desirable sensory characteristics (*Babic Milijasevic et al.*, 2023; *Esteves et al.*, 2021). This trend has led to the development of an efficient modified atmosphere packaging (MAP) concept, which ensures a longer shelf life and preserves key freshness parameters (*Gimenez et al.*, 2002).

Gas mixtures with high concentrations of CO_2 and N_2 have attracted the most attention from

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researchers dealing with fish packaging issues over the past decade (*Zhang et al*., 2021). However, the impact of modified atmosphere packaging on the shelf life of fresh fish and the most suitable gas mixture depends on the type of fish being packaged, fat content, initial microbial contamination, handling after catch, the volume ratio of gas to product in the package, and most importantly, the packaging method and storage conditions (*Sivertsvik et al*., 2002; *Bøknæs et al*., 2000). In some cases, MAP can negatively affect the quality of packaged fish due to the dissolution of CO_2 in the fish meat, leading to the formation of carbonic acid (*Babić Milijašević et al*., 2018). At lower pH values, the water-binding capacity of the fish meat decreases, causing the release of meat juice in the package, which serves as an ideal medium for the growth of spoilage microorganisms (*Sivertsvik et al*., 2002; *Goulas and Kontominas*, 2007). For these reasons, it is essential to determine the optimal gas ratio in the mixture based on the characteristics of the packaged product and the packaging system.

The level of total volatile basic nitrogen (TVB-N) is considered a chemical indicator of fish freshness. TVB-N consists of compounds responsible for the unpleasant odour and taste of fish meat, including ammonia, dimethylamine (DMA), trimethylamine (TMA), amines formed through the decarboxylation of amino acids, as well as other nitrogen compounds that become volatile in their alkaline form (*Debevere and Boskou*, 1996; *Ruiz-Capillas and Moral*, 2005). Ammonia is produced by bacterial deamination of proteins, peptides, and amino acids, as well as the autolytic degradation of adenosine monophosphate (AMP). Dimethylamine and trimethylamine result from the breakdown of trimethylamine oxide (TMAO), a compound that plays a significant role in osmoregulation and whose presence has been confirmed in all marine and many freshwater fish species. Due to the activity of endogenous fish enzymes, TMAO is degraded, leading to the formation of DMA and formaldehyde (*Huss*, 1995).

The aim of this study was to examine the impact of modified atmosphere packaging and vacuum packaging on changes in the TVB-N in the meat of perch (*Stizostedion lucioperca*) and common silver carp (*Hypophthalmichthys molitrix*), and to determine the most suitable gas mixtures for packaging these two freshwater fish species.

2. Materials and methods

The perch (*Stizostedion lucioperca*) used in the experiment were raised under identical conditions and originated from a semi-intensive farming pond. For the study, 40 one-year-old perch with a mean weight of 320 g were selected. The fish were transported alive from the farm to the processing facility, where they were placed in a reception tank and then stunned using electric current. Slaughtering and evisceration were performed using an automatic device, while carcasses were washed manually under running water.

The silver carp (*Hypophthalmichthys molitrix*) came from a fish farm where a semi-intensive farming method was applied. For the study, 10 two-year-old silver carp with a mean weight of 2.5 kg were used. The silver carp were transported alive to the processing facility, where they were stunned, slaughtered, and descaled.

Fish carcasses were then cut into 4 cm thick fillets, with each carcass yielding 6 fillets. Three sample groups were formed, each consisting of 18 cleaned perch or silver carp fillets. The first two groups were packed using MAP with different gas ratios: 70% CO_2 + 30% N_2 (Group I) and 50% CO_2 + 50% N_2 (Group II), while the third, control group was vacuum-packed. The samples were packaged using a Variovac machine (Variovac Primus, Zarrentin, Germany). The packaging material was OPA/EVOH/PE film (oriented polyamide/ethylene vinyl alcohol/polyethylene, Dynopack, Polimoon, Kristiansand, Norway) with low gas permeability (O_2 permeability: 3.2 cm^3/m^2/day at 23 °C; N_2: 1 cm^3/m^2/day at 23 °C; CO_2: 14 cm^3/m^2/day at 23 °C; and water vapor: 15 cm^3/m^2/day at 38 °C). The gas-to-sample ratio in the packaging was 2:1. Throughout the study, all packages of fish were stored under identical conditions at 3 °C. Determination of TVB-N was performed on days 1, 4, 7, 9 and 14 of storage.

The total volatile basic nitrogen (TVB-N) was determined using the EU reference method, as specified in *Commission Regulation (EC)* (2005).

For statistical analysis (mean value, variation measures, variance analysis, and t-test), the Microsoft Office Excel 2016 software package was used.

3. Results and discussion

The mean TVB-N levels in perch packed in the modified atmospheres and in vacuum packaging are presented in Figure 1. Based on the displayed

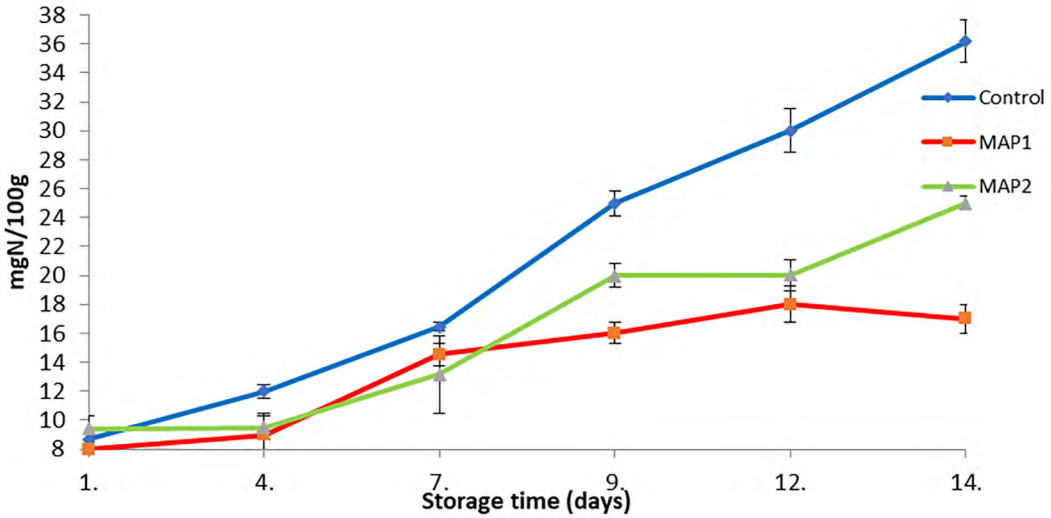

Figure 1. Mean TVB-N levels in perch packaged in modified atmosphere (MAP) and vacuum (control).

results, it can be observed that on day 1, the mean TVB-N content in all three groups of perch (8.00±0.32, 9.40±0.46, and 8.70±0.31 mg N/100 g) did not differ significantly ($p > 0.05$). The mean TVB-N content in group I (14.55±0.60 mg N/100 g) and group II (13.15±0.98 mg N/100 g) after seven days of storage showed a statistically significant difference ($p < 0.001$) compared to the mean TVB-N content in group III (16.46±1.00 mg N/100 g). No statistically significant difference ($p > 0.05$) was found between TVB-N levels in groups I and II on day 7. On day 14, a statistically significant difference ($p < 0.001$) was established between the mean TVB-N levels in group I (17.00±0.93 mg N/100 g), group II (25.00±0.81 mg N/100 g), and group III (36.18±2.65 mg N/100 g).

The mean TVB-N levels in silver carp fillets packed in the modified atmosphere and vacuum packaging are presented in Figure 2.

On day 1, no statistically significant difference ($p > 0.05$) was found between the mean TVB-N (16.15±0.84, 15.45±0.78, and 15.05±0.27 mg N/100 g) in silver carp fillets. On day 7, the mean TVB-N value in group I (19.01±0.78 mg N/100 g) was the lowest and showed a statistically significant difference ($p < 0.05$) compared to group III (24.90±1.74 mg N/100 g). The difference between TVB-N levels in group II (20.86±0.75 mg N/100 g) compared to group III was also statistically significant at $p < 0.001$. No statistically significant difference ($p > 0.05$) was found between the mean TVB-N levels in groups I and II on day 7. On day 14, the lowest mean

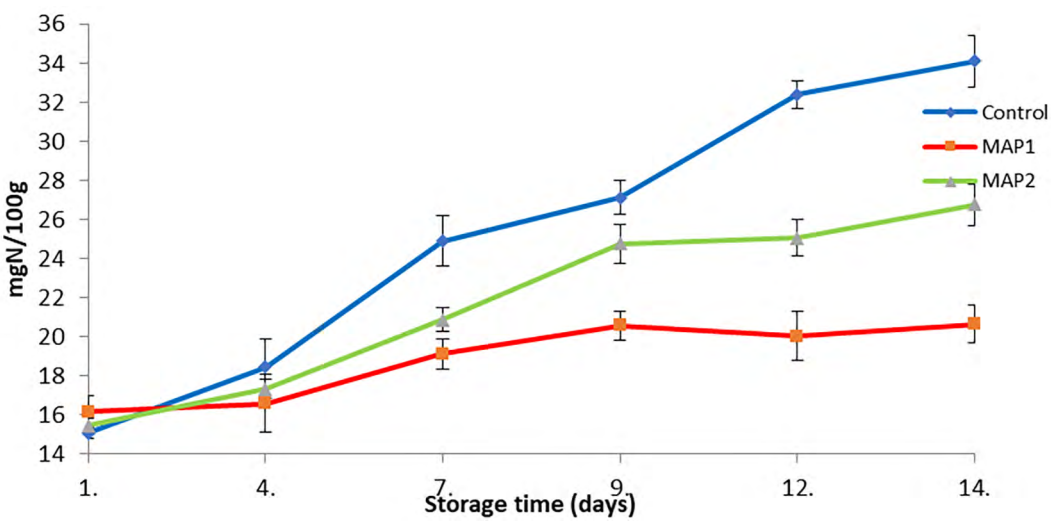

Figure 2. Mean TVB-N levels in silver carp packaged in modified atmosphere (MAP) and vacuum (control).

TVB-N level was found in silver carp from group I (20.64±1.48 mg N/100 g), whereas the highest was observed in group III (34.10±1.75 mg N/100 g). The mean TVB-N level in group II on day 14 was 26.74±0.31 mg N/100 g. The differences between the mean TVB-N levels in all three groups of silver carp fillets on day 14 were statistically significant ($p < 0.001$).

Arahisar et al. (2004) found that the initial TVB-N level in trout fillets was 12 mg N/100 g, while *Ježek and Buhtova* (2010) reported an initial TVB-N of 16.25±0.79 mg N/100 g in carp fillets. The differences in TVB-N levels in the fish compared to those measured in our study could be attributed to variations in the non-protein nitrogen content of fish meat, which depends on diet, time of catch, fish size, and microbiological quality (*Connell*, 1990).

The observed differences in TVB-N levels between our MAP test groups can be explained by the higher percentage of CO_2 in the gas mixture used to pack group I. The primary role of CO_2 in MAP packaging technology is to inhibit microbial growth, especially spoilage bacteria, which produce volatile nitrogen compounds through their metabolic activity.

There is no established limit for TVB-N in perch meat in the literature, but we consider 25 mg N/100 g as a critical threshold. In our study, TVB-N levels in MAP-packed perch (groups I and II) remained below this threshold throughout the storage period, whereas in vacuum-packed perch (group III), the mean TVB-N reached 36.18±2.65 mg N/100 g on day 14, significantly exceeding the recommended limit for perch freshness.

Milijašević et al. (2010) examined the inhibitory effect of CO_2 on TVB-N content in carp (*Cyprinus carpio*) fillets, confirming that the lowest TVB-N level was recorded in carp fillets packed in a modified atmosphere containing 100% CO_2. Similarly, *Ježek and Buhtova* (2010) found lower TVB-N in carp (*Cyprinus carpio*) fillets packed in MAP (69% N_2 + 25% CO_2 + 5% O_2 + 1% CO) compared to fillets stored under refrigeration at 2 °C. These authors recommend a maximum acceptable TVB-N limit of 20 mg N/100 g in carp meat.

In our study, TVB-N values in MAP-packed silver carp fillets (groups I and II) reached this recommended limit on day 14, while in vacuum-packed fish (group III), the limit was reached on day 7.

4. Conclusion

Based on the obtained results, it can be concluded that, in terms of the selected chemical parameter, TVB-N, the most suitable gas mixture for packaging fresh perch and silver carp fillets was the mixture containing 70% CO_2 and 30% N_2.

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Original Scientific Paper

Quality attributes of chilled common carp (*Cyprinus carpio*) cuts packaged in vacuum and modified atmosphere

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ABSTRACT

The objective of this study was to investigate the impact of vacuum and modified atmosphere packaging (MAP1: 60%CO_2 + 40%N_2 and MAP2: 40%CO_2 + 60%N_2) on selected microbiological and sensory attributes of common carp (*Cyprinus carpio*) cuts stored at 3±0.5°C, and to establish the shelf life of the products. Samples were assessed on days 1, 4, 7, 9, 12, and 15. An increase in the APC during the storage period was observed in all types of packaging, with the most exceptional changes occurring in the vacuum packaged fish. Carp cuts stored in a CO_2-enriched atmosphere exhibited lower APC number throughout the entire storage period. Panellists rejected VP fish on day 9, MAP2 fish on day 12, while MAP1 fish was evaluated as unacceptable from a sensory point of view on day 15. The shelf-life of carp cuts was extended from 7 days in VP to 9 days in MAP2 (40% CO_2 + 60% N_2) and to 12 days in MAP1 (60% CO_2 + 40 % N_2).

1. Introduction

Modern consumers demand fresh fish for consumption rather than frozen or processed fish. This requirement is largely met by packaging the products in vacuum or modified atmosphere. Vacuum packaging (VP) and modified atmosphere packaging (MAP) are considered established technologies for food preservation, and they work by changing the gas proportions in a food environment, respectively, by withdrawing O_2 or by replacing the atmosphere inside the package by a mixture of gases, such as carbon dioxide (CO_2) and nitrogen (N_2) gas (*De Witt et al*., 2016). MAP with high levels of CO_2 generally promote fresh fish storage stability (*Olatunde & Benjakul*, 2018). Evaluation of different quality parameters, such as microbial, chemical, physical, and sensory attributes, have been used to determined the shelf-life of fish (*Lerfall et al.*, 2018). The shelf-life of fresh, chilled fish is relatively short, and at refrigeration temperatures of 2 ± 2 °C, it is about 2 to 3 days. MAP can be applied as a preservation method to extend the shelf-life of fresh chilled fish (*Masniyom*, 2011).

In Serbia, fish are traditionally marketed for human consumption as whole fish, freshly slaughtered on flaked ice, or as live fish. For slaughtered fish, the preferred type of fish packaging is VP. There are generally very few studies on freshwater fish produced in Serbia and packaged under modified atmospheres. Therefore, the aims of this research were to monitor changes of selected microbial and sensory parameters of common carp (*Cyprinus carpio*) cuts

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packaged in vacuum and modified atmosphere during storage at 3±0.5°C, and to determine the shelf-life of the packaged fish.

2. Materials and methods

Two-year-old marketable carp (*Cyprinus carpio*) of average body weight of 2.5 kg were used in the experiment. Carp originated from a fishpond located in the lowland region of Serbia, where semi-intensive farming is used. Fish were transported live to the fish slaughtering and processing facility, where they were stunned, slaughtered, scaled, and each carcass was cut into steaks 2 cm thick. Three sample groups of carp cuts were formed. Firstly, two groups were packaged in a modified atmosphere with different respective gas ratios: 60%CO_2+40%N_2 (MAP1) and 40%CO_2+60%N_2 (MAP2), whereas the third group was vacuum packaged. The packing machine was a Variovac (Variovac Primus, Zarrentin, Germany), and the packaging foil was OPA/EVOH/PE (oriented polyamide/ethylene vinyl alcohol/polyethylene, Dynopack, Polimoon, Kristiansand, Norway) with low gas permeability (degree of permeability for O_2 – 3.2 cm^3/m^2/day at 23°C, for N_2 – 1 cm^3/m^2/day at 23°C, for CO_2 – 14 cm^3/m^2/day at 23°C, and for steam – 15 g/m^2/day at 38°C). The gas:fish ratio in MAP packages was 2:1. All packaged fish were stored in the same conditions at 3±0.5°C, and on days 1, 4, 7, 9, 12, and 15 of storage, microbiological and sensory testing was performed. The last examination for each group was conducted on the day when sensory changes were revealed.

2.1. Microbiological analysis

For each test group, fish from three packages was analyzed each sampling day. For the analyses, from each package, 20 g of fish muscle was removed aseptically with a sterile scalpel and tweezers, transferred into a stomacher bag, diluted with 180 mL of 0.9% NaCl solution, and homogenized for 2 min (Steward Stomacher 400 Lab Blender, London, UK). For microbial enumerations, ten-fold serial dilutions were prepared and 1 mL from each dilution was used.

Aerobic plate count (APC) was determined in plate count agar (PCA, Merck) using the pour plate technique with agar tempered to 44°C. Plates were incubated at 30°C for 72 h. All plates were prepared in duplicate and examined visually for typical colony types and morphological characteristics, and counts were expressed as logarithms of the number of colony-forming units per gram (log cfu/g).

2.2. Sensory assessment

Sensory analysis was conducted by six experienced judged from laboratory staff, in the laboratory for sensory evaluation, at room temperature (20 °C) and adequate lighting. Fish was removed from the packaging 10 min before trial and presented on the tray. These trays were coded by randomly chosen 3-digit numbers. Each panellist analyzed the samples individually for overall acceptability, with regard to odour, flesh colour and texture using 1-7 intensity scale, with 7 corresponding to the most liked sample and 1 corresponding to least liked sample. The product was defined as unacceptable when it achieved a score less than 3 points recorded by at least of 50% of the judges. Fish from each test group was evaluated throughout the storage period on each sampling day.

2.3. Statistics

The mean values and standard deviations were calculated by using column statistics with the processing of six values for each analyzed group. Significant differences between groups were calculated using one-way ANOVA. When a significant F was found, additional post-hoc tests with Tukey's adjustment were performed. Statistical significance was set at the ($p<0.05$) level of confidence. All analyses were performed using the program Microsoft Office Excel (2016).

3. Results and discussion

The APCs of common carp cuts, expressed as log cfu/g (mean ± SD), are presented in Figure 1. The initial low numbers of mesophilic bacteria imply good microbial quality of the fish. Good hygiene practices during growing and handling of the fish could also help limit bacterial contamination as inferred by these findings. An increase in the APC during the storage period was observed in all types of packaging, with the most exceptional changes occurring in VP fish, with a significantly higher ($p<0.05$) APC from day 4 of storage compared to MAP fish. From day 4, the APC was significantly lower ($p<0.05$) in the group with the highest percentage of CO_2 in the packaging, and this trend was observed until the end of storage. Our results indicated a strong bacteriostatic effect of

CO_2 and a consequent reduction in the growth of mesophilic bacteria during the entire storage period. This effect depended on the applied CO_2 concentration and is in agreement with numerous results from the literature. The results of *Provincial et al*. (2010) indicated that there was significant reduction in the APC of fresh sea bass fillets packaged in a gas mixture with high CO_2 content compared to control fillets. Similar results were published by *Rodrigues et al.* (2016), who investigated the shelf-life of trout fillets packaged in MAP and in MAP with applied UV-C radiation, as well as *Esteves et al.* (2021), who examined grey triggerfish fillets packaged in vacuum and MAP.

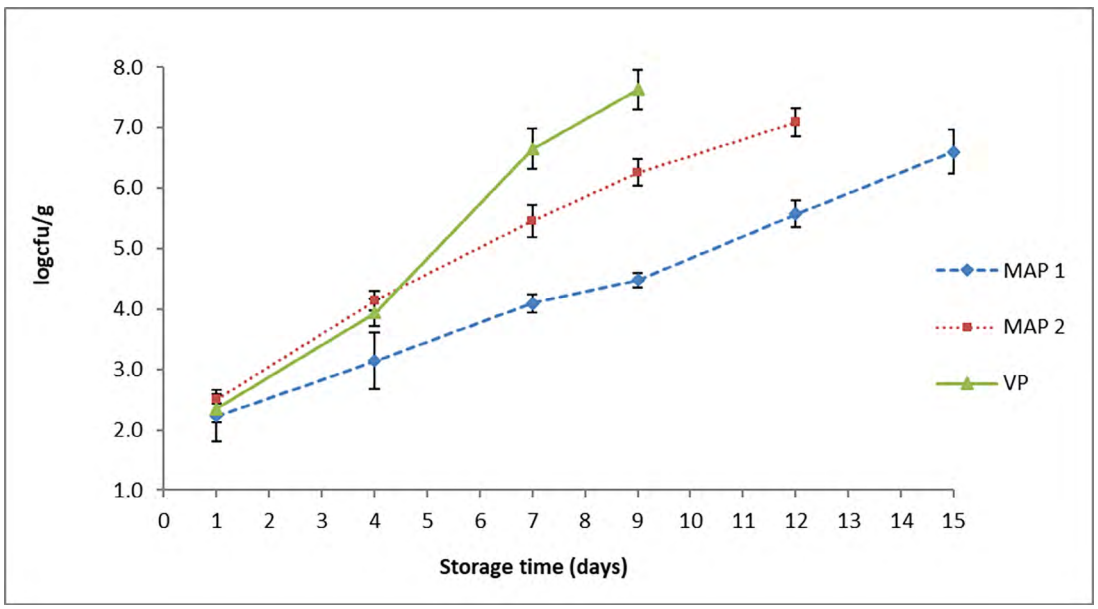

Figure 1. Aerobic plate count of common carp cuts expressed as log cfu/g (mean ± SD) packaged under different conditions during the storage period

According to ICMSF recommendations, the APC in fresh fish should not exceed 7 log cfu/g (*ICMSF*, 1986). This limit was also considered as a microbiological criterion that indicated the shelf-life of fish during the storage. In our study, in VP fish, APC reached numbers above the recommended limit on day 9 and in MAP2 fish on day 12; this corresponded with sensory unacceptability, i.e., spoilage. In MAP1 fish, APCs never exceeded 7 log cfu/g over the 15 days of the study.

Our research showed there was a good correlation between the aerobic plate counts of carp cuts packaged in vacuum and MAP2 and overall sensory acceptability. Similar findings were reported by *Fletcher et al.* (2002) who concluded that the aerobic plate count in salmon fillets packaged in a modified atmosphere (40% CO_2 + 60% N_2) was a good indicator of fish spoilage. In salmon fillets packaged in a modified atmosphere (60% CO_2 + 40% N_2) or vacuum, *Hansen et al.* (2009) also found a high level of correlation between APC and odour, as the main sensory attribute of fish freshness.

On the other hand, our research showed that although higher CO_2 concentration in the MAP1 gas mixture significantly slowed down the growth of APC, fish was not acceptable from the sensory point of view at the end of storage period (see below). *Stamatis & Arkoudelos* (2007) proved that the APC did not exceed 10^7 log cfu/g at the time of the first signs of spoilage of fresh sardine fillets packed in a modified atmosphere, supporting the results of the present study.

Sensory scores decreased over time in all experimental groups. At the beginning of storage (day 1), the quality of the fish was characterized by a uniform rosy colour and elastic texture of flesh and fresh, neutral odour. Sensory scores did not change significantly until day 4, so on days 1 and 4, fish from all groups were assessed as fresh and of very good quality. From day 4 on, as expected, sensory parameters changed differently in the different packaging conditions.

The greatest decrease of all sensory parameters was established in VP fish. A closer analysis showed the attributes that had the greatest impact on overall acceptability in VP fish were odour and flesh colour. At the moment of sensory rejection after 9 days of storage, the odour of VP fish was

sour and unpleasant and discolouration of fish flesh was detected.

In the MAP1 and MAP2 fish, the sensory attributes that most influenced the overall acceptability were odour and flesh texture. The odour changed from fresh at the start to neutral and ammoniacal during the extended the storage. At the end of the shelf-life, the odour was unpleasant, sour (MAP2) and rancid (MAP1). Metabolic activities of microorganisms at the end of the storage period could be a reason for the unpleasant odour in MAP2 fish. Since a rancid odour was detected in MAP1 fish when samples were evaluated as unacceptable, chemical changes could be a reason for this. Carbon dioxide-rich packaging effectively inhibited microbial spoilage, but did not prevent chemical changes, especially lipid oxidation. For this reason, some antioxidants would have to be applied in combination with MAP to achieve an extended shelf-life in terms of microbial and chemical changes. On the other hand, dissolution of the CO_2 in the aqueous phase of the fish muscle packaged in MAP1 and MAP2 led to a drop in the pH and consequent loss of meat juice, which negatively affected the texture of the fish flesh. Panellists rejected MAP2 fish on day 12, while MAP1 fish was evaluated as unacceptable from a sensory point of view on day 15.

4. Conclusion

Based on sensory and microbiological parameters, MAP has great potential for preserving fish quality and extending the shelf-life of carp cuts from 7 days in VP to 9 days in MAP2 (40% CO_2 + 60% N_2) and to 12 days in MAP1 (60% CO_2 + 40 % N_2).

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Brief of Preliminary Paper

Does sanitation best practice effectively minimize the risk of undeclared components in food products?

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ABSTRACT

In modern food production, food safety is one of the most important aspects that must be taken into consideration at all stages. Sanitization at food industry enterprises is a key procedure for the production of high-quality and safe food products. Successful implementation of this procedure protects the food products from any pollution and from the contamination with the undeclared components, which helps to increase the stability of the food product and increase its shelf life. This paper presents the results of a study to determine undeclared components in swabs taken from the equipment of a meat processing plant certified for compliance with the requirements of the FSSC 22000 certification scheme, after cleaning and disinfection.

1. Introduction

Currently, one of the most acute issues of quality and safety management in the food industry of the Russian Federation is the issue of food product falsification, which among other things, is caused by the detection of undeclared components (TR CU 022/2011). The current *Technical Regulations of the Customs Union* (2011a) "Food Products in Terms of Their Labelling" and *Technical Regulations of the Customs Union* (2011b) "On the Safety of Food Products" do not set up maximum permissible levels (MPLs) for undeclared components in finished food products. Thus, any detected amount of an undeclared component can be interpreted as a violation of the requirements of the relevant technical regulations, and the food product can be stipulated as non-compliant or falsified. As a result, when conducting research on food products, if regulatory authorities and public organizations detect, for example, insignificant amounts of DNA of various types of meat not declared in the composition on the food label, or genetically modified organisms (GMOs) in corn, they can transmit information to the mass media about the finding of compositional discrepancies without further analysis of the reasons, which causes heavy economic and reputational damage to the industry (*Kryuchenko & Kuzlyakina*, 2021). In addition, data on such studies are published spontaneously and unsystematically, which does not allow society to draw a general conclusion about the real situation (*Kuzlyakina et al*., 2023).

For example, starch, meat and some other ingredients can get into food products by accident, since traces of their DNA can be only completely removed

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from equipment with hydrochloric acid. Regulatory authorities consider even a tiny share of another type of meat DNA in meat products to be a violation (*Minaev*, 2017). The presence of undeclared ingredients can be unintentional and related to the inclusion of residues from a previous batch of food products processed on the same line, but with proper sanitary and hygienic practices, those risks are reduced to a minimum (*Jackson et al*., 2008).

The aim of this study was to investigate the efficiency of sanitization aimed to minimize the risk of undeclared components occurring in the food products. Over six months in 2024, experts from the V.M. Gorbatov Federal Research Center for Food Systems of the Russian Academy of Sciences analyzed the swabs from the various facilities of a meat processing plant certified for compliance with the FSSC 22000 certification scheme in order to find the residual availability of recipe components after the washing of equipment. This plant has implemented best practices for cleaning and disinfection.

2. Materials and methods

The objects of the study were swabs from equipment, auxiliary inventory, and clothing of the personnel of a meat processing plant certified according to FSCC 22000 in order to find the residual presence of components of the recipe of sausages produced in the plant. Three swabs were collected from 20 control points. In total, 60 swabs from different objects were examined.

To understand the probable spots of the food product cross-contamination with undeclared components, it is important to note that some allergenic components specified in TR CU 022/2011 are ingredients in the plant's food product recipes. For example, mustard is included in the list of ingredients of the frankfurters "Adzharian frankfurter with spicy herbs for frying", milk is added into the frankfurters "Milky" and into frankfurters "Creamy". In addition, some complex food additives included in the sausage recipes contain mustard, soy, peanuts, or gluten, but these are not directly listed as recipe ingredients of those sausages.

Swabs from equipment, auxiliary tools, clothing of the personnel were collected in accordance with the swab sampling plan for control of undeclared components.

2.1 Methods of study

The content of gluten, soy, peanuts, and mustard in the swabs was defined by:

a) PCR method;

b) ELISA (AgraQuant® Gluten test kit from Romer Labs) according to the test kit manual.

All laboratory analyses were conducted strictly according to the rules of averaged samples, so each sample was tested three times to facilitate calculation of the average value. The reliability of the obtained results was confirmed by threefold repetition of the experiments, as well as statistical processing of their results, carried out using Microsoft Excel 2019 and STATISTICA 10 software. The results of product studies were presented as mean ± SD, statistical significance was calculated using variance analysis (ANOVA) with the Tukey criterion. The probability of 0.05 was chosen as a significant level. The results of swab studies were presented as median ± SD and percentiles [P 25 - 75]. Statistical significance was calculated using the nonparametric Mann-Whitney U criterion. The probability of 0.1 was chosen as a significant level.

3. Results and discussion

Based on the conducted risk analysis, the swab sampling plan was developed, of which a representative part is shown below in Table 1.

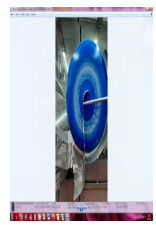
The results of the study of swabs from the company's production line for the presence of DNA indicating soy, gluten, mustard, and peanut using the PCR method are presented in Figures 1 and 2.

Ct values for positive controls were 15.57 for gluten, 14.65 for mustard, and 13.24 for both peanut and soy. It is worth noting that both gluten and mustard DNA were found on the inner surface of the knackwurst stuffing hopper.

Since gluten and mustard DNA were found on the equipment used for food product manufacturing, and were also found in food products produced on the line (data not shown), we assume that cross-contamination of these products occurred because of the equipment. However, we cannot exclude the occurrence of the hazards via unintentional introduction of the undeclared components in the food additives included in the recipes of the above-mentioned food products.

The presence of gluten in the swabs taken from the manufacturing equipment was confirmed by us, including confirmation with the ELISA method. The results of detecting gluten in the swabs with the ELISA method are presented in Table 2 and Figure 4.

Table 1. Representative part of the swab sampling plan to detect undeclared components in cooked sausages.

Subdivision	Tested points – name of the object	Designated place for swabbing	Range of food products manufactured with the equipment
Department for mince stuffing and shaping	1. Unloading disc of the cutter for preparing minced meat for cooked sausages		Cooked sausages: “Veal” frankfurters “Milky” frankfurters “Creamy” knackwursts “Doctor” frankfurters
	2. Unloading disc of the cutter for preparing minced meat for cooked-smoked sausages		Cooked-smoked sausage “Cervelat”
	3. Scraper for cleaning the surfaces of the cutter for preparing minced meat for cooked sausages		Cooked sausages: “Veal” frankfurters “Milky” frankfurters “Creamy” knackwursts “Doctor” frankfurters
	4. Internal surface of the bin for minced meat in the frankfurter line of AL-system		Cooked sausages: “Milky” frankfurters “Creamy” knackwursts

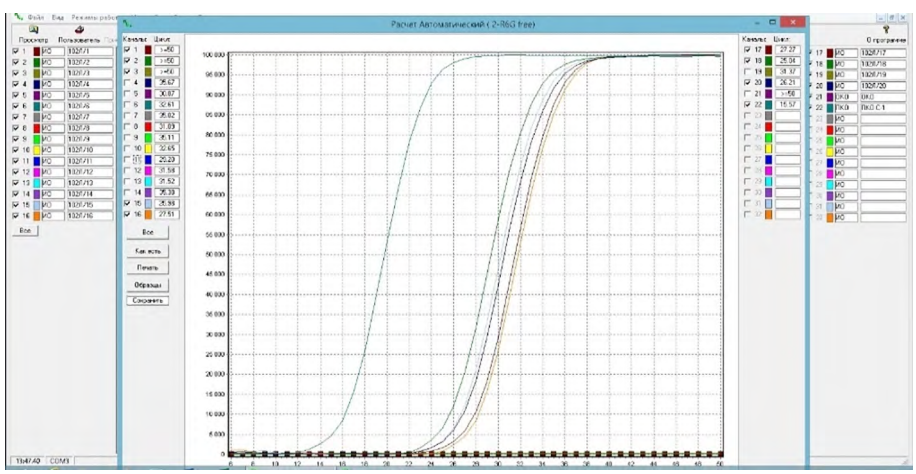


Figure 1. DNA amplification curves of the positive control and the swab samples that were positive. Gluten was regularly detected in the swabs from the inner surface of the horn of the AL-system frankfurter line (< 0.01%, Ct=39.35), the hopper for minced meat of the knackwurst forming stuffer (< 0.01%, Ct=30.12), the inner surface of the horn of the cooked-and-smoked sausage forming stuffer (< 0.01%, Ct=33.16), the inner surface of the horn of the cooked sausage forming stuffer (< 0.01%, Ct=33.14), the inner surface of the horn of the cooked sausage forming stuffer (< 0.01%, Ct=32.18).

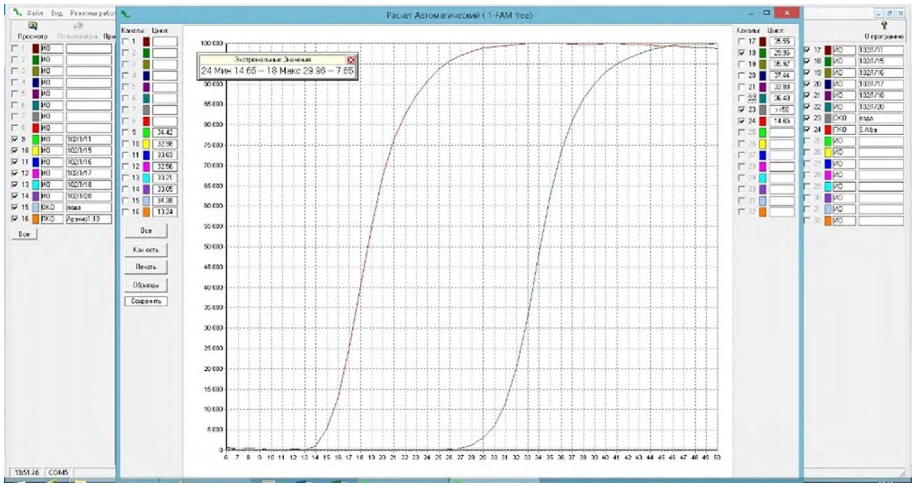


Figure 2. Typical DNA amplification curves of positive control and a swab samples that was positive. Mustard was regularly detected in the swabs taken from the inner surface of the knackwurst stuffing hopper (< 0.001%, Ct=37.18).

Table 2. Results of swab analysis to quantitatively detect the presence of gluten via the ELISA method.

Swab sample		Gluten content, ppm
1 Inner surface of the mince hopper in the knackwurst stuffer	Mean ± SD	9.565±1.074
	Median± SD	**9.697±1.074б***
	[P25 – P75]	[8.431 – 10.566]
2 Inner surface of the inner surface of the stuffer horn for shaping the cooked-and-smoked sausages	Mean ± SD	50.515±6.3981
	Median± SD	**48.474±6.3981**
	[P25 – P75]	[45.387–57.685]
3 Inner surface of the mince hopper for the stuffer for shaping the cooked sausages	Mean ± SD	177.689±121.608a
	Median± SD	**181.886±121.608**
	[P25 – P75]	[54.037–297.145]
4 Inner surface of the stuffer horn for shaping the cooked sausages	Mean ± SD	4.237±0.453 б*
	Median± SD	**4.366±0.453**
	[P25 – P75]	[3.734–4.612]
5 Inner surface of the horn in the sausage line of the AL-system	Mean ± SD	46.412±50.219
	Median± SD	**32.441±50.219**
	[P25 – P75]	[4.671–102.147]

Legend: a-b – significant difference between the sampling locations (ANOVA, Tukey, P<0.05), * – significant difference between columns (nonparametric test, Mann-Whitney U, P<0.1)

It is noteworthy that the gluten concentration varied significantly in different areas of the inner surface of horn on the AL-system sausage line, from which it can be concluded that residual quantities of the components were distributed unevenly along the equipment surface. Naturally, this undoubtedly complicates the process of managing the undeclared components, since an uncontrolled yet high concentration of an undeclared component could show up in one piece of the food product only.

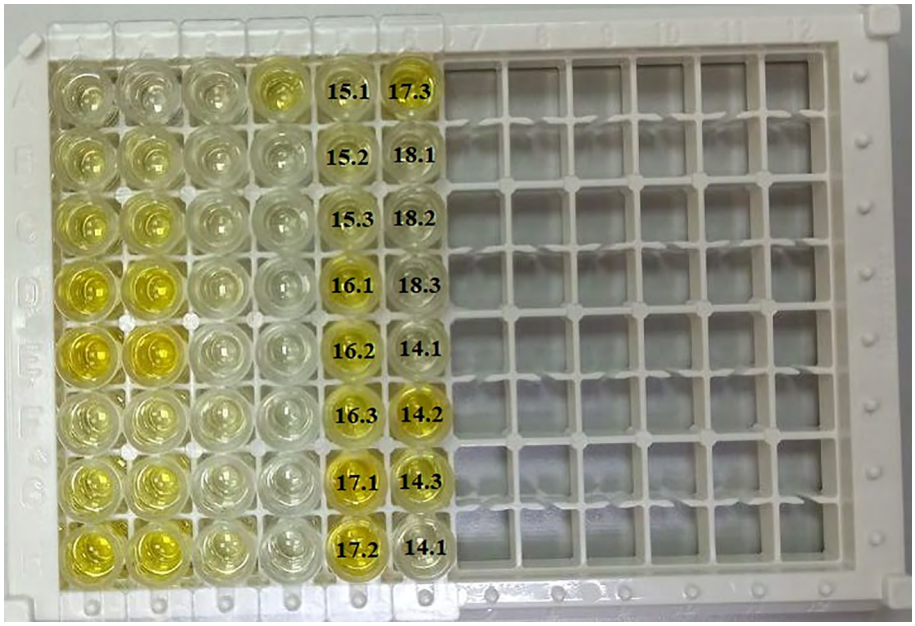


Figure 4. Representative ELISA detection of the presence of gluten in swabs taken from the of AL-system sausage production line. Gluten was regularly detected in the swabs taken from the inner surface of the stuffer horn on the sausage line, the mince hopper of the stuffer for sausage shaping, the inner surface of the stuffer horn for shaping the cooked-and-smoked sausages, the inner surface of the mince hopper of the stuffer for shaping the cooked sausages, and the inner surface of the stuffer horn for shaping cooked sausages.

4. Conclusion

This study shows that the implementation of food safety management systems—including those in accordance with the requirements of the FSSC 22000 certification scheme, which provides for extended and more complex requirements—cannot be considered as efficient methods for reducing or even eliminating the residual amounts of undeclared components from the surface of equipment and auxiliary tools. In this regard, it is necessary to develop and introduce more advanced practices targeted at eliminating the very risk of cross-contamination of the food product with undeclared ingredients after cleaning. This is especially important given that a number of components are allergens, even a minor amount of which can be harmful to a consumer's health.

In conclusion, it should be noted that cleaning of the process equipment is one of the most important stages in food production, and must be done regularly and thoroughly. Each processing method features its own advantages and disadvantages, so it is necessary to choose a method depending on the specific type of the particular equipment.

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Review Paper

Propolis as a bioindicator of elemental contamination

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ABSTRACT

Bioindicators are organisms used to predict the level of environmental pollution in a selected geographical area. Some of these live organisms and their products are very sensitive to surrounding changes in the concentrations of heavy metals, whether due to geographical or anthropogenic origins. High agricultural and industrial activity can be sources of chemical and other contaminants. Honeybee products are natural mixtures produced by a honey bee. The honeybee products are rich with biologically active compounds, such as carbohydrates, proteins, amino acids, lipids, vitamins, phenolics, minerals and enzymes. The most popular bee products are honey (unifloral and multifloral), bee venom, propolis, bee pollen, bee bread and royal jelly. Propolis is a honeybee product produced by honeybees using plant resins (more than 50%), beeswax, essential oils, pollen and other organic substances (alcohols, acids and their esters, benzofurans, benzopyrenes, chalcones, flavonoids and their esters, glycerol and its esters, lignans, phenylpropanoids, steroids, terpenes and terpenoids). Propolis is a very popular honeybee products with high biological activities, such as anti-oxidant, antimicrobial, anti-inflammatory, immunomodulatory and anticancer activities. Some studies shown in vitro antibacterial activity of propolis against different types of Gram-positive and Gram-negative bacteria and data of synergism exhibited among the various propolis compounds. The chemical composition of propolis is complex, and depends with geographical area and season. The chemical variability presents several challenges to its standardization and quality control. The present review is aimed at highlighting the presence of heavy metals in propolis, produced by the species Apis mellifera, from different geographical areas.

1. Introduction

Propolis, also known as bee glue, is a resinous substance collected by bees from plant juices. It has been demonstrated to possess a wide range of beneficial pharmacological properties, including antibacterial, antiviral, antioxidant, anti-inflammatory, immunostimulatory, hepatoprotective, and cytotoxic activities. Today, propolis is widely used across the globe and is available in its raw form or in combination with other natural products. The demand for commercial products containing propolis continues to grow steadily. Extraction plays a crucial role in enabling the utilization of the bioactive constituents of propolis (*Matuszewska et al*., 2021).

Over the past decade, numerous studies from various countries have actively investigated the

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concentration of heavy metals in different bee products—including honey (both unifloral and multifloral), bee venom, propolis, bee pollen, bee bread, and royal jelly—using inductively coupled plasma atomic emission spectroscopy (ICP-AES). These studies suggest that quantifying heavy metals in bee products is an effective method for environmental monitoring (*Ćirić et al*., 2020, *Spirić et al*., 2019, *Hodel et al*., 2020). Among bee products, propolis has been identified as one of the bee-derived substances containing the highest concentrations of heavy metals (*Vakhonina et al*., 2021). Furthermore, raw propolis materials sometimes fail to meet safety standards required for the production of pharmaceuticals and cosmetics. Several researchers attribute the elevated levels of heavy metals—particularly lead (Pb) and cadmium (Cd)—in propolis to its high content of lipid-like substances and waxes, which may facilitate the accumulation of such contaminants.

Extensive research has been dedicated to analysing the chemical and biological composition of propolis. To date, over 300 distinct compounds have been identified in propolis collected from various geographic regions. The main chemical components of propolis are presented in Figure 1. Propolis is primarily composed of the following constituents: plant resins (50%), wax (30%), essential oils (10%), pollen (5%), and other organic compounds—including sugars, amino acids, vitamins, and minerals—accounting for the remaining 5%. Additionally, propolis contains a variety of phenolic compounds (e.g., flavonoids, polyphenols, phenolic acids and other phenolic aromatic substances (*Devequi-Nunes et al*., 2018; *Galeotti et al*., 2019). Propolis also contains esters, terpenes and terpenoids, steroids, aromatic acids and esters, aldehydes, alcohols, sugars, sugar alcohols and acids, amino acids, vitamins, fatty acids, hydrocarbons, mineral elements and other alcohols (*Stojanović et al*., 2020). On the other hand, some chemical constituents, such as alkaloids and iridoids, have not been detected in propolis, a feature often attributed to its botanical sources.

According to *Stojanović et al.* (2020), the major group of phenolic compounds present in propolis is the flavonoid group, which includes flavones, flavanols, flavanones, flavanonols, chalcones, dihydrochalcones, isoflavones, isodihydroflavones and neoflavonoids. These compounds significantly contribute to the biological and pharmacological activities of propolis. In addition to flavonoids, propolis contains various phenolic acids, the most commonly identified being ferulic, cinnamic, caffeic, benzoic, salicylic and p-coumaric acids (*Huang et al*., 2014; *Pasupuleti et al*., 2017). Volatile compounds—particularly terpenes and terpenoids—are also present in propolis, although they make up only about 10% of its composition. Nevertheless, they play a significant role in its biological activity. Several studies (cited in *Sforcin*, 2016) have reported the presence of various terpenoids in propolis, including terpineol, camphor, geraniol, nerol and farnesol. Propolis also contains B-complex vitamins, as well as vitamins C and E (*Stojanović et al.*, 2020). Hydrocarbons such as alkanes, alkenes, monoesters, diesters, aromatic esters, fatty acids and steroids have been identified in various types of propolis. Additionally, several enzymes—such as succinic dehydrogenase, glucose-6-phosphatase, adenosine triphosphatase and acid phosphatase—have been reported in propolis.

Numerous studies have identified various macro- and microelements in propolis, including calcium (Ca), potassium (K), sodium (Na), magnesium (Mg), manganese (Mn), iron (Fe), silicon (Si),

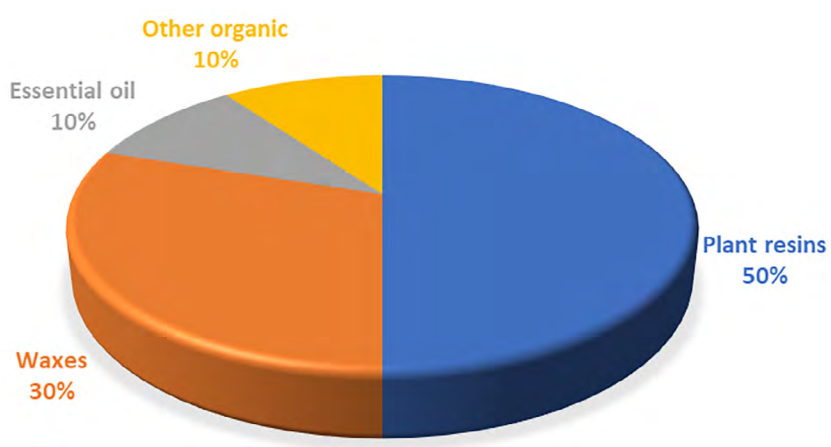

Figure 1. Chemical composition of propolis (adapted from *Stojanović et al*., 2020)

zinc (Zn), selenium (Se), copper (Cu), nickel (Ni), aluminium (Al), boron (B), barium (Ba), chromium (Cr), and strontium (Sr) (*Sforcin*, 2016; *Vakhonina et al*., 2021; *Hodel et al.*, 2020). The trace element profile of propolis can be used as a marker for determining its geographical origin. However, some heavy metals—such as arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb)—have also been detected in propolis.

The interest in bioindicator-based techniques for the detection and evaluation of environmental contaminants has increased during the past 10 years. It is evident that selection of a suitable organism as a bioindicator is a critical step in the overall biomonitoring activity. As far as the biomonitoring of atmospheric pollution is concerned, the honeybee (*Apis mellifera*) has been the subject of various investigations and may be considered an 'ideal bioindicator' as defined by *Stöcker* (1980). Honeybees are continuously exposed to contaminants present in the (usually widespread) area surrounding the apiary for the duration of their foraging activity (i.e., from spring to autumn). As such, honeybees and their products can supply a suitable amount of biological material to be easily sampled and analysed throughout the year. According to data from ScienceDirect (https://www.sciencedirect.com/), and using the research query "Contaminants in Propolis", the number of published articles has grown from 2003 to 2023. The total number of published articles in the last 20 years ranged from 2003 (four articles) to 2023 (76 articles) (Figure 2).

2. Honeybee products as bioindicators

In Europe, trace elements and heavy metals deposition in soil and plants is one of the most pressing concerns in the science of food safety and food quality (CEC Thematic Strategy for Soil Protection). Different studies (*Jovanović et al*., 2017; *Ćirić et al*., 2020; *Matović et al*., 2018) show the impact of different trace elements and heavy metals on human health as well as the mechanisms of uptake and bioaccumulation of trace elements and heavy metals by plants and other foods for human nutrition. Some of the heavy metals are considered potential carcinogens and are associated with the aetiology of a number of internal diseases. On the other hand, some elements are essential elements for human health (*Jovanović et al*., 2017). Honey bees and their products can be contaminated with heavy metals from different sources. The origin of contamination can be the environment (air, water, plants and soil) and beekeeping practices (*Ćirić et al*., 2020).

The beneficial properties of propolis have stimulated growing interest in investigating its chemical composition in relation to its botanical origins.

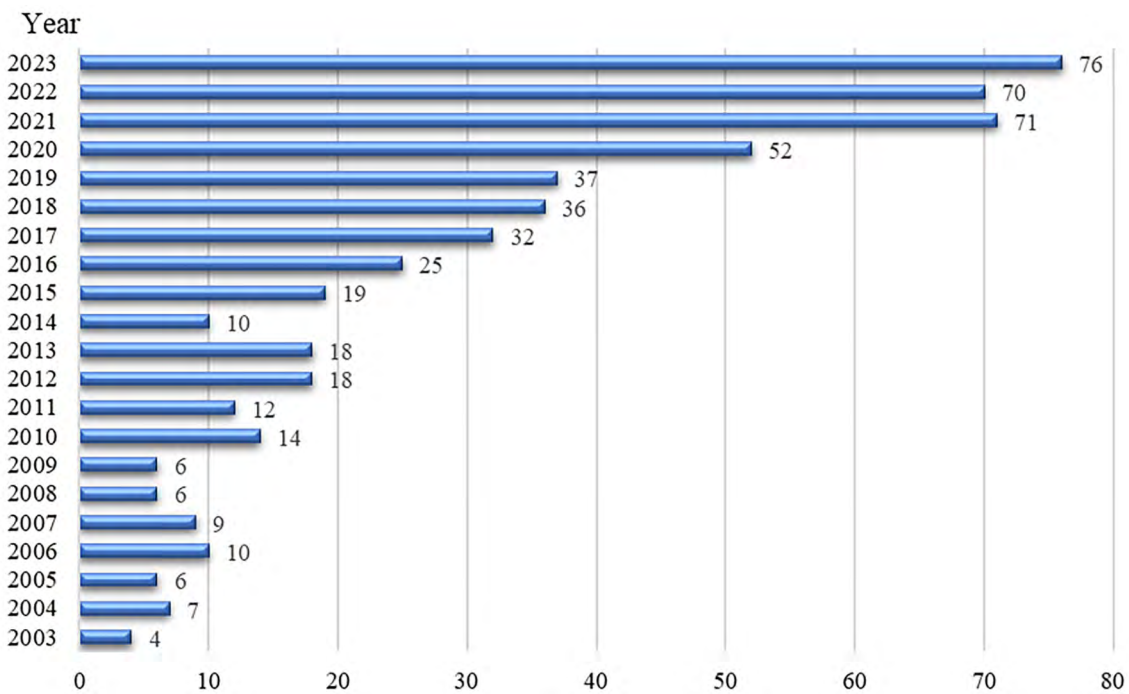

Figure 2. Number of published articles over the past 20 years (data retrieved from the ScienceDirect database using the search query "Contaminants in Propolis").

Propolis produced by *Apis mellifera* contains polyphenolic compounds, with flavonoids—its primary polyphenols—being significantly influenced by the botanical source and the surrounding ecological environment in which the bees reside. The raw materials available to honeybees for the production of propolis primarily consist of plant ingredients secreted in response to injuries, including lipophilic substances from leaves and leaf buds, resins, adhesives, gums and plant ingredients (*Ristivojević et al*., 2015). The botanical composition of these plant-derived substances determines the chemical makeup of propolis, which varies by geographic region. In general, the composition of propolis exhibits high variability due to the diversity of plant species around the hive, from which bees collect the necessary ingredients (*Drescher et al.*, 2019). Furthermore, propolis' chemical profile is influenced by many factors, such as altitude, sunlight exposure, seasonal changes and the characteristics of the bees' foraging areas (*Silva-Carvalho et al*., 2015).

3. Conclusion

Over 500 compounds have been identified in propolis through numerous studies. These include flavonoids, phenylpropanoids, terpenoids, stilbenes, lignans, coumarins and their prenylated derivatives, as reported in various studies. Future research should prioritize the influence of bee species and behaviour on the composition of propolis, which will enhance our understanding of its chemical composition and quality. Comprehensive characterization of propolis from different geographical regions and plant origins is necessary to establish acceptable quantitative standards for various types of propolis. Moreover, the biological activities of propolis need to be correlated with its chemical composition, and standardized products should be used in such studies. Additionally, propolis has potential as a bioindicator for environmental heavy metal deposition. Several studies have confirmed that propolis tends to accumulate heavy metals to a significantly greater extent than honey.

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Review Paper

Application of environmental, social, and governance (ESG) standards in the food industry

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ABSTRACT

This review examines the main environmental, social, and governance (ESG) standards applied by the food industry. The United Nations has adopted the 2030 Agenda that is generally focused on achieving a more sustainable future. This agenda increased the attention of the public on the application of ESG standards by various industries, including food. The most important group of these are the Global Reporting Initiative (GRI) standards. GRI standards play a pivotal role in shaping sustainability reporting practices globally by establishing a comprehensive framework for organizations to disclose their ESG impacts. As one of the most recognized and widely adopted frameworks, GRI emphasizes transparency and accountability in corporate operations, ensuring that organizations report relevant, accurate, and comparable sustainability data. This review covers the application of ESG standards in the meat sector. Generally, the meat sector is considered to be relatively big contributor of greenhouse gas emissions and a large water consumer. Implementation of ESG standards is essential, as they help organizations demonstrate transparency, align with stakeholder expectations, and meet regulatory obligations while promoting sustainability and ethical business practices.

1. Introduction

The 2030 Agenda for Sustainable Development, adopted by United Nations Member States in September 2015, marks a significant commitment to addressing the global challenges faced by humanity, encompassing issues of poverty, inequality, climate change, environmental degradation, peace, and justice. Comprising 17 Sustainable Development Goals (SDGs) and 169 targets, the agenda underscores an integrative and inclusive approach to development that aspires to improve the quality of life for all, while ensuring the sustainability of the planet (*United Nations*, 2015). Notably, the agenda emphasizes that the path to achieving these goals requires collaboration among multiple stakeholders, including governments, civil society, private sectors, and citizens, thereby fostering shared responsibility for sustainable progress and well-being. Furthermore, the interconnected nature of the SDGs implies that actions taken to advance one goal can have positive effects on others, reinforcing the need for cohesive strategies that are context-specific and responsive to local challenges. Ultimately, the successful

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realization of the 2030 Agenda is contingent upon a collective effort towards transformative actions that ensure equitable resource distribution and uphold the principles of sustainability for current and future generations.

Since adoption of the 2030 Agenda, application of environmental, social, and governance (ESG) standards in the food industry has garnered increasing attention due to increased consumer awareness and shifting regulatory landscapes surrounding sustainability. As global markets evolve, the adoption of ESG practices within this sector not only affects operational efficiency but also significantly impacts stakeholder perceptions and financial performance. The interrelationship between ESG factors and corporate outcomes is particularly important in environmentally sensitive industries like meat and dairy production, where the implications of operational practices on resource sustainability and social responsibility are profound.

Research indicates that food companies that integrate ESG principles into their business strategies can experience improved financial performance as a result of enhanced risk management and reputation (*Sandberg et al.*, 2022). These dynamics are articulated within the context of various industries, including food (*Ahmedet al.*, 2023), where the relationship between sustainability practices and financial benefits has become more pronounced. Specifically, the meat and dairy industry, characterized by its carbon emissions and resource-intensive operations, must navigate the complex landscape of ESG standards to mitigate risks associated with environmental and social governance (*Stojkovic et al*., 2025).

Moreover, technological advancements, such as the implementation of Industry 4.0 technologies, are reshaping operational paradigms in the meat sector. Such innovations not only streamline processes but also enhance compliance with ESG standards by optimizing resource use and reducing waste (*Echegaray et al.*, 2022). The interplay between technological integration and ESG compliance serves as a pivotal factor in driving sustainability within the industry, as companies strive to meet both regulatory standards and consumer expectations.

Furthermore, understanding the specific ESG risks and opportunities in the meat industry forms the cornerstone of effective sustainability reporting and strategy. Companies are increasingly adopting a tailored approach to navigate the unique challenges they face, which range from animal welfare concerns to environmental impacts related to production practices (*Boiral et al.*, 2024). This shift towards a comprehensive ESG strategy reflects a broader transformation within the agricultural sector, underscoring the need for robust frameworks that align business objectives with sustainability goals.

2. Standards covering ESG reporting

ESG reporting is covered by several standards and frameworks that guide disclosure and assessment practices across various industries. These standards are essential as they help organizations demonstrate transparency, align with stakeholder expectations, and meet regulatory obligations while promoting sustainability and ethical business practices.

2.1. Global reporting initiative (GRI)

One prominent framework for ESG reporting is the Global Reporting Initiative's (GRI's) standards, which provide comprehensive guidelines that underline the importance of sustainability reporting by mandating organizations to disclose their economic, environmental, and social impacts (*Baraibar-Diez & Odriozola*, 2019). GRI encourages consistency and comparability in reporting, fostering a deeper understanding of corporate responsibilities and performance in aligning with sustainable development goals (*Park & Jang*, 2021). As one of the most recognized and widely adopted frameworks, GRI emphasizes transparency and accountability in corporate operations, ensuring that organizations report relevant, accurate, and comparable sustainability data (*Leeson & Kuszewski*, 2023; *Yehezkiel et al.*, 2023). This framework promotes stakeholder engagement by positioning stakeholders as central to the sustainability reporting process. The GRI actively involves affected parties in its standard-setting activities, allowing for a collaborative approach that reflects the diverse interests and concerns of these stakeholders (*Leeson & Kuszewski*, 2023). Furthermore, the GRI standards are designed to be adaptable across various sectors, enabling organizations of all types and sizes to tailor their disclosures to accurately reflect their sustainability practices and performance (*Yehezkiel et al.*, 2023). The thoroughness of the GRI framework ensures that organizations not only communicate their sustainability efforts but also foster a culture of continuous improvement and ethical responsibility, thus

enhancing their overall credibility and societal trust (*Villiers et al.*, 2022).

2.2. International sustainability standards board (ISSB) standards

The International Sustainability Standards Board (ISSB) plays a critical role in establishing a global framework for sustainability reporting, aiming to enhance the transparency and comparability of sustainability-related financial disclosures. Launched under the auspices of the International Financial Reporting Standards (IFRS) Foundation, the ISSB's primary objective is to develop standards that address the evolving expectations of investors and other stakeholders regarding ESG factors (*van Wyk & Els*, 2023). The ISSB has introduced two key standards, IFRS S1 and IFRS S2, which focus on general sustainability reporting and climate-related disclosures, respectively. These standards underscore the importance of detailed disclosures on sustainability risks and their financial implications, promoting a double materiality perspective that reflects both how sustainability issues affect an organization and how the organization's activities impact the environment and society (*Zdolšek & Beloglavec*, 2023). The initiative also seeks to harmonize sustainability reporting with existing financial reporting standards, thereby fostering a more integrated approach to corporate reporting (*Sabauri & Kvatashidze*, 2023). As the ISSB develops its standards, it continuously engages with stakeholders and industry experts to ensure that the frameworks remain relevant and practically implementable, thus addressing the complexities of sustainability reporting in an ever-changing global landscape (*Kulik & Dobler*, 2023). Consequently, the ISSB stands at the forefront of driving the shift towards more sustainable business practices by providing clear guidelines that encourage companies to disclose and manage their sustainability impacts effectively.

2.3. Other standards

Another group of standards was introduced by the Sustainability Accounting Standards Board (SASB), which focuses on sector-specific ESG metrics that highlight material sustainability information for investors. By establishing industry-targeted standards, SASB aids companies in identifying and reporting on the most relevant ESG issues that could impact their financial performance, thus bridging the gap between traditional financial reporting and sustainability metrics. This approach allows for cross-sector comparisons and ensures that investors are well-informed regarding the sustainability risks associated with their investments.

Another significant framework is the United Nations Global Compact, which outlines ten principles covering human rights, labour, the environment, and anti-corruption. Companies that align with these principles are encouraged to report on their advancements and challenges in fulfilling them, thereby integrating ESG into their core business strategies. This helps businesses proactively manage their social and environmental impacts while fostering a culture of accountability and ethical governance and consequently positively affecting reputation of the company (*Praveen & Mohammad*, 2022) .

3. Application of ESG standards in the meat industry

The global meat sector is currently facing increasing scrutiny regarding its ESG practices. This heightened attention is driven by consumers becoming more conscious of the ethical, environmental, and health impacts associated with meat production, prompting shifts within the industry toward adherence to ESG guidelines. These frameworks aim to prioritize sustainability, animal welfare, and robust governance systems while enhancing the public image of the meat sector and ensuring its long-term viability. Given the urgent concerns related to climate change, water scarcity, deforestation, and the environmental repercussions of livestock farming, the meat industry is under significant pressure to adopt greener and more ethical practices (*Briscoe*, 2022). The study of *Rajic et al*. (2021) examined the application of ESG data by the 75 largest food companies in the world according to total revenue and compared compliance of ESG practices of plant and animal origin food companies. The results showed that even the world's largest companies have gaps in ESG reporting, which demonstrates the complexity and complexity of implementing standards in the industry. On the other hand, small- to medium-scale (SME) meat producers face significant challenges in applying environmental, social, and governance (ESG) standards due to their limited administrative, technical, and financial resources. This limitation hinders their ability to effectively implement and adhere to these standards compared to larger corporations, which

typically have more robust infrastructures in place for ESG compliance (*Lam et al*., 2024; *Yip et al*., 2024).

From the climate perspective, the meat industry ranks among the largest of industrial contributors to global greenhouse gas emissions, primarily through methane production from livestock, which poses a significant environmental threat (*Nalon et al.*, 2021). The complicated task of accurately calculating emissions from various phases of meat production—spanning farming, feed supply, transportation, and processing—is complicated by a lack of consistent, globally accepted metrics (*Coleman et al.*, 2022). Additionally, the substantial water consumption involved in meat production, particularly in beef production, raises concerns due to its extensive water footprint, with irrigating feed crops and livestock hydration contributing to this issue (*Mekonnen & Hoekstra*, 2011).

Deforestation linked to meat production, especially linked to cattle ranching in areas like the Amazon region, poses another severe environmental challenge. This practice leads to biodiversity loss and exacerbates climate change (*Mekonnen & Hoekstra*, 2010). However, companies often struggle to track their deforestation impacts accurately, particularly when sourcing from regions with weak regulatory frameworks (*Sinclair et al.*, 2017). Overall, the meat industry's move toward incorporating ESG practices reflects recognition of the need for sustainable development that balances productivity with responsible environmental stewardship (*Howell et al.*, 2016).

4. Conclusion

In conclusion, the 2030 Agenda for Sustainable Development is a pivotal framework advocating for holistic progress across various sectors, including the imperative of adopting ESG standards within the food industry. As the meat sector navigates increased consumer awareness regarding ethical, environmental, and health issues, the integration of ESG principles has become crucial in addressing these concerns and enhancing corporate accountability. The interconnectedness of the SDGs emphasizes the necessity for collaborative action and tailored strategies that align with local contexts and stakeholder needs. In response to the mounting pressures of climate change, resource scarcity, and social responsibilities, it is incumbent upon the meat industry to embrace innovative practices and technologies that facilitate compliance with ESG standards and promote sustainable operations. Furthermore, the rise of frameworks such as GRI and ISSB underscores the importance of transparency and accountability, guiding companies in their sustainability reporting efforts and providing a framework, that is, most importantly, comparable globally. Ultimately, achieving progress towards the 2030 Agenda necessitates a concerted commitment to sustainability that is not only reflected in established procedures but also embedded in the corporate culture of the meat industry. By fostering a comprehensive approach to ESG implementation, the industry can secure its legitimacy, enhance its resilience, and contribute to a more sustainable future for all.

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Review Paper

Key parameters affecting the sensory quality of beef: a review

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ABSTRACT

The sensory quality of beef is one of the most important factors by which consumers assess their satisfaction, and it plays a crucial role in influencing future purchasing decisions. Color, tenderness, juiciness and flavor are the most valorized sensory attributes of beef. The sensory characteristics of beef are influenced by various factors related to the animal, such as breed, sex, age, and growth potential, as well as technological factors, including feeding systems, rearing methods, transport, and slaughter procedure. Consumer preferences regarding sensory quality parameters are shaped by geographic regions, cultural norms and individual differences. Consumers in some regions favor a bright red color, while others associate a darker red color with higher quality of beef. European consumers tend to prefer the more intense flavor of grass-fed beef, while U.S. consumers favor the flavor of grain-fed beef. This review focuses on the key parameters for the sensory quality of beef from a consumer perspective, as well as the main variables that affect these parameters.

1. Introduction

Sensory attributes, perceived through the senses, play a central role in shaping consumer food preferences and satisfaction. The principal sensory quality parameters of beef that define satisfaction in consumers include color, tenderness, juiciness, and flavor. However, consumer perception of these sensory attributes is not uniform and can vary significantly based on geographic region, cultural norms, educational background, and other sociodemographic factors (*Clinquart et al.*, 2022). The sensory characteristics of beef are influenced by a complex combination of different factors, including breed, age, sex, and rearing systems, as well as technological factors. Moreover, diet, growth strategy, and technological procedures ranging from transport to slaughterhouse and meat preparation are critical in determining the final sensory quality of beef (*Pogorzelski et al.*, 2022).

The objective of this review is to describe the sensory attributes of beef that are most valued by consumers, and outline the main factors that influence these attributes.

2. Beef color

Color is commonly regarded as a critical visual indicator of freshness, taste, texture, and overall quality

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(*Smith et al*., 2000). A bright, cherry-red hue is typically associated with freshness and superior eating quality, whereas a brown discoloration is often interpreted as a sign of spoilage or lower quality (*Lukić et al*., 2013). Meat color is predominantly determined by the concentration of myoglobin and its chemical state, particularly its level of oxygenation or oxidation. The main chemical forms of myoglobin responsible for the appearance of beef are: Oxymyoglobin (OMb; Fe^{2+}in the presence of O_2), which imparts a bright cherry-red color and positively influences consumer acceptance; Metmyoglobin (MMb; Fe^{3+} in the oxidized state), associated with a brown coloration and commonly regarded as a primary indicator of freshness loss, often resulting in product rejection; Deoxymyoglobin (DMb; Fe^{2+} in the absence of O_2), which gives the meat a purplish-red appearance. The susceptibility of myoglobin to oxidation is affected by several interrelated factors, including breed, sex, nutrition, muscle type, pre-slaughter stress, and postmortem metabolic changes. Female animals tend to accumulate pigment more rapidly with age compared to males. Nonetheless, meat from intact males generally appears darker than that from females or castrated males, which is attributed to higher physical activity levels and greater myoglobin concentrations (*Liu et al*., 2022). Variations in beef color are significantly affected by the production system and feeding regime. It is well established that grass-fed, particularly pasture-raised, cattle produce meat with a darker color. This effect is primarily attributed to increased physical activity, which induces a shift in muscle fiber composition toward oxidative metabolism. Such a physiological adaptation is characterized by enhanced capillarity, a higher proportion of slow oxidative (red) muscle fibers, and elevated myoglobin concentrations (*Scollan et al*., 2014). The visible portion of intramuscular fat (IMF), commonly referred to as marbling, is widely recognized as a key indicator of beef quality. Marbling is influenced by several factors, including diet, age at slaughter, muscle type, and particularly differences among cattle breeds. *Park et al.* (2018) reported a wide range in IMF content, from as low as 1.9% in Brahman cattle to as high as 37.8% in Japanese Wagyu beef. Generally, grain-fed cattle reared in feedlot systems accumulate greater amounts of IMF, which contributes to a lighter meat appearance due to increased fat dispersion. A comparative study involving fifteen European cattle breeds revealed significant breed-dependent variation in beef color, allowing classification into four distinct groups. Highly specialized beef breeds, such as Limousin, Charolais, Piemontese, and Marchigiana, tend to produce meat with a bright, pale-red color. South Devon, Danish Red, Asturiana de los Valles, Pirenaica, Aberdeen Angus, and Holstein are associated with meat of a similarly bright but pale appearance. Simmental, Avileña-Negra Ibérica, Highland, and Casina produce beef with a more intense red color, while the Jersey breed (a dairy breed) yields meat with a darker and dull red hue (*Ripoll et al*., 2018). European consumers generally prefer beef with a bright or light red color, a preference that is also observed among consumers in Australia and Japan. U.S. consumers tend to favor a cherry-red color. In contrast, consumers in Scotland and Ireland perceive bright red beef as less acceptable, exhibiting a clear preference for darker red meat (*Santos et al*., 2021).

3. Beef tenderness

Consumer satisfaction with meat tenderness is primarily determined on the basis of the interaction between the physical and textural properties of the meat and the perceived oral sensations during mastication, commonly referred to as mouthfeel (*Liu et al*., 2022). Beef tenderness is strongly influenced by genetic factors. It is well established that meat from *Bos indicus* cattle tends to be less tender than that from *Bos taurus* breeds. This difference is primarily attributed to elevated levels of calpastatin enzyme in *Bos indicus*, which inhibits postmortem proteolysis (*Wright et al*., 2018). Breed-specific differences in tenderness can occur independently of intramuscular fat content. *Chambaz et al.* (2003) demonstrated that cattle from the Angus and Limousin breeds consistently produced more tender meat compared to Charolais and Simmental, even when marbling levels were equivalent. Post-slaughter carcass cooling is a critical factor influencing the meat tenderization process, and it can negatively impact final meat quality. Elevated carcass temperatures with insufficient or delayed carcass cooling, combined with a rapid postmortem pH decline can lead to a condition known as heat-induced toughening, which significantly compromises tenderness. Conversely, if the carcass is chilled too rapidly before the completion of rigor mortis, excessive muscle contraction can occur, resulting in irreversible toughness—a phenomenon referred to as cold shortening. Compared with other meats, beef is particularly susceptible to cold shortening due to its relatively high proportion of oxidative red muscle fibers, which are more reactive to sudden temperature drops postmortem

(*Thomson et al*., 2008). Beef tenderness is inversely related to the age of the animal, with tenderness decreasing as age increases. This decline is primarily attributed to age-related changes in connective tissue. The impact of age on tenderness becomes especially pronounced when the age differences among animals are substantial. Sex category also influences meat tenderness. Heifers generally produce more tender meat than bulls and steers, due to the females' lower intramuscular connective tissue content and smaller muscle fiber diameters (*Duarte et al*., 2022). Feeding strategy is another critical determinant of tenderness. High-energy diets in intensive beef production systems promote greater deposition of intramuscular fat, which reduces the mechanical strength of intramuscular connective tissue and improves tenderness (*Santos et al*., 2021).

4. Beef juiciness

The primary determinants of juiciness are the meat's water-holding capacity and its intramuscular fat content. Accordingly, sensory perception of juiciness is typically divided into two phases: initial juiciness, which is primarily affected by the water content of the meat and perceived during the first few chews; and sustained juiciness, which is associated with the fat content and its effect of stimulation of salivary flow during continued mastication (*Winger and Hagyard*, 1994). Previous research has demonstrated that significant differences in meat juiciness exist among cattle breeds. *Chambaz et al.* (2003) evaluated the meat quality of four European breeds—Limousin, Angus, Simmental, and Charolais—raised under semi-intensive fattening systems and slaughtered at comparable levels of marbling. The study found that the Limousin breed produced meat with superior juiciness relative to the other breeds. Further evidence of the relationship between intramuscular fat and juiciness was provided by a study involving U.S. and Australian consumers, which identified a strong positive correlation between fat content and perceived juiciness. Maximum juiciness scores were observed when IMF content reached approximately 20% (*Thompson*, 2004).

5. Beef flavor

Flavor results from a complex integration of olfactory and gustatory sensations, encompassing both basic taste and aroma perceptions. As a complex sensation, flavor develops primarily during cooking, and it is affected by the chemical composition of the meat. Both lipids and water-soluble compounds undergo degradation during cooking, producing numerous volatile aromatic components (VACs) that contribute to the development of meat flavor. The quantity and composition of intramuscular lipids, the oxidative status of these lipids, and their fatty acid profiles are critical factors in determining the extent of lipid oxidation and the diversity of VACs produced. These variables collectively affect the overall flavor balance (*Fu et al.*, 2022). Seven major classes of VACs have been identified as key contributors to lipid-derived flavor development in cooked beef: aldehydes, alcohols, carboxylic acids, furans, hydrocarbons, pyrazines, and sulfur-containing compounds (*Lee et al*., 2025). In addition to lipid-derived VACs, water-soluble compounds such as peptides, amino acids, reducing sugars, and nucleotides, formed after slaughter through the breakdown of proteins and glycogen, enhance the flavor development. The Maillard reaction, which involves interactions between reducing sugars (e.g., ribose, glucose) and free amino compounds (e.g., amino acids and peptides), is a critical pathway for the generation of flavor-enhancing heterocyclic compounds (*Kosowska et al*., 2017). Flavor is predominantly an inherent attribute of fresh meat that is not substantially modifiable through postmortem handling. Instead, it is primarily determined by pre-slaughter factors such as feeding regime, breed, sex, and age at slaughter. Early research has demonstrated that the feeding strategy plays a pivotal role in influencing the intramuscular fat content and fatty acid composition. Beef from grass-fed animals contains higher levels of polyunsaturated fatty acids (PUFAs), such as linoleic acid (C18:2n-6) and α-linolenic acid (C18:3n-3), compared to beef from grain-fed animals. The high oxidative susceptibility of PUFAs is essential in development of off-flavors, commonly described as grassy or pastoral. These undesirable flavor notes are largely attributed to degradation products of C18:3n-3, including compounds such as skatole, indole, (Z)-4-heptenal, and 4-methylphenol. Conversely, in most studies, beef from grain-fed cattle, which is typically richer in saturated fatty acids (SFAs), has been consistently associated with more favorable flavor characteristics, such as juicy, fatty, sweet, and umami (*O'Quinn et al*., 2016; *Piao et al*., 2019).

However, European consumers tend to prefer the more intense flavor of grass-fed beef over that of concentrate-fed cattle. This preference is likely

influenced by traditions, cultural norms, and individual taste perceptions. In contrast, U.S. consumers generally perceive grass-fed beef as less acceptable, favoring the flavor of grain-fed beef, which is typically associated with higher IMF content and characterized by buttery/beef fat flavor notes (Realini et al., 2013). Differences in flavor intensity among cattle breeds can be explained by variations in fat content and marbling. For instance, beef from Wagyu cattle is reported to exhibit more intense flavor and greater juiciness compared to Angus beef, primarily due to its significantly higher IMF content and marbling score (*Frank et al*., 2016).

Meat from the Hereford breed has been reported to exhibit a more rancid flavor profile compared to that of Aberdeen Angus cattle. This difference is primarily attributed to the higher PUFA content in Hereford meat, making it more susceptible to oxidative degradation (*Warren et al.*, 2008). Heifers and steers generally receive higher flavor scores than bulls, largely due to their higher IMF content. Furthermore, flavor quality has been shown to improve with the animal's age, a trend that could be associated with increased fat deposition and greater physiological maturity (*Therkildsen et al*., 2017).

6. Conclusion

Studies evaluating beef eating quality have consistently identified color, tenderness, flavor, and juiciness as the primary sensory attributes affecting consumer satisfaction. Consumer experiences with these sensory characteristics during consumption play a critical role in shaping future beef purchasing behavior. Importantly, consistency in eating quality has been recognized as a pivotal factor in promoting increased beef consumption.

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Review Paper

Salt reduction in cooked sausages

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ABSTRACT

In modern times, the daily intake of sodium chloride is relatively high, especially in industrially developed countries. Increased consumption of sodium chloride can lead to essential hypertension, which is associated with other cardiovascular and renal diseases. Reducing the sodium content in meat products has become an increasingly important trend in contemporary nutrition due to the adverse health effects of excessive salt intake. In the production of cooked sausages, sodium chloride affects both the functional properties of meat products, such as water-binding capacity, and the sensory characteristics of the product, including tenderness, juiciness, and textural attributes. Therefore, one of the main objectives in reducing sodium chloride in such products is to maintain product stability during thermal processing, while preserving a sufficiently salty and desirable taste, as well as the mentioned textural properties.

1. The role of salt in meat products

Sodium chloride is the first known food preservative, especially for meat, and it has remained in use to this day. In addition to its role in food preservation, the desirable salty taste is one of the most important effects that sodium chloride imparts to food (*Multhauf*, 1987). A widely accepted theory is that sodium ions play a primary role in the perception of salty taste, while chloride ions act as modulators (*Bartoshuk*, 1985).

Adding salt to food increases the desire to consume that food to a certain extent. Sodium chloride not only creates a salty taste but also plays other important roles in many types of food. It affects the perception of firmness, enhances sweetness, masks metallic and undesirable flavors, and rounds out the overall taste, contributing to its intensity (*Gillette*, 1985).

The main source of sodium in food products comes from sodium chloride, that is, table salt, and its intake significantly exceeds nutritional recommendations, especially in modern and industrially developed countries. The consumption of table salt is influenced not only by physiological needs (such as in athletes) but also by habits formed early in childhood, as well as by dietary traditions (climate conditions, food preparation, livestock resources, etc.).

At the beginning of the twentieth century, salt consumption reached its peak and continued to grow as industrial food production developed. Salt was once a source of life, but today it has started to pose a high health risk to modern humans. Nowadays, the daily intake of sodium chloride is quite high, especially in developed countries, that is, in populations that primarily consume industrially produced food. Of the total daily salt intake through usual quantities of food (homemade dishes, bread, bakery products,

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cheese), meat products belong to the top five food categories that contribute the most to this intake (*Dunteman et al*., 2022).

Excessive sodium intake is recognized as the leading cause of essential hypertension, which is one of the primary risk factors for the development of cardiovascular and renal diseases (*Diehl and Ludington*, 2007; *He and MacGregor*, 2013; *O'Donnell et al*., 2021; *Xiao et al,* 2022).

Research has shown that high levels of sodium in the diet increase fluid retention in the body, which can put strain on the heart and blood vessels (*MacGregor and Ashby*, 2013). Despite this, salt consumption peaked at the beginning of the twentieth century and continued to rise as industrial food production developed.

Reduction of salt content in meat products can be achieved in several ways:

(1) by reducing the amount of added sodium chloride (*Sofos*, 1983); (2) by replacing part of the sodium chloride with other salts (*Sofos*, 1983; *Terell*, 1983; *Guàrdia et al*., 2006); (3) by using flavor enhancers, and masking agents (*Desmond*, 2006); (4) by combining the aforementioned methods (*Sofos*, 1983; *Terell*, 1983); (5) by adding herbs or spice extracts to meat products (*Lilić and Matekalo-Sverak*, 2007); (6) by optimizing the physical form of the salt (*Angus et al*., 2005) and; (7) by using alternative processing techniques (*Claus and Sørheim*, 2006).

However, reducing sodium in meat products presents a major challenge.

2. Possibility of reducing salt in cooked sausages

According to research conducted in Serbia, the salt content in cooked sausages ranged from 1.28-2.03g/100 g, with a mean of 1.66g/100 g (*Vranić et al*., 2009). Reducing sodium in the most commonly consumed meat products, such as cooked sausages, can contribute to lowering excessive sodium intake (Petit et al., 2019).

In the production of cooked sausages, salt does not play a significant role in preservation due to the temperature regime to which these sausages are subjected (pasteurization). However, sodium chloride affects the functional properties of meat products in terms of water binding, as well as the sensory characteristics of the product, such as tenderness, juiciness, and textural properties. One way to reduce its use is by substituting sodium chloride with other salts, in order to maintain the product's stability during thermal processing, as well as to preserve a sufficiently salty or desirable taste and the aforementioned textural characteristics.

Potassium chloride is most commonly used salt substitute; however, complete replacement of sodium chloride is not possible because, according to many studies, even at 50% substitution, it results in an increased bitter taste and a reduced perception of saltiness. The use of potassium salts is often contested due to the potential sensitivity of certain segments of the population, such as people with type I diabetes, chronic renal insufficiency, those in the final stage of kidney diseases, and individuals with heart and adrenal insufficiency (*FSAI*, 2005). In the guide *Dietary Guidelines for Americans* (2005), it is specified that a diet rich in potassium reduces the effects of salt on blood pressure and recommends a daily potassium intake of 4700 mg. Dietary salts that are a mixture of sodium chloride and potassium chloride, usually with the addition of L-lysine hydrochloride, which masks the bitter taste of the salt and promotes the excretion of sodium from the body, are already available on the market. (*Ruusunen end Poulanne*, 2005).

Phosphates have also proven to be effective in reducing the salt content in meat products, as they act synergistically with sodium chloride. They increase the water-holding capacity (WHC) by increasing ionic strength, where free negatively charged groups allow proteins to bind more water (*Trout and Schmidt*, 1984). However, phosphates are also carriers of sodium. Thus, sodium polyphosphate contains 31.24% sodium compared to 39.34% found in sodium chloride; however, sodium polyphosphate use is limited to about 0.5% in the product.

Ruusunen et al. (2002) determined that it is possible to produce Bologna sausage and cooked ham with a lower salt content (1.0–1.4%) and that it is possible to reduce sodium content by using potassium salts. *Lilić et al.* (2008) established that substitution of sodium chloride with potassium chloride in an amount up to 40% in cooked sausages is possible without a significant impact on taste acceptability. The substitution did not affect the product's appearance, nor the acceptability of color and texture.

One of the possible ways to reduce the salt content in meat products is the use of flavor enhancer and masking agent blends. There are many commercial blends that typically contain yeast extract, lactates, monosodium glutamate, and nucleotides. Flavor enhancers work by activating receptors in the oral cavity, which help compensate for the reduced salt content in the product (*Brandsma,* 2006).

Some authors (*Pasin et al*., 1989) have determined that it is possible to reduce sodium chloride addition by up to 75% in cooked sausages by combining potassium chloride with a ribotide preparation (a commercial mixture of 5′-ribonucleotides IMP and GMP). In such cases, any addition of monosodium glutamate leads to a decrease in product flavor acceptability by up to 50%, due to the enhancement of the bitter taste caused by potassium salts. Linguagen, a U.S. company, has patented a bitter taste blocker, adenosine 5′-monophosphate, which works by blocking the activation of gustducin in taste receptor cells and thus prevents the stimulation of the nerve that innervates the taste receptors (*McGregor*, 2004). This blocker can be used to improve flavor when using combinations of potassium chloride and sodium chloride.

Other combinations, such as lysine and succinic acid, are used as salt substitutes (*Turk*, 1993). These substances have a salty taste and possess some antimicrobial and antioxidant properties, and can be used to replace up to 75% of salt. For technological purposes, particularly water-binding capacity, phosphates, starches, and gums can also be used. The degree of saltiness also depends on the physical form of the salt. Flake salt has proven to be more functional in terms of water-binding capacity, increasing pH, and enhancing protein solubility in emulsion model systems (*Campbell*, 1979). Salt in flake form is better and more quickly soluble than granulated salt, which can be an issue in formulations where water is not used. Therefore, flake salt can be suitable for products that do not involve added water, such as dry-cured meat. Leatherhead Food International has researched the optimization of the physical form of salt and monitored the changes in its physical state, which make it more bioavailable and thus usable in smaller quantities. This includes increasing salt efficiency, altering its structure, and modifying salt perception (*Angus et al*., 2005).

Alternative processing techniques involve the use of pre-rigor meat in the production of meat products or the application of high-pressure technology in manufacturing (*Claus and Sørheim*, 2006). High-power ultrasound (HPU) has the ability to alter protein structure and improve the distribution of ingredients within the meat matrix (*Gomez-Salazar et al*., 2021). It can also be used to reduce sodium content in meat products. The combination of HPU with a low level of potassium chloride (0.5%) can overcome technological issues caused by a 50% reduction of sodium chloride in meat emulsions (*Pinton et al*., 2020). However, these products exhibited a reduced intensity of salty taste.

3. Conclusion

Reducing the salt content in food is becoming an increasingly important trend in modern nutrition due to the negative effects that excessive salt intake can have on human health.

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Review Paper

Probiotic and prebiotic properties of honey: a review

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ABSTRACT

Honey is widely recognized for its diverse health-promoting properties, including antibacterial, antioxidant, anti-inflammatory, antimutagenic, anticarcinogenic, and bacteriostatic effects. Additionally, it has been effectively utilized in the treatment of wounds and sunburns. The chemical composition of honey comprises a variety of minor bioactive constituents—such as enzymes, minerals, amino acids, polyphenols, and vitamins—alongside major components predominantly consisting of monosaccharides, namely glucose and fructose. The content of bioactive compounds in honey is largely influenced by the botanical origin of the nectar and the geographical location in which it is produced. Among its numerous health-promoting effects, honey exhibits notable antioxidant, anti-inflammatory, and antimicrobial properties. In recent years, increasing scientific attention has been directed toward the probiotic and prebiotic potential of honey. Studies have revealed the presence of lactic acid bacteria in honey, which are capable of producing bioactive metabolites that can persist in varying concentrations in mature honey. Additionally, honey contains oligosaccharides—recognized as prebiotic compounds—that promote the growth and activity of probiotic bacteria, while also enhancing their survival during gastrointestinal transit and product storage. Moreover, honey's antioxidant constituents are believed to play a crucial role as prebiotic agents by protecting and stimulating the viability of probiotic microorganisms. Therefore, the objective of this paper is to present recent scientific findings on the probiotic and prebiotic properties of honey, highlighting its potential as a functional food.

1. Introduction

Honey is a natural sweet substance produced by honeybees through enzymatic transformation of floral nectar or secretions. Since ancient times, honey has been used not only as a natural sweetener but also as a therapeutic agent. Its composition is highly complex and variable, comprising at least 181 distinct components (*Baltić et al*., 2023; *Alvarez-Suarez et al*., 2010). From a chemical point of view, it could be defined as a natural food mainly composed of sugars and water together with minor constituents, such as minerals, vitamins, amino acids, organic acids, flavonoids and other phenolic compounds, and aromatic substances (*Tafere*, 2021). These minor constituents confer the functional properties of honey, and can positively influence human health. Depending on the botanical origin of the nectar, honey can contain various antimicrobial and prebiotic compounds, positioning it as a functional food beneficial to both human and animal health (*Grabowski and Klein*, 2017).

The unique composition of honey provides a selective environment conducive to the growth and

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development of specific microorganisms. The natural microbiological and physicochemical characteristics of honey are influenced by its floral source, seasonal variability, climatic conditions, geographical origin, and processing methods (*Mărgăoan et al*., 2021; *Wright et al*., 2018; *Martinello and Mutinelli*, 2021; *Jia et al*., 2020). Thus, honey might contain fungi, algae, yeasts, and lactic acid bacteria (LAB) (*Olofsson et al.*, 2014a). The presence and activity of microorganisms in honey directly impact its safety and quality.

Owing to its unique composition, honey is classified as a functional food that, in addition to its nutritional value, could exert beneficial effects on human health. Recent studies have demonstrated that honey possesses antioxidant, hepatoprotective, hypolipidemic, hypoglycaemic, and anti-obesity effects, as well as anticancer, anti-atherosclerotic, hypotensive, neuroprotective, and immunomodulatory activities (*Afrin et al*., 2020; *Wusiman et al*., 2019). These health-promoting effects are largely attributed to honey's diverse nutritional constituents, including polysaccharides and polyphenols, which contribute significantly to it's bioactive properties. The characteristics and biological activities of honey can vary depending on its botanical and geographical origin. Furthermore, scientific evidence has confirmed the prebiotic properties of honey (*Schell et al*., 2022). Accordingly, the objective of this review is to present recent findings on the probiotic and prebiotic potential of honey, which may contribute to its growing application in health and nutrition.

In addition, a literature review was conducted to identify recent scientific findings on the probiotic and prebiotic compounds present in honey and their effects on human health. The reputable scientific database, *ScienceDirect*, was utilized for the search (Figure 1). The following keywords were applied individually and in various combinations as inclusion criteria for selecting articles relevant to this review: honey, prebiotics, and probiotics.

2. Probiotic properties of honey

Recent studies have demonstrated that honey can serve as a source of probiotic bacteria, primarily originating from the gut microbiota of honeybees, although environmental sources also contribute. Probiotics are live microorganisms and substances that contribute to the maintenance of intestinal microbial balance (*Mustar and Ibrahim*, 2022). The primary criteria for the selection of probiotic bacteria are resistance to gastrointestinal conditions; clear taxonomic identification at the genus, species, and strain levels along with their origin; antimicrobial activity; ability to adhere to the intestinal epithelium; interaction with the host's intestinal microbiota; and absence of pathogenicity and infectivity. Additional selection criteria encompass bile salt metabolism, lack of haemolytic activity, and absence of antibiotic resistance genes. From a safety perspective, probiotic

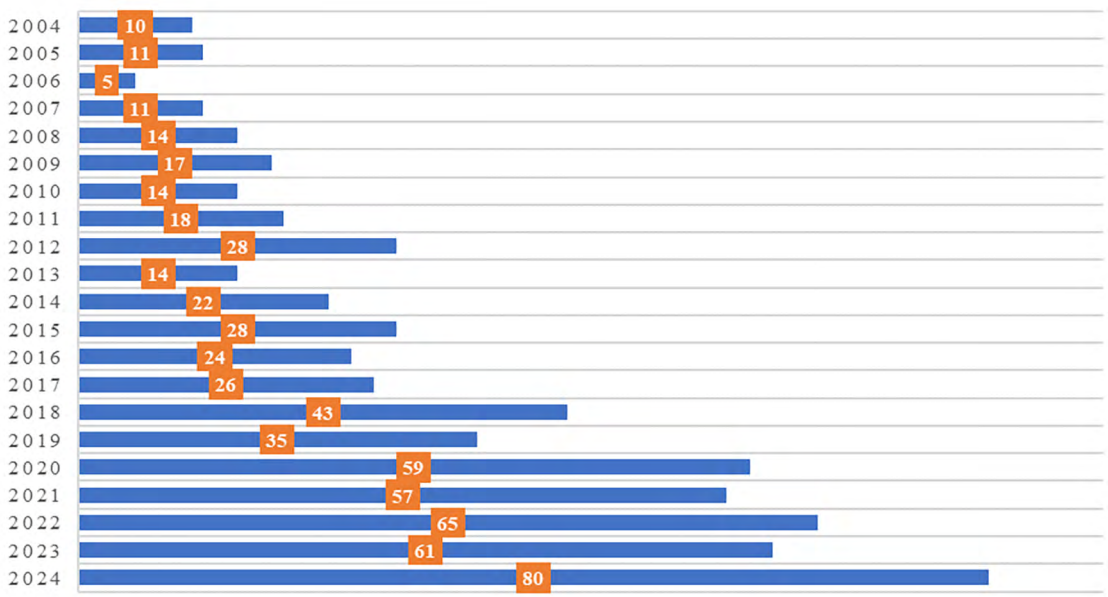

Figure 1. Number of published studies over the past 20 years (data retrieved from the ScienceDirect database using the search query "Probiotics and Prebiotics in Honey")

bacteria must be non-pathogenic, have no history of association with disease, not deconjugate bile salts or produce toxins, and must neither harbour nor transfer antibiotic resistance genes (*Schell et al*., 2022).

Recent studies have shown that lactic acid bacteria (LAB) are naturally present in the stomach of honeybees and are, therefore, introduced into honey during its production (*Suraiami and Nurliayana*, 2022). During the honey-making process, the enzyme glucose oxidase catalyses the conversion of glucose into gluconic acid, leading to the acidification of honey, which plays a crucial role in its preservation. This acidic environment inhibits the growth of most pathogenic and spoilage microorganisms. As a result of honey's acidity, the predominant microbial populations are yeasts and lactic acid bacteria. Among the LAB, several strains are recognized as probiotic microorganisms. The dominant probiotic bacterial populations belong to the genera *Lactobacillus* and *Bifidobacterium*. The most commonly species isolated from the stomach of the giant honeybee *Apis dorsata* include *Lactobacillus apis, Lactobacillus insectis, Lactobacillus alvei, Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus parabuchneri, Lactobacillus kunkeei, Lactobacillus kefiri* (*Tajabadi et al.*, 2011), and *Lactobacillus acidophilus*. Within the *Bifidobacterium* genus, novel species, such as *Bifidobacterium asteroides* and *Bifidobacterium coryneforme*, have also been identified in honeybees (*Olofsson et al*., 2014b). *Olofsson et al.* (2014b) reported the presence of different lactic acid bacterial strains originating from the gut of honeybees (*Apis mellifera*) in fresh honey. According to these authors, many of honey's unexplained therapeutic and antimicrobial properties may be attributed to these LAB symbionts. Their research also demonstrated that all tested LAB strains produced organic acids, albeit in varying amounts.

3. Prebiotic properties of honey

The beneficial effects of probiotic bacteria can be further enhanced by the presence of specific compounds, molecular groups, extracts, or functional ingredients in their environment—collectively referred to as prebiotics. Prebiotics are substances that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of probiotic bacteria in the colon (*Coppola et al*., 2025).

Honey oligosaccharides have potentially prebiotic activity. These compounds selectively stimulate the growth of beneficial microorganisms, such as *Lactobacillus* and *Bifidobacterium* (*Coppola et al*., 2025). *Sanz et al.* (2005) conducted a study on how honey oligosaccharide affects the bacteria population in human gastrointestinal tract and found honeys that contain higher amount of oligosaccharide resulted in prolific growth of beneficial bacteria. The main oligosaccharides in honeys were the disaccharides, turanose, nigerose, melibiose, sucrose, isomaltose and four trisaccharides, maltotriose, panose, melezitose, and raffinose. The highest amounts of maltulose and turanose (0.66–3.52 g/100 g and 0.72–2.87 g/100 g of honey, respectively) were in samples of honey from different regions of Spain and commercially available nectar and honeydew honeys. The trisaccharides, melezitose and panose, were the most abundant oligosaccharides in New Zealand honey (*Sanz et al*., 2005).

Bacteria from the *Lactobacillus* and *Bifidobacterium* genera are benefited in environments with low redox potential, and the presence of antioxidant compounds in honey is important in this regard. Flavonoids, amino acids, and phenolic acids are the main antioxidant compounds in honey. Most valuable and superior antioxidant compounds of honey, such as some phenolic compounds and glutathione, are unstable over time and thermolabile. Thus, its final quality is compromised when raw honey goes through conventional thermal processing (*Mustar and Ibrahim*, 2022). While it is well-documented that honey can positively influence gut microbial balance (*Mustar and Ibrahim*, 2022), the specific interaction between Rosaceae-type honey and probiotic *Lactobacillus* strains remains insufficiently explored. It can be hypothesized that the complex mixture of bioactive compounds in such honey varieties supports the growth of certain probiotic bacteria strains.

These findings are consistent with earlier studies that reported a stimulatory effect of honey on the growth of specific probiotic microorganisms. For instance, *Rosendale et al.* (2008) observed enhancements in the biological activity of *Lactobacillus* and *Bifidobacterium* species, including increased production of certain organic acids. Moreover, monofloral honeys have also been shown to promote the growth and activity of probiotic bacteria. Similarly, various types of *Eucalyptus* honey have been shown to stimulate the growth of *Lactobacillus plantarum* and *Lactobacillus rhamnosus*, while lime honey has been reported to influence the cytotoxicity of *Lactobacillus* strains cultured in its presence (*Abadi et al*., 2023).

4. Conclusion

Based on the literature review, there is a limited amount of experimental data on the probiotic and prebiotic properties of honey published within the last decade, highlighting a clear opportunity for further research. Additional studies are necessary to investigate probiotic strains in honey, with strong resistance to pathogenic microorganisms, intended for both human and animal consumption or as potential therapeutic agents.

A similar gap exists in honey prebiotic research, where the majority of studies have been conducted *in vitro*, typically using normal intestinal microorganisms derived from human faecal samples exposed to various concentrations of selected honey types. In conclusion, honey and honeybees—which show considerable potential as sources of probiotics and prebiotics—warrant greater scientific attention and more comprehensive investigation. Advancing this field of study could significantly contribute to the development of alternative therapeutic strategies, particularly against antibiotic-resistant pathogens.

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Original Scientific Paper

Occurrence and species-level identification of thermophilic Campylobacter in retail poultry meat in Serbia

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ABSTRACT

Species within the *Campylobacter* genus, notably *Campylobacter jejuni* and *Campylobacter coli*, are among the primary bacterial agents responsible for foodborne illnesses worldwide. Poultry meat frequently serves as a key vector of transmission, owing to the high prevalence of these bacteria in the gastrointestinal tracts of birds. The purpose of this study was to assess both the occurrence and levels of *C. jejuni* and *C. coli* in raw chicken meat available in Serbian retail markets. In 2023, a total of 118 fresh poultry samples were obtained from various retail outlets and analyzed in accordance with ISO standards for qualitative and quantitative microbiological evaluation. Results indicated a notably high contamination rate, with *Campylobacter* detected in 75% of the samples. Among these, 63 samples exhibited quantifiable bacterial loads ranging from 20 to 7,600 CFU/g. Molecular testing further confirmed the presence of thermotolerant *Campylobacter* species in 98% of the positive samples. Specifically, *C. jejuni* was detected in 75%, and *C. coli* in 85% of cases, with over half of the positive samples containing both species. The highest bacterial concentrations were found in drumstick and thigh meat cuts. These results emphasize the considerable public health concern associated with *Campylobacter* contamination in poultry meat sold in Serbia, and they reinforce the necessity of stricter hygiene practices and comprehensive monitoring across the poultry production and distribution chain.

1. Introduction

Species of the genus *Campylobacter*, particularly *Campylobacter jejuni* and *Campylobacter coli*, are recognized as leading contributors to bacterial gastroenteritis transmitted through food on a global scale. Estimates suggest that approximately 96 million infections occur annually as a result of these pathogens (*Majowicz et al.,* 2020). These thermophilic, Gram-negative organisms are commonly found in the intestinal tracts of poultry, where they typically do not induce symptoms in the host. However, during slaughtering and processing, they can be transferred from the intestines to the surface of poultry carcasses, creating a significant potential for contamination of meat destined for retail markets (*Sahin et al*., 2015; *Lopes et al.,* 2021).

Human exposure is frequently linked to the consumption of raw or undercooked poultry products, as well as to inadequate handling practices during food preparation (*Lopes et al.,* 2021). Due to the low infectious dose—often fewer than 500

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viable cells—even minimal levels of *Campylobacter* in meat can pose a notable threat to public health (*Black et al.,* 1988).

Although *Campylobacter* has been consistently reported as the most prevalent foodborne pathogen in the European Union since 2005 (*EFSA,* 2023), data from non-EU countries such as Serbia remain sparse. Serbia introduced surveillance of campylobacteriosis in humans in 1997 and implemented mandatory testing of poultry carcasses in slaughterhouses starting in 2019. Nevertheless, only a limited number of studies have explored the presence of *Campylobacter* spp. specifically in chicken meat at the retail level—an important point of consumer exposure.

Reliable baseline data on the occurrence and levels of *Campylobacter* in chicken meat sold through retail channels are crucial for evaluating consumer risk and informing appropriate control strategies (*Stella et al.,* 2017). Unlike farm-level testing, retail surveillance reflects the cumulative impact of contamination throughout the entire poultry production and distribution process. Furthermore, since *Campylobacter* does not multiply under refrigeration, quantification at the point of sale offers a realistic estimate of the microbial load to which consumers may be exposed (*Habib et al.,* 2022).

Reports from the EU demonstrate substantial variability in the prevalence of *Campylobacter* in retail poultry—ranging from 1.8% in Estonia to nearly 100% in Italy—indicating the need for country-specific investigations (*Wieczorek et al.,* 2012; *Belkacem et al.,* 2023). In Serbia, the incidence of human campylobacteriosis was reported at 4.04 cases per 100,000 inhabitants in 2022 (*IPHS,* 2023), yet the degree of exposure through retail poultry products remains insufficiently documented. While prior research has identified contamination levels as high as 85% in broiler carcasses at the slaughter stage (*Jovanović et al., 2020*), comprehensive data from the retail sector are lacking.

In light of these gaps, the present study aims to assess both the prevalence and levels of *C. jejuni* and *C. coli* in raw chicken meat obtained from retail stores across Serbia. The findings are expected to contribute to a deeper understanding of poultry meat safety and support the development of targeted interventions to mitigate public health risks associated with *Campylobacter* contamination.

2. Materials and methods

2.1. Sample collection

In 2023, a total of 118 fresh poultry meat samples were aseptically collected from retail outlets across Serbia. Sample types were drumsticks, thighs, fillets, wings, backs, breasts, leg quarters, whole grill chickens, and schnitzels. All samples were transported to the laboratory under controlled temperature conditions for microbiological analysis.

2.2. Detection and enumeration of Campylobacter spp.

Isolation and quantification of *Campylobacter* spp. were performed according to SRPS EN ISO 10272-1:2017/A1:2023 (qualitative; *ISO*, 2023a) and SRPS EN ISO 10272-2:2017/A1:2023 (quantitative; *ISO*, 2023b), with a detection limit of 10 CFU/g.

2.2.1. Qualitative detection

For enrichment, 25 g of sample was homogenized in 225 mL of Bolton broth and incubated under microaerophilic conditions: 4 h at 37 ± 1°C followed by 44 h at 41.5 ± 1°C. A 10 µL aliquot from the broth was streaked onto CASA agar (*bioMérieux, France*) and incubated at 41.5 ± 1°C for 48 h. Presumptive colonies were confirmed as per standard protocol.

2.2.2. Quantitative detection

For enumeration, 25 g of meat was homogenized in 225 mL of buffered peptone water. Aliquots (3×0.33 mL and 0.1 mL) were plated on CASA agar, including decimal dilutions. Plates were incubated at 41.5 ± 1°C for 48 h under microaerophilic conditions. Colony counts and identification followed the standard procedures.

2.3. Molecular confirmation of Campylobacter spp.

DNA was extracted from 91 samples, including 88 poultry isolates and 3 reference strains (*C. jejuni* ATCC 33291, *C. coli* ATCC 43478, *C. lari* ATCC 35223). The extraction involved lysis with buffer and Proteinase K, followed by isopropanol precipitation, ethanol washing, and resuspension in DNA storage buffer. DNA concentration and purity (A260/A280) were measured by spectrophotometry and standardized to 100 µg/mL.

2.3.1. Genus-level identification (Real-Time PCR)

Genus-level identification was conducted by targeting the 16S rRNA gene using species-conserved primers and a fluorogenic probe. PCR was performed using the Aria MX system (*Agilent Technologies*) and Brilliant III Ultra-Fast QPCR Master Mix (*Agilent Technologies*), following thermal cycling conditions from SRPS EN ISO 10272-1:2017/A1:2023, Annex D.

2.3.2. Species-level confirmation

Species-specific Real-Time PCR assays were used to identify *C. jejuni*, *C. coli*, and *C. lari* using primers and probes targeting mapA, ceuE, and gyrA genes respectively, according to Annex E of the ISO standard.

3. Results

A total of 118 fresh poultry meat samples were analyzed during the study. Of these, 88 samples tested positive for *Campylobacter* spp., corresponding to an overall prevalence of 75%. The distribution of positive samples across different poultry meat categories is presented in Table 1.

Enumeration of *Campylobacter* spp. using ISO 10272-2 (*ISO*, 2023b) revealed quantifiable contamination in 63 positive samples, with bacterial counts ranging from 20 to 7600 CFU/g. The highest contamination levels were observed in the drumstick and thigh category, ranging from 20 to 7600 CFU/g, whereas no colonies were detected in the chicken schnitzel group. Notably, the chicken back category showed a high contamination level of 4200 CFU/g despite the small number of samples. Qualitative detection of *Campylobacter* spp. using ISO 10272-1 (*ISO*, 2023a) revealed an additional 25 positive samples with contamination levels below 10 CFU/g.

Molecular analysis confirmed the presence of thermotolerant *Campylobacter* species in 98% of positive samples. Species-level identification revealed that *C. jejuni* was present in 75% of samples, while *C. coli* was identified in 85% of samples. *Campylobacter lari* was not detected in any chicken sample. Co-occurrence of *C. jejuni* and *C. coli* was found in 58% of samples. Additionally, *C. coli* was identified as a single species in 27% of samples, and *C. jejuni* alone was found in 15% of samples (Figure 1).

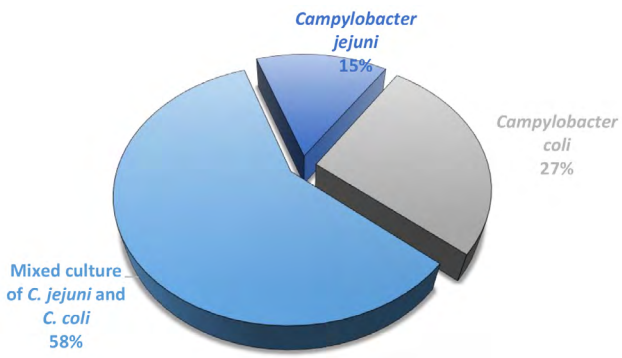

Figure 1. Species identification of *Campylobacter* isolates

Table 1. Prevalence of Campylobacter spp. in poultry meat by product category

Sample category	No. of samples	Negative samples	Positive samples	Prevalence (%)
Chicken drumstick	14	3	11	79%
Drumstick and thigh	30	4	26	87%
Chicken fillet	30	13	17	57%
Chicken wings	11	0	11	100%
Chicken back	2	1	1	50%
Chicken breast	10	3	7	70%
Chicken hindquarter	3	2	1	33%
Whole grill chicken	6	0	6	100%
Chicken thigh	8	2	6	75%
Chicken schnitzel	4	2	2	50%
Total	**118**	**30**	**88**	**75%**

4. Discussion

The findings of this study indicate a considerably high prevalence of *Campylobacter* spp. in retail poultry meat in Serbia, with 75% of the analyzed samples testing positive. Comparable results have been reported in nearby countries. For example, research in Croatia found a prevalence rate of 73.86% in chicken meat sold at retail level (*Mikulić et al.,* 2016). These regional similarities could suggest that common agricultural practices, meat processing systems, and conditions in the retail chain collectively contribute to the widespread contamination with *Campylobacter*. At the European level, *Campylobacter* has consistently been recognized as one of the most frequently encountered foodborne pathogens. According to the European Food Safety Authority's (EFSA) One Health Zoonoses Report for 2023, this bacterium was the most commonly detected zoonotic agent in the EU, particularly in broiler chicken carcasses, with a prevalence of 12% and in live broilers, with the prevalence of 18.1% (*EFSA,* 2023). These statistics reflect the need for rigorous and ongoing monitoring across poultry supply chains throughout Europe. Prevalence rates differ between countries. For instance, a study from France reported a 76% contamination rate in broiler meat products (*Guyard-Nicodème et al.,* 2015), and findings from Greece indicated a prevalence as high as 90% in chicken products sold at retail (*Kostoglou et al.,* 2023). In Poland, a study found a prevalence of 70% in chicken meat samples (*Szosland-Fałtyn et al.,* 2018). In contrast, some countries reported lower rates, such as 50% in Spain (*Perez-Arnedo & Gonzalez-Fandos,* 2019) and 60% in the United Kingdom (*FSA,* 2018). These variations could arise due to differences in surveillance approaches, local farming systems, or sampling strategies.

Within this study, prevalence also differed by product type. Chicken wings and whole grilled chickens were the most frequently contaminated (100%), while chicken schnitzels had the lowest rate (50%). A study conducted in Nova Scotia, Canada, found that chicken thighs had the highest prevalence of *Campylobacter* contamination among various retail poultry cuts, with 73.9% of thigh samples testing positive. In contrast, chicken wings had a lower prevalence of 58% (*Hodges et al.,* 2019). Similarly, research in Italy reported that chicken thighs had a higher occurrence of *Campylobacter* compared to other cuts, including breasts and wings (*Mezher et al.,* 2016). That study highlighted the anatomical proximity of thighs to the digestive tract as a potential factor influencing higher contamination rates (*Mezher et al.,* 2016). Factors such as anatomical location, handling practices, and storage conditions likely influence contamination levels.

Quantitative analysis of the samples in this study revealed bacterial loads ranging from 20 to 7,600 CFU/g, indicating considerable variability. *Campylobacter* spp. loads were also measured in Estonian broiler chicken products. Enumeration data reported by *Mäesaar et al.* (2014) indicated an overall arithmetic mean of 1.6×10^3 CFU/g of product. In contrast, the quantitative results of the study published by *Szosland-Fałtyn et al.*, (2018), demonstrated a low level of *Campylobacter* spp. contamination in the examined poultry meat samples. Specifically, *Campylobacter* spp. counts were below 10 CFU/g in 68% of the positive samples. Additionally, 22% and 26% of the samples exhibited pathogen concentrations ranging from ≥10 to <100 CFU/g. These results clearly shown needing for improved hygiene measures across all stages of production and distribution to reduce microbial risks.

Molecular identification confirmed *C. jejuni* and *C. coli* as the dominant species in the current study, present in 75% and 85% of positive samples, respectively. In Croatia, *C. jejuni* and *C. coli* were isolated from 53.53 and 15.35% of the samples, respectively (*Mikulić et al.,* 2016). In North Macedonia, *C. jejuni* was found in 39.2 of examined samples from broiler meat production (*Angelovski et al., 2021*). These findings demonstrate the widespread distribution of these pathogens in poultry meat across the continent.

5. Conclusion

The high prevalence and differing contamination levels of *Campylobacter* spp. in retail poultry meat in Serbia pose significant public health risks, as these bacteria are major causes of foodborne gastroenteritis worldwide. The European Food Safety Authority (EFSA) has emphasized the need for continuous monitoring and control of *Campylobacter* in the food chain to protect public health (*EFSA,* 2023). Implementing stringent hygiene practices in poultry processing and retail environments, along with consumer education on proper meat handling and cooking, are essential steps in mitigating the risks associated with *Campylobacter* contamination.

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Original Scientific Paper

Novel autochthonous strains from *Cyprinus carpio* as candidates for probiotic use and microplastic-degrading properties

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ABSTRACT

In the modern era, identifying and characterizing novel bacterial strains with possible probiotic potential and environmental bioremediation capabilities is an emerging focus in microbiology and biotechnology. This study analysed the cultivable gut microbiota of the freshwater fish, *Cyprinus carpio*, and identified six different bacterial genera, including *Citrobacter*, *Serratia*, *Bacillus*, *Enterococcus*, and *Kocuria*. Among these, two novel autochthonous strains—*Hafnia alvei* UUNT_MP41 and *Hafnia paralvei* UUNT_MP29—were isolated and selected for further investigation due to their promising probiotic traits and potential to degrade microplastics in aquatic ecosystems. Both strains were evaluated for antibacterial activity against pathogens and susceptibility to a broad spectrum of antibiotics. Whole-genome analysis using next-generation sequencing (NGS) revealed the presence of genes potentially associated with probiotic properties, such as *ClpB*, as well as genes potentially involved in the biodegradation of common microplastics, including the *tesA* gene, a homolog of the *PpEst* gene from the genome of *Pseudomonas pseudoalcaligenes*, and the *lipR* gene, a homolog of the *EstC9* gene from the genome of *Acidocella* sp. Here, we performed a more in-depth analysis of the similarity between the genes/proteins we identified as potentially involved in plastic biodegradation and previously described ones. Notably, the identified strains' potential to degrade microplastics under conditions relevant to the human gastrointestinal system positions them as candidates for a new generation of dual-function probiotics, supporting both human health and microplastic detoxification. These findings lay the groundwork for future development of multifunctional probiotic formulations with environmental and therapeutic benefits.

1. Introduction

The microbiota plays a crucial role in maintaining health by supporting digestion, providing essential molecules, modulating the immune system, and protecting against certain diseases. Probiotic strains, such as *Lactobacillus* species and *Bifidobacterium*, can enhance health status by eliminating harmful substances. On the other hand, exposure to various pollutants, including water, soil, and air contamination,

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can lead to serious health issues. Plastic, as one of the primary materials in use, pollutes the complete environment, contaminating water, soil, and air. The mechanisms by which microbes degrade plastic are currently under active investigation *(Cai et al.*, 2023; *Payanthoth et al*., 2024). Microbial plastic biodegradation likely occurs through biofilm formation and enzymatic degradation. Studying plastic biodegradation by microorganisms from aquatic ecosystems is essential for developing effective strategies. The discovery of bacterial strains with probiotic and plastic-degrading properties could be a game-changer in biotechnology and the healthcare sector.

2. Materials and methods

2.1 Isolation of autochthonous species from the gastrointestinal tract of Cyprinus carpio

Bacterial strains from *Cyprinus carpio* were identified from fish samples collected at the DTD fish market in Serbia. The fish samples were thoroughly cleaned and dissected, and samples of gut were homogenized before being plated onto de Man Rogosa Sharpe (MRS) agar plates. The bacteria isolated were grown overnight in MRS broth at 37°C, and glycerol stocks were created. Details of the procedure are published in our previous paper (*Dragacevic et al*., 2025).

2.2 Genomic DNA extraction from the bacteria strains isolated from Cyprinus carpio and NGS Sequencing and Bioinformatics Analysis

Following the manufacturer's protocol, DNA was extracted using the GeneJet Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA, USA). The purity and concentration of the extracted DNA were assessed with the Nanodrop 2000c spectrophotometer (Thermo Scientific), and finally, samples were analysed for genomic DNA integrity using agarose gel electrophoresis. The extracted genomic DNA was sequenced on the Illumina Short Reads platform (2 × 250 bp) with 30X target coverage. The phylogenetic tree was constructed using the Type (Strain) Genome Server (TYGS) bioinformatics platform *(Meier-Kolthoff et al., 2022; Meier-Kolthoff & Göker*, 2019).

2.3 Antibiotic susceptibility

The strains were assessed using the disk diffusion method (Kirby-Bauer test) on the Mueller-Hinton agar (MHA) reference medium to determine their antibiotic susceptibility. Overnight cultures were inoculated aerobically into Mueller-Hinton broth at 37°C. Antibiotic discs were placed onto MHA, incubated at 37°C for 48 hours, and inhibition zone diameters were measured in millimetres.

3. Results

3.1 Identification of autochthonous strains using 16S rRNA gene sequencing, isolated from C. carpio

Thirty-nine bacterial strains were isolated from the gut of healthy *C. carpio* and were identified using 16S rRNA gene amplification and sequencing. The bacterial strains that were identified at the species level using standard/universal 16S rRNA gene primers (27F-AGAGTTTGATCCTGGCTCAG and 1492R-ACGGYTACCTTGTTACGACTT) are presented in Table 1.

Table 1. Identified bacteria species isolated from the gastrointestinal tract of *Cyprinus carpio* using 16S rRNA sequencing

Identified genus	Identified species	No of strains
Citrobacter	*Citrobacter freundii*	13
Serratia	*Serratia liquefaciens*	10
	Serratia plymuthica	1
	Serratia marcescens	1
Bacillus	*Bacillus pumilus*	3
Hafnia	*Hafnia alvei*	1
	Hafnia paralvei	1
Enterococcus	*Enterococcus pseudoavium*	1
Kocuria	*Kocuria rhizophila*	8

Among 39 strains, six genera were identified in the gastrointestinal tract of *C. carpio* (Table 1). Genome sequences of two novel strains, denoted as *H. alvei* UUNT_MP41 and *H. paralvei* UUNT_MP29, were deposited in the National Library of Medicine (NIH), https://www.ncbi.nlm.nih.gov/biosample/SAMN44705350, and https://www.ncbi.nlm.nih.gov/biosample/SAMN44705351. These strains were then selected for further research due to their potential classification as next-generation probiotics (NGP), making them particularly appealing now. Genomic DNA was extracted from these

strains in three independent replicates, and 16S rRNA gene sequencing was carried out, followed by whole-genome next-generation sequencing (NGS). The assembled genomes have been deposited in the NCBI database (*Sayers et al*., 2022) under BioProject numbers PRJNA1185451 for strain UUNT_MP41 and PRJNA1185452 for strain UUNT_MP29, using NGS sequencing of both *Hafnia* strains. Based on the Kraken analysis results, strain UUNT_MP29 is closely related to *H. paralvei*, while strain UUNT_MP41 is most closely associated with *H. alvei.* The phylogenetic tree was published in our previous paper (*Dragacevic et al.*, 2025).

3.2 Antibiotic susceptibility of H. alvei UUNT_MP41 and H. paralvei UUNT_MP29

The study found that *H. alvei* UUNT_MP41 and *H. paralvei* UUNT_MP29 exhibited the highest sensitivity (among the isolated bacteria) to imipenem, ciprofloxacin, enrofloxacin, ampicillin, ceftazidime, and five other medications, suggesting potential probiotic activity. *H. alvei* UUNT_MP41 demonstrated greater sensitivity than *H. paralvei* UUNT_MP29. A figure depicting the disc diffusion method for assessing antibiotic susceptibility was published in our previous paper (*Dragacevic et al*., 2025).

3.3 Search and analysis of potential candidate genes for microplastic degradation

To identify genes responsible for plastic biodegradation in both *Hafnia* species, a local sequence alignment was performed using the BLAST tool (blastp) (*Altschul et al*., 1990), comparing annotated proteins from *Hafnia* genomes against protein sequences from the PMBD database. Two proteins, encoded by the *tesA* and *lipR* genes, showed the highest percentage of identity to the proteins PpEst (UniProt ID: AMW89397.1) and EstC9 (UniProt ID: AOR05752.1), respectively (*Uniprot Consortium*, 2025). PpEst is an esterase capable of hydrolysing the co-aromatic-aliphatic polyester poly(1,4-butylene adipate-co-terephthalate) (PBAT), characterized initially in *Pseudomonas pseudoalcaligenes* (*Wallace et al*., 2017). EstC9 was isolated from the

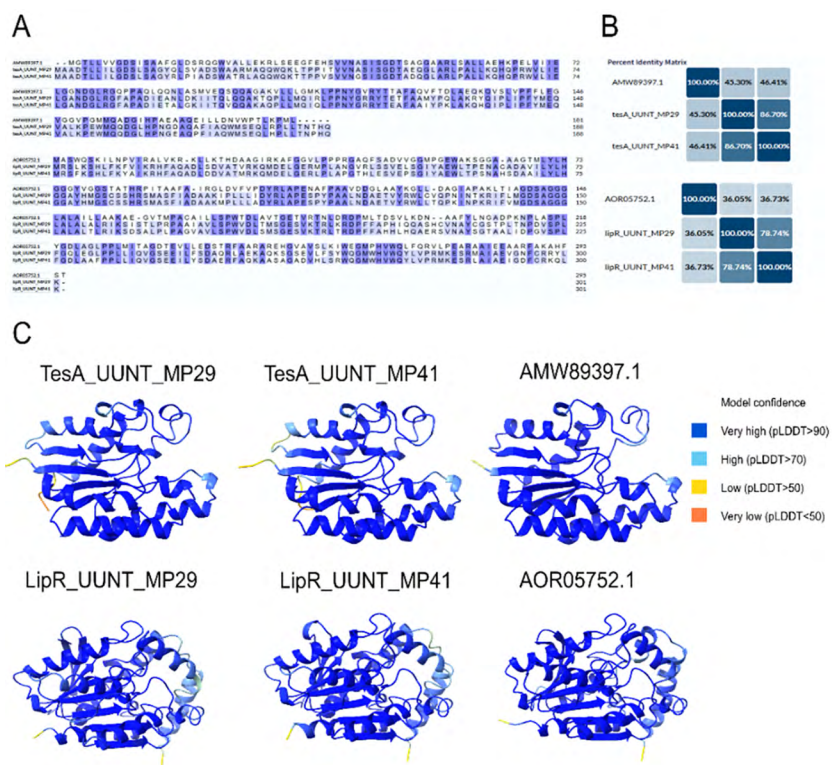

Figure 2. Comparison of proteins that may be involved in plastic biodegradation from *Hafnia* genomes with the characterized proteins AMW89397.1 and AOR05752.1. A – Alignment of the primary amino acid sequences. B – Percent identity matrix, with color intensity showing the degree of sequence identity. C – AlphaFold2 structural prediction of the compared proteins.

microbiome of the moss *Sphagnum magellanicum* and is presumed to be encoded in the genome of an *Acidocella* species (*Müller et al.*, 2017). It has also been shown to hydrolyse PBAT (*Müller et al.*, 2017). We analysed the sequences of these proteins (Figure 2) and found that for TesA, the percent identity between the *Hafnia* strains exceeded 86%, while the identity between *Hafnia* TesA and the protein from *P. pseudoalcaligenes* was just over 45%. All three proteins were predicted to contain an SGNH hydrolase-type esterase domain (InterPro ID: IPR013830) (*Blum et al*., 2025). Esterases in this group are known to share a similar fold with flavoproteins—specifically, a three-layer α/β/α structure where the β-sheet consists of five parallel strands. Enzymes containing this domain function as esterases and lipases but exhibit low sequence homology to true lipases (*Akoh et al.*, 2004; *Mølgaard et al.*, 2000). We predicted the protein structures using AlphaFold2 via ColabFold (*Mirdita et al.*, 2022), and structurally, the proteins were nearly identical. For the LipR protein, the percent identity between *Hafnia* strains was around 78%, and between *Hafnia* LipR and EstC9, slightly over 36%. All three proteins were predicted to contain the Alpha/beta hydrolase fold-3 domain (InterPro ID: IPR013830), and the structures predicted by AlphaFold2 also appeared highly similar in all three cases. Based on these findings, *Hafnia* strains may prove to be promising candidates for use as microorganisms in plastic biodegradation, particularly for plastics like PBAT.

4. Discussion

The *H. alvei* UUNT_MP41 and *H. paralvei* UUNT_MP29 strains, which potentially possess probiotic properties, underwent a more in-depth analysis. A genomic study was conducted to identify antibiotic resistance genes, and antibiogram tests were performed and analysed. Bioinformatics analysis revealed that the genomes of *H. alvei* UUNT_MP41 and *H. paralvei* UUNT_MP29 contain numerous genes responsible for antibiotic resistance. Additionally, genes encoding products that may degrade plastics, primarily PBAT-type plastic, were identified and characterized through bioinformatic approaches. Therefore, the described *Hafnia* strains represent interesting and promising candidates for further investigation.

5. Conclusion

The results showed that various antibiotics significantly resist *H. alvei* UUNT_MP41 and *H. paralvei* UUNT_MP29. Taking into consideration the results obtained in this study, we suggest that these two novel bacteria strains could be considered as a candidate for a potential new generation of probiotics that could also have role in microplastic biodegradation. Further investigation will focus on a complete analysis of the safe use of these strains and potential genes for plastic biodegradation.

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Original Scientific Paper

Chlorine content in meat products

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ABSTRACT

Chlorine is a halogen element that appears in the human body as a free anion, and it plays important role in physiological processes. The main source of chlorine in food is sodium chloride. Data regarding the salt content in meat products were collected from the nutrition labels of 339 meat product from retail markets. The study encompassed 39 pates, 71 cooked sausages (41 finely minced and 30 coarsely minced), 66 pasteurized hams, 42 smoked meat products, 60 dry fermented sausages, 41 dry meats and 20 samples of bacon. The highest average chlorine contents were determined in dry fermented sausages and dry meat (2.30 g/100 g and 2.69 g/100 g, respectively) in accordance with their highest salt contents among the studied meat products. Due to table salt having a major role in meat processing, it can be concluded that meat products are important source of chlorine in consumers.

1. Introduction

Chlorine is a halogen element and has two stable isotopes, ^{35}Cl and ^{37}Cl. It appears in human body as a monoatomic free anion, and in the combination with cations of sodium, calcium and magnesium, it has important roles in physiological processes. After sodium, chloride is the second most abundant ion in the human body (*Wang et al*., 2017), making up 70% of all anions in extracellular fluid (*Berend et al*., 2012).

Chloride is transported through several chloride channels, such as channels dependent on voltage (the chloride channel (ClC) family), the cystic fibrosis transmembrane conductance regulator (CFTR), Ca^{2+} activated chloride channels, anion channels that are volume regulated and ligand-regulated channels (*Berend et al.*, 2012, *Kondratskyi et al*., 2014). These channels maintain and regulate membrane polarity and osmotic and acid-base balance between intracellular departments and the cytoplasm as well as between the cytoplasm and extracellular fluid (*Berend et al*., 2012). Also, they contribute to the generation of electric signals in muscles and nerves as well as to secretion and reabsorption of fluids, particularly in the lungs (*Hollenhorst et al*., 2011). Water secretion in lungs and exocrine organs is dependent on chloride that make mucus more wet and fluid, regulated by CFTR channels, defects in which cause cystic fibrosis (*Johnson et al*., 2006). The channel for exchange of Cl^-/HCO^{3-} ions enables oxygen intake and the release of CO_2 in the lung, but converse exchange in peripheric tissues. This channel releases the CO_2 that is taken in as bicarbonate and enables the intake of chloride

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ions, which increases haemoglobin affinity to oxygen (*Fischer et al*., 2007). Chloride secretion from gastric cells is a main factor in the secretion of HCl (*Berend et al*., 2012). In general, human food is low in chlorine, so the most significant source of chlorine is table salt (NaCl).

Since table salt is the most common ingredient in meat processing, this study aimed to investigate the chlorine content derived from meat products

2. Materials and methods

Data on the salt content in meat products were collected from the nutrition labels of 339 meat products from retail markets. The study encompassed 39 pates, 71 cooked sausages (41 finely minced and 30 coarsely minced), 66 pasteurized hams, 42 smoked meat products, 60 dry fermented sausages, 41 dry meats and 20 samples of bacon. Salt content is mandatorily declared on the label, along with protein content, carbohydrate and sugar content, fat and saturated fatty acid content and energy value per 100 g or 100 mL of food, according to Serbian legislation. Label data are also expressed with reference to a requirement for 2000 kcal of energy per day and a salt intake of 6 g/day. Labels can also contain data about monounsaturated fats, polyunsaturated fats, polyols, starch, fibre, vitamins and minerals. Sodium and chlorine contents were calculated from their atomic masses in the declared sodium chloride content of the products examined.

3. Results

The declared sodium chloride contents of the meat products are shown in Table 1, while the calculated sodium and chlorine contents are presented in Table 2. Therefore, the sodium and chlorine contents were directly dependent on the salt content.

According to technological use and sensory characteristics of products, the lowest mean salt contents were determined in pate and finely minced cooked sausages, while salt contents were higher in coarsely minced cooked sausages, pasteurized ham

Table 1. Sodium chloride content in meat products, g/100 g

Thermally treated products	Mean ± SD
Pate (n = 39)	1.17±0.06 (1.10-1.30)
Finely minced cooked sausages (n = 41)	1.90±0.35 (1.40-2.60)
Coarsely minced cooked sausages (n = 30)	2.26±0.40 (1.70-3.30)
Pasteurized ham (n = 66)	2.10±0.38 (1.20-2.85)
Smoked meat products (n = 42)	2.68±0.53 (1.70-3.30)
Thermally untreated products	
Dry fermented sausages (n = 60)	3.78±0.37 (3.10-4.50)
Dry meat (n = 41)	4.40±1.21 (2.60-6.40)
Bacon (n = 20)	2.80±0.83 (2.00-5.10)

Table 2. Sodium and chlorine content in meat products, g/100 g

Thermally treated products	Sodium (range)	Chlorine (range)
Pate (n = 39)	0.47 (0.44-0.52)	0.72 (0.67-0.79)
Finely minced cooked sausages (n = 41)	0.76 (0.56-1.04)	1.16 (0.85-1.58)
Coarsely minced cooked sausages (n = 30)	0.91 (0.68-1.32)	1.38 (1.03-2.01)
Pasteurized ham (n = 66)	0.84 (0.48-1.14)	1.28 (0.73-1.73)
Smoked meat products (n = 42)	1.07 (0.68-1.32)	1.63 (1.03-2.01)
Thermally untreated products		
Dry fermented sausages (n = 60)	1.51 (1.24-1.80)	2.30 (1.89-2.74)
Dry meat (n = 41)	1.76 (1.04-2.56)	2.69 (1.58-3.90)
Bacon (n = 20)	1.11 (0.80-2.04)	1.69 (1.22-3.10)

and smoked meat products. The highest salt contents were, according to the labels, in dry fermented sausages and dry meat.

4. Discussion

Deficiency of chloride rarely occurs and is defined as a low chloride level in the blood, mostly 97-107 mmol/L. It can be caused by gastrointestine and kidney losses and inherited and acquired metabolic disorders (*Berend et al*., 2012). One study cited that deficiency occurs in infants that are fed breast milk with a naturally low chloride concentration (*Hill and Bowie*, 1983) and in infants fed with milk replacers. It can occur in children and adults fed on fluid foods with low chloride content (*Miyahara et al*., 2009). Hypochloridaemia in infants is linked to growth retardation, lethargy, toughness, anorexia, gastrointestinal symptoms, weakness, loss of potassium, metabolic alkalosis and haematuria (*Grossman et al*., 1980).

Excessive chloride intake through food is not common. A chloride concentration in serum above 107 mmol/L, hyperchloremia, mainly appears due to loss of bicarbonates through faeces, for example in the case of severe diarrhoea that causes metabolic acidosis. It can appear as a consequence of all conditions that cause loss of fluid through skin and kidneys, and is also seen if there is a reduced volume of extracellular fluid, an increased tubular chloride reabsorption, an excessive intake of chloride salt, particularly NaCl, and as a consequence of therapy with corticosteroids and acetazolamide. A chlorine level in drinking water up to 4 mg/L is considered as safe and unlikely to cause health problems, according to the US Center for Disease Control and Prevention (CDC), while the World Health Organization (*World Health Organization*, 2013) recommends the guideline value of 5 mg/L for long-term human consumption of drinking water.

In 2005, the European Food Safety Authority (EFSA) still had not set a tolerable upper intake level for chloride, then only recommending that salt consumption be limited to 6 g daily. However, in 2019, EFSA set safe and adequate daily intake levels for chloride: For infants aged 7–11 months 0.3 g per day, for toddlers of 1–3-years 1.7 g per day, for children of 4–6 years 2 g per day, for children of 7–10 years 2.6 g per day, and for people aged 11 years and more 3.1 g per day.

EFSA considers that 5 g of table salt per day is adequate intake in adults. Extrapolated adequate intakes for sodium are 0.2 g/day for infants (0–6 months) and for infants (7–12 months), 1.1 g/day for children from 1–3 years and 1.3 g/day for children from 4–6 years, 1.7 g/day for children aged 7–10 years and 2 g/day for children 11–17 years old, just as for adults (*EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA)*, 2019; *Turck et al*., 2019). However, the estimated mean adult salt intake (g/day) in some countries was: China 10.9 g, Montenegro 10.7 g, Portugal 10.5 g, Benin 9.9 g, Italy 9.7 g, India 9.1 g, Australia 9.0 g, the United States 9.0 g, New Zealand 8.5 g, Canada 8.3 g, England 8.1 g, Samoa 7.3 g, and Barbados 6.6 g (*Armstrong*, 2019). *Rybicka and Nunes* (2022) reported on salt intake in European countries. The average daily salt intake was: 6–7 g in Germany and Latvia; 7–8 g in Bulgaria; 8–9 g in the United Kingdom, France, Switzerland, Austria, Slovakia, the Netherlands, Denmark, Norway, Finland and Lithuania; 9–10 g in Ireland, Spain and Italy; 10–11 g in Belgium, Sweden and Estonia; 11–12 g in Poland and Romania; 12–13 g in Slovenia and Portugal; 13–14 g in the Czech Republic, 14–15 g in Hungary (*Rybicka and Nunes*, 2022) and; in Serbia 10.01 g/day (*Kwong et al.*, 2022).

5. Conclusion

The lowest average salt contents were determined in pate and finely minced cooked sausages, while salt levels were higher in coarsely minced cooked sausages, pasteurized ham and smoked meat products. The highest salt contents were, according to the product labels, in dry fermented sausages and dry meat. Sodium and chlorine levels are directly linked to the salt content in the products. Meat products constitute a significant source of dietary chloride.

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Review Paper

Investigation of parameters relevant to the pheasant population in Serbia from 1999 to 2023

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ABSTRACT

The aim of this study was to present data on the number of pheasants, their harvested numbers due to hunting, the volume of obtained meat, and the numbers of pheasantries, feeding grounds for small feathered game, and hunters in Serbia from 1999 to 2023, based on data from the Statistical Yearbooks of Serbia, which report these parameters for each odd-numbered year. The average number of pheasants during the study period was 373 thousand, with 146.2 thousand harvested (39.28% of the total population). From the harvested pheasants, 102.4 tons of meat was obtained. The average number of pheasantries was 500.5, with varying production capacities. For supplementary feeding of pheasants from late autumn until spring, an average of 23,880 feeding grounds were installed, maintained by an average of 83,622 hunters. All examined parameters showed a slight increasing trend from 1999 to 2023, except for the number of pheasants, which showed a decline.

1. Introduction

The pheasant (*Phasianus colchicus L.*) is a non-migratory bird species from the order *Galliformes*, family *Phasianidae*. It originates from East Asia, from where it spread through Central and Western Asia to the Roman Empire and the Balkans. Today, it is distributed across all continents except Antarctica. Climatic and spatial conditions, along with dietary diversity, have contributed to minor genetic but more pronounced phenotypic variations within this bird species, which is now classified into 42 subspecies (*Davidović et al*., 2023), or 31 subspecies according to *Ashrafzadeh et al.* (2021). In Serbia, besides *Phasianus colchicus*, four additional subspecies are present in smaller numbers.

From the eighth to the twelfth week of life, pheasants exhibit clear sexual dimorphism. The primary differences between males and females are in body weight and body length (both greater in males), as well as in plumage color, which is especially vivid in males on the breast, neck, and head. In the wild, pheasants live in small groups (up to 20 birds), with one male typically accompanied by 5–6 females. Pheasants face numerous natural predators (foxes, cats, martens, hedgehogs, hawks, crows, magpies, wild boars), as well as threats from humans (hunters)

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and farmers (agricultural machinery) (*Ashrafzadeh et al*., 2021). In addition to predators, adverse weather conditions and food shortages also threaten their survival in the wild, leading to population declines, which are compensated for by pheasants bred in pheasantries. Pheasantries provide the necessary conditions for raising pheasants and preparing them for life in the wild.

The aim of this paper is to analyze pheasant population numbers and the harvest due to hunting, estimate the volume of meat obtained, and analyze the number of pheasantries, feeding grounds, and hunters in Serbia from 1999 to 2023.

2. Materials and methods

Data on the number of pheasants, number of harvested individuals, number of pheasantries, and number of hunters in Serbia from 1999 to 2023 were collected from the *Statistical Yearbook* of the Republic of Serbia, published by the Statistical Office of the Republic of Serbia. In the Statistical Yearbook, the data are presented from 1999 to 2023 for each odd-numbered year (n=13). Based on the total number of pheasants and the total number of harvested pheasants for each year, the percentage of harvested pheasants was calculated. The quantity of meat obtained was calculated from the number of harvested pheasants and the estimated carcass weight (based on literature data, the average carcass weight is 700 g), considering processing as for poultry carcasses prepared for roasting.

Statistical analysis of the results was performed using GraphPad Prism software version 7.00 for Windows (GraphPad Software, San Diego, CA, USA (http://www.graphpad.com)). All parameters were described using means and standard deviations. Trend lines were created for each examined parameter (Microsoft Excel 2010). The results are presented in tables and graphs.

3. Results and discussion

Data on the total number of pheasants, number of harvested pheasants, percentage of harvested pheasants relative to the total population, as well as the quantity of pheasant meat obtained through hunting in Serbia from 1999 to 2023 for each odd-numbered year (n=13), are presented in Table 1, while the trends for the examined parameters are shown in Figure 1.

In Serbia, from 1999 to 2023, the average number of pheasants was 373±30.06 thousand, with a relatively low coefficient of variation (Cv) of 8.06%. A slight decline in the pheasant population during the observed period can be noted from the graphical representation of population changes. The number of pheasants harvested annually ranged from 122 thousand (in 1999) to 173 thousand (in 2009), with an average of 146.2±17.05 thousand, showing a slight increasing trend. On average, 39.38±5.17% of the total pheasant population was harvested annually, also showing an upward trend. The quantity of meat obtained annually from harvested pheasants averaged 102.4±11.95 tons, following the trend of the number of harvested pheasants.

Among landfowl species, two orders—*Galliformes* and *Columbiformes*—are the most significant for hunting. In Europe, 52 million birds from these two orders are hunted annually, accounting for 42.12% of the total number of landfowl species birds that are hunted. Among them, the pheasant is the most frequently hunted species, being widely distributed across Europe and the most economically important non-migratory bird species (*Quaresma et al.,* 2016). The population of wild pheasants in Europe is estimated to be between 9,700 and 16,300 adult individuals. The total number of male pheasants in Europe ranges from 4,140,000 to 5,370,000, with the trend being further increase.

The introduction of pheasants into hunting grounds is a widely accepted practice in Europe. Introduction refers to the release of pheasants raised in pheasantries into hunting grounds when they are sufficiently mature (10–12 weeks old) and adapted to survive in the wild. In France, 10 to 15 million pheasants are released into the wild annually from pheasantries, with a smaller portion intended for export. Finland imports about 15,000 pheasants annually from France (*Chiatante and Meriggi*, 2022). Between 10 and 57 million birds annually are exported from the European Union to the United Kingdom (*Madden***,** 2022).

The highest natural population of pheasants is in Eastern and Central Asia, Russia, Georgia, and to a lesser extent Turkey, Armenia, and Greece (*Chiatante and Meriggi*, 2022). In Hungary, the pheasant population in 2016 was 630,435 individuals, with around 300,000 harvested (47.59%), which is higher both numerically and proportionally than the harvest in Serbia. In Hungary, the pheasant is considered the most important wild bird species, with its meat pri-

Table 1. Pheasant population parameters in Serbia from 1999 to 2023 (measured in odd-numbered years, n=13)

Parameter	$\bar{X}$	Measures of variation				
		Sd	Se	Min	Max	Cv (%)
Total number*	373	30.06	8.337	334	421	8.06
Harvest*	146.2	17.05	4.73	122	173	11.66
%	39.38	5.17	1.434	28.74	48.07	13.13
Meat**	102.4	11.95	3.314	85.4	120.1	11.66
Number of pheasantries	505.2	263.6	73.12	51	763	52.18
Feeding stations	23880	2761	765.7	19103	27901	11.56
Hunters	83622	5553	1540	74544	93078	6.64

* thousand; ** ton.

marily consumed by hunters themselves (*Fernye et al*., 2017).

Hunting of game species in Serbia also includes feathered game, among which the pheasant is the most commonly hunted species, primarily for its meat. Considering the volume of harvest, the estimated pheasant carcass weight (700 g), and the human population in Serbia (6.5 million), the calculated per capita meat consumption is approximately 17 g per year. In Hungary, this figure is slightly higher, around 22 g. The estimated carcass weight of pheasants, used to calculate meat consumption in Serbia and Hungary, was taken from literature data reporting average carcass weights of pheasants and pheasant hens. These values for male pheasants are 898 g, 900 g, 759 g, 720 g, 896 g, and 915 g, while for hens they are 644 g, 732 g, 583 g, 755 g, 660 g, and 895 g, mostly depending on age and diet (*Kokoszynski et al*., 2014; 2018; 2024).

Pheasant meat cannot be found in Serbia's retail stores (unless imported), not even in the best-supplied shops. Occasionally, during the peak hunting season (autumn), pheasant meat may be available at local markets in Vojvodina (Novi Sad, Sombor, Subotica). There are no registered slaughterhouses for pheasants in Serbia. This could potentially be addressed by the introduction of mobile slaughterhouses, which are registered for animal slaughter in some countries (*Baltić et al*., 2024).

Pheasant meat has exceptional nutritional value due to its high protein content (over 25% in breast meat) and lower fat content (below 0.5%) compared to other types of meat. The protein content in thigh and drumstick meat ranges from 22% to 23%, with fat content not exceeding 2% (*Kokoszynski et al*., 2024; 2018; *Franco and Lorenzo*, 2013; *Tucak et al*., 2008; *Fernye et al*., 2017; *Strakova et al*., 2011).

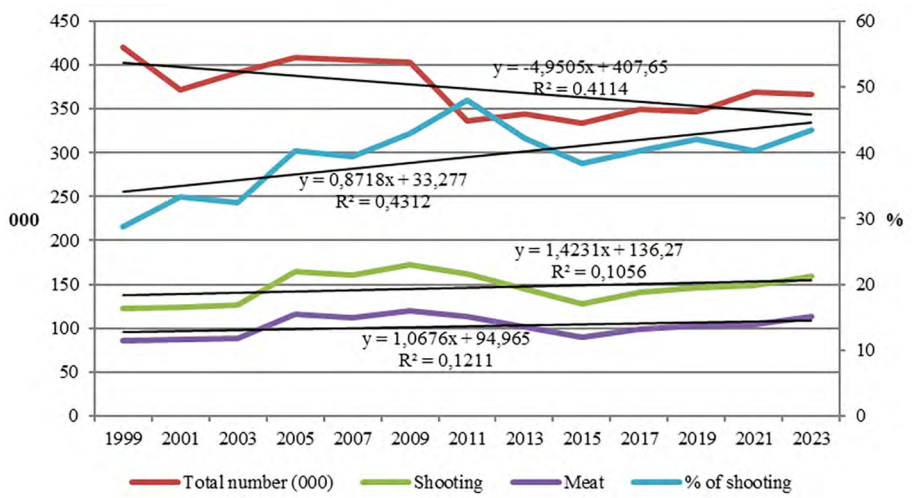

Figure 1. Trend of pheasant population parameters in Serbia from 1999 to 2023

In natural conditions, pheasant mortality is extremely high (75% to 78%) due to numerous predators, food shortages, adverse weather conditions, and the large number of hunters. Therefore, pheasants raised in pheasantries are released into the wild. These facilities have breeding flocks, incubators, and the ability to provide optimal housing and feeding conditions for birds of different ages, as well as gradual adaptation for survival in the wild.

Since a decline in the wild pheasant population was noticed even before 2000, increasing attention has been given to establishing new pheasantries and expanding the capacities of existing ones. The number of pheasantries in Serbia has notably increased since 2003. The trend of changes in the number of pheasantries in Serbia from 1999 to 2023 is shown in Figure 2. The average number of pheasantries in Serbia during this period was 505.5±263.6. Pheasantries in Serbia have different production capacities, ranging from several thousand to over 200,000 birds (as in the pheasantry in Bač). The total capacity of pheasantries in Serbia is close to one million (*Popović and Stanković*, 2009).

To maintain pheasant populations in the wild, supplementary feeding is essential, especially from late autumn through early spring, as well as during the summer when pheasants are released from pheasantries into the wild (*Đorđević et al*., 2008). For this purpose, feeding stations are established in open areas or under shelters, where food is provided for the birds. The trend of increasing numbers of feeding stations for small feathered game (pheasants, quails) from 1999 to 2023 is shown in Figure 3. The same figure also presents the change in the number of hunters during the same period. The ratio of the average number of feeding stations (23,880±2,761) to the average number of hunters (83,622±5,553) indicates that three to four hunters are responsible for providing food at one feeding station for feathered game.

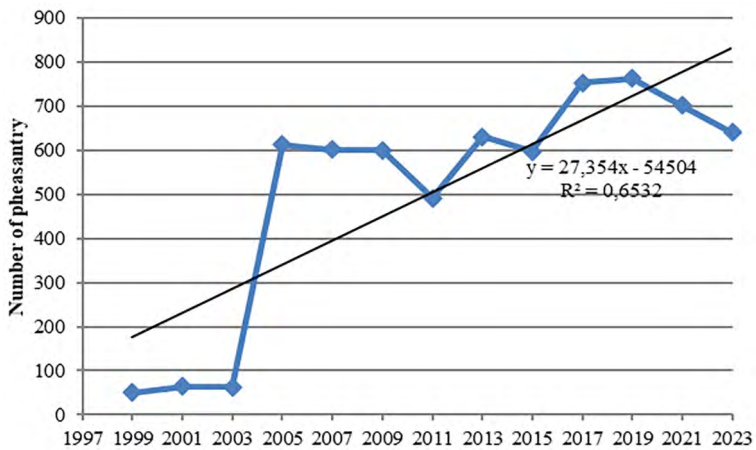

Figure 2. Trend of the number of pheasantries in Serbia

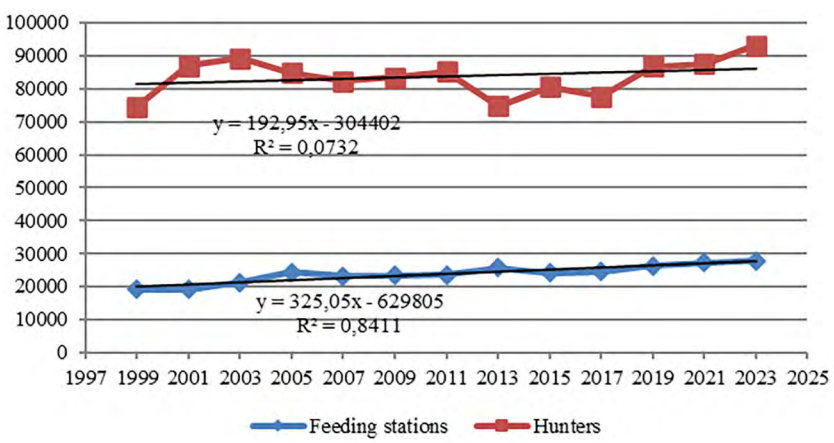

Figure 3. Trends of the number of feeding stations and hunters in Serbia

4. Conclusion

The pheasant population in Serbia has been declining from 1999 to 2023, which could be a result of increased hunting harvest and the growing number of hunters. An increase in pheasant numbers in hunting grounds can be expected through greater introduction of pheasants from pheasantries, an increased number of feeding stations and the larger amount of food provided for small feathered game, as well as a reduction in the number of predators near the hunting grounds.

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Review Paper

Zero-tolerance *Listeria* in EU: preparing for the stricter controls

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ABSTRACT

From July 1, 2026, significant amendments to Regulation (EC) No. 2073/2005 will come into effect, tightening microbiological criteria for food safety, particularly concerning *Listeria monocytogenes*. The revision, introduced through Regulation (EU) 2024/2895, responds to both a rise in listeriosis incidence across Europe between 2019 and 2023 and an identified legal loophole that allowed uneven enforcement among Member States. The updated framework extends responsibility across the entire food supply chain, holding all food business operators-from producers to retailers-accountable for compliance. Ready-to-eat foods must either demonstrate absence of *L. monocytogenes* in a 25 g sample or provide scientific evidence that levels of this pathogen remain below 100 CFU/g throughout the product's shelf life.

The rising burden of listeriosis, with EU-confirmed cases and fatalities increasing steadily, underscores the urgency of these measures. Vulnerable populations, particularly the elderly, remain at greatest risk, while the prevalence of *L. monocytogenes* in fish, fishery, and meat products highlights the need for strengthened controls. Key regulatory changes include stricter environmental monitoring, comprehensive shelf-life and challenge testing, and expanded obligations covering storage, distribution, and retail. Compliance will require enhanced HACCP programs, increased testing capacity, staff training, and systematic documentation, with substantial financial and operational implications for the food industry.

Adaptation strategies center on predictive microbiology and challenge testing, guided by ISO 20976-1:2019 and the EURL Lm Technical Guidance Document, ensuring reproducibility and scientific robustness. Ultimately, these reforms aim to reduce consumer exposure and improve food safety outcomes, albeit with anticipated costs, product reformulations, and market adjustments.

1. Introduction

On July 1, 2026, significant amendments to *Regulation (EC) No. 2073/2005* will be implemented, emphasizing microbiological criteria for food safety, particularly concerning *Listeria monocytogenes* (*Regulation (EU) 2024/2895*). The amendments can be attributed to (i) the rise in listeriosis incidence within the European Union during 2021 to 2022 and (ii) a legal loophole in Point 1.2 of Chapter 1 of Annex I to Regulation No. 2073/2005, as recognized by the European Court of Justice (CJEU) in

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2022 (*CJEU*, 2022), which could be interpreted differently across EU countries, so the CJEU allowed national authorities to enforce stricter measures than those laid down in respective EU legislation.

The constriction exerts a considerable influence on the comprehensive food supply chain, encompassing all stakeholders from producers to retailers. All food businesses operators (FBOs) within the chain will be held accountable for adhering to the established standards. This underscores the significance of conducting comprehensive shelf life and challenge tests, to ensure reliability and performance. In contrast to prior practices where the manufacturer held the primary responsibility, it is now imperative for all entities within the supply chain to demonstrate that their products comply with the established requirements for the entirety of their shelf life. The updated regulation stipulates that there must be either evidence demonstrating the absence of *L. monocytogenes* in a 25-gram sample of the product, or that any *L. monocytogenes* growth must be scientifically substantiated to remain below the threshold of 100 CFU/g. This indicates that food enterprises are required to enhance their procedural rigor while simultaneously improving their comprehension of the design and execution of microbiological testing methodologies.

2. *Listeria monocytogenes* –- a continual hazard and alarming statistics

L. monocytogenes is a Gram-positive pathogen associated with foodborne illnesses and is widely distributed in various environments. The organism's capacity to endure in a range of environments is attributed to several factors, including its robustness against different conditions found in food processing facilities, such as cold storage, as well as its ability to form biofilms (*Swaminathan and Gerner-Smidt*, 2007). Listeriosis has the potential to result in gastroenteritis or meningoencephalitis, with particularly severe outcomes observed in individuals with compromised immune systems (*Wiktorczyk-Kapischke et al*., 2023). It is also a continual challenge for the food industry, especially when it comes to ready-to-eat (RTE) foods that are consumed without further cooking.

In the European Union, the data show a statistically significant upward trend in *L. monocytogenes* infections from 2019 to 2023, with this pathogen ranking fifth among the most frequently reported foodborne infections. According to a 2022 report by the European Food Safety Authority (EFSA), cases of listeriosis increased by 15.9% compared to 2021 and the number of deaths was one of the highest in the last 10 years. In 2023, there was a documented increase of 5.8% in the listeriosis notification rate relative to 2022, culminating in a total of 2,952 confirmed invasive human cases identified from a pool of 5,691 foodborne outbreak cases and leading to 335 fatalities (*EFSA*, 2024). In 2024, 179 cases of listeriosis were reported in England and Wales, and incidence rates of listeriosis were highest in people aged 80 years and over (*UKHSA*, 2025). Death was reported for 28 cases, of whom 9 were known to have listeriosis recorded as a cause of death on the death certificate.

These data indicate a notable escalation and underscore the critical necessity for enhanced food safety protocols. The EFSA reports indicated a higher prevalence of *L. monocytogenes* in specific categories of RTE foods, specifically fish, fishery products, and meat products. The data appear to have been a crucial factor in instigating the recent legislative modifications designed to improve control measures.

The recent increase in listeriosis cases throughout Europe presents a multifaceted problem with numerous contributing elements grouped into these four major categories:

- Alterations in consumer behavior: The increasing prevalence of RTE foods, although advantageous in terms of convenience, elevates the likelihood of exposure due to possible mishandling and storage complications.
- Complex supply chains: The phenomenon of globalization has resulted in the development of intricate supply chains, thereby complicating the process of tracing ingredients and identifying sources of contamination.
- Persistence and adaptability: *L. monocytogenes* exhibits remarkable resistance, demonstrating the ability to survive in diverse environments, including those at refrigeration temperatures. Additionally, it possesses the capability to form biofilms on surfaces, which poses significant challenges to effective control measures.
- Poor awareness and knowledge issues: Insufficient awareness regarding safe food handling practices, especially within vulnerable populations, may facilitate the transmission of foodborne pathogens. The role of education is fundamental in the context of prevention strategies.

3. Key changes in upcoming regulatory framework

Key changes which will be introduced from 2026 include stricter criteria and extension of FBO responsibility. The initial regulation concentrated on the management of *Listeria* during the production phase. The amendment broadens this responsibility to encompass all phases of the food chain, thereby guaranteeing a uniform level of protection across the entirety of the product's shelf life. The amendment establishes more stringent criteria, mandating that *L. monocytogenes* must be absent in RTE foods available on the market, unless the producer can provide evidence that the levels will consistently remain below the established safety threshold for the duration of the product's shelf life. In more straightforward language: Should a producer be unable to demonstrate that their RTE food will maintain a *L. monocytogenes* level below 100 CFU/g for its entire shelf life, it is imperative that *L. monocytogenes* is entirely absent (i.e., not detected in 25 g).

The recent regulatory update necessitates prompt consideration of each FBO's environmental monitoring program. Although the reference methods EN ISO 11290-1 (*ISO*, xxxx) and EN ISO 11290-2 (*ISO*, xxxx) have not undergone alterations, it is imperative that the frequency and scope of testing be considerably increased. Current environmental monitoring protocols are presumably centered around critical control points and surfaces that come into direct contact with food. The newly established regulation (*European Commission*, 2024) mandates thorough monitoring of both food contact and adjacent non-food contact surfaces, ensuring that assessments are conducted with adequate frequency to identify persistent contamination prior to its escalation into a systemic issue. Documentation will serve as a critical resource for ensuring compliance. All products that can facilitate the proliferation of *L. monocytogenes* must undergo comprehensive shelf-life investigations, which encompass physico-chemical assessments, predictive modeling, and frequently, challenge testing. The HACCP and/or quality assurance teams will be required to conduct thorough trend analyses that document continuous control measures and to maintain detailed records of corrective actions for each positive finding. This is not solely a matter of fulfilling regulatory obligations; it involves constructing a robust food safety framework capable of enduring regulatory examination.

The regulation further expands each FBO's obligations beyond the confines of their respective facilities. The interconnected nature of storage, distribution, and retail operations necessitates adherence to compliance obligations. It is essential to acknowledge that temperature abuse or cross-contamination occurring during transport and display can undermine even the most stringent production controls. The supplier agreements and customer specifications will certainly require revision to accurately represent the delineated shared responsibilities, but also to address considerable expenses associated with compliance.

There has been a lot of negative perception in the food industry sector ever since the EU established the zero-tolerance policy for *L. monocytogenes* in late 2024. Even during the vote on the Regulation, unanimity was not attained, because 25 member states voted in favor of the modification, Finland abstained, and Belgium voted against. The primary obstacle lies in the necessity for rigorous scientific validation. Manufacturers will be required to provide scientific evidence that products consistently exhibit a "not detected in 25g" status for *L. monocytogenes* over the food's shelf-life, or that *L. monocytogenes* will not surpass the 100 CFU/g threshold if asserting this exception. This has been understood by certain national authorities as necessitating challenge testing, wherein products are inoculated with *L. monocytogenes* and observed throughout their shelf-life. Conversely, other authorities adopt a more pragmatic stance, permitting FBOs to utilize existing historical data on prevalence of *L. monocytogenes* at the end of product shelf-life. Also, a significant number of researchers express skepticism regarding shelf-life testing, arguing that the artificial conditions of laboratory environments fail to accurately replicate real-world scenarios. Next, regular microbiological testing could result in a considerable increase in the financial costs involved due to the need for increased environmental monitoring. Considerable modifications to FBOs' HACCP plans will be needed, such as elimination of potential harborage points, the implementation of enhanced hygienic zoning with stricter separation between raw food and RTE food areas, an increase in testing capacity, staff training focused on improved procedures, and the establishment of data management systems for trend analysis. The modifications in regulatory frameworks are anticipated to improve consumer safeguards against listeriosis, especially among susceptible groups where mortality rates could escalate to 30%. Manufacturers must

anticipate market repercussions, which could include potential price escalations as compliance expenditures are integrated, the likelihood of product discontinuation for items that may no longer be economically feasible, and reduced shelf-lives as reformulation strategies are enacted.

4. Adaptation to the new legislation

In order to comply with this new legislation by July 1, 2026, FBOs can demonstrate that their RTE products do not exceed the 100 CFU/g threshold by means of various tools. Among these options, challenge tests and predictive microbiology are most frequently used. Predictive microbiology involves use of mathematical models that forecast bacterial behavior depending on variables such as pH level, water activity, salt content, inhibitory components, adjuvants, background microbiota and temperature. On the other hand, challenge tests involve the deliberate contamination of a product to assess the proliferation of *L. monocytogenes* over a designated timeframe and inside a particular RTE product. It is important to highlight that these two tools are not mutually substitutable, but rather complementary, since the application of predictive microbiology modeling enables the forecasting of bacterial behavior in various environments, eliminating the need for laboratory experimentation, while a bacterial challenge test is essential for acquiring precise data concerning bacterial activity in a specific product under defined storage conditions.

The implementation of shelf-life and challenge tests aimed at evaluating the growth of *L. monocytogenes* has been detailed in international standard ISO 20976-1:2019 (*ISO*, 2019). The EU reference laboratory for *L. monocytogenes* (Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail, France) has systematically documented various protocols for challenge and durability tests for *L. monocytogenes* in a technical guidance document (TGD) (*EURL Lm*, 2021). The TGD is especially critical in enhancing the reliability and reproducibility of microbiological testing, thereby ensuring that results are both consistent and of high quality. It anticipates use of selected *L. monocytogenes* strains that are pertinent to the specific product type, and pre-testing a minimum of three distinct production batches in advance of the challenge test. Furthermore, it stipulates analysis of no fewer than five samples from each batch for parameters such as water activity (aW), pH, and salt concentration to address variability both within individual batches and across different batches. The TGD also incorporates simulation scenarios of temperature variations in production, distribution, and consumer storage.

For the purposes of pre-analysis or research and development by the FBO, challenge testing, as well as predictive modeling, can be conducted at the FBO's own laboratory, provided it exists. However, for regulatory self-inspections, these tests must be conducted in accredited laboratories that have been recognized by local authorities for the purpose of performing challenge tests and which possess the requisite expertise, infrastructure and knowledge potential to conduct these intricate tests. This aspect is important not only for the reliability of the results but also for compliance with legal frameworks and regulatory standards.

5. Conclusion

The forthcoming amendments to Regulation (EC) No. 2073/2005 represent a decisive shift towards strengthening consumer protection against *L. monocytogenes*. By extending accountability across the entire food supply chain, Regulation 2024/2895 (*European Commission*, 2024) compels all stakeholders to adopt more rigorous monitoring, testing, and documentation practices. While compliance will entail significant financial and operational challenges, it also fosters the development of more robust and scientifically validated food safety systems. Ultimately, these measures are expected to reduce listeriosis incidence, safeguard vulnerable populations, and enhance trust in the European food market.

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Review Paper

Major foodborne zoonotic bacteria and their role in antimicrobial resistance

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ABSTRACT

Foodborne zoonotic bacteria present a major public health concern worldwide, responsible for hundreds of millions of infections and hundreds of thousands of deaths annually. These organisms not only cause severe illness but are increasingly exhibiting resistance to critically important antimicrobials, limiting treatment options and complicating outbreak control. The rise of antimicrobial-resistant (AMR) strains, particularly in low- and middle-income countries, is closely linked to the misuse of antibiotics in both human medicine and food-producing animals. Resistance arises through multiple mechanisms, including enzymatic drug inactivation, target site modification, reduced membrane permeability, efflux pump activation, and the horizontal transfer of resistance genes via mobile genetic elements.

This paper presents an overview of the global burden of foodborne zoonoses, highlighting the prevalence and AMR patterns of major zoonotic bacterial pathogens (*Campylobacter* spp., *Salmonella*, Shiga toxin-producing *Escherichia coli*, *Listeria monocytogenes*, and methicillin-resistant *Staphylococcus aureus*) across different regions. It also addresses the substantial economic costs associated with these infections. Drawing on recent data from WHO, EFSA, ECDC, CDC, and OECD, the paper underscores the urgent need for coordinated, cross-sectoral strategies. Emphasis is placed on the One Health approach as a comprehensive framework to address the interconnected risks to human, animal, and environmental health. Strengthening surveillance, promoting responsible antimicrobial use, and improving food safety systems are essential to mitigate the growing threat of AMR along the food chain.

1. Introduction

Foodborne zoonotic bacteria pose a growing global public health concern, causing an estimated 600 million illnesses and 420,000 deaths annually (WHO, 2015). Among the most significant bacterial pathogens are *Campylobacter* spp., *Salmonella*, Shiga toxin-producing *Escherichia coli* (STEC), *Listeria monocytogenes*, and methicillin-resistant *Staphylococcus aureus* (MRSA). These bacteria not only trigger acute infections but increasingly contribute to the spread of antimicrobial resistance (AMR), complicating treatment and disease control efforts (*Vesković*, 2025).

AMR is recognised as one of the most pressing global threats to human and animal health. Key

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drivers include the intensive use of antibiotics in animal production, inappropriate therapeutic and prophylactic use, and weak regulatory oversight (*Nastasijevic et al*., 2023; *Milijašević et al.*, 2024). The food chain acts as a major reservoir of resistant bacteria due to antibiotic use in food-producing animals, while global food trade accelerates the transboundary spread of resistance *(Founou et al*., 2016). Additional factors such as inadequate hygiene practices, low biosecurity standards, and direct or indirect contact with contaminated food or animals contribute to human exposure (*Mc Nulty et al*., 2016; *Vesković*, 2025). Resistance emerges through selection of naturally resistant strains, genetic mutations, and horizontal gene transfer, and may also be driven by antimicrobial residues in animal-derived foods (*Serwecińska*, 2020; *Muteeb et al*., 2023). The confined conditions of intensive farming foster rapid spread of resistance genes within and between bacterial populations (*Manyi-Loh et al*., 2018).

The burden of AMR is staggering. In 2019, an estimated 4.95 million deaths were associated with AMR globally, with 1.27 million directly attributable to resistant infections (*Antimicrobial Resistance Collaborators*, 2022). In the European Union (EU), AMR is responsible for more than 35,000 deaths and economic losses exceeding €11.7 billion each year (*ECDC*, 2024). Similar estimates apply to the United States (US), with over 2.8 million infections and 35,000 deaths annually linked to resistant bacteria, and healthcare costs exceeding US$4.6 billion (*CDC*, 2019). Without effective mitigation, AMR could cause more than 10 million deaths annually by 2050, surpassing cancer, and lead to a cumulative economic loss of US$100 trillion (*O'Neill*, 2016; *OECD/WHO*, 2022). In response, international organisations such as the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (WOAH), and the United Nations Environment Programme (UNEP) advocate for a One Health approach that integrates human, animal, and environmental health. Core strategies include prudent antimicrobial use, improved animal husbandry, biosecurity, vaccination, and hygiene protocols (*WHO*, 2017).

This paper provides an overview of the major foodborne zoonotic pathogens—*Campylobacter* spp., *Salmonella* spp., STEC, *L. monocytogenes*, and MRSA—with emphasis on their prevalence, AMR patterns, and impact on public health. The aim is to contribute to understanding their role in AMR transmission through the food chain and support the development of effective prevention and control strategies.

2. Literature search strategy

This review is based on peer-reviewed scientific literature and official reports addressing AMR in major foodborne zoonotic bacteria. Relevant publications were identified through a comprehensive search of electronic databases including PubMed, Scopus, Web of Science, and Google Scholar. In addition, documents and surveillance data from international organisations, such as the WHO, FAO, ECDC, and OECD were reviewed. The literature search focused on publications published between 2015 and 2025, using a combination of keywords such as "antimicrobial resistance", "foodborne bacteria", "zoonoses", "One Health", and specific bacterial names (e.g., *Campylobacter*, *Salmonella*, STEC, *L. monocytogenes*, MRSA). Sources were selected based on their scientific relevance, methodological quality, and alignment with the thematic scope of this review.

3. Overview of major foodborne bacteria and their role in AMR transmission

Foodborne zoonotic bacteria play a critical role in the transmission of AMR along the food chain. Their ability to persist throughout various stages of food production, processing, and distribution, as well as to colonise and cause infections in humans, makes them particularly relevant within the One Health framework. This section provides an overview of their prevalence, resistance patterns, and public health impact.

3.1. Campylobacter spp.

Campylobacter are leading bacterial causes of foodborne diarrhoea, responsible for an estimated 400–500 million cases of gastroenteritis annually (*Igwaran and Okoh*, 2019). In the US, approximately 1.5 million infections are reported each year, with an associated economic burden ranging from US$1.3 to 6.8 billion (*Kaakoush et al*., 2015), while annual costs in the EU are estimated at €2.4 billion (EFSA, 2024). Although most cases are mild and self-limiting, severe or systemic infections require antibiotic therapy, primarily macrolides and fluoroquinolones, with tetracyclines and aminoglycosides, such as gentamicin, occasionally used for invasive forms (*Shen et al*., 2018). In the US, 29% of *Campylobacter* isolates

exhibit reduced susceptibility to these classes (*CDC,* 2019). Multidrug-resistant (MDR) strains are increasingly prevalent in low- and middle-income countries, where unregulated antibiotic use in animal production facilitates resistance spread through the food chain (*Igwaran and Okoh*, 2019; *Gahamanyi et al*., 2025). Ciprofloxacin resistance is particularly high in both humans and food-producing animals across the EU (EFSA/ECDC, 2025). In addition to *gyrA* point mutations, several MDR genes, *erm(B)*, *arsP*, *fosXCC*, and *cfr(C)*, have been identified (*Tang et al*., 2017; *Shen et al*., 2018). Horizontal gene transfer further complicates resistance monitoring and control (*Guernier-Cambert et al*., 2021).

3.2 Salmonella

Salmonella causes approximately 1.35 million infections annually in the US, leading to 26,500 hospitalisations and around 420 deaths (*CDC*, 2023). Of these, an estimated 212,500 cases are linked to antimicrobial-resistant strains, resulting in approximately 70 deaths and US$400 million in treatment costs each year (*CDC*, 2019; 2023). Similarly, in the EU, 65,208 human salmonellosis cases were confirmed in 2022, with rising levels of AMR observed in isolates from humans, animals, and food, particularly against fluoroquinolones and third-generation cephalosporins (*EFSA/ECDC*, 2025), which complicates effective treatment. Commonly used antibiotic classes for treating *Salmonella* infections include β-lactams, fluoroquinolones, tetracyclines, aminoglycosides, and trimethoprim-sulfamethoxazole (*Akinyemi and Ajoseh*, 2017; *San Millan*, 2018). Resistance mechanisms in *Salmonella* involve alterations in antibiotic targets, overexpression of efflux pumps, and the acquisition of resistance genes, such as *blaCTX-M*, *qnr*, and *aac(6')-Ib-cr*, which are also prevalent among other *Enterobacteriaceae* (*WHO*, 2023; *Zhou et al*., 2023; *Monte et al*., 2023). These resistance factors reduce therapeutic efficacy. The ability of *Salmonella* spp. to persist throughout the farm-to-fork continuum, along with its pronounced genetic adaptability, underscores its role as a critical vector in the spread of AMR under the One Health framework.

3.3. Shiga toxin-producing Escherichia coli (STEC)

STEC is a major foodborne pathogen responsible for severe gastrointestinal illness, including haemorrhagic colitis and haemolytic uremic syndrome (HUS), particularly in children and the elderly. In 2022, 7,117 STEC cases were confirmed in the EU/EEA, with the highest rates in Ireland and Sweden (*EFSA/ECDC*, 2023). In the US, STEC causes an estimated 265,000 infections annually, leading to over 3,600 hospitalisations and 30 deaths (*CDC*, 2022). The most virulent serotype, *E. coli* O157:H7, is commonly linked to contaminated ground beef, raw milk, and fresh produce (*Stager et al*., 2023; *Loor-Giler et al*., 2025). Due to the risk of HUS, antibiotic treatment is generally avoided; however, AMR in STEC strains remains a significant public health concern. Resistance, particularly to ampicillin, tetracyclines, and trimethoprim-sulfamethoxazole, has been reported in both O157:H7 and non-O157 serogroups (*Pan et al*., 2021; *EFSA/ECDC*, 2025). Resistance genes, such as bla_{TEM}, $bla_{\text{CTX-M}}$, *tet*(A/B), $sul_{1/2}$, and $dfrA_1$, are often plasmid- or integron-associated (*Zhou et al*. 2021; *Chaudhary et al*., 2023). MDR STEC strains have been detected in livestock, food, and the environment, with prevalence rates of 30–50%, particularly in areas with poor antibiotic regulation (*Nada et al*., 2023). While antimicrobial therapy is not standard for uncomplicated cases, MDR emergence poses risks in severe infections requiring treatment and contributes to the broader reservoir of resistance genes within the One Health interface.

3.4. Listeria monocytogenes

L. monocytogenes is a major zoonotic foodborne pathogen and the causative agent of listeriosis—a severe infection with high fatality rates among vulnerable populations. Though less common than other foodborne pathogens, it is associated with the highest hospitalisation and case-fatality rates in the EU. In 2022, 2,993 confirmed cases were reported across EU/EEA countries, with a case-fatality rate of 18.1% (*EFSA/ECDC*, 2023). In the US, approximately 1,600 cases and 260 deaths are recorded annually (*CDC*, 2024). While *L. monocytogenes* remains generally susceptible to first-line antibiotics like ampicillin and gentamicin, the emergence of resistant strains from food, clinical, and environmental sources is increasingly reported (*Moura et al.*, 2023; *Rippa et al*., 2024; *Sołtysiuk et al*., 2025). Key resistance mechanisms include plasmid- and transposon-mediated gene acquisition, biofilm and persister cell formation, and efflux pump activity (*Bashiry et al*., 2020; *Nikolaou et al*., 2025). These are often driven by antimicrobial use in both human and animal sectors (*FAO/VMD*,

2022). MDR strains have been detected in seafood, ready-to-eat meats, and dairy, complicating treatment and posing food safety risks (*Menon et al*., 2021; *Nikolaou et al*., 2025). *L. monocytogenes* can persist in food-processing environments and survive refrigeration, underscoring the need for stringent hygiene and AMR surveillance. Regional differences in resistance patterns, shaped by antibiotic use and regulatory practices, necessitate targeted monitoring strategies (*Sołtysiuk et al*., 2025). In the EU, Regulation (EC) No 2073/2005, as amended by Regulation (EU) 2024/2895 (applicable from July 2026), introduces a zero-tolerance requirement for *L. monocytogenes* in RTE foods supporting pathogen growth, aligning EU policy more closely with the US zero-tolerance approach.

3.5. Methicillin-resistant Staphylococcus aureus (MRSA)

MRSA plays a significant role in the transmission of AMR through the food chain. The emergence of livestock-associated MRSA (LA-MRSA) has further complicated AMR surveillance, especially in countries with intensive animal production systems (*EFSA/ECDC*, 2025). MRSA has been frequently detected in raw meat, milk, and dairy products, with contamination typically originating either from colonised animals or from infected food handlers (*González-Machado et al*., 2024). Transmission can also occur via direct contact with infected animals or humans, as well as through contaminated food preparation surfaces (*EFSA/ECDC*, 2025). In the US, MRSA is responsible for over 323,700 infections and approximately 10,600 deaths annually, with associated healthcare costs exceeding US$1.7 billion (*CDC*, 2019; *Nelson et al*., 2022). In contrast, the EU recorded a decline in MRSA bloodstream infections, with an estimated 4.64 cases per 100,000 population in 2023, a 17.6% decrease compared to 2019 (*ECDC*, 2024). While recent EU cost estimates are limited, modelling studies suggest that MRSA infections affect around 150 000 patients yearly and impose an added burden of approximately €380 million on healthcare systems (*Rocha et al*., 2020). Beyond the significant morbidity risk, particularly for immunocompromised individuals, MRSA infections are very challenging and expensive to treat due to resistance to multiple antibiotic classes, including β-lactams, fluoroquinolones, and macrolides (*Lade et al*., 2022; *Abebe and Birhanu*, 2023). Resistance is primarily mediated by the *mecA* gene, which encodes an altered penicillin-binding protein (PBP2a), conferring resistance to methicillin and other β-lactams. MRSA strains often carry additional resistance determinants, such as *erm* genes (macrolide resistance) and norA-associated efflux pumps (*Abebe and Birhanu*, 2023).

4. Mitigation of the AMR problem

The rising threat of AMR among major foodborne zoonotic bacteria underscores the need for an integrated and coordinated response. A sustainable solution to AMR requires the adoption of the One Health approach, which recognises the interconnection between human, animal, and environmental health. To address this complex challenge, the following elements are essential: *a)* rational use of antimicrobials in both human medicine and food-producing animals, supported by clear regulatory frameworks and stewardship programs; *b)* strengthening surveillance systems through harmonised monitoring protocols across sectors and countries, enabling early detection and timely intervention; *c)* investment in research focused on alternatives to antibiotics—such as vaccines, probiotics, phage therapy, and improved farming and hygiene practices; *d)* raising awareness and education of all stakeholders, including healthcare providers, veterinarians, farmers, policymakers, and consumers; *e)* implementation of national and regional action plans aligned with the Global Action Plan on AMR and adapted to local epidemiological realities; *f)* sustainable financing mechanisms, especially in resource-limited settings, to ensure continuity of AMR mitigation efforts; *g)* enhanced international collaboration and policy coherence across the human-animal-environment interface.

Only through a comprehensive and cross-disciplinary effort can we preserve the efficacy of life-saving antimicrobials and ensure a safer future for generations to come.

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Original Scientific Paper

Generation and Pareto optimization of sterilized meat-and-plant canned food formulations using the R programming language

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ABSTRACT

This study outlines key trends in modern software for food formulation development. An algorithm that enables the generation of a set of formulations for meat-and-plant sterilized canned products and identifies a Pareto-optimal subset thereof based on selected criteria is presented. The generation is carried out based on a developed mathematical model of a formulation that complies with the requirements of current standards. The algorithm was used to generate 1,000 formulations, of which 44 were identified as Pareto-optimal.

1. Introduction

The development of multicomponent food formulations is a labor-intensive process that requires both creativity and knowledge of the chemical composition and technological properties of ingredients. Additionally, it involves numerous calculations, particularly when accounting for coefficients and biological value indicators. Currently, specialized software has been developed that significantly simplifies and automates the formulation process, increases its speed, and eliminates potential errors in mathematical calculations (*Lisitsyn et al.*, 2021; *Musina et al.*, 2017). Most modern recipe formulation systems typically include the following features and functionality: built-in standardized parameters from current regulatory documentation for specific product categories; integrated databases containing chemical composition of ingredients and selected technological properties of ingredients; capability to calculate chemical composition and economic indicators of formulations; functionality for predicting certain technological properties of designed products; optimizing existing formulations; ability to maintain a registry of active formulations (*Khabibullin et al.*, 2014). Some software solutions also include functionality for generating labeling information blocks. Modern-generation systems are typically characterized by implementation of cloud computing technology (*Foodworks.online, n. d.*), utilization of artificial intelligence for recipe optimization (*FoodSolver, n. d.*), and prediction of product taste characteristics (*NIQ, n. d.*).

In Russia, the first software enabling the design of multicomponent food formulations was the "System for Designing and Assessing the Quality of Multicomponent Food Compositions", developed

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in 1997 by Academician Lipatov N. N. (Jr.) in collaboration with Bashkirov O. I. It was Academician Lipatov who laid the foundations for computer-aided design of multicomponent food products based on the theoretical framework of food combinatorics (*Lipatov and Rogov*, 1987; *Lipatov et al*., 1990; *Lipatov et al*., 2001). This software allows for calculating the chemical composition of a product, the minimum amino acid score, the utility coefficient, and the comparable redundancy coefficient. However, the program has significant limitations, such as the lack of a formalized ingredient composition database, the manual data entry for each ingredient, its restricted functionality, and the inconvenient user interface. These constraints substantially limit its applicability for broader scientific research and practical applications. Currently available Russian software for designing multicomponent food formulations includes Generic 2.0, MultiMeat Expert, Recipe Constructor, and others (*Drofiche*va, 2023; *Nikitina et al*., 2016). Internationally, widely used solutions are NutriCalc, Genesis R&D, and Emydex Recipe Formulation (*Khabibullin et al*., 2014). However, key limitations of existing solutions are their inability to adapt to specific niche requirements, the lack of an option to add custom quality assessment parameters not pre-programmed in the software, and the paid access model.

A promising solution to personalize software functionality for food product development is by using programming languages, such as Python or R. Both languages are widely employed in research due to their open-source nature, extensive libraries for optimization and data visualization, relative simplicity of use, and abundance of reference and educational materials. The ultimate choice of language and environment depends on the user's individual preferences and skill level (*Hackenberger*, 2020).

The objective of this study was to perform automated generation of a set of synthetic formulations of sterilized meat-and-plant canned products using the R programming language based on a constructed mathematical model, and, through multicriteria optimization, to identify a subset of Pareto-optimal formulations.

2. Materials and methods

The object of this study is the process of generating a set of formulations for sterilized meat-and-plant canned products compliant with the Russian GOST 32245-2013 (*Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing*, 2013) standard and its Pareto optimization. The subject is an algorithm in the R programming language that implements the generation and optimization of formulations.

Formulation generation, optimization and data visualization were performed in the R programming language (R Core Team, 2025) version 4.4.3 (2025-02-28 ucrt) in the R-Studio environment (*Ushey et al.*, 2024). The following packages were used: NutrienTrackeR (*Rodriguez-Martinez et al*., 2023), readxl (*Wickham and Bryan*, 2025), writexl (*Ooms*, 2025), ggplot2 (*Wickham*, 2016), and plotly (*Sievert*, 2020).

As the database (DB) of ingredient chemical composition, we used the Canadian Nutrient File (CNF) database of Health Canada, accessed through the R package NutrienTrackeR (*Rodriguez-Martinez et al*., 2023).

Since it was necessary to find a subset of optimal formulations by more than one criterion in the generated set of formulations, we used Pareto optimization (*Nikitina and Chernukha*, 2018; *Becker et al*., 2023). According to the definition of the Pareto-optimal set, in the set of formulations Y, a Pareto-optimal (dominant) formulation $y_i \in Y$ relative to formulation $y_k \in Y$ is one for which all evaluation criteria are no worse, and one of the criteria is better (Equation 1). In the study, we used Pareto dominance without weight coefficients for the criteria.

$$(y_i, y_k) \in Y \rightarrow \forall_j : [f_j(y_i) \geq f_j(y_k)] \Delta [f(y_i) \neq f(y_k)], \begin{cases} f_1(x) \rightarrow max \\ f_2(x) \rightarrow max \end{cases} \quad (1)$$

For optimization we selected the following three criteria: 1) amino acid adequacy criterion (the rationality coefficient of amino acid composition, RAAC (*Lisitsyn et al*., 2021; *Lisin et al*., 2012)). For the calculations, we used the amino acid composition reference standard from the *Food and Agriculture Organization [FAO]*, 2013); 2) the fatty acid adequacy criterion (Euclidean distance of fatty acids, EDFA) (Equation 2):

$$EDFA = \sum_{i=1}^{5} \sqrt{(j_{ref,i} - (i_{ji} \cdot 9 \cdot 100 \div E_j)^2} \quad (2)$$

where $j_{ref,i}$ – reference content of the i-th fatty acid fraction, %, according to MR 2.3.1.0253-21 (*Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing*, 2021); i_{ji} – content of the i-th fatty acid fraction in the j-th formulation, g/100g; E_j – energy value of the j-th formulation, kcal; 9 – energy conversion factor for fats; 100 – percentage conversion factor. Here, we calculate the distance between the reference point $j_{ref,i} = (p_1, p_2, p_3, p_4, p_5)$, where $p_1, \dots, p_5$ are the reference percentages of fatty acid fractions relative to daily caloric intake, and the formulation point $i_j = (q_1, q_2, q_3, q_4, q_5)$, where $q_1, \dots, q_5$ are the actual percentages of fatty acid fractions relative to the formulation's caloric value. Thereby, formulations with minimal EDFA values are nutritionally closer to the reference fatty acid ratio; 3) formulation cost criterion, calculated as the sum of average ingredient costs. For each ingredient, at least three price values were obtained.

Since equation (1) represents a solution to a two-criterion maximization problem, we reformulated the Pareto-optimality condition for the three selected minimized criteria (equation 3):

$$(y_i, y_k) \in Y \rightarrow \forall_j : [f_j(y_i) \le f_j(y_k)] \Delta [f(y_i) \ne f(y_k)], \begin{cases} f_1(x) \rightarrow min \\ f_2(x) \rightarrow min \\ f_3(x) \rightarrow min \end{cases} \quad (3)$$

where f_1 – RCAAC criteria, which ought to be minimized; f_2 – EDFA criteria, which ought to be minimized (2); f_3 – Formulation cost criteria, which ought to be minimized.

The mathematical model for meat-and-plant canned food formulations (equation 4) was developed in compliance with GOST 32245-2013 (*Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing*, 2013).

$$\begin{cases} \sum_{i \in I} x_i = 1, \\ 0{,}3 < \sum_{i \in I_{meat}} x_i \le 0{,}6, \\ \sum_{i \in I} x_i \cdot p_i \ge 0{,}06, \\ \sum_{i \in I} x_i \cdot f_i \le 0{,}25, \\ x_i \ge 0 \ \ \forall i \in I. \end{cases} \quad (4)$$

where I – set of all canned food formulation ingredients; x_i – mass fraction of the i-th ingredient in the formulation, %; $I_{meat} \subset I$ – subset of all meat ingredients; p_i – protein mass fraction of the i-th ingredient in the formulation, %; f_i – fat mass fraction of the i-th ingredient in the formulation, %.

3. Results and discussion

The algorithm for generating a set of formulations and their Pareto optimization consisted of three blocks (Figure 1). The input data for the algorithm is an XLSX file containing a list of selected ingredients and their chemical composition (protein, fat, carbohydrates, vitamins, micro- and macronutrients, fatty acids, etc.; 152 parameters total). In Block 1, data are prepared for subsequent calculations: the contents of methionine with cysteine and phenylalanine with tyrosine are summed, as required by the reference standard (*FAO*, 2013).

Block 2 starts with the determination of the number of recipes to be generated and the ingredient group type, which specifies the mechanism for assigning mass fraction to an ingredient. There are two types of this mechanism. For some ingredients, such as salt and pepper, the mass fraction is constant in every formulation. For others, the mass fraction varies. For example, equation 4 assumes the content of meat ingredients is in the range of 30.00 to 60.00%. The mass fraction within specified ranges is determined using a pseudorandom number generator (PRNG). The second subtype of the variable mechanism is when the mass fraction of an ingredient is defined by another ingredient. For example, the type of plant ingredient defines the amount of added water by a specific coefficient. We then established rules for selecting ingredients from different groups or ingredients. It was assumed that one or two meat ingredients and one or two plant ingredients could be selected in each recipe. After this, the process of assigning mass fractions to the ingredients starts. First of all, obligatory ingredients with constant mass fraction are assigned. Then, the mass fractions of ingredients determined by the first subtype of the second mechanism (meat ingredients, fat ingredients). The unoccupied reminder of one (first line in equation 4) is distributed by the second subtype of the second mechanism between the plant ingredients and water. The chemical composition of

the recipes is calculated by matrix multiplication of the established mass fractions and chemical composition of the ingredients. Formulations falling outside the acceptable range, as well as those with an amino acid score below 100%, are excluded from further consideration.

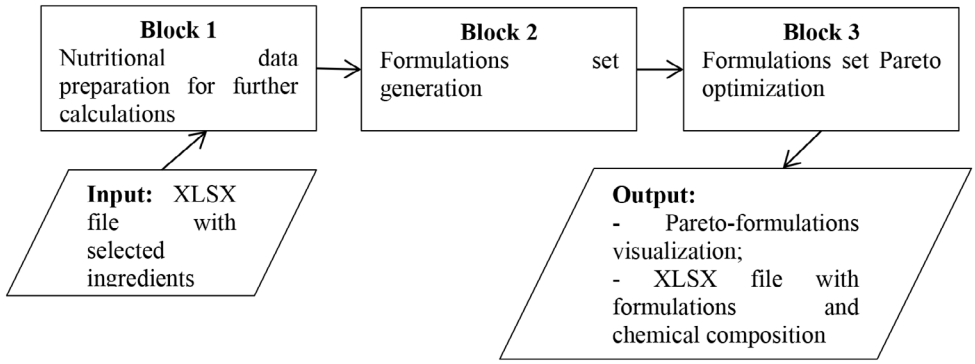

Figure 1. Algorithm for generation and Pareto optimization of meat-and-plant canned food formulations

Block 3 performs Pareto optimization of the generated set of formulations (equation 3) using three minimization criteria. The Pareto-optimal subset is then visualized in a 3D plot using the ggplot2 (Wickham, 2016) and plotly (*Sievert*, 2020) packages. Figure 2 displays 1,000 generated formulations, of which 44 were Pareto-optimal. The block outputs an XLSX file with four sheets: complete set of generated formulations; chemical composition of each formulation; Pareto-optimal formulations; chemical composition of Pareto-optimal formulations. Two examples of Pareto-optimal formulations are presented in Table 1, while Table 2 shows the optimization criteria values.

Table 1. Examples of generated Pareto-optimal formulations.

Ingredient	Mass fraction, %	
	Formulation 1	Formulation 2
Pork heart	31.65	–
Pork liver	–	15.93
Turkey liver	–	14.08
Millet groats	23.25	20.92
Buckwheat groats	–	10.05
Onion	2.87	2.36
Pork fat	4.97	8.16
Vegetable oil	3.28	–
Salt	1.40	1.40
Black pepper	0.04	0.04
Water	32.54	27.06
TOTAL	100.00	100.00

Table 2. Values of optimization criteria for two generated Pareto-optimal recipes

Criterion	Formulation 1	Formulation 2
Rationality coefficient of amino acids (RAAC)	30.25 %	29.72 %
Euclidean distance of fatty acids (EDFA)	13.04	10.51
Formulation cost per 100 g	6.81 RUB	8.73 RUB

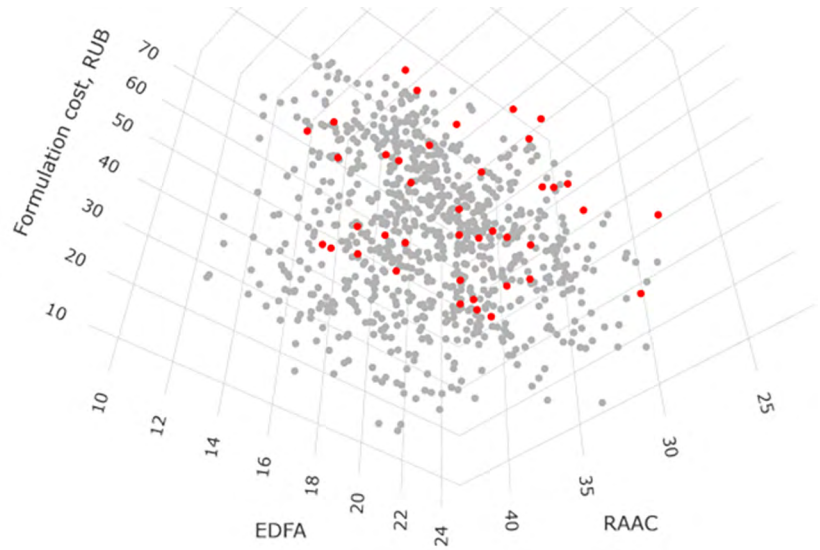

Figure 2. 3D visualization of all generated (gray points, N = 1,000) and Pareto-optimal (red points, N = 44) formulations across three optimization criteria, i.e., the cost (in rubles), the rationality coefficient of amino acids (RAAC), and the Euclidean distance of fatty acids (EDFA).

4. Conclusion

Food formulation development involves various costs, including time, financial resources, and other expenditures. Modern computational technologies help mitigate these costs by enabling rapid processing of large datasets with specified precision. Depending on the objectives, practitioners can utilize either specialized software or broader programming tools. The latter approach allows for personalization of the formulation process and incorporation of custom quality metrics during multi-criteria optimization of the final product.

The R-based algorithm presented in this study generates multiple meat-and-plant canned food formulations compliant with regulatory standards for this product category. Through multi-criteria optimization, it identifies a Pareto-optimal subset of formulations based on selected criteria.

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Original Scientific Paper

Valorisation of pumpkin leaf by-product proteins through enzymatic modification in a biorefinery concept for meat emulsion application

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ABSTRACT

The valorisation of agricultural and industrial side-streams within integrated biorefinery concepts is gaining increasing importance in the context of sustainable protein production and the circular bioeconomy. In this context, waste green biomass is emerging as an alternative protein source with potential applications in food formulations. This work investigated the possibility of improving the techno-functional and antioxidant properties of protein isolated from pumpkin leaves by partial enzymatic hydrolysis.

The white protein fraction, mainly composed of RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) was isolated from green juice obtained by pressing pumpkin leaves through thermocoagulation, acid precipitation, subsequent resolubilisation and lyophilisation, and further treated with endopeptidase from *Bacillus licheniformis* in a stirred batch reactor under the reported optimal conditions (pH 8, 55 °C). Enzymatic treatment at the optimal enzyme-to-substrate ratio (*E/S* = 6.5 wt.%, 75 min, degree hydrolysis ~ 15%) markedly improved protein solubility, emulsifying capacity, interfacial functionality, and antioxidant activity, supporting the potential application of pumpkin leaf protein as natural emulsifiers and antioxidants in meat products.

1. Introduction

Nowadays, due to steadily increasing population growth and the rising demand for meat production, the search for a new generation of renewable protein sources has become highly important (*Malila et al.*, 2024). Press cakes and other residues obtained after oil extraction from stone fruit and oilseeds represent a valuable source not only for lipids for biodiesel production, but also of proteins and other high-value compounds that can be recovered and valorised. In this framework, green leaf biomass like pumpkin leaves represents an underutilised protein source with potential applications in food formulations (*Perović et al.*, 2024).

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In addition to their nutritional value, plant proteins play a key technological role in food systems (*Baig et al.*, 2025). In the meat industry, they are increasingly used as natural emulsifiers and functional ingredients, which contribute to stabilisation of emulsions, texture improvement and water and fat retention (*Bukarbayev et al.*, 2025). Their use can achieve a dual effect: reducing synthetic additives in line with clean-label trends and enriching products with proteins that enhance nutritional value. It is particularly attractive that such proteins can be obtained from waste plant biomass, promoting full utilisation of raw materials within biorefinery system and reducing waste.

Pumpkin leaves (Curcubita sp.) are one of the underutilised plant resources, although they are rich in proteins, especially chloroplast and membrane proteins that have favourable emulsifying and foaming properties. This biomass is most often discarded as agricultural waste during harvesting or processing, but within integrated biorefinery concepts, it can represent a valuable source of food protein (*Korićanac et al.*, 2025). However, some limitations such as poor solubility or insufficiently developed techno-functional properties in the native form often hinder their direct application in food systems. In this context, enzymatic modification stands out as an effective approach for improving the functional properties of native proteins including emulsifying activity, foaming ability, and water and oil binding capacity. In addition, a partial degradation of protein by proteolytic enzymes can produce oligopeptides exerting biological functions, particularly antioxidant activity. Thus the obtained hydrolysates may have significant potential as pure-declared natural antioxidants in meat emulsions, where they can partially or completely replace synthetic additives and contribute to the nutritional value of the product (*Mensah et al.*, 2024). Comparison with soy protein, which is most commonly used as a protein standard in the industry, allows for the assessment of the technological applicability of these new ingredients.

In this work, the potential of proteins isolated from pumpkin leaves to use as functional antioxidants in meat emulsion was investigated. To improve their techno-functional properties, the isolated protein fraction was subjected to partial enzymatic hydrolysis. The antioxidant and techno-functional properties of the obtained hydrolysates including solubility, emulsifying and interfacial properties were evaluated and compared with those of the unmodified pumpkin leaf protein and commercial soy protein concentrate.

1. Materials and methods

2.1. Preparation of leaf protein isolate

The extraction of protein from fresh pumpkin leaves (*Cucurbita pepo* var. *oleifera*; September 2023 harvest, JS&O d.o.o., Novo Miloševo, Serbia) involved mechanical screw-pressing, thermal coagulation and isoelectric precipitation, as described with slight modification (*Perović et al*., 2024). Fresh leaves (150 g) were pressed using a twin-screw press (Angel Juicer 8500) to obtain green juice and a fibrous press cake. The juice was incubated at 55 °C for 30 min, cooled, and centrifuged (7800 rpm, 10 min, 4 °C) to remove pigments and membrane proteins. The supernatant ("brown juice") was adjusted to pH 4.5 with 2 M HCl and stored at 2-8 °C for 12-16 h without stirring. The precipitate, white protein fraction, was collected by centrifugation, neutralised to pH 7, and freeze-dried to obtain the protein powder. The total nitrogen content was determined by the Kjeldahl method (AOAC 2001.11).

2.2. Enzymatic hydrolysis

The white protein fraction was hydrolysed with Alcalase® 2.4 L (EC 3.4.21.62, endoprotease from *Bacillus licheniformis*, 2.4 Anson Units (AU)/g; Sigma Aldrich, St. Louis, USA) in a stirred batch reactor at pH 8 and 55 °C. The protease was added to 200 mL of the reaction mixture at different enzyme-to-substrate ratios (E/S = 0.5-10 wt%). The degree of hydrolysis (*DH*), defined as the percentage of peptide bonds cleaved, was calculated based on base consumption using the pH-stat method (*Knežević-Jugović et al*., 2022).

2.3. Evaluation of leaf protein interfacial properties

Emulsifying properties were analysed turbidimetrically (*Moure et al.*, 2002). Oil-in-water emulsions (1 wt.% protein) were formulated with sunflower oil in a 3:1 volume ratio and homogenised at 9500 rpm for 1 min. The absorbance of the 100-fold diluted emulsions in 0.05 M phosphate buffer (pH 7, 0.1% SDS) was measured at 500 nm. The emulsion activity index (*EAI*) was calculated as follows:

$$EAI\ \left(\frac{m^2}{g}\right) = \frac{2 \cdot T \cdot A_0 \cdot d_f}{\theta \cdot c \cdot 10{,}000} \qquad (1)$$

where T is turbidity (2.303 × 1 cm^2); A, absorbance; θ, oil phase volume fraction; c, protein mass

per unit volume of aqueous phase (g); and d_f = 100, the dilution factor.

For determining emulsion stability index (*ESI*), the prepared emulsions were kept for 10 min at 20 °C and analysed as previously described. *ESI* was calculated using the formula:

$$ESI\ (h) = \frac{A_0}{(A_0 - A_t) \cdot t} \qquad (2)$$

where t represents the time interval observed for emulsion stability.

Surface and interfacial tension were measured using a Krüss K100 tensiometer (Wilhelmy plate method) to assess protein adsorption and protein-lipid interactions at the interface. Interfacial tension was determined between the aqueous protein solution and sunflower oil, while surface tension was monitored at the air-water interface. A platinum Wilhelmy plate was immersed 3 mm into 10 g of the aqueous phase, followed by gentle addition of 25 g of the oil phase. Measurements were conducted after 10 min of stabilisation.

2.4. Determination of protein solubility at various pH

Protein samples were dispersed in water and stirred for 30 min. The pH was adjusted to 2, 4, 6, 8, and 10 using 0.2 M NaOH or HCl, then diluted to a protein concentration of 0.5 mg/cm^3, and stirred (250 rpm, 30 min). After centrifugation (12,000 rpm, 10 min), supernatants were collected. Solubility was expressed as the percentage of soluble protein relative to the initial concentration. Protein content was determined by the modified Lowry method (*Hartee*, 1972).

2.5. Determination of antioxidant activity of leaf protein and hydrolysates

The antioxidant capacity was evaluated through ABTS radical scavenging and ferrous ion chelating assays, as described (*Knežević-Jugović et al*., 2022). Lyophilised powders were dissolved in deionized water to a concentration of 2 mg/cm^3. For the ABTS assay, 0.01 cm^3 of the protein solution was mixed with 1 cm^3 of freshly prepared $ABTS^+$ radical solution, incubated for 5 min in darkness, and absorbance measured at 734 nm.

For the metal-chelating assay, 0.2 cm^3 of the protein solution was mixed with 0.8 cm^3 of water and 0.10 cm^3 of 2 mM iron (II) chloride, incubated for 3 min, followed by addition of 0.20 cm^3 of 5 mM ferrozine and a further 10-min incubation. Absorbance was measured at 562 nm.

2.6. Statistical analysis

All measurements were performed in triplicate, and results are expressed as mean ± SD. Statistical analysis was performed using OriginPro 9.0, and differences between means were evaluated by one-way ANOVA followed by Tukey's test at the 5% significance level.

2. Results and discussion

3.1. Enzymatic hydrolysis of the white protein fraction

The green juice was separated into two protein fractions after thermocoagulation and centrifugation: a water-soluble (white protein), and a water-insoluble (green protein) fraction. The Sankey chart illustrating the protein flow and fraction distribution during the protein isolation is shown in Fig. 1.

The lyophilised pumpkin leaves contained 28.63±1.54% protein (dry weight basis), which is comparable or slightly higher than that of other leaf sources like spinach (28%) or sugar beet leaves (19.4% to 24.02±0.18%) (*Akyüz et al.*, 2021). The protein recovery yield was 10.20% (total yield of 2.15%), similar to other plant sources (*Liese et al*., 2023), but considerably lower than that reported for duckweed (*Nieuwland et al.*, 2021). The obtained white protein fraction contained 94.43% dry matter, mainly consisting of soluble RuBisCO protein with 84.45% protein on a dry mass basis. This high protein contents confirms the efficiency of the isolation method. Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC.4.1.1.29; RuBisCO) is an enzyme that plays a vital role in photosynthesis, representing the major soluble protein fraction in leaves, accounting for 80–90% of total protein. In higher plants, it predominantly occurs as form I, composed of eight large (L, 52 kDa) and eight small (S, 14-15 kDa) subunits, designated as the L8S8 structure. Although RuBisCO has been reported to possess good gelation and emulsifying properties (*Martin et al.*, 2019; *Gracio et al*. 2023), our preliminary results indicated that its solubility and emulsifying capacity were insufficient for direct implementation in food products. Therefore, the protein was subjected to enzymatic hydrolysis.

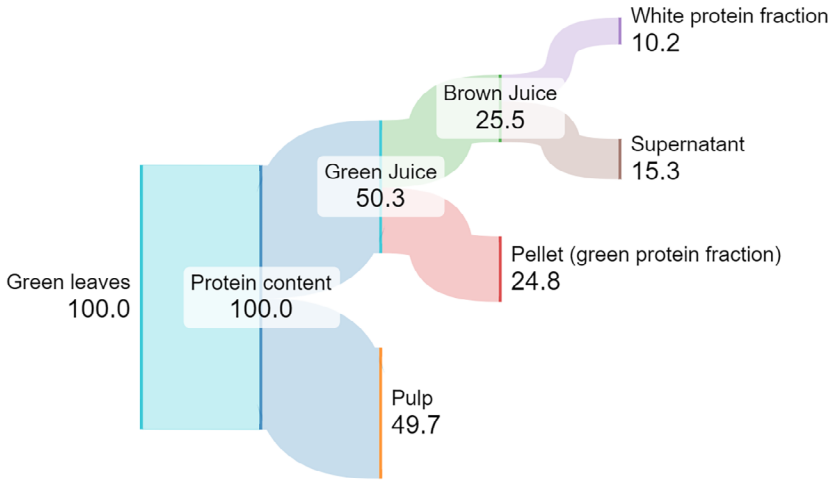

Figure 1. The Sankey chart illustrating the protein flow and fraction distribution during the protein isolation

Enzymatic hydrolysis is a key method for producing protein hydrolysates and peptides, offering precise process control, high specificity, and solvent-free, non-toxic products. Besides improved functional properties, the obtained hydrolysates may also exhibit antioxidant activity due to the release of cryptic bioactive peptides from the parent protein chain. The kinetics of enzymatic hydrolysis of the white protein fraction at *E*/*S* ratio ranging from 0.5 to 10 wt% is presented in Fig. 2. The antioxidant activity of the obtained hydrolysates was also examined to select the optimal degree of hydrolysis. This property is particularly relevant for meat products, as oxidative changes that occur during storage or as a result of microbial contamination reduce the quality, safety and shelf life of products. In this context, the use of antioxidants as food additives is a common approach in the meat industry. However, consumers' increasing concern over synthetic additives like butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT), due to their potential health risks, has driven the search for natural alternatives.

The hydrolysis curves indicate a high enzyme specificity toward the leaf protein, as a relatively high degree of hydrolysis (6.3-16.7%) was achieved, depending on the *E*/*S* ratio. The used enzyme, Alcalase, is known for its endopeptidase activity, meaning that it attacks peptide bonds within the interior of the molecule, randomly producing polypeptides of lower molecular mass. It preferentially cleaves peptide bonds between hydrophobic and aromatic amino acid residues (*Jovanović et al.*, 2018). The results revealed that with an increasing amount of enzyme (*E*/*S* ratio), the initial reaction rate also increased proportionally up to 0.28 mAU, and continued to rise further to 0.33 mAU, but not in a linear manner. The release of soluble proteins increased with reaction time and correlated with the degree of hydrolysis, reaching a plateau after around 120 min. The curve of initial reaction rate obtained from the tangents to the hydrolysis curves, showed a maximum, indicating enzyme saturation, thus *E*/*S* ratios of 6.5% and 8% were optimal, with 6.5% selected as the most efficient and economical.

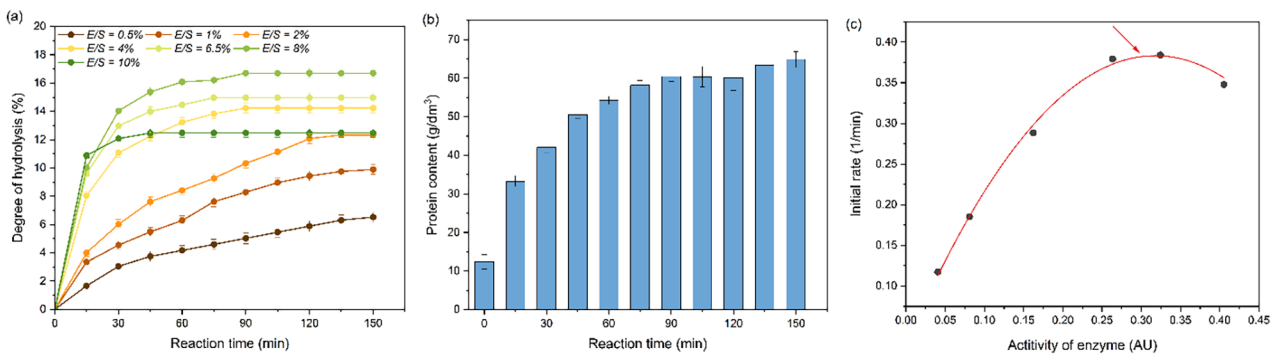

Figure 1. Time course of enzymatic hydrolysis at different *E*/*S* ratios: (a) degree of hydrolysis and (b) protein content as a function of reaction time, and (c) initial reaction rate (*V*o) as a function of enzyme activity (AU).

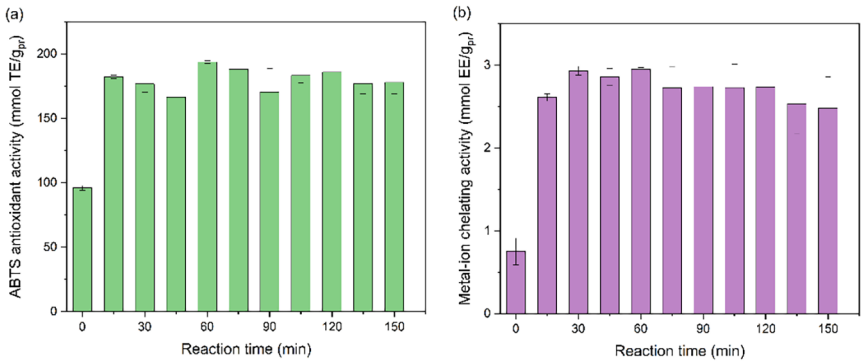

Figure 3. Antioxidant activity of leaf protein hydrolysates determined by (a) ABTS method, and (b) Methal-chelating ion method

These findings agree well with the antioxidant activity results obtained by both methods (Fig. 3). All hydrolysates obtained at different degrees of hydrolysis showed higher ABTS and metal ion chelating activity compared to the initial leaf protein. The antioxidant activity was closely related to the degree and extent of hydrolysis, as prolonged hydrolysis generated shorter peptide fragments capable of electron transfer (*Cotabarren* et al., 2019). The ABTS activity increased significantly after only 15 min of hydrolysis and remained stable up to 150 min. The hydrolysates obtained after 75 min showed the highest chelating activity compared to the native protein. However, no significant differences among the hydrolysates were observed in their metal ion chelating values during the first 120 min of hydrolysis, whereas the values decreased significantly thereafter. Based on these results, the hydrolysate obtained after 75 min and *E*/*S* = 6.5% was selected for further analysis of functional properties.

3.2. Functional properties of leaf protein hydrolysates

Compounds with strong antioxidant activity can extend the shelf life of meat products, enhance food safety and enrich them with bioactive ingredients. To be applied in meat formulations, hydrolysates must exhibit favourable solubility, emulsifying and related functional properties. Solubility is the key parameter influencing all other functionalities, including emulsification. The solubility results of the white protein fraction before and after enzymatic hydrolysis are presented in Fig. 4, while emulsifying and interfacial properties are summarised in Table 1.

It appeared that solubility increased significantly at pH 4-12 compared to the native protein. This enhancement may result from the reduction in molecular weight and the increase in polar groups caused by enzymatic hydrolysis, leading to the formation of smaller, more hydrophilic and soluble oligopeptide units. Since the isoelectric point of pumpkin leaf protein is around 4.6, the observed improvement in solubility at acidic pH values is particularly important, as many meat products have pH levels in this range. Furthermore, the obtained hydrolysate exhibited improved emulsifying properties, in terms of both *EAI* and *ESI*, compared to the intact leaf protein. Although these values are still lower than those achieved with soy protein concentrate (*Jovanovic et al*., 2018), the *EAI* values cannot be directly compared, as different protein concentrations were used in the experiments. The improved emulsifying properties of the hydrolysates correlated well with increased surface activity and reduced interfacial tension, indicating that smaller peptides were able to diffuse rapidly toward the interface. Overall, the enzymatic hydrolysis enhanced both solubility and interfacial activity, confirming its potential for application in meat emulsion system.

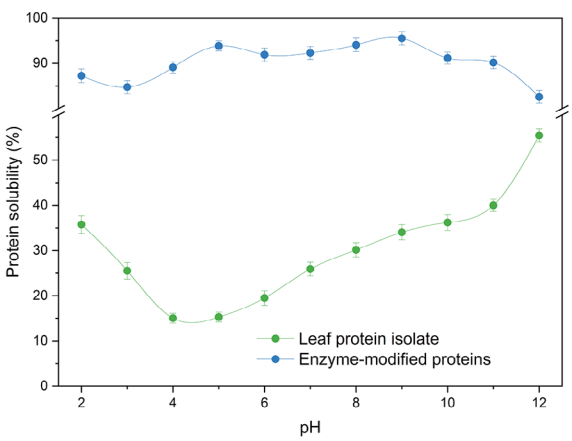

Figure 4. Protein solubility as a function of pH for leaf protein isolate and its enzymatic hydrolysate

Table 1. Emulsifying capacity and interfacial properties of leaf protein isolate and its enzymatic hydrolysate at two protein concentrations

Samples	*c*, mg/ml	*EAI*, m^2/g	*ESI*, min	Surface tension, mN/m	Interfacial tension, mN/m
Leaf protein-white fraction	1	12.95 ± 0.44	24.90 ± 3.45	23.7 ± 0.11	11.6 ± 0.02
	5	15.22 ± 0.52	26.40 ± 4.25	1.6 ± 0.33	5.8 ± 0.07
Enzymatically modified leaf protein	1	60.98 ± 0.69	28.44 ± 2.52	29.0 ± 0.09	10.7 ± 0.04
	5	64.85 ± 1.87	36.30 ± 3.04	29.0 ± 0.08	10.3 ± 0.13

3. Conclusion

Green biomass, such as waste pumpkin leaves generated after oilseed processing, has enormous potential as an alternative protein source. Enzymatic hydrolysis of the isolated white protein improved its solubility, interfacial functionality and antioxidant activity. The hydrolysate produced under optimal conditions (*E*/*S* ratio = 6.5%; DH ~ 15%; time 75 min) demonstrated suitability as a natural emulsifier and antioxidant, supporting its use as a value-added ingredient in functional meat products within a sustainable biorefinery concept.

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Original Scientific Paper

Effect of marbling on pork nutritional value

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ABSTRACT

In the context of the dilemma between tasty and healthy food, obtaining and analyzing information on the nutrients composition of commercial marbled pork make significant interest from the point of view of the right choice of a long-term strategy for the pork producers. This study considers the influence of marbling degree of commercial pork on the nutritional parameters, fatty acid composition, content of selenium, zinc, iron, copper, and vitamins, B1, B2, B5, B6, B12, B3, and D3. It was shown that a high degree of marbling affects only the energy value, producing no effect on the protein content in the meat. The increase of fat content depending on the pork marbling degree was noted, and a correlation between the degree of marbling and the ratio of omega-3:omega-6 fatty acids was found, which proves the high biological value of marbled meat and allows consideration of its possible use in manufacturing functional food products. Analysis of the vitamin and mineral composition of the marbled meat showed that the degree of pork marbling does not affect their quantitative content in the meat.

1. Introduction

Nowadays, pork is the second most commonly consumed meat all over the world (*OECD-FAO Agricultural Outlook*, 2021). In the future, the level of pork consumption will depend on the degree to which the consumers' expectations are met, and it may change over time with the emergence of new trends in human nutrition, social and economic considerations as well as social and cultural factors (*Chernukha et al*., 2023; *Vitale et al*., 2020). In this regard, large-scale pork producers (agricultural holdings) are interested in the correct choice of long-term strategies to increase the market competitiveness of pork, and thus, maintain a high level of its consumption in various countries.

It is known that marbling significantly improves such sensory characteristics of meat as juiciness, tenderness, aroma, taste perception, so pork marbling can be attractive for consumers (*Simunović et al*., 2024). Accordingly, the production of marbled pork can provide certain market competitive advantages for the pork producers. However, pork marbling is associated with an increased content of intramuscular fat, including visible fat. With the rapidly growing desire in society for healthy eating, plus the global trends for reducing fat consumption and the overall caloric content of the diet, the long-term trend for marbled pork consumption is now in doubt. Meat marbling can lead to a deterioration of the pork nutrient profile, and, accordingly, consumers can refuse to buy such meat, instead favouring

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less fatty meats, including those recommended by nutritionists.

In Russia, according to official statistics, in recent years the number of pigs and their commercial husbandry have grown significantly, which has led to the country's complete self-sufficiency in pork meat supply, with no imported pork. On the contrary, in recent years, agricultural holdings have been actively increasing pork exports, considering new strategies for expanding the range of pork and satisfying consumer demand in the domestic and foreign markets (*Kovalev*, 2023). In the context of the dilemma between tasty and healthy food, obtaining and analyzing information on the nutrient composition of commercial marbled pork makes sense in terms of choosing the right long-term strategy for the pork producers.

The purpose of the work was to study selected nutritional parameters, i.e., fatty acid composition, selenium, zinc, iron, and copper content, and vitamins, B1, B2, B5, B6, B12, B3, and D3, depending on the degree of marbling of the commercial pork.

2. Materials and methods

The studied meat was musculus L. dorsi with varying degrees of marbling, samples of which were obtained from pigs aged 185-195 days as a result of slaughter at an commercial enterprise (AGROEKO-YUG LLC) located in Voronezh region of the Russian Federation.

Twenty-four hours after slaughter, the chilled pork carcasses were selected by the company's employees, who were specially trained to assess the degree of marbling using a visual scale of five marbling categories. Three categories were selected, i.e., the maximum, the medium and the minimum levels of marbling. The back-rib cut excised from the selected carcasses, deboned and vacuum-packed. Cuts were then delivered to the laboratory for testing.

To ensure greater variability in the composition of the meat cuts, those with the maximum degree of marbling (group 1) were taken from the carcasses obtained by slaughtering a herd of Duroc pigs that had been fed with additional carbohydrate nutrition for 51 days. The rest of the meat cuts were selected from the carcasses obtained by slaughtering hybrid pigs that were fed with a standard diet; these hybrids were typically used in the commercial production of pork at the enterprise. The cuts obtained from the hybrid pigs were visually classified by the degree of marbling as having the medium degree of marbling (group 2) or the minimum degree of marbling (group 3).

Thus, each group of meat cuts comprised three samples of m. L. dorsi, categorized into the relevant group according to the degree of marbling (9 samples, in total).

From the middle part of m. L. dorsi, three 2 cm thick steaks were cut out, their cross-sections were photographed and the steaks were used to prepare an average sample sent for the chemical analysis.

To objectively assess the marbling, the photographs of the steak were processed using an image analyzer. The amount of visible intramuscular fat was determined in the software ImageJ (https://imagej.net/download.html) using photographs of the "loin eye" (m. L. dorsi). The number of fat inclusion dots was calculated as a percentage of the area of the entire loin eye.

The nutritional value parameters were determined using standard methods accepted in Russia and relevant to general laboratory practice: mass fraction of protein was determined according to *GOST 25011* (2017); mass fraction of fat – according to *GOST 23042* (2015); fatty acid composition – according to *GOST 31663* (2012); mass fraction of zinc, copper according to *GOST 30178* (1996); mass fraction of selenium – according to *GOST 31707* (2012); mass fraction of vitamins – according to *GOST 323072* (2013), *GOST R 55482* (2013).

The analyses were repeated three times. The data were processed by analysis of variance (variation statistics) using the Microsoft Excel 2010 software, as well as using the Statistica 10.0 software. The results were compared using one-way analysis of variance (ANOVA) and the Tukey-Kramer test. The differences were considered reliable and the correlation between the parameters was considered, when the probability level did not exceed 0.05.

3. Results and discussion

The amount of visible intramuscular fat in the different groups of m. L. dorsi was rated in the laboratory environment with the help of an image analyzer (Table 1).

The content of the visible intramuscular fat differed significantly between the groups ($p<0.05$), which generally proved the correctness of the sample selection by the company's specialists. Meanwhile, the cuts with the maximum degree of marbling (group 1) exceeded the degree of marbling in

groups 2 and 3 by 2.5 and 4.9 times, respectively, i.e., the ratio of marbling in groups 1, 2, and 3 was 1.0:0.4:0.2. This supports the conclusion that in the future it should be possible to improve the marbling assessment and increase its objectivity by switching to an instrumental assessment method.

Table 1. Visible intramuscular fat content in m. L. dorsi

Parameter	Groups of meat cuts selected according to the degree of marbling		
	Group 1	Group 2	Group 3
Representative photo of the loin eye (m. L. dorsi)			
Content of the visible intramuscular fat (%, mean ± SEM)	13.56±1.19	5.52±0.5	2.79±0.94

The study of the main parameters of the cuts' nutritional value (Figure 1) showed that all three groups featured a high level of protein, and there were no statistically significant differences in this parameter. At the same time, in terms of fat content, the marbling groups differed reliably ($p<0.05$). In terms of fat content, the ratio of groups 1, 2 and 3 was 1.0:0.6:0.5. This means the difference in total fat between the groups was less than in visible fat, although in general the values of these parameters correlated.

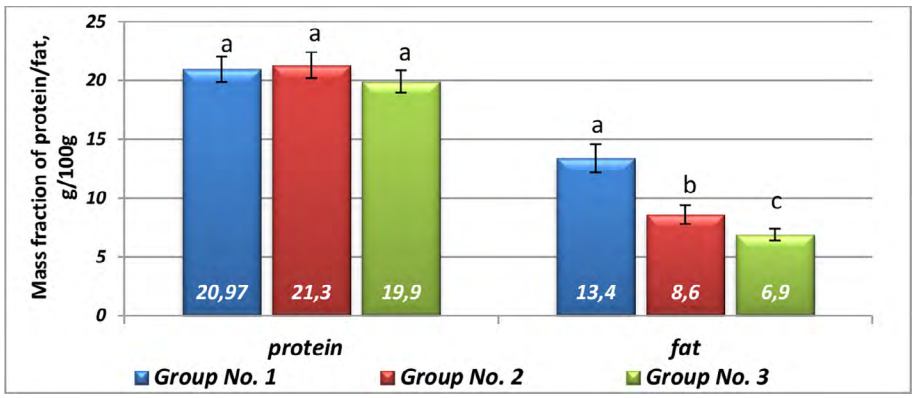

Figure 1. Protein and fat content in m. L. dorsi depending on the marbling degree. *a, b, c - values without a common superscript letter differ ($p < 0.05$).*

The energy value (caloric content) of the cuts with different degrees of marbling also showed significant differences. Thus, the caloric content of the samples from group 1 was 204.5±19.7 kcal, group 2 – 162.6±11.6 kcal and group 3 – 141.7±8.3 kcal. Meanwhile the difference in caloric ratios between the groups was even less than for the total fat content (1.0:0.8:0.7).

Thus, pork with the highest degree of marbling, due to its higher fat content, featured the highest energy value. However, an increase in marbling did not cause proportional changes in the fat content and caloric value. At the same time, a high degree of marbling did not affect the protein content of the meat.

Analysis of effect on the marbling degree on the fatty acid composition in the meat cuts (Table 2) showed that the samples of group 3 with the least degree of marbling had the lowest values of polyunsaturated fatty acid content, including the omega-3 and omega-6 acids families.

In terms of the content and ratio of saturated, monounsaturated and polyunsaturated fatty acids, which characterize the balance of the fatty acid composition from the point of view of healthy nutrition (*Briggs et al*., 2017), the best parameters were found in the meat cuts with the maximum and the medium degrees of marbling. The higher content of PUFA in these cuts was provided mainly by linoleic (omega-6)

and linolenic (omega-3) fatty acids. Meanwhile, in terms of the ratio of polyunsaturated fatty acid content, i.e., the ratio of the omega-3 to omega-6 acid families, only the group 1 cuts featured a positive difference—in contrast to the cuts in groups 2 and 3—and matched the standards of physiological needs of the human body established in Russia (*Methodological recommendations MR*, 2021). According to many researchers, a high PUFA content, including the omega-3 family, provides beneficial effects on consumers' health (*Therkildsen et al*., 2021; *Aboagye et al*., 2020).

Table 2. Fatty acid composition of m. L. dorsi depending on the degree of marbling

Parameter	Group 1	Group 2	Group 3
Myristic C14:00	1.40 ±0.37 [a]	-	-
Palmitic 16:0	24. 55 ±2.95 [a]	25.00±3.00 [a]	26.20±3.10 [a]
Palmitoleic C16:1	2.30 ±0.57 [a]	1.80±0.40 [ab]	1.50±0.40 [b]
Margaric C17:0	0.40 ±0.08 [a]	-	-
Heptadecenoic C17:1	0.40 ±0.09 [a]	-	-
Stearic C18:0	11.30 ±1.36 [a]	13.90 ±1.70 [b]	15.00 ±1.80 [b]
Oleic C18:1	39. 25 ±4.72 [a]	38.40 ±4.60 [a]	40.30 ±4.80 [a]
Linoleic C18:2ω6	16.60 ±1.99 [a]	17.30 ±2.10 [a]	13.90 ±1.70 [b]
Linolenic C18:3ω3	2.00 ±0.49 [a]	1.60 ±0.40 [a]	1.00 ±0.30 [b]
Arachidic C20:0	0.40 ±0.12 [a]	0.40 ±0.10 [a]	0.40 ±0.10 [a]
Eicosadienoic C20:2ω6	0.70 ±0.18 [a]	0.80 ±0.20 [a]	0.70 ±0.20 [a]
Gondoinic C20:1ω9	0.70 ±0.17 [a]	0.80 ±0.20 [ab]	1.00 ±0.20 [b]
∑SFA	38.0 5 ±4.88 [a]	39.30±4.80 [a]	41.60±5.00 [a]
∑ MUFA	42. 65 ±5.55 [a]	41.00±5.20 [a]	42.80±5.40 [a]
∑ PUFA	19.30±2.66 [a]	19.70±2.70 [a]	15.60±2.20 [b]
SFA:MUFA:PUFA	2.0:2.2:1	2.0:2.1:1	2.7:2.7:1
∑ ω3	2.00±0.49 [a]	1.60±0.40 [a]	1.00±0.30 [b]
∑ ω6	17.30±2.17 [a]	18.10±2.30 [a]	14.60±1.90 [b]
ω3: ω6	1.0:8.6	1.0:11.3	1.0:14.6

a, b - values in rows without common superscript letter differ ($p<0.05$).

It is necessary to note that the fatty acid composition also depends on the pig's fattening, the farming conditions, and genetic characteristics of the pig's breed, which can also have some effect on the pig's lipogenesis (*Yashin et al*., 2020). However, the effect of marbling on a higher content of monounsaturated and polyunsaturated fatty acids, including omega-6 and omega-3 groups, is also underlined in the other studies (*Joo et al*., 2017; *Shlykov*, 2016).

Despite the important role of meat in providing some vitamins and minerals, the studies on the effects of marbling have generally been limited to fatty acid composition. In this regard, the results on vitamin and mineral content (Table 3), obtained by us, are of additional interest.

As is visible from Table 3, the studies conducted did not reveal a correlation between the degree of marbling of pork muscle tissue and the quantitative content of vitamins and minerals in the meat. According to the data obtained, among the three groups, the greatest potential for healthy nutrition was found for the group 1 cuts that had the maximum degree of marbling, in which a high content (more than 30% of the daily requirement) of selenium and vitamins B6, B3 and D3 was noted. In the cuts of groups 2 and 3, a high content of selenium was noted, as well as vitamin B6 (group 2) and vitamin B3 (group 3), the content of which was possibly related to the additives in the diet of the Duroc variety pigs.

In modern literature databases, most research devoted to the analysis of trace elements accumulation in muscles have found no variations at the level of genetic manipulations. Thus, a study (*Tomović et al*., 2011) conducted in Serbia showed that the content of minerals in pork muscle tissue (m. Semimembranosus), obtained from two purebred pigs

Table 3. Content of vitamins and minerals in m. L. dorsi depending on the marbling degree

Parameter	Group 1	Group 2	Group 3
Selenium, mg	0.035±0.011 [a]	0.036±0.011 [a]	0.028±0.008 [b]
Zinc, mg	2.353±0.479 [a]	1.734±0.369 [b]	2,220±0,455 [a]
Iron, mg	0.989±0.375 [a]	0.705±0.336 [b]	0.750±0.342 [b]
Copper, mg	0.090±0.029 [a]	0.025±0.017 [b]	0.022±0.017 [b]
Vitamin B1, mg	0.15±0.03 [a]	0.14±0.03 [a]	0.10±0.02 [b]
Vitamin B2, mg	0.02±0.007 [a]	0.14±0.06 [b]	0.04±0.01 1 [c]
Vitamin B5, mg	0.50±0.10 [a]	0.66±0.15 [b]	0.47±0.09 [a]
Vitamin B6, mg	0.72±0.18 [a]	0.64±0.16 [a]	0.41±0.10 [b]
Vitamin B12, µg	LESS THAN 1.00	LESS THAN 1.00	LESS THAN 1.00
Vitamin B3, mg	17.00±3.40 [a]	5.33±1.07 [b]	16.40±3.28 [a]
Vitamin D3, µg	1.50±0.40 [a]	LESS THAN 1.00	LESS THAN 1.00

a, b, c - values in rows without common superscript letter differ ($p < 0.05$).

and eight crossbred pigs, did not depend on genetic lines. It was noted that the fortification of meat with selenium and copper can be achieved by regulating and enriching the feed for the pigs with these elements. It was also noted that the copper content in meat obtained from different pig breeds varied within the same range from 0.03 to 1.40 mg/100 g of product (*Kurbanova et al*., 2023).

4. Conclusion

Research into the effect of marbling on the nutritional value of m. L. dorsi of pigs did not reveal any statistically significant differences in the protein content in the analyzed cuts that had various degrees of marbling. An increase in fat content was noted directly depending on the degree of pork marbling, and a correlation was found between the degree of marbling and the ratio of omega-3:omega-6 fatty acids. In the meat cuts with the maximum degree of marbling, optimal values of this parameter were noted, and which correspond to the norms of physiological needs of the human body established in Russia (*Methodological recommendations MR*, 2021). The analysis of the vitamin and mineral composition of the meat cuts showed that the level of marbling of pork does not affect their quantitative content in the meat.

In general, the results obtained confirm the data on the high content of individual vitamins and minerals in pork. In addition, despite the increase in the quantitative content of fat depending on the meat marbling degree, its biological value also increases. The use of this marbled raw meat, including for the production of functional food and specialized nutrition, will provide not only appealing taste characteristics, but also will ensure high quality of the food products in terms of their nutritional and biological value. This will help in solving the issue of eliminating health problems associated with the consumption of fatty pork. Thus, the choice of a strategy for marbled pork consumption from the point of view of its assessed nutritional value should not be in conflict with the trend for healthy eating.

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Original Scientific Paper

Fat and fatty acid content as indicators of declared origin and composition of fermented sausage

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ABSTRACT

An attempt to confirm the declared type of meat from which fermented sausages were manufactured was made. The fat content of the sausages was determined by reference method, and the fatty acid composition of fat was analyzed by gas chromatography. The obtained data were used for statistical processing and confirmation of the declared animal origin of the meat. Also, the data obtained by the above methods were used to verify the compliance of the declared composition. Fermented sausages were obtained from retail, manufacturers and importers in the period 2021-2025, and the research included 189 samples, made with pork meat (n= 151), beef (n=15) and mixed pork and beef (n=23). The fat amount in the sausages varied from 23.70 to 54.10% in pork sausages, 34.80 to 46.00% in beef sausages and 34.80 to 49.30% in mixed pork & beef sausages. Fatty acid content ranges were similar to the reported results for the same products. Principal component analysis provided significant information about origin of raw materials used to make fermented sausages.

1. Introduction

Fermented sausages are highly treasured traditional foods that are widely consumed in many countries of the world, especially in Mediterranean, Middle East and Southeast European countries (*Stajic et al.*, 2013, *Paulos et al.*, 2015). A large number of distinct sausages with widely differing properties are produced from various animals, using different recipes and processing procedures often lacking uniformity (*Pleadin et al.*, 2020). Moreover, the sausages are prepared by mincing animal, one or more species, lean and fatty parts, to which are added spices, additives and permitted condiments. Sausages undergo a drying process which is accompanied by a maturation (temperature ranging from 15 to 25°C, relative humidity in the 65-80 % range during the fermentation process) where some biochemical reactions occur that induce the development of particular organoleptic properties of sausages (*Pavli et al.*, 2020). The world consumption of sausages was estimated to 19.58 million kg by 2019 and is expected to continue growing by 3.3 % per year (*Zhu et al.*, 2020). Average annual production of sausages in Serbia in 2023 was 73803 tons (*Statistical Yearbook*, 2024). Nowadays, the demand for these types of products is rising globally. Traditional recipes used for the

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production of traditional fermented sausages greatly differ, not only across production regions, but across producing households as well (*Lešić et al*., 2020).

The sausages are a complex food matrix, composed mainly of water, proteins, lipids, carbohydrates and spices, so therefore, microbial and chemical alterations can occur during their storage (*Tirado-Gallegos et al*., 2021). The high lipid content and packaging in oxygen semi-permeable materials lead to the main deterioration of the sausage lipids by oxidation (not the microbiota), which impacts the food's nutritional and sensory properties *(Bolívar-Monsalve et al.*, 2019). Fats are important from the physiological point of view because they contain a number of important nutrients, like proteins, liposoluble vitamins and essential fatty acids, and are important source of energy.

Nowadays, consumers are increasingly concerned about the link between diet and health in terms of the quality of foodstuffs offered by the food industry, in particular meat and meat products, which have nutritional high value. However, over recent years, fermented sausage consumption has been associated with potential health hazards due to their high contents of fat, saturated fats, high NaCl content, the presence of nitrite and its degradation products such as nitrosamines, and the use of smoking which can lead to the formation of toxic compounds, e.g., polycyclic aromatic hydrocarbons, in the products. The high levels of fat and salt used to process meat products are known to be associated with the development of obesity, cancer, heart related disease and high blood pressure, respectively (*Brewer*, 2012, *World Health Organization*, 2003, *Alshahrani et al*., 2019, *Virtanen et al.*, 2019). Hazards can also be both of direct microbiological nature, the sausages potentially being contaminated with food pathogens, and of indirect microbiological nature by metabolic activity of microorganisms causing presence of biogenic amines and mycotoxins (*Holck et al*., 2017).

According to national regulation on food labelling (*Official Gazette of the Republic of Serbia*, 2024), as well as EU regulation No 1169/2011 (*EU Regulation*, 2011), it is obligatory for nutrition labels to contain information on the presence of energy and specified nutrients, like fat content, saturated fatty acids (SFA), carbohydrates, sugars, proteins and salt. Part B, Annex 12 of the national regulation, and Annex XIII, Part B of the EU regulation, have defined the reference energy and nutrient intakes as 8400 kJ/2000 kcal, and 70 g of total fat per day.

In this study, an attempt was made to confirm the declared type (species) of meat from which the tested fermented dry sausages were produced. The fat content of the sausages was determined and the fatty acid (FA) composition of that fat was analysed (saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA)). The data obtained by these methods of chemical composition analysis were used as a basis for statistical processing and confirmation of the declared animal origin of the meat from which the sausages were made. Also, the data obtained by the above methods were used to verify the compliance of the declared composition.

2. Materials and methods

2.1. Samples

Fermented sausages were part of regular control of food quality and safety parameters, obtained from retail and from producers and importers in the period 2021-2025. The research included 189 fermented sausage samples, made with pork meat (n=151), beef (n=15) and mixed pork & beef (n=23).

2.2. Analytical procedure

Fat content determination was performed according to *ISO 1444*,1996. Briefly, homogenised sausages were dried, and afterwards, dried homogenates were subjected to Soxhlet extraction using n-hexane. Extracted components in n-hexane were dried and measured, based on which, the free fat contents were calculated.

FA determination was performed by gas chromatography according to *ISO 5509* (2000). Fat extract obtained in the previous step by ISO 1444 (1996) was converted to FA methyl esters (FAMEs) by using 0.25 mol·l-1 trimethylsulfonium hydroxide (TMSH) in methanol (*ISO 5509*, 2000), as described by *Spirić et al*., 2010. FAMEs were determined by capillary gas chromatography on a Shimadzu 2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a capillary HP-88 column 100 m × 0.25 mm × 0.20 μm (J&W Scientific, Folsom, California, USA). The chromatographic peaks in the samples were identified by comparing relative retention times of FAME peaks with peaks in Supelco 37 Component FAME mix standard (Supelco, Bellefonte, Pennsylvania, USA).

2.3. Statistical analysis

Principal component (PCA) analysis of fat and FA content of fermented sausages were performed using the JMP Statistical Discovery 10 software (SAS Institute, Cary, North Carolina, USA). Descriptive statistics and preparation of data for multivariate statistical analysis were performed in MS Office 2016 Excel.

2. Results and discussion

Table 1 presents distribution intervals of fat and FA content (SFA, MUFA and PUFA) in the analysed fermented sausages. Table 2 presents distribution intervals of FA, expressed as % of total fatty acids. The number of noncompliant results according to the fat and SFA content as determined in the fermented sausages, and according to the allowed tolerances between analytically determined and declared values of these nutritional parameters, is shown in Table 3.

The amount of fat present in fermented sausages generally varied widely: from 23.70 to 54.10% in pork sausages, 34.80 to 46.00% in beef sausages and 34.80 to 49.30% in pork & beef sausages. Such variations can be attributed to the differences in the amount of added backfat and the choice of more or less fatty meat made by individual manufacturers (*Pleadin et al*., 2014). These presented results are similar to those obtained for fat content in fermented sausages of similar geographic origin (*Branković et al*., 2019, *Simunović et al*., 2021), but were considerably higher than results for three types of Portuguese fermented sausages, alheira, a certified brand with Protected Geographical Indication (*Teixeira et al*., 2020).

The fat content observed was characteristic for the origin of the raw materials, and differences could be attributed to the production processes, which often are region-specific and increase gradually throughout ripening as a result of water loss (*Lešić et al*., 2020; *Kovačević et al.*, 2014; *Soyer*, 2005; *Polumbo et al*, 1976).

Knowledge of the FA profile of the fat in fermented sausages is an important quality attribute. An unfavourable lipid profile of processed meat products is generally associated with harmful cholesterol levels and heart disease. The content of specific fatty acids plays an important role in prevention and treatment of many chronic disorders, especially cardiovascular diseases. The *World Health Organization* (2003) proposed optimal fat intake, which should be between 15-30% of the total energy intake, while the SFA intake should be up to 10%, PUFA intake between 6 and 10% (n-6: 5-8%, n-3: 1-2%), and an intake of less than 1% trans fatty acids.

Table 1. Fat and fatty acid content in fermented sausages

	PORK (g/100g)	BEEF (g/100g)	PORK & BEEF (g/100g)
FAT	23.70 - 54.10	34.80 – 46.00	34.80 - 49.30
SFA	9.10 - 21.42	16.32 - 23.76	13.73 - 18.10
MUFA	11.56 - 25.21	16.39 - 21.38	13.61 - 25.57
PUFA	1.89 – 12.00	0.60 - 3.82	0.07 - 8.08

Table 2. Fatty acid content, expressed as % of total fatty acids in fermented sausages

	PORK (%)	BEEF (%)	PORK & BEEF (%)
SFA	30.39 - 44.16	42.9 - 51.65	36.32 - 43.52
MUFA	39.28 - 54.27	45.78 - 53.39	36.78 - 58.37
PUFA	3.90 - 24.44	1.65 - 9.46	0.16 - 21.51

Table 3. Number of noncompliant fermented sausages

	PORK	BEEF	PORK & BEEF	TOTAL
FAT	8 (5.3 %)	2 (13.3 %)	0	10 (5.3 %)
SFA	7 (4.6 %)	2 (13.3 %)	0	9 (4.8 %)*

*Number in parenthesis denotes percent of samples per each group

Traditional fermented sausages, prepared from pork meat, were earlier reported to contain SFA from 39.30±1.86% to 43.63±3.04%, MUFA from 47.64±3.27% to 49.93±3.32% and PUFA from 10.72±2.85% to 10.92±2.07% as % of total fatty acids (*Vulić et al*., 2023). These data were similar to the current obtained results: 30.39% to 44.16% (SFA), 39.28% to 54.27% (MUFA), and with much wider range for PUFA of 3.90% to 24.44%. The greater variability ranges in our study were very likely a consequence of the considerable variety of fermented sausage types included in our study.

The quality of fermented sausages' raw material is influenced by animals' genotype, their keeping and feeding, and pre- and post-slaughter conditions. Technological processes also influence the properties of the final product (*Pleadin et al*., 2020). Particular significance is due to the processes of fat lipolysis, free fatty acid formation, and degradation and oxidation of short-chain fatty acids, since these key reactions, taking place during ripening, affect the formation of specific odour and taste of the final product (*Kozačinski et al*., 2006; *Miličević et al*., 2014; *Marušić et al*., 2014). Due to the application of various technological processes, the activity of technological microbiota, especially during the fermentation process and maturation of sausage, complex microbiological, physicochemical and biochemical changes take place in fundamental building materials (fats, proteins and carbohydrates), resulting in water loss and increase in dry matter weight (*Kovačević*, 2014).

Fat content was over the permitted tolerance value (declaration/analytically obtained) in eight samples of pork and two beef fermented sausages (5.3% and 13.3%, respectively per group), and SFA contents were beyond permitted tolerance in seven pork and two beef fermented sausages (4.6% and 13.3% respectively per group). Fermented sausages made from beef & pork meat mix were within permitted tolerances of declared values for contents of fat, SFA, MUFA and PUFA.

Principle component analysis results are shown in Figure 1. The first two principal components (PC1 and PC2) accounted for 94.2 % of the data total variance (68.1% and 26.1% respectively). The confidence ellipses (at a 95% confidence interval) showed complete separation of the data for pork & beef sausages. The pork & beef mixed sausages, in the middle, overlapped with both groups, which is consistent with the composition of these products.

From the PCA results, a high degree of correlation of fat, SFA and MUFA content can be observed (*Pećina et al*., 2018). PUFA content can be considered as the main factor for the separation of our sausage sample groups, while the other three factors were responsible for the variability of data within groups. Sausages declared as prepared from only beef or only pork were distinctly separated from each other, except

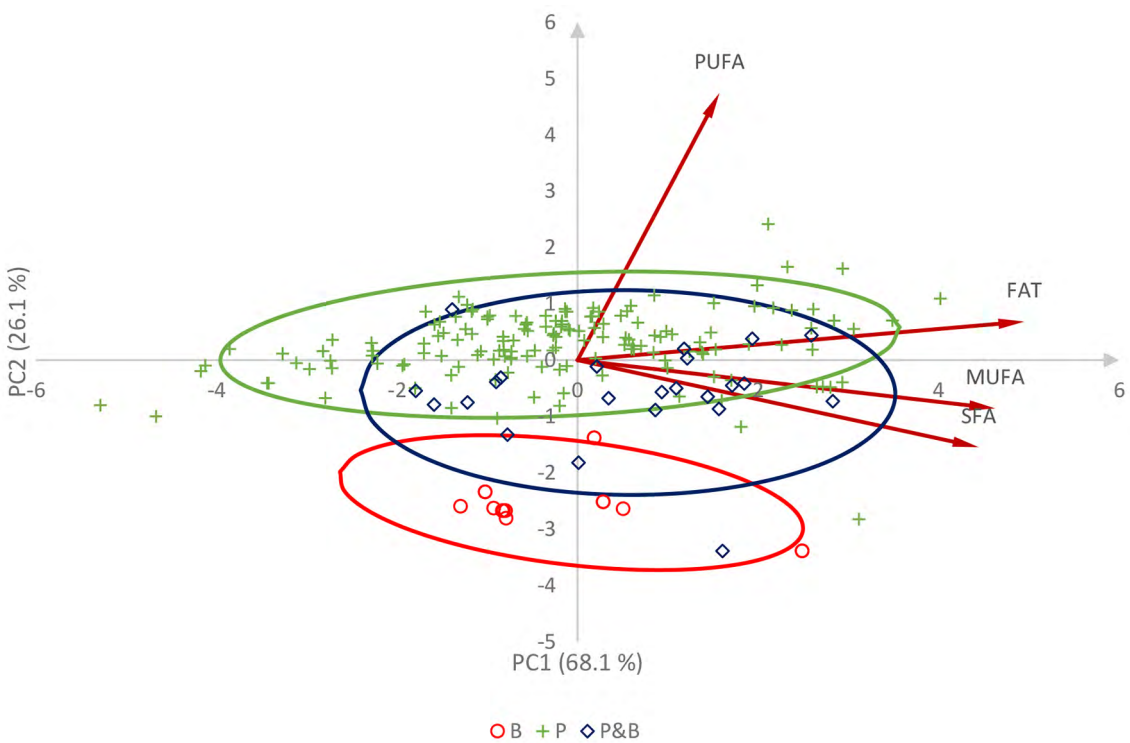

Figure 1. Potential effects of meat-derived BPs on food quality

two sausages, one of which was grouped as beef by our PCA, but was declared as pork only (green cross in lower right quadrant of Figure 1) and one mixed pork & beef (navy blue diamond in the same quadrant). These outlier results could be considered as misdeclaration by the manufacturer for the sausage that was declared as pork only, or, either misdeclaration by manufacturer, or erroneous homogenization of the sample in our laboratory during analysis, in the case of the mixed meat sausage. On the other hand, a sausage declared as made from a mix of pork & beef (navy blue diamond in the upper left part of Figure 1) obviously contained only pork fat, and hence, had a dubious declaration. Declared composition scrutiny based alone on analyses of ingredients cannot achieve such information. Therefore, the introduction of additional, statistical, analysis of the content of measured and declared components is necessary, both to detect economically motivated food fraud and to check the declared composition and nutritional value of products. This is the way to ensure implementation of legal regulative requests, improve the safety and quality of fermented sausages and protect the interests of all subjects in the chain, from manufacturers to retailers to consumers.

3. Conclusion

Along with protein and moisture, fat content is one of the most important quality parameters of dry fermented sausages. The results obtained show that the amount of fat and the FA content in fermented sausages varies widely, but these two parameters were similar to their previously reported contents for similar products. Differences in fat contents could be attributed to the differences in formulations and production processes, which often are region-specific, as well as the nature of the raw materials.

From the point of view of the declared contents of fat and FA, the present results show that fermented sausages sampled from the Serbian market, generally, follow the declared fat and SFA composition. Approximately 5% of all sausages were non-compliant regarding the declared content of both parameters. Fat content analysis showed that the permitted tolerance limit was transgressed in eight pork and two beef fermented sausages (5.3 % and 13.3 %, respectively per group). SFA contents were beyond the tolerance limit in seven pork and two beef fermented sausages (4.6 % and 13.3 % respectively per group). Fermented sausages made from mixed beef & pork meat were in compliance with declared composition of fat and SFA.

PCA results show a high degree of correlation of fat, SFA and MUFA content. PUFA content was main factor for the separation of sausage sample groups. Fat, SFA and MUFA contents were responsible for the variability of data within groups. PCA separated the beef and the pork sausages from each other, with some exceptions. Outlier results could be considered as misdeclaration or, either misdeclaration or errors in sample preparation in the case of a mixed meat sausage. One sausage sample declared as mixed pork & beef, after PCA, was clearly grouped in the pork fat group, and this indicates intentional misdeclaration. This information cannot be achieved without additional, statistical, analysis of the measured content and comparison with the declared components. Thus, it is necessary to implement complementary analytical procedures to detect food fraud, examine the declared composition and nutritional value of products, improve implementation of legal regulative requests, safety and quality of fermented sausages and protect the interests of all subjects in chain, from manufacturers to retailers to consumers.

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Original Scientific Paper

The concept of intelligent control system for mincing meat with specified dispersion

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ABSTRACT

The use of a digital twin (DT) in modelling the technological process of single-stage grinding of frozen meat in real time is described. The DT concept provides for the use of its "digital shadow". Due to the significant heterogeneity of meat, the parameters of chemical composition and dispersion are determined in ranges of values with fuzzy boundaries. Fuzzy control based on the theory of fuzzy sets was applied. The developed DT ensures the stabilization of a given degree of dispersion of the grinding meat and calculates the predicted particle size. The hardware design of the process involves its use in the operation of a continuous production line for meat products. The control system operates without operator involvement, which reduces the risks of producing low-quality products and minimizes possible economic losses.

1. Introduction

The food industry faces many challenges, including inefficient production systems. To cope with this, digital twins (DT) are used, which create a digital representation of physical objects by integrating real-time and real-world data into digital space (*Saranya & Subhashini*, 2023, *Tao et al*., 2018). The application of the DT concept with the development and digitalization of Industry 4.0 has increased significantly, as evidenced by the exponential growth of publications on this topic in recent years (*Cimino et al*., 2019, *Emmert-Streib,* 2023a). DT has been widely used in agriculture (*Verdouw & Kruize,* 2017); applications focus on plant growth control (*Skobelev et al*., 2020, *Turgay et al*., 2022, *Verdouw et al*., 2021), systems for growing plants in greenhouses and fields (*Ghandar et al*., 2021, *Jans-Singh et al*., 2020), and monitoring animal conditions (*Alves et al*., 2019). A number of authors note the successful use of DT at the stages of raw material processing or packaging, as well as in the food supply chain (*Defraeye et al*., 2019, Kampker *et al*., 2019, Shoji *et al*., 2022). *Vignali et al*. (2017) developed a DT pasteurizer taking into account the dynamic parameters and properties of a non-Newtonian fluid. *Gericke et al*. (2019), considered the creation of a DT to optimize a water bottling plant on a production line. *Koulouris et al.* (2021) presented a DT for modelling the production and planning process of a brewery, taken as an example.

The DT of the process of grinding meat (GM) on continuous production lines should contain a predictive computational model identified using data obtained as a result of measuring and calculating

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model parameters during the operation of technological equipment (*Ivashov et al*., 2018). This approach will allow for continuous forecasting and measures to be taken to prevent problems before they become a serious factor affecting the quality of the final product. The mechanism of fuzzy solutions using the results of the DT provides great opportunities for implementing optimization processes in a flexible production environment (*Amrita & Ashok,* 2024). The DT of the process provides continuous learning in the production system and self-regulation (*Emmert-Streib,* 2023b). Fuzzy mathematical models, developed on the basis of fuzzy set theory (*Azeem*, 2012), have proven useful in the management of biotechnological and food processes (*Birle et al*., 2013, *Mavani et al*., 2025).

Thus, the DT in food production provides data availability and advanced analytics in real time, helping to make more informed, balanced and rational decisions. The use of DT leads to a more effective risk assessment and mitigation strategies based on an "IF...THEN" analysis as a result of modelling. The purpose of this study was to develop a concept for the use of DT to control the technological process of single-stage grinding of frozen meat.

2. Materials and methods

Frozen meat blocks (beef, 70 × 75 × 300-400 mm, core temperature -8°C to -12°C) were chopped with a laboratory single-stage grinder with capacity of 400 kg/h (Fig. 1). Mathematical calculations and plotting were carried out in the MATLAB (Fuzzy Logic Toolbox) (MathWorks, USA, https://www.mathworks.com/) computing environment (*Eshkabilov*, 2022, *Xue & Pan*, 2024). Fuzzy interval analysis (FIA)—a hybrid method combining fuzzy logic and interval mathematics—was used.

3. Results

The virtual part of the DT—a virtual model—consists of two parts: digital master (DM) and digital shadow (DS). The DM contains comprehensive information about the object necessary for its manufacture and operation. The DS that a physical object "casts into virtual space" is a set of data obtained from sensors embedded in the object, and a mathematical model that can calculate a forecast of the results of the functioning of a real object in specific operating conditions. The developed concept of a DT provides for the use of the DS of this process. The physical object in our case was the real process of single-stage grinding of frozen meat. The actuator of the chopper grinding mechanism was equipped with devices (sensors) to measure the speed of rotation of the cutting shaft, as well as the electromagnetic torque (stator current) developed by the drive motor. The mathematical model of the DS was presented in *Kapovskiy et al*. (2023).

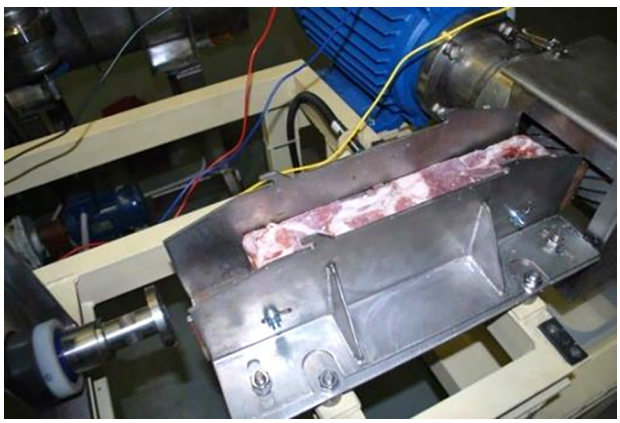

Figure 1. Laboratory single-stage frozen meat block grinder

The moment of resistance (M_c) to GM, brought to the cutting shaft of the meat chopper, is a random process due to the heterogeneity of the processed meat. Our research has shown that this process has the characteristics of a stationary ergodic random process. To solve the problem of stabilizing the established degree of grinding, we proposed hybrid control (clear and fuzzy) of the grinding process, using the mathematical model specified in *Kapovskiy et al*. (2023). This control takes into account the change in the plasticity of the meat as its temperature increases (*Kapovskiy et al*., 2020). The control computer that completes the processing line, using the DS, conducts a computational experiment of grinding virtual blocks of frozen meat using the statistical test method (Monte Carlo method), expanding the statistical base for calculating the predicted particle size of the GM (*Ivashov et al*., 2018). The computational experiment is carried out based on measurement data and calculation of a real random process $M_c(t)$. The calculated prediction of meat particle size ultimately makes it possible to more accurately determine the generalized quality criterion of the formulation mixture, taking into account the degree of its dispersion and mixing, with computer control of further processing.

Processed meat is characterized by significant heterogeneity, and as a result, the range boundaries of values of the fuzzy variables need to be

determined—parameters of the chemical composition, as well as the dispersion of GM. Therefore, it is advisable to apply fuzzy control of the mixing process based on the theory of fuzzy sets (*Pujaru et al*., 2024). As a result of simulation modelling of single-stage grinding using the Monte Carlo method, a probabilistic range of particle size values with selected confidence limits was obtained. With fuzzy modelling of the process, a large width of the final fuzzy interval (range) was obtained, which leads to an increase in the uncertainty of the simulation results. At the same time, fuzzy modelling takes into account all factors, including the boundary values of the initial clear and fuzzy intervals, when describing the uncertain parameters of the model. Obtaining such guaranteed estimates using the traditional Monte Carlo approach is impossible due to the probabilistic nature of this type of modelling.

Thus, it was proposed to combine both the abovementioned approaches to formalize the uncertain parameters of the single-stage grinding. These approaches were suggested to formalize the uncertainties of the technological process consistently: 1) Conduct a simulation of the Monte Carlo single-stage grinding using the DT of the process, solving the problem of determining the boundaries of the range of finding the particle size of GM with a given accuracy and reliability. This problem is solved in the area of probability distribution (frequency domain); 2) Formalize the solution obtained in the first stage using the field of fuzzy interval analysis.

Next, the task of multi-criteria optimization of the process of composing and mixing a multicomponent mixture in a fuzzy area should be solved. It is advisable to use as criteria: a) meat chemical composition (protein, fat, moisture); b) dispersion; c) pH; d) viscosity; e) meat temperature, etc.

In the field of probability, only numerical characteristics of distributions are used (mathematical expectation, variance, median, mode, etc.). This can lead to a loss of initial information, or to a distortion of the qualitative picture of the phenomena under study. That is why, at the second stage, it is proposed to proceed to a fuzzy interval analysis. In this case, the differential distribution function $f(x)$ of the particle size of GM in the frequency domain is transformed into a membership function $\mu(x)$ of a fuzzy set by piecewise linear approximation. This will make it possible to operate with the advanced mathematical apparatus of fuzzy set theory. For the specified transformation $(f(x) \to \mu(x))$, it is advisable to use the description of membership functions of fuzzy sets using $\alpha -$ levels. This will preserve the bulk of the information provided by the differential probability distribution function $f(x)$. The $\alpha -$ level set of a fuzzy set A in X is called the set $A_{\alpha,}$ consisting of $x \in X$, whose degrees of membership in the fuzzy set A are not less than α (*Eshkabilov*, 2022, *Xue & Pan*, 2024):

$$A_\alpha = \{x(x \in X), \mu_A(x) \geq \alpha\} \quad (1)$$

The decomposition of a fuzzy set into a set of $\alpha -$levels can be written as $\bigcup_\alpha \alpha A_\alpha$.

Let us consider the differential function of the probability distribution of the particle size of GM obtained in a laboratory single-stage grinding (Fig. 2). The graph of the function $f(x)$ is obtained as an alignment of the histogram of the measured particle sizes determined as a result of microstructural analysis of GM. The specified histogram was given in *Kapovskiy et al*. (2023). It was hypothesized that the experimental statistical distribution of particle sizes obeys a normal law:

$$f(x) = \left[1/\left(\sigma^*(x) \cdot \sqrt{2\pi}\right)\right] \cdot exp[-(x - m_x^*)^2/(2 \cdot 251.54)] \quad (2)$$

In our particular case, m_x^* = 42.28 (μm) – estimation of the statistical average meat particle size across all measurement ranges; $\sigma^*(x)$ = 15.86 (μm) – estimation of the mean square deviation for all measurement ranges. The plausibility of the hypothesis was confirmed by testing according to Pearson's criterion χ^2. Let us carry out the transformation $f(x) \to \mu(x)$. We transform the differential probability distribution function (2) into a trapezoidal fuzzy interval membership function $\mu(x)$ with four reference points that correspond to the vertices of the trapezoid. For practical (engineering) calculations, it is sufficient to have information about only two intervals corresponding to $\alpha -$ levels (1): 1) the lower base of the trapezoid $(\mu(x) = 0)$; 2) the upper base of the trapezoid, corresponding to the most possible values of the membership function $(\mu(x) \cong 1)$. For the lower base of the trapezoid, corresponding to the level $\alpha = 0$, we select an interval whose length will be $2|2{,}5\sigma^*(x)|$. Calculate the probability of the size of the meat particles falling into the specified interval:

$$P(|x - m_x^*| < 2.5 \cdot \sigma^*(x) = 2 \cdot F(2.5) \quad (3)$$

where F is the Laplace function. Since $F(2.5)$=0.4938, the value of the desired probability will be 0.9876. The coordinates of the two upper reference points can be determined by calculating

the mean square deviation σ_i corresponding to the selected α_i – level:

$$\sigma_i = \sigma^*(x) \cdot \sqrt{-2ln(\alpha_i)} \quad (4)$$

For $\alpha_i = 0.9$:

$$\sigma_{0.9} = 15.86 \cdot \sqrt{-2ln(0.9)} = 7.28\ (\mu m).$$

Using the symmetry of the graph of the function $f(x)$ relative to m_x^* in our case, we construct the graph $\mu(x)$ in the form of a trapezoid with the bases defined above: the lower, corresponding to $\alpha = 0$, and the upper, corresponding to $\alpha = 0.9$, intervals of meat particle size values (Fig. 3). Note that for other types of distribution *x*, when transforming $f(x) \rightarrow \mu(x)$, the specific type of graph $f(x)$ should be taken into account. The approach proposed in this paper is consistent with other works (*Birle et al*., 2013, *Tao et al*., 2018), where it was noted that modelling and simulation are an integral part of the DT, and the lack of convergence between the physical and virtual space of the product data in the product lifecycle is isolated and useless for production.

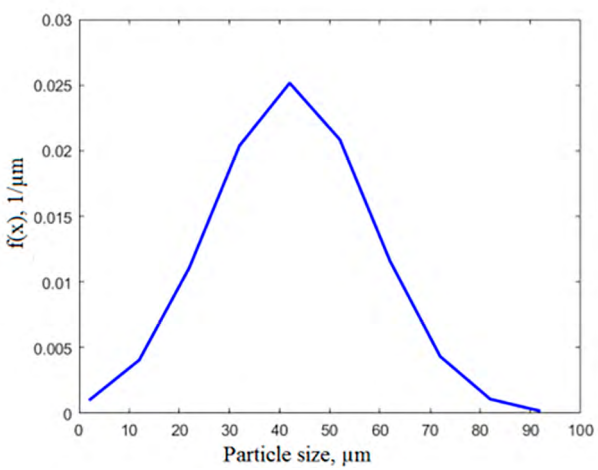

Figure 2. Differential probability distribution function (f(x)) of the particle size of GM as a random variable

Uhlemann et al. (2017) also emphasize the importance of using real-time data in DT work. Thus, as a result of the operation of the DT, the control computer has a forecast of the values of the boundaries of the particle size range of the GM, which is translated into a fuzzy region and further considered as an interval of values with fuzzy boundaries. Using the same method, the control computer processes a range of predicted chemical composition values (protein, fat, water). These parameters are considered as private quality criteria, which the control computer uses in multi-criteria process optimization and to calculate a generalized quality criterion in the form of a convolution of private criteria. Based on the calculation results, the control computer adjusts the process of grinding and composing the minced meat.

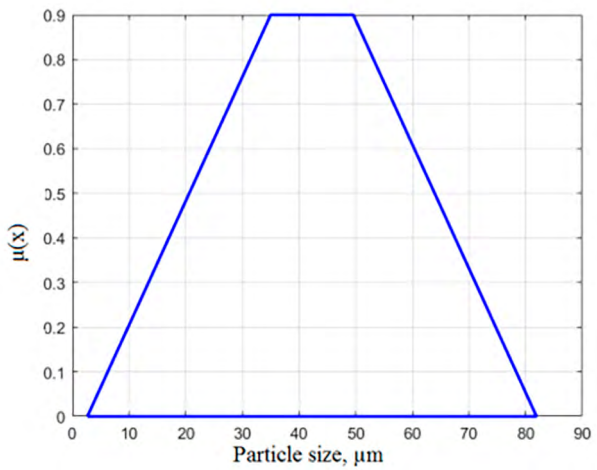

Figure 3. Trapezoidal membership function (µ(x)) of a fuzzy set of particle sizes of GM

4. Conclusion

The result of the work is a scientifically based concept of intelligent (fuzzy) control of the process of producing minced meat with a given nutritional value in the conditions of continuous technological lines. Application of the proposed process control supports on-line automated control of the preparation of minced meat of a given dispersion and chemical composition. The control system operates in real-time production without operator involvement, which reduces the risks of producing low-quality finished products and minimizes economic losses.

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Original Scientific Paper

Aroma evolution in aged horse meat: dry vs. vacuum aging

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ABSTRACT

This study investigates the impact of aging method (dry vs. vacuum) and duration (0, 3, and 6 weeks) on the volatile compound profile of horse loin meat. Twenty-seven Italian Heavy Draft Horse foals were reared under uniform conditions and slaughtered at 17 months. Loin samples were aged under controlled dry or vacuum conditions and analyzed for volatile organic compounds using headspace solid-phase microextraction and gas chromatography–mass spectrometry. Results revealed that, compared with vacuum aging, dry aging led to a significantly higher accumulation of aldehydes, ketones, alcohols, and sulfur compounds, especially after three weeks, reflecting increased lipid oxidation and microbial activity. Notably, hexanal and nonanal levels surged in dry-aged meat, enhancing flavor complexity but also indicating oxidative degradation. Conversely, vacuum aging limited oxidation, preserving a fresher profile with fewer oxidative markers, but saw a moderate increase in certain furans and hydrocarbons, possibly due to anaerobic microbial metabolism. Overall, three weeks of dry aging emerged as the optimal balance, enhancing desirable aroma compounds without excessive oxidation. In conclusion, these findings contribute to the understanding of flavor development in horse meat and suggest that aging strategies can be tailored to optimize sensory quality and product stability.

1. Introduction

Although less commonly consumed than beef or pork, horse meat remains integral to the culinary heritage of several Western European countries, including Italy, France, Belgium, and Spain (*Stanisławczyk et al*., 2023). Its growing popularity is linked to its nutritional value—rich in essential amino acids, B vitamins, iron, and omega-3 fatty acids (*De Palo et al*., 2016; *Lorenzo et al*., 2017; *Maggiolino et al*., 2019; *Marino et al*., 2022). Beyond its nutritional properties, sensory characteristics, such as flavor and aroma, play a crucial role in consumer acceptance and are closely linked to the presence of volatile organic compounds (VOC). Aging, whether by vacuum or dry methods, is commonly used to enhance these sensory traits, with each technique influencing VOC formation differently depending on time and environmental conditions (*Terjung et al*., 2021; *Xu et al*., 2021). Due

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to its biochemical composition, horse meat is particularly susceptible to oxidative reactions during aging, resulting in the production of VOCs, such as aldehydes, ketones, alcohols, and furans (*Forte et al*., 2024). These compounds shape the aroma profile and can have both positive and negative effects on meat quality (*Beldarrain et al.*, 2020; *Xu et al.*, 2021). Despite this, research on VOC evolution in aged horse meat is limited, and comparative studies between vacuum and dry aging are lacking. This study aims to fill this gap by assessing the impact of aging method and duration on the volatile profile of horse loin.

2. Materials and methods

2.1 Animal management and muscle sampling

The trial involved 27 male Italian Heavy Draft Horse foals, all born and raised on the same farm until 17 months of age and fed a uniform diet (35% oat hay, 65% commercial feed: rolled corn 33%, soybean meal 21%, wheat bran 17%, rolled barley 13.5%, rolled oats 13.5%, vitamin/mineral supplements 2%). All animals were transported and slaughtered at an EU-approved abattoir, following *Maggiolino et al.* (2019), in 9 sessions (3 foals/session). From each carcass, both left and right loins (between 13th and 18th thoracic *vertebra*) were collected, totaling 54 loins. Left loins were dry aged (D), right loins vacuum aged (V), with 27 loins per method. Each loin was divided into three sections, randomly assigned to aging times of 0, 3, or 6 weeks; cranial-caudal and caudal sections were randomly distributed. Dry aging occurred in chambers at 2°C, 82% relative humidity (RH), and 0.4 m/s air velocity. Vacuum aging used Besser Vacuum® film (65 µm thick, 63 g/m², O_2 transmission ≤ 65 cm³/m²/day/bar at 23°C, 0% RH; H_2O vapor transmission ≤ 3.5 g/m²/day at 23°C, 85% RH), with storage also at 2 °C.

2.2 Volatile compounds analysis

At each aging time, a 5 g portion was taken from every loin sample to analyze VOC. Samples were cooked for 5 minutes on a thermostatically controlled electric griddle (CG660, Delonghi, Italy) at 130–150°C. Internal temperature was monitored with a copper-constantan thermocouple (Model 5SCTT-T-30-36, Omega Engineering, USA) inserted into the sample center, and the cooked samples were removed at 70°C, following the method of *Forte et al*. (2024). Cooked samples were homogenized with a commercial grinder (Moulinex, Swan Holding, UK), and ~1.00 ± 0.05 g of ground meat was placed in 20 mL headspace vials (Agilent Technologies, USA), spiked with 82 ng of 2-octanol (internal standard), and sealed with Teflon-lined septa. VOC were extracted via solid-phase microextraction (SPME) and analyzed by gas chromatography-mass spectrometry (GC–MS), as per *Maggiolino et al*. (2021). Prior to extraction, samples were equilibrated at 35°C for 15 min using a Triplus RSH autosampler (Thermo Fisher Scientific, Italy). Extraction was done for 30 min at the same temperature with a DVB/CAR/PDMS fiber (50/30 µm; Supelco, USA). Volatiles were thermally desorbed into a Trace 1300 GC (Thermo Fisher Scientific, Rodano, Italy) in splitless mode, coupled to an ISQ Series 3.2 SP1 MS (Thermo Fisher Scientific, Rodano, Italy). Separation used a VF-WAX MS capillary column (60 m × 0.25 mm i.d., 0.25 µm; Agilent) with oven program: 35°C (5 min), 1.5°C/min to 45°C, 4°C/min to 160°C, then 20°C/min to 210°C (7 min hold). MS settings: ion source 250°C, 70 eV, 1700 V, scan range 40–300 amu. Compounds were identified via Xcalibur v2.0 (Thermo Fisher Scientific, Rodano, Italy) using NIST spectral database, and semi-quantified with the internal standard method, expressed in µg/kg.

2.3 Statistical analysis

The data set was tested for normal distribution (Shapiro-Wilk) and variance homogeneity (Bartlett test). Each loin represented an experimental unit. All data (excluding the sensory profile) were analyzed using two-way ANOVA with SAS (SAS, 2018), according to the following model:

$$y_{ijk} = \mu + \alpha_i + A_j + T_k + (A \times T)_{jk} + \varepsilon_{ijkl},$$

where y_{ijk} are dependent variables; μ is the overall mean; α_i is the single block random effect (1,…9); A was the effect of the jth aging method adopted (dry or vacuum) (j = 1, 2), T was the effect of the kth aging time (k = 1, …, 9), A × T was the effect of the interaction of the jth aging method and kth aging time (1, …,18), and ε_{ijkl} was the error term. When not significant, the binary interaction was dropped from the model. A Tukey test was applied to evaluate the differences according to aging time when time effect was significant. All means were expressed as square means and mean standard error. The significance level was set to $P < 0.05$.

3. Results

The results of the VOC analysis are presented in Table 1. Aldehydes in D meat significantly increased at 3 w and decreased thereafter at 6 w ($P < 0.01$), while in V meat, they declined at 6 w compared to 0 and 3 w ($P < 0.01$). Moreover, aldehyde levels were significantly higher in D meat than in V meat at both 3 and 6 w ($P < 0.01$). Among aldehydes, hexanal increased in both aging methods at 3 w ($P < 0.01$); at 6 w, it markedly decreased in V meat ($P < 0.01$) while remaining stable in D meat ($P < 0.01$). Furthermore, at 6 w, hexanal concentration was significantly higher in D meat compared to V meat ($P < 0.01$). Nonanal increased in D meat at 3 w and remained stable thereafter; at both 3 and 6 w ($P < 0.01$), its concentration was significantly higher in D meat than in V meat ($P < 0.01$). Pentanal in

Table 1. Effects of aging method, aging time and their binary interaction on the volatile compound families detected in horse meat.

Family	Aging	Aging week			SEM[1]	*P*-value		
		0	3	6		A[2]	T[3]	A × T
Aldehydes	**D**	**589.62^{A}**	**1139.51^{BX}**	**720.96^{AX}**	**162.054**	**< 0.0001**	**< 0.0001**	**< 0.0001**
	V	**586.48^{A}**	**456.29^{AY}**	**237.00^{BY}**				
Hexanal	D	587.21^{A}	815.64^{B}	459.02^{AX}	92.09	0.0021	< 0.0001	< 0.0001
	V	436.06^{A}	271.20^{B}	65.36^{CY}				
Nonanal	D	58.54^{A}	151.06^{BX}	127.40^{BX}	15.994	< 0.0001	0.0469	0.0101
	V	58.73	52.51^{Y}	44.65^{Y}				
Pentanal	D	30.46^{A}	82.65^{BX}	65.97^{CX}	10.29	< 0.0001	0.0026	0.0011
	V	33.67	20.43^{Y}	3.02^{Y}				
Carboxylic acids	**D**	**248.34^{A}**	**466.53^{B}**	**761.37^{CX}**	**58.376**	**< 0.0001**	**< 0.0001**	**< 0.0001**
	V	**201.55^{A}**	**311.77^{B}**	**287.76^{ABY}**				
Hexanoic acid, ethenyl ester	D	72.89	69.66	72.843^{X}	17.775	0.0030	< 0.0001	0.0012
	V	91.92^{A}	97.20^{A}	34.22^{BY}				
Ketones	**D**	**143.71^{A}**	**411.24^{B}**	**768.18^{CX}**	**94.934**	**0.0007**	**< 0.0001**	**< 0.0001**
	V	**138.70^{A}**	**251.01^{B}**	**388.89^{CY}**				
2-butanone,3-hydroxy-	D	59.84^{A}	128.87^{B}	206.74^{C}	88.033	0.2321	< 0.0001	0.1186
	V	56.63^{A}	81.98^{B}	298.09^{C}				
Alcohols	**D**	**110.34^{A}**	**287.64^{B}**	**315.57^{CX}**	**95.288**	**0.0013**	**< 0.0001**	**0.0011**
	V	**86.91^{A}**	**350.26^{B}**	**230.26^{CY}**				
1-octen-3-ol	D	38.52^{A}	83.30^{B}	89.52^{B}	20.845	0.3531	< 0.001	< 0.001
	V	31.31^{A}	178.85^{B}	81.19^{B}				
1-pentanol	D	20.18^{A}	32.12^{A}	54.79^{BX}	15.0175	0.0036	0.0025	0.0601
	V	10.55	18.98	8.62^{Y}				
Aromatic hydrocarbons	**D**	**15.71**	**14.05**	**13.61**	**2.162**	**0.0801**	**0.0043**	**0.0922**
	V	**11.24^{A}**	**19.74^{B}**	**21.18^{B}**				
Furans	**D**	**5.06**	**5.89^{X}**	**4.95**	**1.637**	**0.0056**	**0.0028**	**0.0042**
	V	**2.04^{A}**	**18.37^{BY}**	**6.51^{A}**				
Sulfur compounds	**D**	**3.70^{A}**	**53.67^{BX}**	**50.15^{BX}**	**7.351**	**< 0.0001**	**0.0043**	**0.0024**
	V	**3.77**	**3.68^{Y}**	**6.64^{Y}**				
Thiols	**D**	**37.92^{A}**	**14.88^{B}**	**11.96^{B}**	**3.794**	**0.0982**	**0.0022**	**0.0019**
	V	**35.24^{A}**	**15.22^{B}**	**13.76^{B}**				
Hydrocarbons	**D**	**22.10**	**27.42^{X}**	**23.02^{X}**	**22.747**	**< 0.0001**	**0.0102**	**0.0032**
	V	**26.98^{A}**	**86.54^{BY}**	**73.89^{BY}**				

Note: [1]SEM: standard error of the mean; [2]A: aging method; [3]T: aging time.

D meat increased at 3 w ($P < 0.01$), decreased at 6 w ($P < 0.01$), and was higher than in V meat at both time points ($P < 0.01$). Carboxylic acids showed a progressive increase in D meat ($P < 0.01$), whereas in V meat, their levels increased at 3 w and then stabilized ($P < 0.01$). Hexanoic acid and ethenyl ester increased in V meat at 6 w compared to earlier time points ($P < 0.01$), although its concentration was lower than in D meat ($P < 0.01$). Both ketones and alcohols exhibited a progressive increase throughout the aging period in both aging methods ($P < 0.01$). At 6 w, the concentrations of carboxylic acids, ketones, and alcohols were significantly higher in D meat compared to V meat ($P < 0.01$). The 2-butanone,3-hydroxy increased progressively over time in both aging methods ($P < 0.01$). The 1-octen-3-ol increased in both methods at 3 w and remained stable thereafter ($P < 0.01$). In contrast, 1-pentanol increased in D meat at 6 w ($P < 0.01$), reaching higher concentrations compared to V meat ($P < 0.01$). Aromatic hydrocarbons and hydrocarbons increased in V meat at 3 w and subsequently remained stable ($P < 0.01$). Moreover, hydrocarbon levels at both 3 and 6 w were significantly higher in V meat compared to D meat ($P < 0.01$). Furans increased in V meat at 3 w ($P < 0.01$), reaching significantly higher concentrations than in D meat ($P < 0.01$), and then decreased again at 6 w, returning to baseline levels ($P < 0.01$). At both 3 and 6 w, D meat showed significantly higher concentrations of furans compared to V meat ($P < 0.01$). Thiols decreased in both aging methods at 3 w and remained constant thereafter ($P < 0.01$).

4. Discussion

The aging process, both in terms of method and duration, significantly influenced the profile of volatile compounds in horse meat. Across the board, dry-aged samples (D) exhibited higher concentrations of several volatile families compared to vacuum-aged (V) counterparts, particularly after extended storage. This aligns with previous literature data indicating that dry aging, due to its oxygen-rich environment, promotes oxidative reactions and microbial activity that contribute to a more complex volatile profile (*Kim et al.*, 2016). Among the aldehydes, hexanal, nonanal, and pentanal were notably more abundant in dry-aged meat over time, especially at week 3, reflecting an increase in lipid oxidation, particularly of polyunsaturated fatty acids. Hexanal is widely recognized as a primary oxidation product of linoleic acid and a marker of oxidative degradation in meat (*Tateo et al*., 2020). In contrast, vacuum-aged samples showed lower levels of aldehydes, consistent with the limited oxygen availability that suppresses lipid oxidation (*Lee et al*., 2021). A significant rise in carboxylic acids, especially in dry-aged meat by week 6, suggests intensified lipolysis and microbial fermentation under aerobic conditions, as also observed by *Ahnström et al.* (2006). Interestingly, some acid levels in vacuum-aged meat increased at week 3 but plateaued or declined thereafter, which may indicate a temporary microbial or enzymatic activity peak followed by stabilization. Ketone levels, such as 2-butanone-3-hydroxy, followed a similar trend, with dry aging showing a steady increase throughout the aging period, indicative of ongoing oxidation and potential microbial metabolism (*Bliznyuk et al.*, 2024). These compounds contribute sweet, buttery, and creamy notes and are considered important in dry-aged flavor development (*Maggiolino et al*., 2019). Alcohols, notably 1-octen-3-ol and 1-pentanol, were more prevalent in dry-aged meat and increased with aging, suggesting intensified oxidative degradation of lipids and amino acids (*Fu et al*., 2022). 1-octen-3-ol, derived from the oxidative cleavage of linoleic acid, contributes to mushroom-like aromas and is often found in oxidized meat (*Mottram*, 1998). In vacuum-aged meat, a spike in 1-octen-3-ol at week 3 may suggest a transient oxidative episode or microbial contribution (*Song et al*., 2021; *Sun et al*., 2024). Aromatic hydrocarbons and furans increased significantly in vacuum-aged meat, particularly at week 3, possibly linked to Maillard reaction intermediates or microbial action under anaerobic conditions (*Tateo et al*., 2020). Notably, furans are often associated with heat treatment, but low levels may also arise from sugar or amino acid degradation (*Liu et al*., 2022). In terms of sulfur compounds and thiols, dry-aged meat showed markedly higher levels over time compared with vacuum-aged meat, likely due to the breakdown of sulfur-containing amino acids (e.g., methionine, cysteine) and microbial activity, which are known to generate potent aroma-active molecules (*Meinert et al*., 2009). These compounds, although present in small quantities, greatly influence the sensory characteristics due to their low odor thresholds (*Vilar et al*., 2022). Lastly, hydrocarbons increased significantly in vacuum-aged meat, especially at weeks 3 and 6. Although less aromatic, they may act as markers of lipid degradation or microbial fermentation (*Fu*

et al., 2022). Overall, the volatile profile suggests that dry aging leads to a more pronounced and rapid development of lipid oxidation products and Maillard-derived volatiles, whereas vacuum aging suppresses oxidation but may enhance fermentation and microbial-derived compounds under anaerobic conditions. These findings corroborate previous studies on beef and lamb, and now extend them to horse meat, highlighting the impact of aging strategies on flavor development and potential consumer preference (*Kim et al*., 2016).

5. Conclusion

This study confirms that both aging method and duration significantly influenced the development of volatile compounds in horse meat, affecting its oxidative stability and flavor profile. Dry aging, particularly for three weeks, led to a higher accumulation of lipid oxidation products such as aldehydes, ketones, and sulfur compounds, resulting in a more complex and intense aroma. In contrast, vacuum aging reduced oxidative reactions and maintained a fresher but less complex volatile profile. A three-week dry aging period proved to be the most effective for enhancing desirable aroma compounds without causing excessive degradation. Vacuum aging, instead, better preserved freshness and could suit products requiring longer shelf-life and milder flavor. The findings highlight how tailoring aging strategies could improve the sensory qualities and marketability of horse meat. Future studies should correlate these chemical patterns with sensory analysis to better understand consumer preferences.

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Original Scientific Paper

Transitioning to a risk-based approach in pork inspection in Serbia: are we ready for Trichinella control reform?

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ABSTRACT

The implementation of risk-based approaches in meat inspection is a key step toward modernizing food safety systems in accordance with international standards. In Serbia, national Regulation 48/2022 introduced the possibility of designating pig farms as "*Trichinella*-free", thereby allowing for reduced testing—only 10% of pigs from such farms are required to undergo routine post-mortem *Trichinella* inspection. However, three years after its adoption, no farm in Serbia has officially obtained this status. This paper critically examines the legal and institutional framework surrounding the application of Regulation 48/2022, with a focus on the readiness of the veterinary system, the level of awareness among stakeholders, and the practical challenges to implementation.

In addition to the regulatory and veterinary aspects, this study also incorporates findings from slaughterhouse-level inquiries. Slaughterhouse operators report that, regardless of official *Trichinella*-free status, they would still prefer to inspect every pig carcass, fearing the legal and reputational consequences of potential oversight. This undermines the core principle of the regulation and reflects a gap between policy design and its real-world acceptance.

Special attention is given to the public health implications of *Trichinella* control, as well as the potential benefits and risks of transitioning to a selective inspection model. The analysis suggests that although the regulatory framework is formally aligned with EU practices, significant barriers—including institutional inertia and distrust—remain that hinder its practical application. The paper concludes with recommendations for improving stakeholder engagement, capacity building, and confidence in surveillance systems as prerequisites for a successful shift toward risk-based *Trichinella* control in Serbia.

1. Introduction

Trichinellosis is among the most widespread zoonotic parasitic diseases globally. Human infection occurs through the consumption of raw or undercooked pork meat containing larvae of nematodes belonging to the genus *Trichinella*. In many countries, individual carcass inspection for pigs at slaughter is mandatory; however, such measures entail significant costs, particularly given the low incidence of positive cases in pigs raised under controlled housing conditions (*Frenssen et al*., 2017).

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Serbia has aligned its national legislation (No. 48/2022; *RS Regulation*, 2022) with European Union Regulation 2015/1375 (*EU Regulation*, 2015), which mandates control measures for the detection of *Trichinella* spp. in meat from pigs and other susceptible domestic and wild animals to protect public health. All pigs slaughtered for commercial purposes must be tested for *Trichinella*, unless they originate from controlled housing systems that comply with stringent biosecurity requirements (e.g., rodent control, prohibition of feeding with animal by-products, and prevention of contact with wildlife) and have been officially approved by the competent authority.

Even under such controlled systems, it is required that at least 10% of pigs from each farm be tested annually to verify the *Trichinella*-free status. Detection is performed using the standard artificial digestion method of muscle tissue (most commonly the diaphragm pillars), which involves the use of pepsin and hydrochloric acid to release larvae from the tissue. This method is considered the reference standard due to its high sensitivity and reliability, while alternative methods can be used only if they are properly validated and officially approved.

These regulatory measures are grounded in risk-based principles, with a strong focus on the prevention of infection at the primary production level, official controls at slaughterhouses, laboratory accreditation, and mandatory traceability of products of animal origin. This integrated approach ensures systematic control and minimizes the risk of human trichinellosis through consumption of contaminated meat (*RS Regulation*, 2022; *EU Regulation*, 2015).

In both Serbia and the European Union, human trichinellosis is a notifiable disease subject to mandatory reporting. In Serbia, a total of 2,922 cases were reported between 2001 and 2022, corresponding to an incidence rate of 38.94 per 100,000 inhabitants. The average annual number of registered cases during this period was 133, ranging from a peak of 577 cases in 2002 to zero cases reported in 2021. A statistically significant downward trend in the incidence rate of trichinellosis has been observed since 2004 (*Plavša et al.*, 2023).

Similarly, a clear reduction in human trichinellosis cases has been documented across the European Union over the past decade, with a decline of more than 85% compared to 2014, as illustrated in Figure 1 (*ECDC,* 2016; 2017; 2018; 2023).

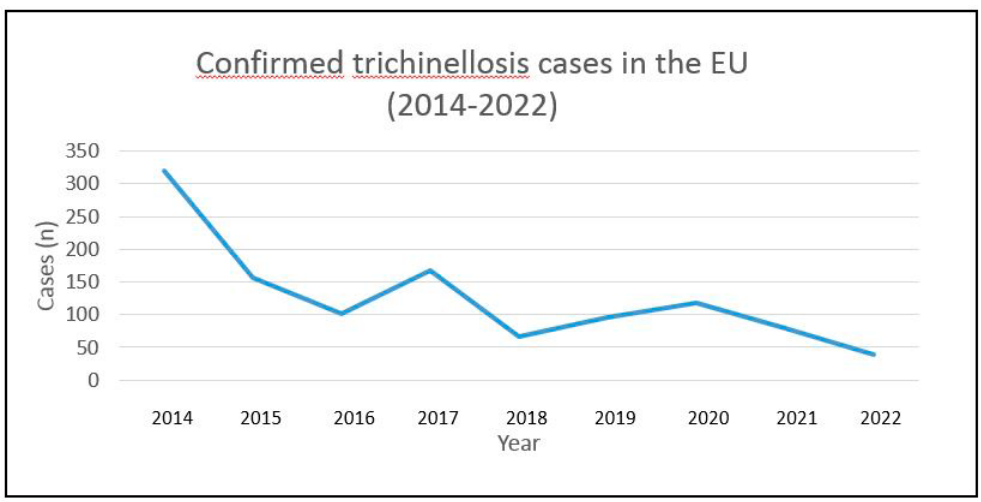

Figure 1. Trend of reported trichinellosis cases in the EU in the period from 2014 to 2022

2. Reform of trichinella control in serbia

Serbia harmonized its national Regulation (48/2022; *RS Regulation*, 2022) with *EU Regulation* (2015) three years ago. According to data obtained from the Veterinary Directorate of Serbia, no farm has yet been granted *Trichinella*-free status (personal communication).

To assess stakeholder awareness and the implementation of the new regulatory requirements, a survey was conducted among nine slaughterhouses in Serbia that process pigs. While the results offer valuable preliminary insights into industry perceptions and practices, the sample is relatively small and not representative of the entire slaughterhouse sector. Future studies should aim to expand the research

Table 1. Survey questions for slaughterhouses on awareness and implementation of Regulation 48/2022 (*RS Regulation*, 2022).

Question	Answer options	Number of slaughterhouses (N=9)	Percentage (%)
1. Are you familiar with the amended legal regulation 48/2022 regarding *Trichinella* inspection?	1) Yes 2) No	7/9 2/9	77.8% 22.2%
2. Are you aware of the key changes introduced by the new regulation?	1) Yes 2) No	3/9 6/9	33.3% 66.7%
3. When evaluating your suppliers, do you consider whether the farm is certified as *Trichinella*-free?	1) Yes 2) No	0/9 9/9	/ 100%
4. If a farm is certified as *Trichinella*-free, would you in the future test only 10% of pigs from that farm?	1) 10% 2) 100%	0/9 9/9	/ 100%

across a wider geographic area and include a larger number of facilities, to ensure more robust and comprehensive findings.

The survey results indicate that although the majority of slaughterhouses (77.8%) is aware of Regulation 48/2022, only one-third (33.3%) understands the key changes it introduces. This discrepancy highlights a lack of adequate information and understanding of the regulatory framework, which could hinder its effective implementation.

None of the slaughterhouses consider the *Trichinella*-free status of farms when evaluating their suppliers, further confirming that the concept of selective testing has not yet been adopted in practice. Moreover, all respondents (100%) stated that even if a farm were officially certified as *Trichinella*-free, they would still continue to test every pig carcass, despite the regulation allowing for 10%-sample testing.

This practice reflects a deep-rooted mistrust in the certification system and concerns about the potential legal consequences of missing an infection. As a result, the core objective of the regulation—transitioning to risk-based inspection—is effectively undermined.

While the current findings offer a preliminary understanding of the challenges in implementing risk-based *Trichinella* control in Serbia, the limited number of surveyed slaughterhouses (N=9) is a study limitation. Broader, nationwide surveys would be necessary to more accurately assess the level of readiness across the sector and identify regional variations in awareness and implementation.

3. Conclusion

Although Regulation 48/2022 in the Republic of Serbia (*RS Regulation*, 2022) has been formally harmonized with Regulation 2015/1375 (*EU Regulation*, 2015), its practical implementation reveals significant shortcomings. The absence of farms certified as *Trichinella*-free, even three years after the regulation came into force, along with the low level of awareness among slaughterhouses regarding the legislative changes, highlights the need to strengthen communication and education across all stakeholders in the pork production chain.

The prevailing practice of testing all pigs—including those from farms that could potentially qualify for *Trichinella*-free status—reflects a deep-rooted lack of trust in the certification system and concerns about reputational or legal consequences in the case of failure. To meet the intended objectives of the regulation and build confidence in the risk-based model, it is essential to enhance institutional support through targeted education, transparent communication, and system-level reforms that facilitate consistent application of the law.

Further research involving a larger and more geographically diverse sample of slaughterhouses is recommended to validate these findings and inform national-level policy.

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Original Scientific Paper

Characterization of commercial fish *pâtés*: toward the development of novel fish-based spreads

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ABSTRACT

This study evaluated the textural and sensory properties of commercial fish *pâtés* available on the Serbian market to identify differences in quality and consumer-relevant characteristics. 14 *pâté* samples made from marine (tuna, salmon, mackerel, hake) and freshwater (trout) fish were analyzed. Texture profile analysis included measurements of firmness and work of shear, while sensory evaluation was conducted by a trained panel, focusing on hardness, spreadability, graininess, color uniformity, taste, and smell. Significant variability ($p<0.05$) was observed among the samples, indicating diverse formulation strategies. Tuna- and salmon-based *pâtés* achieved the highest overall acceptability due to their appealing flavor and appearance. In contrast, trout-based *pâtés* exhibited more balanced texture attributes, with sensory and instrumental data aligning closely with ideal values. These findings provide useful guidance for the development of novel fish *pâtés* using underutilized species such as freshwater bream (*Abramis brama*), which have been identified as key low-trophic species within the HEU project IMPRESS. The results highlight the importance of optimizing textural properties to enhance consumer acceptability.

1. Introduction

The global *pâté* market was valued at US$ 1.74 billion in 2023 and is projected to grow at a compound annual growth rate (CAGR) of 4.9% from 2024 to 2030, reaching an estimated US$ 2.44 billion by 2030 (*Grand View Research*, 2024). This growth is driven by evolving consumer preferences for convenient, ready-to-eat foods, the rise of snacking culture, and the increasing adoption of diverse culinary trends (*Engelen & de Wijk*, 2012). In Serbia, the *pâté* and spread category recorded a 1% increase in volume and a 6.3% increase in value between March 2024 and February 2025, with 43% of total sales generated through promotional activities. Notably, fish *pâtés* account for 11% of total volume sales but contribute 18% to total value sales, indicating strong market potential for premium fish-based products (*Retail Zoom*, 2025).

Fish *pâtés* offer significant nutritional benefits compared to meat-based alternatives. These attributes make them a valuable addition to diets, particularly in regions with low fish consumption. Given that consumers with a strong interest in food quality perceive fish more positively than meat in terms of freshness, taste, and safety (*Torrissen & Onozaka*, 2017), the development of premium fish *pâtés* may

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particularly appeal to this market segment. However, the Serbian market is predominantly supplied with marine fish *pâté*s (e.g., tuna – *Thunnus albacares*, hake – *Merluccius hubbsi*, salmon – *Salmo salar*, Atlantic mackerel – *Scomber scombrus*) and freshwater (trout – *Oncorhynchus mykiss*), while low-trophic species such as bream (*Abramis brama*) and roach (*Rutilus rutilus*) remain underutilized. According to the Food and Agriculture Organization, global per capita fish consumption surpassed 20.6 kg in 2021, more than doubling since the 1960s (*FAO*, 2024). In contrast, fish intake in Serbia remains significantly lower, averaging approximately 7 kg per person annually. Marine fish and seafood accounted for 92.80% of the total fish and fish product supply on the Serbian market, whereas freshwater fish represented only 7.14% (*Janjić et al*., 2024). Freshwater fish, although less studied than marine species, are a valuable nutritional source due to their lower fat and sodium content, lower mercury risk, and richness in high-quality proteins and micronutrients. These attributes make them a safe and nutritionally beneficial choice for diverse populations (*Lámfalusy et al*., 2025; *Regal Springs*, 2025; *Ahmed et al*., 2022; *Mielcarek et al*., 2020).

This study aimed to conduct an evaluation of sensory and textural properties of 14 commercial fish *pâté*s available on the Serbian market to identify their "strengths and weaknesses". The results will serve as a basis for developing of an innovative fish spread from underutilized species (freshwater bream) by guiding the optimization of its formulation and processing parameters in accordance with consumer preferences and current market trends.

2. Materials and methods

2.1.1. Sample collection

A total of 14 commercial fish *pâté* samples were collected from retail markets in Serbia between March and April 2025. The samples were selected to represent a diverse range of products available on the Serbian market, including both marine (tuna – *Thunnus albacares*, hake – *Merluccius hubbsi*, salmon – *Salmo salar*, Atlantic mackerel – *Scomber scombrus*) and freshwater (trout – *Oncorhynchus mykiss*) fish-based *pâté*s. Selection criteria were product availability in major retail channels (hypermarkets, supermarkets, and medium-sized stores), popularity based on sales data (*Retail Zoom*, 2025), and variation in fish species and brands.

2.1.2. Sensory evaluation

Sensory evaluation was conducted by 10 trained assessors (ISO 8586:2023 ; Sensory analysis, 2023) in a standardized sensory lab (SRPS EN ISO 8589:2015 ; Sensory analysis, 2015), using seven-point (7) scales (SRPS ISO 4121:2013 ; Sensory analysis, 2013) to assess color uniformity, taste, smell, surface homogeneity, spreadability, graininess, hardness, and overall acceptability, following the protocol described by *Nikolić et al*. (2018).

2.1.3. Textural analysis

Texture analysis of the top-rated sensory samples was performed using a TA.XT Plus Texture Analyzer (Stable Micro Systems, UK), following the Margarine Spreadability protocol (MAR4_SR.PRJ), as described by *Županjac et al*., 2023. A Spreadability Rig HDP/SR was used at 22 °C with a 5 kg load cell. Test speeds were set to 1.0 mm/s (pre-test), 3.0 mm/s (test), and 10.0 mm/s (post-test). Firmness and shearing work were determined based on the force during probe penetration.

2.1.4. Statistical analysis

Statistical analysis was done using One-way ANOVA (Statistica 14.0.0.15, TIBCO Software Inc., USA). Duncan's post hoc test was performed for comparison of mean values. Differences were considered significant at $p<0.05$.

3. Results and discussion

Statistically significant differences ($p< 0.05$) were observed among the *pâté* samples in both firmness and work of shear (Table 1), indicating a broad textural range among the commercial products. Sample 139 (salmon *pâté*) exhibited the highest firmness (414±16.9 g) and work of shear (417±22.7 g sec), suggesting a dense and cohesive texture that may hinder spreadability. In contrast, sample 571 (tuna and mackerel) had the lowest values (firmness, 49.4±0.52 g; work of shear, 38.1±1.80 g sec), reflecting a very soft and easily spreadable product. The variability in firmness and spreadability among *pâté* samples can be attributed to differences in protein content and composition (*Trindade et al*., 2023; *Zheng et al*., 2022; *Zhang et al*., 2010), fat content and structure (*Martínez et al*., 2025 ; *Botella-Martínez et al*., 2024; *Lima et al*., 2022; *Tiensa*

et al., 2017), water content (*Mancera-Rodriguez et al*., 2022), use of different emulsifiers (*Baechle et al*., 2025; *Youssef & Barbut*, 2011; 2010), and processing methods (*Pětová et al*., 2024; *Lazárková et al*., 2023; *Aydin et al*., 2020), as well as storage and serving temperature, which have been shown to significantly affect the rheological and sensory characteristics of high-fat emulsified products (*Yang et al*., 2024). Proteins derived from different fish species (*Yi-Li et al*., 2025; *Fernandes et al*., 2024; *Traina et al*., 2024; *Ahmed et al*., 2022; *Tilami et al*., 2018) exhibit distinct gelation behavior, emulsifying capacity, and water-holding ability, which significantly influence the texture of fish-based spreads. Higher protein and fat levels generally contribute to a denser and more cohesive texture, while increased moisture and finer particle size favor softer and more easily spreadable products.

Samples 383, 428, and 329 exhibited intermediate firmness (276±16.1 to 288±7.73 g) and work of shear (274±13.4 to 291±15.2 g sec), likely indicating a balanced texture that aligns with typical consumer expectations.

Sample 555, although soft (146± 24.7 g), exhibited high work of shear (245.51 ± 33.93 g sec), indicating a soft yet less spreadable pâté touch (*Di Monaco et al*., 2008; *Szczesniak*, 2002). This is often linked to cohesive or elastic matrices that increase resistance to deformation, as confirmed in studies on fish pâtés and fat-based products (*Ranilović et al*., 2020; *Lončarević et al*., 2013).

Variations in firmness and spreadability were also observed within the same fish *pâté* types. For example, significant differences ($p<0.05$) in firmness and work of shear were observed among tuna *pâté* samples, with firmness ranging from 167±7.11g to 327±21.4 g. Higher values of both parameters indicate a denser, more cohesive texture that may reduce spreadability, while lower values suggest a softer, more easily spreadable product. These variations likely reflect different formulation and processing approaches (*Pětová et al*., 2024; *Lazárková et al*., 2023). Similarly, mixed fish *pâté*s made from tuna and mackerel (samples 149 and 571) showed statistically significant differences ($p<0.05$). Sample 149 exhibited high firmness and work of shear values (310±28.9 g and 301±35.7 g sec, respectively), indicating a dense and cohesive texture, while sample 571 had the lowest values (49.4±0.52 g and 38.1±1.80 g sec, respectively), reflecting a very soft and easily spreadable product. These differences highlight the impact of ingredient ratios and processing methods, emphasizing the need for formulation standardization to optimize textural properties (*Martínez et al*., 2025; *Botella-Martínez et al*., 2024; *Mancera-Rodriguez et al*., 2022; *Tiensa et al*., 2017). Among the salmon *pâté*s, samples 857 (firmness 295±4.55 g; work of shear 262±6.90 g sec) and

Table 1. Texture (firmness and work of shear) for fish *pâté* samples

Sample	Firmness (g)	Work of Shear (g sec)
139 - salmon	414±16.^{9}g	417±22.7^{g}
428 - tuna	276±16.1^{d}	274±13.4^{def}
571 - tuna and mackerel	49.4±0.52^{a}	38.1±1.80^{a}
965 - trout	151±7.11^{b}	152±8.03^{b}
711 - tuna	327±21.4^{f}	335±1.80^{f}
383 - tuna	288±7.73^{de}	286±12.6^{ef}
627 - tuna	172±15.3^{b}	163±20.4^{b}
334 - tuna	167±7.11^{b}	169±13.2^{b}
555 - trout	146±24.6^{c}	245±33.9^{c}
149 - tuna and mackerel	310±28.9^{ef}	301±35.6^{f}
314 - salmon	229±7.25^{c}	223±7.38^{c}
857 - salmon	295±4.5^{5d}e	262±6.90d^{e}
222 - tuna	284±8.13^{de}	299±12.4^{f}
329 - hake	281±12.2^{d}	291±15.2^{ef}

Legend: ^{a,b,c,d,e,f,g} Different letters in the column mean that pâtés are statistically different

314 (firmness 229±7.25 g; work of shear 223±7.38 g sec) showed similar texture and spreadability, while sample 139 (firmness 414±16.92 g; work of shear 417±22.7 g sec) exhibited significantly higher firmness and shear work, indicating a denser and less spreadable product.

Sensory evaluation is a valuable tool for predicting the overall acceptability of spreadable food products. It has been widely applied in the assessment of fish-, meat-, and plant-based spreads (*Mancera-Rodriguez et al*., 2022; *Ranilović et al*., 2020; *Branciari et al*., 2019; *Difonzo et al*., 2019; *Kim et al*., 2018). Figure 1 (panels A-C) presents the sensory evaluation findings described in the following section. Overall scores ranged from 4.4 (samples 329, 571) to 6.4 (222, 428), with tuna and salmon pâtés rated highest, aligning with previous findings on consumer preferences (*Nguyen et al*., 2023; *Budhathoki et al*., 2022).

Texture attributes varied: sample 139 showed slightly excessive hardness (5.4) and spreadability (5.1), while sample 571 had notably low scores (1.3), indicating an overly soft texture and poor homogeneity (2.3). Graininess was near optimal for most, though 383 (4.3) was slightly coarse, and 571 overly smooth (1.9). Color uniformity was generally high, with 139 scoring 7.0, though some samples, like 329 and 627, showed minor surface heterogeneity.

Lightness varied; 329, 383, and 571 appeared too light, suggesting possible dilution, while 334, 428, and 857 were slightly darker. Taste and smell were scored highest for 139, 314, and 965 (≥6.6), especially trout pâtés (555, 965), which also showed ideal hardness and spreadability, though with slightly lower overall acceptability compared to marine fish pâtés (*Lámfalusy et al*., 2025; *Stojanovska et al*., 2022).

Results highlight that, while marine fish pâtés, particularly tuna and salmon, had higher overall acceptability, the freshwater trout pâtés excelled in textural and flavor attributes important for consumer satisfaction.

4. Conclusion

This study provided a detailed evaluation of the textural and sensory properties of commercial fish pâtés available on the Serbian market. The results revealed substantial variability among products, reflecting heterogeneity in formulation strategies and a general lack of standardization regarding texture and consumer-relevant attributes. Instrumental measurements of firmness and spreadability

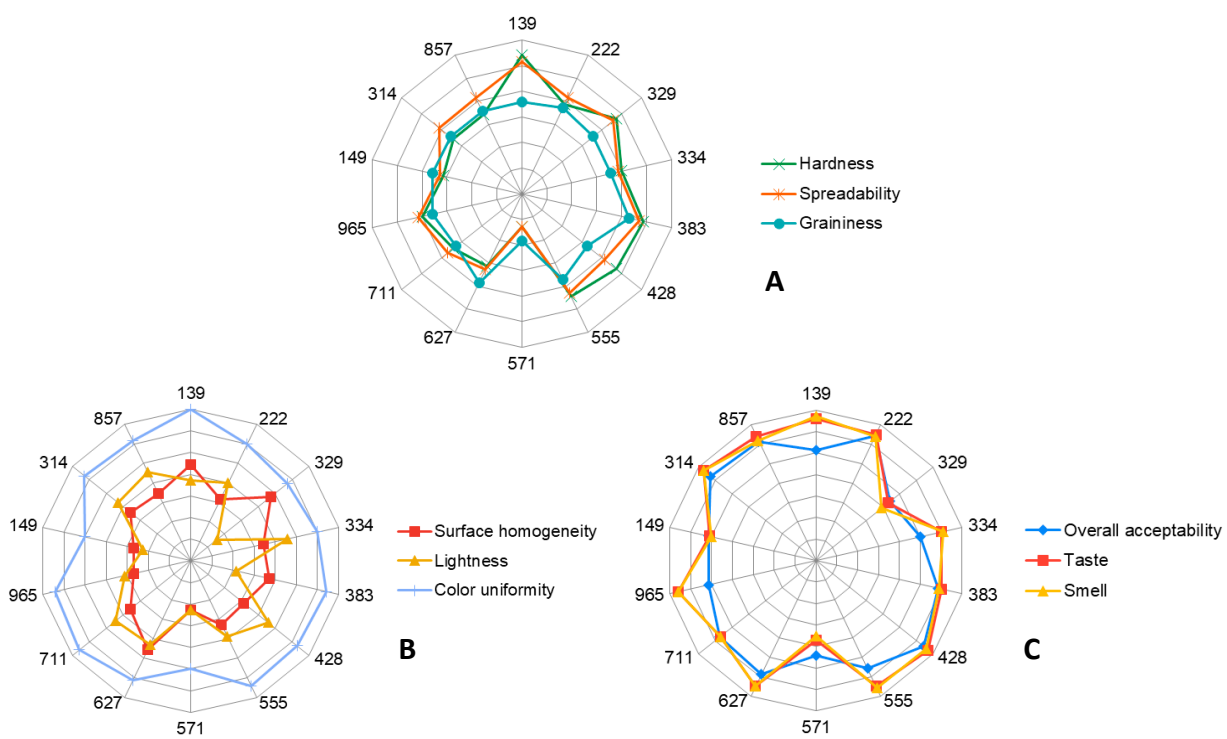

Figure 1. A) Hardness, spreadability and graininess; B) Surface homogeneity, lightness and color uniformity; C) Overall acceptability taste and smell (222 – Tuna; 383 – Tuna; 334 – Tuna; 428 – Tuna; 627 – Tuna; 711 – Tuna; 329 – Hake; 139 – Salmon; 314 – Salmon; 857 – Salmon; 571 – Tuna and Mackerel; 149 – Tuna and Mackerel; 555 – Trout; 965 – Trout.)

showed significant differences between the samples, being largely in agreement with sensory perceptions of hardness and ease of spreading. Marine fish pâtés, particularly those made from tuna and salmon were generally rated higher in terms of overall acceptability, primarily due to favorable taste, aroma, visual appeal, and consumer familiarity with these species. However, pâtés made from freshwater trout demonstrated more balanced textural characteristics, with sensory and instrumental data indicating desirable levels of hardness and spreadability.

These findings offer valuable insights for the development of novel fish pâtés based on freshwater bream *(Abramis brama)*, an underutilized species in food production. Drawing inspiration from the favorable texture profiles observed in trout-based products, freshwater bream pâtés could be formulated to achieve optimal consistency and sensory appeal. Such an approach would not only enhance product quality but also support the sustainable valorization of local freshwater fish through the creation of convenient, ready-to-eat food products.

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Original Scientific Paper

Daily consumption of sodium and nitrite from Serbian meat products: insights from principal component analysis

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ABSTRACT

Sodium chloride and nitrite play important roles in the meat processing industry, significantly influencing the safety, technological, and sensory properties of meat products, with nitrite classified as a food additive and sodium chloride as a food ingredient. Besides this important technological role, excessive intake can lead to human health disorders. Thus, the content of sodium chloride and nitrite in meat products on the Serbian market were analyzed using principal component analysis. A total of 64 meat products produced by the 18 most notable meat processors were collected from the Serbian retail market. The meat products collected belonged to four different groups: dry fermented sausages, finely comminuted cooked sausages, canned meat in pieces, and liver sausages and pates. Mean sodium chloride and nitrite contents for all analyzed meat products were 1.91 g/100g (the highest was for dry fermented sausages 3.67 g/100g; the lowest was for liver sausages and pates 1.19 g/100g) and 7.66 mg/kg (the highest was for chicken hot dogs 13.79 mg/kg; the lowest was for dry fermented sausages 2.17 mg/kg), respectively. The nitrite content in all analyzed meat products was in accordance with the European and Serbian legislation. Considering daily consumption of meat products and dried meat products in Serbia, and based on the obtained results, it can be concluded that consumers in Serbia exceed the recommended average daily consumption for sodium chloride. In conclusion, all possibilities for further reduction of sodium chloride consumption should be considered, especially because Serbia is a high risk country for cardiovascular diseases.

1. Introduction

Sodium chloride (salt) and nitrite are widely used in the meat processing industry due to their significant impact on the safety, technological, and sensory properties of meat products. Sodium chloride has historically been used in meat preservation due to its bacteriostatic effects. Moreover, it contributes to protein hydration and water-binding capacity, improves texture, and intensifies the aroma, flavor, and color of meat products (*Desmond*, 2006; *Haddad et al*., 2018; *Inguglia et al*., 2017; *Matthews & Strong*, 2005; *Pretorius & Schönfeldt*, 2018; *Rede & Petrović*, 1997;

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Ruusunen & Puolanne, 2005; *Wu et al*., 2015). Apart from its important role in the safety and quality of meat products, in the human body, sodium and chloride are essential for nerve and muscle function, fluid balance, and regulation of blood pH and pressure (*Catterall*, 2000; *Jentsch et al*., 2002).

Like salt, nitrite also influences the safety of meat products due to its ability to prevent and control the growth of pathogenic and undesirable microorganisms, especially *Clostridium botulinum* and *Listeria monocytogenes*. Furthermore, it has the ability to prevent lipid oxidation, and it improves the sensory properties of meat products by contributing to the development of their characteristic pink color, taste, and aroma (*Honikel*, 2008; *Parthasarathy & Bryan*, 2012; *Sebranek*, 2009).

Despite their technological benefits, excessive intake of salt and nitrite can adversely affect human health, which is why this topic has been receiving increasing public health attention. High sodium consumption increases the risk of hypertension, cardiovascular diseases, and other conditions (*Aburto et al*., 2013; *WHO*, 2010, 2016; *Wu et al*., 2015). Nitrites can form carcinogenic nitrosamines and contribute to toxic effects, such as methemoglobinemia and increased cancer risk (*Choi et al*., 2007; *EFSA*, 2010; *Gilchrist et al*., 2010; *Zhang et al*., 2018). These risks are particularly relevant in Serbia, where in 2024, 49.72% of deaths were due to cardiovascular diseases and 21.79% to cancer (*Statistical Office of the Republic of Serbia*, 2024). In response to these risks, regulatory frameworks limit nitrite content to 150 mg/kg (100 mg/kg for sterilized canned meats), with exceptions for traditional products (*European Commission*, 2011; *Serbian Regulation*, 2008). In contrast, the sodium chloride content in foods is not strictly regulated at the EU level, although international health authorities have issued recommendations for reduced intake, typically less than 5–6 g of salt (2–2.4 g of sodium) per day (*EFSA*, 2019; *WHO*, 2012; *WHO*, 2023; *FSA*, 2024; *Desmond*, 2006; *Matthews & Strong*, 2005). Instead, the EU Salt Reduction Framework has established non-binding, category-specific targets for sodium reduction, while some countries have introduced their own mandatory national benchmarks. For instance, Norway has defined precise targets for salt in meat products (sausages 1.6 g/100 g, pâté 1.5 g/100 g, dried sausages up to 5.0 g/100 g), whereas Bulgaria has implemented compulsory limits on salt content in processed meat products, such as up to 2 g/100 g for general meat products and up to 3.5 g/100 g for cured sausages. (*World Action on Salt, Sugar & Health*, 2025*; Norwegian Directorate of Health*, 2016; *European Commission; European Public Health Alliance*, 2021).

Considering the importance of monitoring sodium chloride and nitrite intake, this study analyzed their content in 64 meat products from four categories most commonly consumed in the Serbian diet. All samples were produced by the most prominent meat processors on the national market. Principal component analysis (PCA) was applied to provide a representative overview and support potential strategies for improving public health through informed regulatory measures.

2. Materials and methods

2.1. Meat products

A total of 64 meat products, produced by the 18 preeminent meat processors (P1-P18) were collected from the Serbian retail market. The meat products belonged to four different groups, defined by the Regulation on the Quality of Minced Meat, Semi-finished Meat Products, and Meat Products (*Serbian Regulation*, 2023) widely consumed in Serbian diet. The first group comprised dry fermented sausages (n=10) of the product type called "čajna kobasica" (PC). The second group was finely comminuted cooked sausages (n=21), of which 11 were chicken hot dogs (PPV) and 10 pork hot dogs (PSV). The third group consisted of canned meat in pieces (n=20), of which 11 were made of chicken meat called "pileća prsa" (PP) and 9 were pork products called "pizza šunka" (PS). Finally, the fourth group was liver sausages and pâtés (n=13), and all were the liver pâté called "jetrena pašteta" (PJ). After collection, the meat products were homogenized and stored at -18°C until analysis. All determinations were conducted in triplicate.

2.2. Sodium chloride and sodium nitrite content determination

The sodium content was determined using the FINSLab-5.4-3M-037 method for the determination of Na, K, Mg, Ca, and Mn in food by atomic absorption spectrophotometry, in accordance with the Rulebook on Food Declaration, Labeling and Advertising (*Serbian Regulation*, 2024). Based on the determined sodium content, the sodium chloride content was calculated in accordance with the Rulebook on Food

Declaration, Labelling and Advertising (*Serbian Regulation*, 2024) and expressed as grams of sodium chloride per 100 grams of sample. Residual nitrite content was determined according to the international standard (*ISO*, 1975) and expressed as mg nitrite/kg sample (*Peulić et al.*, 2020).

2.3. Statistical analysis

PCA was carried out using Statistica 13.5.0.17 software (TIBCO, Palo Alto, United States). PCA was performed to visualize differences between meat products groups and between samples in sodium chloride and nitrite content.

3. Results

The results of the PCA are presented in Figure 1. Figure 1a displays the PCA loading plot, which illustrates the contribution of individual variables—sodium chloride and nitrite—to the separation of the analyzed meat products. As evident from the plot, both variables significantly influenced the distribution of the meat products within the PCA space. Moreover, the two variables were not correlated, indicating that a higher nitrite content in a sample did not necessarily correspond to a lower sodium chloride content, and vice versa. Most meat products clustered closely around the origin of the PCA score plot (Figure 1b), suggesting a high degree of similarity in sodium and nitrite content across the majority of analyzed products. Only a limited number of meat products deviated significantly from this cluster, reflecting potential product-specific formulation practices or technological differences in processing.

The mean sodium chloride content across all analyzed meat products was 1.91 g/100 g. As shown in the PCA score plot (Figure 1b), samples of čajna kobasica (a dry fermented sausage) were positioned in the negative quadrant of the first principal component (F1), with their distribution strongly influenced by sodium chloride content. This group exhibited the highest mean sodium chloride concentration (3.67 g/100 g), with a particularly elevated level observed in the product produced by manufacturer no. 4. These results are consistent with previously reported data for traditional Serbian dry fermented sausages (*Ikonić et al*., 2020), and exceed levels recorded by *Vranić et al*. (2009) for similar products on the domestic market. In contrast, liver sausages and pâtés exhibited the lowest sodium chloride levels, averaging 1.19 g/100 g. Notably, three meat products from this group (PJ4, PJ11, and PJ18) had sodium chloride contents below 1.00 g/100 g and were located in the positive region of F1, showing a clear difference from the dry fermented sausages. Finely comminuted cooked sausages contained, on average, 2.10 g/100 g (chicken hot dogs) and 1.95 g/100 g (pork hot dogs) of sodium chloride, while canned meat products ranged from 1.35 g/100 g in pileća prsa to 1.41 g/100 g of sodium chloride in pizza šunka.

On average, 183.47 g of meat and meat products are consumed daily in Serbia. Among these, meat products are the third most commonly consumed group, after poultry and pork, contributing 38.06 g per day, whereas dried meat products rank fifth, with 12.9 g consumed daily. The mean sodium chloride content in the studied meat products was 1.91 g/100g, and in dried meat products, obtained by *Vranić et al*., (2009), was 5.09 g/100g, which means that the average consumer in Serbia would intake 0.73 and 0.66 g of sodium chloride daily from meat products and dried meat products, respectively. In many countries, meat and meat products are the second largest contributors to dietary salt intake, after baked goods, and the meats contribute around 20% of the sodium intake (*Desmond*, 2006). From these data, it is clear that the average daily intake of sodium chloride from meat products and dried meat products, added together, is 1.39 g. Moreover, the mean sodium chloride content in meat products observed in this study (1.91 g/100 g) exceeds the WHO global sodium benchmarks for liver sausages (525 mg Na/100 g, equivalent to 1.33 g/100 g of salt), hot dogs (540 mg Na/100 g, equivalent to 1.37 g/100 g of salt), and cured meat (950 mg Na/100 g, equivalent to 2.41 g/100 g of salt), highlighting the need for effective sodium reduction strategies in Serbian meat products.

According to the PCA loading plot (Figure 1a), nitrite content—represented in the positive quadrant of F1—significantly contributed to the separation of five meat products (PPV10, PPV4, PP7, PPV2, and PS9), each exhibiting nitrite concentrations exceeding 19 mg/kg (ranging from 19.16 to 39.12 mg/kg). Group-wise means were as follows: 4.59 mg/kg for liver sausages and pâtés, 7.02 mg/kg for pork hot dogs, and 9.27 mg/kg and 9.41 mg/kg for chicken and pork canned meats, respectively.

Based on the mean nitrite content of 7.66 mg/kg for meat products and 10.7 mg/kg for dried meat products (*Bajčić et al*., 2018), and using the same consumption figures as above (38.06 g and 12.9 g/day), the estimated nitrite intake is 0.29 mg/day from

meat products and 0.10 mg/day from dried meat products, resulting in total nitrite intake of 0.39 mg/day. The acceptable daily intakes (ADIs) for nitrite, as set by the former Scientific Committee for Food (*SCF*, 1997) and the Joint FAO/WHO Expert Committee on Food Additives (*JECFA*, 2002), are 0.06 and 0.07 mg/kg body weight/day, respectively (*SCF*, 1997; *JECFA*, 2002). For an adult weighing 65 kg, the maximum permitted daily intake ranges from 3.9 to 4.55 mg/day. The calculated intake of 0.39 mg/day is therefore well below the established safety thresholds. All measured nitrite levels in this study complied with the maximum levels permitted by European legislation (*European Commission*, 2011) and Serbian national regulations (*Serbian Regulation*, 2018). The results are also consistent with findings from the German market (*Andrée et al.*, 2010; *Honikel*, 2008), indicating that Serbian meat processors generally operate within legally acceptable limits of nitrite additives.

4. Conclusions

The results of this study demonstrate that both sodium chloride and nitrite contents significantly influenced the distribution of meat product samples within the PCA space. The mean sodium chloride and nitrite contents were 1.91 g/100 g and 7.66 mg/kg, respectively. Estimated daily intake of sodium chloride from meat and dried meat products in Serbia was 1.39 g, while nitrite intake was 0.39 mg. Although nitrite levels were well below the acceptable daily intake and within legislative limits, sodium chloride intake exceeded recommended levels. Given the high cardiovascular risk in Serbia, efforts to reduce salt intake should be prioritized. Future research should focus on the development and implementation of reformulation strategies, consumer education, and product innovation aimed at improving the nutritional profile of meat products without compromising food safety or quality.

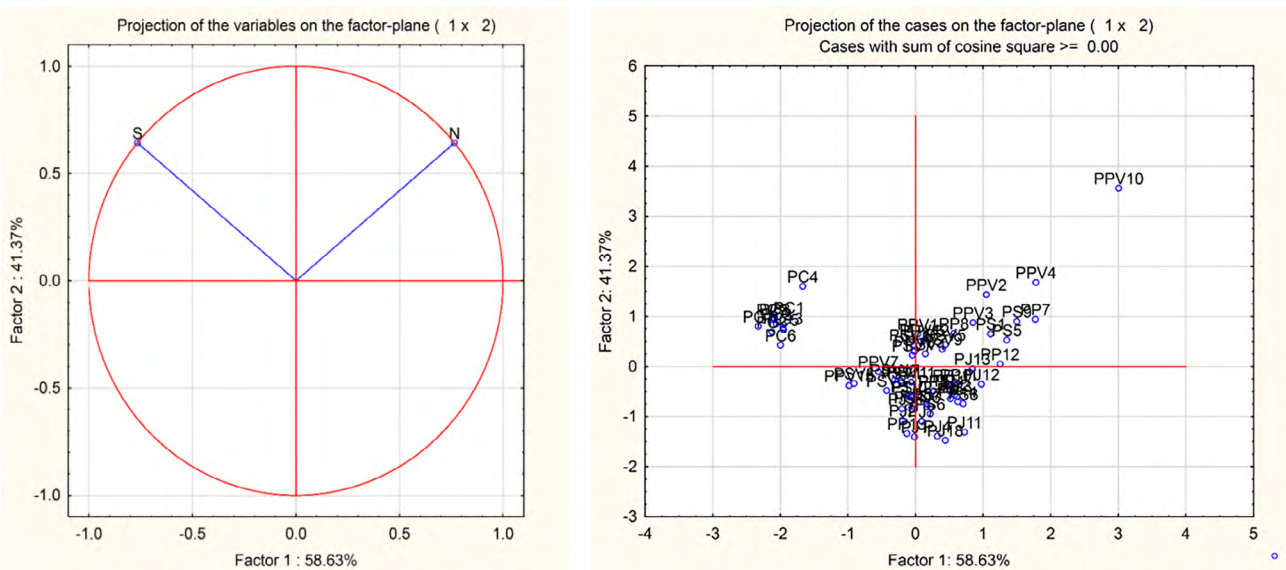

Figure 1 a). PCA loading plot in the plane of factors 1 and 2 – the labels correspond to sodium chloride (S) and nitrite (N) content. **b)** PCA score plot in the plane of factors 1 and 2 – the labels correspond to the meat products given in materials and methods.

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Original Scientific Paper

Development and characterization of dry beef jerky products

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ABSTRACT

In response to growing consumer demand for convenient, high-protein snacks with improved nutritional profiles, three types of beef jerky products were developed and evaluated: basic, sweet and hot. The products were prepared from beef cuts (*M. semimembranosus* and *M. semitendinosus*), marinated with different spice and sauce combinations, and dried under controlled conditions. Microbiological safety and nutritional quality parameters were determined. All products were microbiologically safe for consumption, with no detection of pathogenic microorganisms. Regarding nutritional quality, all products were characterised by a notably high protein content, ranging from 47.61% in the sweet jerky to 49.96% in the hot jerky. Fat content differed slightly between the products, ranging from 5.67% in the basic jerky to 6.29% in the hot jerky, indicating that all products were generally low in fat. Fatty acid profiles indicated a desirable ratio of saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA), with SFA being the most dominant group. The sweet jerky contained significantly higher levels of carbohydrates (8.79%) and sugars (8.13%), which can be attributed to the marinade composition. Although salt levels were relatively high across all variants, they remained within expected ranges for jerky products. These findings suggest that beef jerky can be a nutritious meat-based snack, with potential for further improvement—especially in developing a sweet-flavoured variant with reduced sugar content to meet both sensory and health expectations.

1. Introduction

The modern consumer is increasingly aware of the connection between diet and overall psychophysical well-being. A nutritionally balanced diet—providing all essential macro- and micronutrients while ensuring food safety—is a key foundation for maintaining good health. Proper nutrition positively affects health and quality of life, helps prevent non-communicable diseases, improves immunity, physical fitness, and work capacity, and supports the adoption of a healthy lifestyle (*Chang et al.*, 2024; *Vorster*, 2009). Due to the modern lifestyle, traditional dietary patterns based on three main meals a day consumed at specific times and locations are becoming less common (*Jastran et al.*, 2009). Eating outside the home is becoming increasingly prevalent (*Orfanos et al.*, 2007; *Ribas-Barba et al.*,

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2007), as is the frequency of snacking (*Vandevijvere et al.*, 2009). Snack products have been an integral part of daily diets for many years (*Riaz*, 2004). In fact, two-thirds of respondents in the United States (US) reported regularly consuming snack products between meals (*Sanz et al*., 2017). *Kant and Graubard* (2015) reported a decline in calorie intake from main meals (breakfast, lunch, and dinner) over the past 40 years, with a simultaneous increase in calorie intake through snacking, which some researchers have shown to contribute up to 40% of total daily energy intake (*Rangan et al*., 2008; *Rangan et al*., 2009). In Serbia, the percentage of the population consuming three regular meals a day dropped from 71.9% in 2000 to only 56.6% in 2006. A study conducted among young adults aged 18 to 24 in Australia found that, after taste, the most influential factor in food choice was the convenience of the food product (*Hebden et al*., 2015).

Therefore, the food industry is striving to develop food products that are nutritionally rich, while requiring no preparation or only minimal culinary skills and time. In other words, today's consumer needs food that is both easily accessible and nutritionally valuable. This technical solution describes the development of such a product—a jerky-type product characterized by high nutritional value and ease of access.

Most commercial snack products are characterised by low nutritional quality. Typically, these products are high in carbohydrates and fats, and low in dietary fibre, often falling into the category of high glycaemic index foods. In an effort to develop snacks with improved nutritional profiles, there has been increasing interest in the use of unconventional raw materials rich in proteins, dietary fibre, vitamins, polyphenols, and other valuable components. (*Jozinović et al*., 2021; *Raleng et al*., 2022; *Grasso*, 2020; *Delić et al*., 2020).

To improve the nutritional quality of snack products, some authors have investigated the potential of using animal-based ingredients, which contain all essential amino acids. The most commonly studied animal raw materials include mechanically separated meat, milk and whey proteins, meat from spent laying hens, partially defatted beef, and others (*Cakmak et al*., 2016; *Cho & Rizvi*, 2010; *Delić et al*., 2023; *Lee et al*., 2003; *Rhee et al*., 1999; *Verma et al.*, 2014). In the US, meat-based snack products, such as beef jerky, meat sticks, and meat or meat protein bars, are highly popular. According to a 2014 study, meat snacks were the third most commonly consumed salty snack category in the US. Moreover, these products are gaining popularity in Africa and the Middle East, where their consumption has increased by 25% (*Sanz et al*., 2017). In contrast, Europeans are still more inclined toward traditional meat products, with meat snacks and products containing meat or meat proteins only recently starting to enter the European market.

The aim of this study was to develop and evaluate three types of beef jerky—basic, sweet, and hot—as high-protein, ready-to-eat meat snacks with improved nutritional profiles. The products were assessed for microbiological safety, nutritional quality, and fatty acid composition to determine their potential as healthier snack alternatives.

2. Materials and methods

2.1. Preparation of beef jerky

For preparation of jerky, beef *M. Semimembranosus* (SM) and *M. Semitendinosus* (ST) were purchased in local meat facility. All visible fat and connective tissue from fresh muscles were removed and meat was frozen at -18 °C. Thereafter, the thawed muscles were sliced into pieces 10.0 ×4.0 × 0.5 cm, normal to the direction of the muscle fibre.

Prepared beef slices were marinated for 24 hours in the refrigerator using three different marinades M1 (basic), M2 (sweet) and M3 (hot), consisting of liquid (soy sauce, Worcestershire sauce, etc.) and solids (salt and different spices) in different proportions.

Further, marinated beef slices were uniformly distributed on the chamber shelves and processed to drying in a constant climate chamber (Binder KBF 115, Germany). The drying process lasted for 6.5 h, divided into two phases. During the first, the so-called cooking phase, the chamber was set to 70 °C for 2.25 hours, and relative humidity during this period ranged from 80 to ≥90%. In the second phase, the drying temperature ranged from 65 °C to 60 °C, while the relative humidity was gradually reduced from 70 to 35% over 4.25 hours (*Ikonić et al*., 2022).

2.2. Microbiological safety determination

Microbiological safety was determined by enumeration of the total count of bacteria (*ISO*, 2013), yeasts and moulds (*SRPS ISO*, 2011), *Salmonella* spp. (*ISO*, 2017a), *Enterobacteriaceae* (*ISO*, 2017b), and *Escherichia coli* (*ISO*, 2001).

2.3. Nutritional quality determination

Protein content (*ISO*, 2023a), moisture content (*ISO*, 2023b), ash content (*ISO*, 1998), fat content (*ISO*, 1973) and sodium content (*ISO*, 6869:2000) were determined according to ISO methods. The carbohydrate content was determined using the formula provided by *Omole et al*. (2021), and sugar content was determined using the Luff-Schoorl method (*Serbia, 1983*). The sodium chloride content was calculated from the content of sodium according to the Serbian regulations (*Serbia, 2017 and 2018*). Energy value was calculated according to the EU regulations (*European Union, 2011*).

2.4. Fatty acid determination

Fatty acid methyl esters were prepared from the extracted lipids using boron trifluoride/methanol solution and were analyzed by a GC Agilent 7890A system with FID, autoinjection module for liquid, equipped with fused silica capillary column (*Vidosavljević et al.*, 2025).

3. Results and discussion

Safety parameters and nutritional quality of the three developed types of jerky products, basic, sweet, and hot, were analyzed and the results are presented in Tables 1 and 2.

As shown in Table 1, all developed products were microbiologically safe for consumption, as no pathogenic bacteria were detected (including *Salmonella* spp., *Enterobacteriaceae*, and *E. coli*), nor were any yeasts or moulds present (*Županjac et al.*, 2022).

Regarding nutritional quality (Table 2), all beef jerkies were characterized by a notably high protein content, ranging from 47.61% in the sweet jerky to 49.96% in the hot jerky (*Županjac et al.*, 2022). Moisture content differed slightly between the products, with the basic jerky containing the highest moisture content (32.94%) and the hot jerky containing the lowest (30.50%). Moisture content across the beef jerkies was within acceptable limits for dried meat products, indicating effective dehydration. The slight differences could be attributed to variations in formulation or seasoning blends that influence water retention. Ash content was highest in the basic jerky (7.48%) and lowest in the sweet jerky (6.70%). The

Table 1. Indicators of microbiological safety in developed jerky-type products

Microbiological safety parameters	Basic	Sweet	Hot
Total number of bacteria (cfu/g)	390000	64000	140000
Yeasts and moulds (cfu/g)	<100	<100	<100
Salmonella spp. (not detected in 25 g)	not detected	not detected	not detected
Enterobacteriaceae (cfu/g)	<10	<10	<10
E. coli (cfu/g)	<10	<10	<10

Table 2. Indicators of nutritional quality in developed jerky-type products

Nutritional quality parameters	Basic	Sweet	Hot
Protein content (%)	49.61	47.61	49.96
Moisture content (%)	32.94	31.21	30.5
Ash content (%)	7.48	6.7	7.28
Total carbohydrates (%)	4.3	8.79	5.97
of which sugars (%)	2.39	8.13	3.71
Fat content (%)	5.67	5.69	6.29
Saturated fatty acids (in fat %)	46.8	45.4	45.7
Monounsaturated fatty acids (in fat %)	41.3	46.2	45.6
Polyunsaturated fatty acids (in fat %)	11.9	8.4	8.7
Salt content (%)	4.80	4.36	4.52

content of carbohydrates and sugars was, as expected, the highest in the sweet jerky, given that the sweet marinade formulation included the addition of honey and sugar.

Fat content differed slightly among the meat products, ranging from 5.67% in the basic jerky to 6.29% in the hot jerky, indicating that all products were generally low in fat. Regarding the fatty acid profile, the basic jerky contained the highest proportion of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) with 46.8% and 11.9%, respectively, while the sweet jerky had the highest proportion of monounsaturated fatty acids (MUFA) with 46.2%.

Salt content was the highest in the basic jerky (4.80%), followed by the hot (4.52%) and sweet (4.36%) jerky. The salt content, although relatively high, was typical for jerky products, due to its role in preservation and flavour enhancement.

4. Conclusion

The analysis of three beef jerky snack types confirmed that all products were high in protein, and low in carbohydrates and fat, making them nutritionally suitable as meat-based snack options. The basic jerky was characterised by the most balanced nutritional profile, while the sweet jerky had elevated sugar and carbohydrate levels. Future improvements should aim to reduce sugar and salt content while maintaining sensory quality.

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Original Scientific Paper

Nutritional status and dietary habits of 15–17-year-old adolescents in Bosnia and Herzegovina: findings from the EU menu national survey

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ABSTRACT

Proper nutrition during adolescence is essential for healthy growth, development, and long-term well-being. From 2018 to 2021, the Food Safety Agency of Bosnia and Herzegovina conducted a national dietary survey of the population, following the EU Menu methodology. For this study, a subsample of adolescents aged 15–17 years was analyzed, given the physiological similarity of this age group to adults, which allows for the application of standard adult BMI classification. A total of 250 participants were assessed using standardized tools, including the Diet Assess and Plan (DAP) software, collecting data on anthropometry and dietary intake. The majority of adolescents had a normal BMI; however, a concerning proportion of females were underweight. Furthermore, fat intake exceeded recommended levels in both sexes, suggesting potential future health risks. These findings highlight the importance of targeted nutritional education and interventions to promote balanced dietary habits and address both undernutrition and excessive fat intake among adolescents in Bosnia and Herzegovina.

1. Introduction

Adolescence is a critical stage of development during which lasting lifestyle habits are established. Adopting healthy behaviours, such as regular physical activity and a balanced diet, can offer numerous advantages, including enhanced academic performance (*Doku et al*., 2013). Adolescence is a critical developmental stage bridging childhood and adulthood, marked by profound physiological changes associated with puberty, as well as accelerated emotional, cognitive, and social maturation. This period is embedded within a broader, lifelong continuum of human development that originates in foetal life and is shaped throughout childhood by a range of environmental and sociocultural factors—both protective and adverse. The culmination of adolescence involves the attainment of complete somatic and sexual maturity, the consolidation of individual personality, the achievement of economic independence, and integration into the broader social structure (*Heald*, 1985).

However, adolescence is also a period of increased vulnerability to nutritional deficiencies and the adoption of unhealthy dietary patterns. These factors may have long-term health implications,

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including an elevated risk of developing osteoporosis, obesity, hyperlipidaemia, delayed sexual maturation, and failure to reach full adult stature. Extensive research over the years has consistently demonstrated a strong connection between nutrition and overall health. Evidence continues to grow, indicating that following a healthy dietary pattern as part of a balanced lifestyle can support long-term well-being and lower the risk of chronic illnesses at every stage of life (*USDA*, 2020–2025). Nutrition and dietary habits are of critical importance during adolescence, a period characterized by accelerated physical growth and increased physiological demands. To support optimal development, it is essential for adolescents to adopt healthy eating behaviours and adhere to evidence-based dietary recommendations.

Estimating dietary intake of foods and nutrients is a commonly employed indirect method for evaluating an individual's nutritional status. Ultimately, such assessments aim to safeguard and improve overall human health (*Šatalić & Alebić*, 2008). National dietary surveys are a critical source of evidence that support the development of nutrition and health policies. They offer essential population-level insights into eating habits, nutrient intake and adequacy, diet-related health risks, and contribute to the formulation of dietary guidelines and recommendations (*Zhang et. al*., 2019). The aim of this study was to determine the body mass index (BMI) and nutritional status of a select adolescent group in Bosnia and Herzegovina, based on data collected from the Bosnia and Herzegovina Menu national survey.

2. Materials and methods

The methodology used for conducting the food consumption survey was aligned with the European Food Safety Authority's guidelines (*EFSA*, 2014). The national study on dietary habits in Bosnia and Herzegovina (B&H MENU) aimed to establish a system and database on the population's dietary habits, as well as related indicators such as health status, anthropometric characteristics, and physical activity, following the EU Menu methodology (*Arar et. al*., 2021).

The Food Safety Agency of Bosnia and Herzegovina served as the main coordinating institution, responsible for the development of survey tools, project management, and ensuring full compliance with contractual obligations and project timelines. The sampling framework was developed in collaboration with the Agency for Statistics of Bosnia and Herzegovina, based on their established methodological standards.

The broader study included individuals from households across Bosnia and Herzegovina, including adolescents aged 10 to 17 years, who were divided into two age groups: 10–14 and 15–17 years. However, for the purposes of this paper, a subsample of adolescents aged 15–17 years was selected for analysis. This age group was chosen because the 15–17 year age group is physiologically more comparable to adults, allowing the use of standard adult BMI categories. A total of 250 adolescents aged 15–17 years participated in this subgroup analysis, comprising 124 females and 126 males.

For dietary intake assessment, data storage, and processing, the study employed the Diet Assess and Plan (DAP) software platform, a standardized tool developed in accordance with EU Menu guidelines, ensuring accuracy and consistency in data handling (*Gurinović et al*., 2018).

The B&H MENU study was conducted at the individual level, with households selected through a two-stage stratified random sampling method. The sampling design ensured a representative cross-section of the population by including participants from both urban and rural areas, all geographic regions, and major ethnic groups. All age and sex groups were proportionally represented.

Data collection was conducted over the course of a full year, covering all four seasons. The scheduling of interviews was structured to capture seasonal and weekly variations in dietary intake, with appropriate distribution across weekdays, weekends, and public holidays. Anthropometric data (body weight and height) were self-reported during face-to-face interviews, rather than directly measured.

Body mass index (BMI) was determined by dividing weight in kilograms by height in meters squared, with classification according to WHO criteria: underweight (<18.5 kg/m²), normal weight (18.5–24.9 kg/m²), overweight (25.0–29.9 kg/m²), and obesity (>30.0 kg/m²) (*Connor et al*., 2023).

3. Results

The dietary habits database for residents of Bosnia and Herzegovina includes data from 250 adolescents (124 females and 126 males) aged 15-17 years. The nutritional status of male and female adolescents, as determined by BMI classification, is summarized in Table 1.

Table 1. Nutritional status of adolescents, BMI classification

Gender	Underweight	Normal weight	Overweight	Obese
Female adolescents	15 (12%)	101 (81%)	8 (6%)	0 (0%)
Male adolescents	8 (6%)	105 (83%)	10 (8%)	3 (2%)

Respondents were grouped by sex and age group. The results show that the majority of adolescents in Bosnia and Herzegovina had a normal weight, both among males and females. Specifically, 101 female adolescents (81%) and 105 male adolescents (83%) were classified as having a normal weight according to the BMI. The prevalence of underweight was higher among females (12%) compared to males (6%). Conversely, the proportions of overweight and obesity were slightly higher in males (8% overweight and 2% obese) compared to females (6% overweight and 0% obese). The distribution of BMI categories among adolescents by gender is illustrated in Figure 1, highlighting the higher prevalence of underweight among females and greater overweight/obesity in males.

These results suggest that underweight is more common among female adolescents, whereas excess body weight (overweight and obesity) is more prevalent among male adolescents. In addition to nutritional status, the study evaluated median daily energy intake and the distribution of macronutrients by sex, as shown in Table 2.

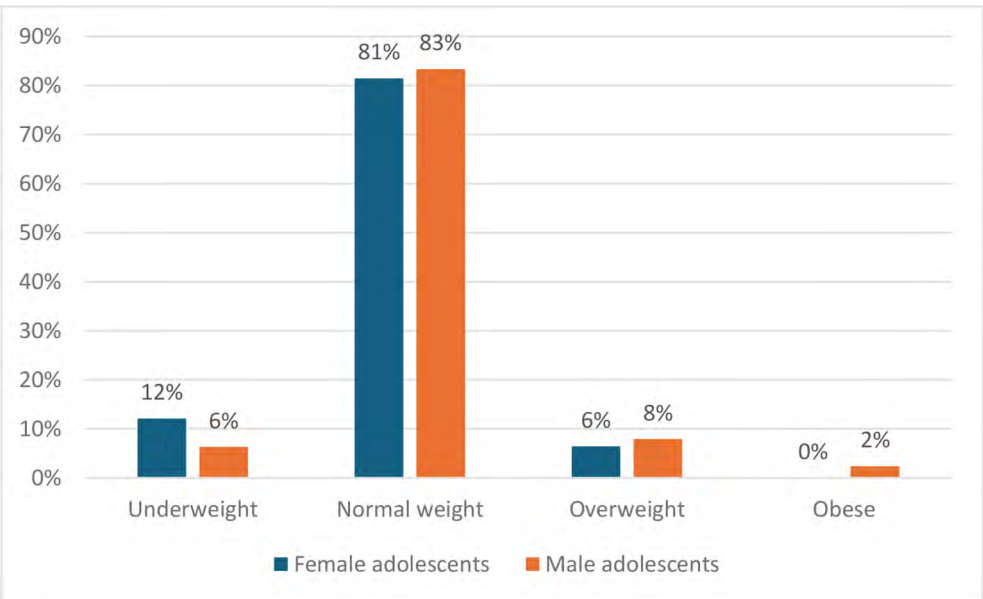

Figure 1. Distribution of BMI categories among adolescents by gender

Table 2. Median daily energy intake and macronutrient distribution among adolescents by sex

Sex	Median Energy Intake (kcal)	Protein (%)	Carbohydrates (%)	Fat (%)
Male	2230.75 kcal	15.00%	41.15%	43.85%
Female	1899.49 kcal	13.82%	43.66%	42.51%

Median daily energy intake was higher in males (2230.75 kcal) than in females (1899.49 kcal). Regarding macronutrient distribution, males derived 15% of their energy from protein, 41.15% from carbohydrates, and 43.85% from fat. Females obtained 13.82% of energy from protein, 43.66% from carbohydrates, and 42.51% from fat. While protein intake was similar across genders, females consumed a higher proportion of carbohydrates, and males had a slightly higher fat intake. Figure 2 presents median daily energy intake and macronutrient distribution by sex, visually illustrating these differences.

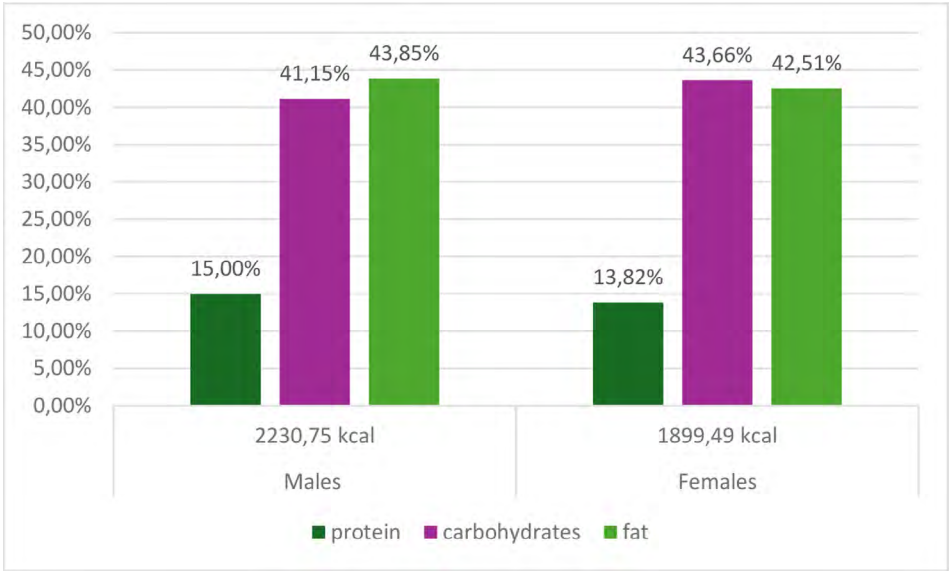

Figure 2. Median daily energy intake and macronutrient distribution by sex

4. Discussion

This study provides a comprehensive overview of the nutritional status and dietary intake of adolescents aged 15-17 years in Bosnia and Herzegovina, based on a representative national survey following the EU Menu methodology. It is important to note that due to insufficient detailed data on the exact ages of participants within the adolescent age range, BMI classification was applied according to adult standards rather than age-specific BMI-for-age references. The subgroup of older adolescents (15-17 years) was selected because their physiological characteristics closely approximate those of adults, making the adult BMI classification appropriate for this analysis.

The findings reveal that the majority of adolescents maintain a normal BMI. However, the higher prevalence of underweight among female adolescents is a notable concern, as it may indicate issues related to inadequate nutritional intake. Conversely, overweight and obesity were more prevalent among male adolescents, consistent with global trends that suggest a rising burden of excess body weight among youth.

The dietary intake analysis revealed that median daily energy intake was higher in males than females, which is expected given physiological differences and energy requirements. The macronutrient distribution showed that fat intake exceeded the recommended 35% of total daily energy intake (*WHO*, 2023) in both sexes, with males consuming slightly more fat and females more carbohydrates. Excessive fat consumption in adolescence is concerning due to its association with future cardiovascular risks and metabolic disorders.

These results underscore the urgent need for targeted nutritional interventions and educational programs that address both undernutrition among females and the prevention of overweight and obesity among males. Promoting balanced dietary habits and increasing awareness about healthy food choices can contribute to better long-term health outcomes for Bosnian and Herzegovinian adolescents.

Limitations of this study include reliance on self-reported anthropometric data, which may introduce bias.

5. Conclusion

This study highlights important aspects of adolescent nutrition in Bosnia and Herzegovina. While most adolescents aged 15-17 fall within the normal BMI range, a significant proportion of females are underweight, and males show higher rates of overweight and obesity. It is important to emphasize that BMI classification was based on adult standards, due to limited data on participants' exact ages, and the subgroup of older adolescents was chosen because their physiological characteristics closely resemble those of adults, making this approach appropriate.

Dietary assessments indicate that fat intake surpasses recommended guidelines in both sexes, posing potential health risks. Addressing these issues requires comprehensive public health strategies focusing on nutritional education and lifestyle modifications tailored to the needs of adolescents.

By fostering healthy eating habits and balanced nutrient intake, it is possible to improve growth, development, and long-term well-being in this vulnerable population.

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Review Paper

Enterobacteriaceae in the food chain – modern strategies for detection and control

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Antibiotic resistance
Pathogen control
PCR
Biosafety

ABSTRACT

Members of the *Enterobacteriaceae* family, such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp., and *Yersinia* spp., are significant contributors to foodborne diseases worldwide. This paper presents a comprehensive overview of their transmission through the food chain, resistance profiles, and modern strategies for detection and control. Particular emphasis is placed on advanced diagnostic approaches, including PCR-based techniques, isothermal amplification, and nanotechnology-driven methods. Control interventions ranging from physical decontamination (e.g., irradiation, pulsed light) to the use of natural antimicrobials such as bacteriophages and algal compounds are also discussed. The review underlines the necessity of integrated approaches in reducing the prevalence of foodborne pathogens and improving public health outcomes.

1. Introduction

Foodborne diseases are a global concern, with *Enterobacteriaceae* often implicated as primary agents. Due to poor hygienic practices and insufficient control measures, particularly in low-resource environments, the transmission of these bacteria through contaminated food continues to pose significant health risks. This work aims to consolidate current knowledge about transmission routes, genetic adaptability, resistance trends, and available tools for detection and control (*Eng et al*., 2015).

2. Routes of transmission of foodborne pathogens in the food chain

The dissemination of foodborne pathogens within the food chain occurs through various pathways, including insect vectors, faecal-oral routes, contaminated food and water, and direct animal-to-human transmission. Some pathogens, such as *Escherichia coli* and *Salmonella* Enteritidis, are zoonotic and can be transmitted from animal reservoirs to humans. In contrast, *Salmonella* Typhi lacks an animal host and is particularly pathogenic to humans (*Mezal et al*., 2014).

Insects are significant vectors of foodborne pathogens due to their habits and ecological niches. Their attraction to decaying organic matter, frequent

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contact with human environments, and physiological features enable them to act as mechanical carriers of microbial agents. Studies have shown that ants in a Brazilian hospital carried *E. coli* and *Salmonella*, while cockroaches sampled from hospitals, restaurants, and retail environments in Spain harboured *E. coli*, *Salmonella*, and other *Enterobacteriaceae* (*Mezal et al.,* 2014). In the South Canary region of India, over 4% of cockroaches collected from various sites tested positive for *Salmonella* spp. Despite these evidences, the role of flies in foodborne pathogen transmission remains under-researched. Houseflies are particularly risky due to their frequent defecation, approximately every 4–5 minutes, which facilitates the spread of pathogens through body hairs, feet, regurgitation, and faecal excretion (*Song et al*., 2021). Research suggests that flies may also transmit pathogens vertically to their offspring. A study by Alexandre Lamas found that Australian bush flies from cattle farms carried a significantly higher bacterial load compared to those collected in urban areas (*Lamas et al*., 2019). Alarmingly, 94% of *Salmonella* and 87% of *Shigella* strains isolated from these flies exhibited multidrug resistance, indicating that flies could serve as vectors for antimicrobial resistance dissemination (*Steele & Odumeru*, 2004).

Water plays a crucial role in the food production continuum and also poses a substantial risk as a medium for microbial contamination. The survival and transmission of pathogens via irrigation water depend on the level of contamination. Pathogens such as *E. coli* and *Salmonella* can be transported via water or soil to plant surfaces, and some strains, like *E. coli* O157:H7, can even penetrate plant tissues, including the stomata of radish seeds and lettuce leaves. Manure-derived *E. coli* has been shown to persist in soil or grassland environments for up to six months, creating long-term reservoirs for contamination (*Avery et al*., 2004).

Animals are also direct sources of foodborne pathogens. Livestock, such as pigs, cattle, and poultry, frequently harbours *E. coli*, including Shiga toxin-producing strains (STEC), which can be transmitted to humans through undercooked meat or poor handling procedures. In a South Korean study, 131 *E. coli* strains were isolated from 334 pork samples obtained from slaughterhouses and markets (*Todd et al*., 2009). Additionally, a UK supermarket survey reported that 23 out of 99 chicken samples contained *E. coli*, emphasizing the ongoing risk at the consumer level (*Heo et al.*, 2020).

Finally, food processing environments and kitchen hygiene practices are critical control points for pathogen entry and spread. Contaminated raw materials, insufficient cleaning of food-contact surfaces, and improper storage or thermal treatment can facilitate cross-contamination and allow pathogens to persist throughout the food supply chain (*Hall et al.,* 2008).

3. Antimicrobial resistance, genetic diversity, and molecular epidemiology of foodborne *Enterobacteriaceae* pathogens

The *Enterobacteriaceae* family includes several critical foodborne pathogens, notably *Escherichia coli*, *Salmonella* spp., *Shigella* spp., and *Yersinia pseudotuberculosis*. These organisms not only contribute significantly to gastrointestinal infections globally but also represent a growing threat due to their contribution to increasing antimicrobial resistance and their high genetic variability, which complicates detection, treatment, and prevention strategies.

Escherichia coli is commonly found in the gut microbiota of humans and animals. While non-pathogenic strains are usually harmless, pathogenic variants can lead to serious illnesses, ranging from gastroenteritis to haemolytic uremic syndrome. Initially susceptible to most antibiotics, *E. coli* has developed resistance mechanisms, including the production of extended-spectrum β-lactamases (ESBLs) and carbapenemases, which hydrolyse a wide range of β-lactam antibiotics. Resistance genes, such as *aadA1, aadA2, mcr-1, blaTEM-1,* and *crf*, are frequently reported in *E. coli*. Molecular typing techniques, such as multilocus sequence typing (MLST), reveal distinct sequence types (STs) among isolates from poultry, cattle, and humans, highlighting the complex epidemiology of foodborne *E. coli* transmission (*Ramadan et al*., 2020). *Salmonella* spp. are also significant foodborne pathogens, with both typhoidal and non-typhoidal strains presenting considerable health burdens. Multidrug resistance is common in *Salmonella*, with resistance genes like *macB, macA,* and *ampE* identified through comparative genomic approaches. Molecular epidemiological tools, including serotyping and MLST, are employed to trace the genetic diversity of *Salmonella* strains. For instance, over 300 isolates have been classified into more than 25 distinct STs. Outbreak investigations emphasize the importance of plasmid profiling in linking clinical cases to contaminated food products, particularly raw meat (*Zhang*, 2021).

Shigella species, especially *S. dysenteriae* and *S. boydii*, are major contributors to childhood diarrhoea, particularly in developing regions. High levels of resistance to fluoroquinolones and third-generation cephalosporins have been observed, with *blaTEM-1*, *blaCTX-M*, *blaOXA-1*, and *blaSHV-12* genes implicated in resistance phenotypes. Serotyping and pulsed-field gel electrophoresis (PFGE) have shed light on outbreak dynamics and strain evolution, while longitudinal studies indicate shifts in dominant serotypes and the emergence of less virulent, sporadic strains (*Wang et al.*, 2016).

Yersinia pseudotuberculosis, though less commonly reported, is a notable enteropathogen with rising antimicrobial resistance. Isolates from both environmental and clinical sources have shown resistance to tetracyclines, beta-lactams, and sulphonamides, driven by genes such as *tetA, tetD, blaTEM-1B,* and *sul2.* Virulence is closely linked to the presence of a 70-kb plasmid and chromosomally encoded toxins like YPMs, as well as high-pathogenicity islands (HPIs), which enhance its infectivity and survival (*Fukushima*, 2003).

4. Emerging technologies for detection and control of *Enterobacteriaceae* in food

Advancements in analytical technologies have significantly improved the speed and accuracy of detecting foodborne pathogens. Among the most promising approaches are those utilizing nanomaterials, nucleic acid-based amplification, and hybridization techniques.

- Nanotechnology-Based Detection: Nanomaterials offer enhanced biosensing capabilities due to their high surface area and unique physicochemical properties. Gold and silver nanoparticles (5–110 nm) are commonly employed to amplify detection signals through optical changes, such as color shifts induced by aggregation. For instance, gold nanoparticles (AuNPs) have been used to detect *Salmonella* and *E. coli* O157:H7 at concentrations as low as 98.9 CFU/mL and 1–10 CFU/mL, respectively. Magnetic nanoparticles (e.g., iron, nickel) and quantum dots also contribute to sensitive pathogen detection via magnetic and fluorescent signals. Additionally, carbon nanotubes (SWNTs and MWNTs) demonstrate excellent conductivity and can be used in field-effect transistors to detect bacteria at extremely low levels, down to 1 CFU/mL. Despite their sensitivity, cost and functionalization challenges limit widespread application (*Gilmartin & O'Kennedy*, 2012).
- PCR-Based Methods: PCR remains a cornerstone of molecular diagnostics. Its capacity to amplify pathogen-specific DNA sequences with high specificity enables rapid identification. Variants such as loop-mediated isothermal amplification (LAMP) and multiplex PCR (mPCR) further extend its utility. LAMP offers high sensitivity, rapid amplification at a constant temperature (60–65°C), and visual result interpretation. However, the risk of aerosol contamination and complex primer design are limiting factors. Multiplex PCR enables simultaneous detection of multiple pathogens by targeting different genes, enhancing diagnostic throughput and cost-efficiency. Technologies like the GeXP system integrate chimeric primers and capillary electrophoresis for high-throughput, fluorescent detection (*Chen et al*., 2012).
- Nucleic Acid Hybridization: Fluorescence in situ hybridization (FISH), using fluorescently labelled rRNA probes, enables direct visualization of pathogens under a fluorescence microscope. Variants such as line probe assays (LIPA) and peptide nucleic acid (PNA) probes have further refined specificity. Due to their uncharged and hydrophobic nature, PNA probes exhibit greater stability and cell permeability than DNA probes, making them suitable for improved analytical performance (*Rho et al*., 2009).

5. Innovative strategies for controlling *Enterobacteriaceae* foodborne pathogens

Food contamination and poisoning caused by *Enterobacteriaceae* pathogens have gained increasing attention in food safety management. Modern control strategies include a range of physical, chemical, and biological methods designed to ensure microbial safety while maintaining product quality.

Irradiation: Ionizing radiation is widely used for food decontamination, particularly in meat and poultry processing. It disrupts microbial DNA, induces protein denaturation, and damages cell membranes. A 4 kGy dose has been shown to eliminate *Salmonella* and significantly reduce *E. coli* and other *Enterobacteriaceae*, without compromising sensory properties, thereby extending shelf life (*Bard*, 2004).

Pulsed Light (PL) Treatment: PL induces DNA damage through photochemical reactions, forming pyrimidine dimers that inhibit replication. Studies have demonstrated reductions of up to 4.5 log CFU/g in *Salmonella* and *E. coli* on fresh produce. However, the effectiveness of PL decreases with uneven surfaces and extended pulse intervals and may cause browning in fruits due to polyphenol oxidase activation (*Ares et al*., 2014).

Microwave Sterilization: Microwave energy disrupts cell membrane permeability and causes protein denaturation, leading to microbial death. Water-assisted microwave treatment at 63°C effectively inactivates *Salmonella* on leafy vegetables. The technique's effectiveness varies depending on the food matrix, and it can complement other physical treatments like cold plasma and cavitation jets for enhanced control (*Shahla et al*., 2013).

Slightly Acidic Electrolyzed Water (SAcEW) and Fumaric Acid: SAcEW, especially when combined with 0.6% fumaric acid and mild heating (40°C, 5 min), significantly reduces *E. coli* and *Salmonella* in fresh meat. This combined treatment improves bactericidal efficacy and extends the shelf life of meat stored at refrigeration temperatures by up to 8 days (*Ahmad et al*., 2015).

Emerging Biocontrol Approaches: Biological agents, such as antimicrobial peptides from *Bacillus* spp. and algae-derived bioactives, offer promising alternatives. These substances demonstrate inhibitory effects against *E. coli* and other pathogens, although further studies are required to understand their mechanisms, effective dosages, and interactions in complex food systems. Algal compounds like fucoidans and ulvans also contribute to antimicrobial defence and may enhance overall food safety and nutritional value (*Neetoo et al*., 2010).

6. Conclusion

The persistence and transmission of *Enterobacteriaceae* foodborne pathogens and their resistance genes throughout the food production continuum, from animal husbandry environments to human consumption, pose a serious threat to global public health. Innovative detection technologies, including molecular and immunoaffinity-based approaches, provide increased sensitivity and specificity for early pathogen identification. At the same time, advanced control measures such as irradiation, pulsed light, microwave sterilization, slightly acidic electrolyzed water, and bioactive compounds from natural sources show significant potential in reducing microbial contamination while maintaining food quality. Integrating these emerging technologies into comprehensive food safety management systems is essential. To effectively mitigate risks, coordinated global efforts through regulatory frameworks, policy implementation, and continuous monitoring are crucial. A multidisciplinary approach that combines scientific innovation, regulatory oversight, and industry application will be vital in ensuring food safety and public health.

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Original Scientific Paper

Cadmium bioaccumulation in the liver and muscle tissue of mallards (*Anas platyrhynchos*) as bioindicators of environmental pollution in Serbia

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ABSTRACT

The aim of this study was to assess the level of cadmium (Cd) contamination in the liver and muscle tissue of mallards (*Anas platyrhynchos*) from three different locations in Serbia: Belegiš, Pločica, and the Vršački ritovi. A total of 55 samples were analyzed, collected during 2024. Cadmium concentrations were determined using the ICP-MS method following microwave digestion of the samples. The highest mean concentration in the liver was recorded at the Belegiš site (0.643 mg kg^{-1}), while the highest average value in muscle tissue was measured in Pločica (0.021 mg kg^{-1}). Although no statistically significant differences were found between locations, 40.7% of liver samples exceeded the reference value of 0.5 mg kg^{-1} established for poultry, indicating a potential risk to human health. This study highlights the importance of wild birds as bioindicators of heavy metal pollution and the need for continued monitoring of cadmium presence in wildlife.

1. Introduction

Toxic metals such as cadmium (Cd) are naturally present in the Earth's crust; however, elevated levels in the environment are largely a consequence of intensive industrial activities over the past centuries, which have led to significant pollution of air, water, and soil (*Durkalec et al.,* 2015*; Aloupi et al.,* 2017). Cadmium belongs to group XII of the periodic table. It is a soft, silvery-white metal with physicochemical properties similar to those of zinc and mercury. It is corrosion-resistant, insoluble in water, and non-flammable, which makes it widely used as a protective coating in industrial applications (*Genchi et al.,* 2020). Naturally, cadmium enters the environment primarily through volcanic eruptions; however, natural sources account for only about 10% of the total cadmium emissions. The majority of cadmium pollution originates from anthropogenic activities, particularly mining, metallurgy, and the combustion of coal in the energy sector. It is also widely used in the production of nickel-cadmium batteries, pigments, fluorescent coatings, solders, alloys, cadmium rods, plastic stabilizers, and fireworks (*Bombik et al.,* 2023).

Cadmium released into the environment can persist for decades. It enters the food chain through

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plants that absorb it from the soil (*Govind & Madhuri,* 2014). The main routes of human and animal exposure are contaminated food, water, and tobacco smoke (*Genchi et al.,* 2020). Waterfowl are particularly vulnerable to metal exposure due to the incidental ingestion of soil and sediment during feeding, especially in heavily polluted areas (*Aloupi et al.,* 2017). Studies have shown that cadmium exposure in birds negatively affects their physiological condition, reduces reproductive capacity, impairs egg quality, and causes developmental disorders during embryogenesis (*Bombik et al.,* 2023).

Cadmium is a toxic heavy metal with no known physiological role in the body. It induces oxidative stress by increasing the production of reactive oxygen species (ROS), which leads to damage of DNA, proteins, and membrane phospholipids, thereby impairing mitochondrial function and reducing ATP synthesis. The key mechanisms of its toxicity include interference with antioxidant enzymes, inhibition of DNA repair, epigenetic modifications, and the activation of apoptosis via the mitochondrial pathway. Chronic exposure to cadmium has been associated with the development of lung, breast, prostate, pancreatic, kidney, and other cancers, as well as with renal, cardiovascular, reproductive, and neurological disorders (*Genchi et al.,* 2020).

Game and wild birds, as free-living species, independently select their diet and are an integral part of the soil-plant-animal food chain (*Nikolić et al.,* 2017). The level of cadmium contamination in wild animals is often used as an indicator in ecological monitoring of cadmium poisoning (*Gasparik et al.,* 2010). Based on these facts, the mallard was used in this study to assess cadmium contamination at three sites in Serbia: Belegiš (Srem District), Pločica (South Banat District), and the Vršački ritovi (South Banat District). These locations were selected to investigate the effects of contamination on wild birds as part of an ecological cadmium pollution monitoring program.

2. Materials and methods

Cadmium levels were measured in liver and muscle tissues samples of mallards at three different locations: Belegiš (Srem District) and Pločica (South Banat District), along the course of the Danube River, and in a fishpond near the Vršački ritovi during the 2024 calendar year. The total number of analyzed samples was 55, including both liver and muscle tissues of mallards. A total of 23 samples were collected from the Belegiš site (12 muscle tissue samples and 11 liver samples), 12 samples from the Pločica site (6 liver and 6 muscle samples), and 20 samples from the Vršački ritovi site (10 liver and 10 muscle tissue samples).

The samples were stored at -18 °C until the time of analysis. One day prior to laboratory processing, the frozen samples were gradually thawed at 4 °C and subsequently homogenized. Approximately 0.3 g of tissue (± 0.001 g) was weighed for each sample and transferred into a Teflon vessel of a microwave digestion system. Nitric acid (67% Trace Metal Grade, Fisher Scientific, Bishop, UK) and deionized water (0.063 µS) obtained from a water purification system (Purelab DV35, ELGA, Buckinghamshire, UK), were added to the sample in quantities of 5 mL each. The microwave digestion system (MARS 6, CEM Corporation, Matthews, NC, USA) was programmed as follows: 5 min from initial temperature to 180°C, hold at 180°C for another 10 min, cooling and venting for 20 min. Digested samples were quantitatively transferred into 100 mL polypropylene volumetric flasks and diluted with deionized water (0.063 µS/cm).

The determination of the ^{111}Cd isotope was performed using inductively coupled plasma mass spectrometry (ICP-MS) on an iCap Q instrument (Thermo Scientific, Bremen, Germany), equipped with a collision cell and operated in kinetic energy discrimination (KED) mode. Quantitative analysis was based on a five-point calibration curve, including a zero point. A multielement internal standard (^{6}Li and ^{45}Sc at a concentration of 10 ng ml^{-1}; ^{71}Ga, ^{89}Y, and ^{209}Bi at a concentration of 2 ng mL^{-1}) was introduced via an additional peristaltic pump line to ensure measurement accuracy. Each sample was measured in duplicate, and the mean value was used, corrected for internal standard response factors. The quality of the analytical procedure was verified by the analysis of certified reference material (NIST 1577c – bovine liver, Gaithersburg, MD, USA), which was prepared in the same manner as the samples, using microwave digestion. Repeated measurements of the reference material yielded results within the range of certified values.

2.1 Statistical analysis

Statistical analysis was performed using the Minitab 17 software package. Descriptive statistics were conducted for each location to determine basic distribution parameters, including mean, standard

deviation and range. To determine statistically significant differences between locations for muscle tissue and liver samples, a one-way analysis of variance (ANOVA) was applied.

3. Results and discussion

Tables 1 and 2 present the results of descriptive statistics for cadmium concentrations in liver and muscle tissue samples of mallards from different locations. The tables include arithmetic means, ranges (min–max), standard deviations, and the number of analyzed samples (n) for each sampling site.

As can be seen from Tables 1 and 2, the concentration of cadmium is significantly higher in the liver. This can be explained by the fact that the liver intensively produces the protein metallothionein to which cadmium binds (*Binkowski & Sawicka-Kapusta, 2015)*. Mean cadmium concentrations in the liver of mallards varied depending on the sampling site. The highest mean value was recorded at the Belegiš location (0.643 ± 0.566 mg kg^{-1}), while slightly lower values were observed at Pločica (0.583 ± 0.423 mg kg^{-1}) and the Vršački ritovi (0.496 ± 0.337 mg kg^{-1}), and the range of liver cadmium concentrations spanned from 0.098 to 2.134 mg kg^{-1}. In contrast, cadmium concentrations in muscle tissue were significantly lower compared to those in the liver. The highest mean value was recorded in samples from the Pločica site (0.021 ± 0.007 mg kg^{-1}), while slightly lower concentrations were observed in Belegiš (0.017 ± 0.016 mg kg^{-1}) and Vršački ritovi (0.008 ± 0.006 mg kg^{-1}). The cadmium concentration ranged in muscle tissue was from 0.002 to 0.071 mg kg^{-1}.

The results of the one-way ANOVA test indicated no statistically significant difference in cadmium concentrations in the liver of mallards among the studied locations ($p = 0.772$). In the case of muscle tissue, although the p-value of 0.077 suggested a possible trend, the difference between sites did not reach statistical significance. Nevertheless, descriptive statistics showed that the mean cadmium concentration was lowest in samples from the Vršački ritovi (0.008 mg kg^{-1}), while higher values were recorded at the Belegiš (0.017 mg kg^{-1}) and Pločica (0.021 mg kg^{-1}) sites. This difference in mean values, although not confirmed by statistical analysis, may indicate the influence of local pollution sources and justifies the need for an increased sample size in future studies.

A comparative analysis with previous studies conducted in Serbia and Croatia provides additional context for interpreting the obtained cadmium concentrations. *Nikolić et al*. (2017), analyzed cadmium levels in the liver and leg muscle tissue of mallards and reported average values of 0.186 mg kg^{-1} in the liver and 0.005 mg kg^{-1} in muscle tissue. *Borjan et al.* (2022), examined cadmium presence in the liver and leg muscles tissue of pheasants over four consecutive years (2018–2021), with mean liver concentrations of 0.354, 0.247, 0.306, and 0.296 mg kg^{-1}, and corresponding muscle tissue concentrations of 0.009, 0.006, 0.011, and 0.011 mg kg^{-1}, respectively. Additionally, *Florijančić et al.* (2009) from Croatia reported an average cadmium concentration of 0.332 mg kg^{-1} in the liver of mallards. The results of the present study are comparable with the aforementioned studies in terms of muscle tissue, where cadmium concentrations are generally low and consistent. On the other hand, as expected, cadmium concentrations were significantly higher in the liver, indicating potential bioaccumulation of this metal. This finding is significant as it highlights the need for more frequent monitoring, as well

Table 1. Cadmium Concentration in Liver Tissue of Mallards

Location	Number of Samples (n)	Range (mg kg^{-1})	Mean ± standard deviation (mg kg^{-1})
Belegiš	11	0.098-2.134	0.643±0.566
Pločica	6	0.195-1.212	0.583±0.423
Vršački ritovi	10	0.125-1.156	0.496±0.337

Table 2. Cadmium Concentration in Muscle Tissue of Mallards

Location	Number of Samples (n)	Range (mg kg^{-1})	Mean ± standard deviation (mg kg^{-1})
Belegiš	12	0.005-0.065	0.017±0.016
Pločica	6	0.010-0.026	0.021±0.007
Vršački ritovi	10	0.002-0.017	0.008±0.006

as the potential influence of local pollution sources on cadmium levels in wild species. Furthermore, the results underscore the necessity of conducting additional ecological investigations, including studies of sediments, water, soil, and air, in order to better understand the sources and pathways of cadmium entry into the environment and food chain.

According to the current Regulation on Maximum Levels of Certain Contaminants in Food (Official Gazette of the Republic of Serbia, Nos. 73/2024, 90/2024, 47/2025 and 61/2025), maximum permissible levels (MPLs) for cadmium are not specified for the meat and organs of wild game, but are defined for domestic animals. In this study, the MPLs established for poultry were used as reference values, given the biological similarity to mallards, with limits of 0.5 mg kg^{-1} for liver and 0.05 mg kg^{-1} for muscle tissue. Out of the 28 analyzed muscle tissues samples, only one sample (3.6%) from the Belegiš site exceeded the reference value, with a concentration of 0.065 mg kg^{-1}. In the case of liver tissue, 11 out of 27 samples (40.7%) had cadmium concentrations above the MPL. By location, at the Vršački ritovi, 4 out of 10 samples (40%) exceeded the threshold. At Belegiš, exceedances were recorded in 5 out of 11 samples (45.5%), while at Pločica, 2 out of 6 samples (33.3%) showed concentrations above the permitted limit, with values of 0.818 and 1.212 mg kg^{-1}. These findings indicate a potential health risk associated with the consumption of wild bird liver and highlight the need for continued monitoring of cadmium levels, as well as consideration of establishing regulatory limits for wild game.

4. Conclusion

The obtained results indicate the presence of cadmium in the liver and muscle tissue of mallards (*Anas platyrhynchos*) at all three investigated sites in Serbia, with notably higher concentrations in the liver compared to muscle tissue. Although differences in cadmium concentrations between the locations were not statistically significant, certain variations suggest a potential influence of local pollution sources. The highest mean cadmium level in the liver was recorded at the Belegiš site, while the lowest values in muscle tissue were found in the Vršački ritovi. A significant proportion of liver samples (40.7%) exceeded the reference value established for poultry, potentially indicating a health risk associated with the consumption of these organs. The results of this study highlight the need for continued monitoring and consideration of the introduction of specific regulatory limits for cadmium in the meat and organs of game, in order to protect human health and maintain ecological balance.

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Original Scientific Paper

Consumer attitudes toward egg consumption, food safety, and animal welfare

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ABSTRACT

Hen eggs are a common component of the human diet, and the egg industry today represents an important branch of animal husbandry. Consumer preferences regarding egg characteristics are evolving, with increasing attention placed on production conditions and laying hen welfare, while issues of food safety are often less clearly understood. In addition, the impact of egg consumption on human health continues to be questioned. The aim of this study was to assess consumer attitudes in the municipality of Zrenjanin, Serbia, with a focus on factors influencing egg purchasing decisions. Respondents emphasized production practices and animal welfare, whereas the physical characteristics of eggs were considered less important. Eggs were generally perceived as healthy food, and most consumers did not associate their consumption with an increased risk of cardiovascular disease. However, many respondents showed limited understanding of the concept of egg safety. These findings underline the importance of strengthening consumer education and awareness regarding both the benefits and potential risks of egg consumption.

1. Introduction

Eggs are a low-cost, nutrient-dense food. Almost all of their nutrients are efficiently utilized by the body, and eggs are recognized as a high-quality protein source alongside breast milk (*Park et al.*, 2018). Due to their rich nutrient content, eggs are susceptible to contamination by various pathogens, making their microbial integrity essential. In Serbia, testing of laying hens for *Salmonella* spp. is mandatory as part of the producer's self-control every four weeks. However, facilities producing table eggs for personal consumption are not subject to this legal requirement (*Vidaković Knežević et al.*, 2017). The Zrenjanin Veterinary Specialist Institute regularly conducts tests for *Salmonella* spp. in eggs and egg products, but the primary responsibility for monitoring and submitting eggs for testing lies with the food business operator.

Animal welfare is the state of an organism, which reflects how well animals adapt to the living conditions provided by humans (*Vučinić*, 2016). *Bonnefous et al.* (2022) note that conventional cages for laying hens were developed after World War II to increase egg production. In 2012, conventional cages were banned in the European Union, leaving only

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enriched cages and aviary system as permitted alternatives. The enriched cage system provides 750 cm² of space per hen. In addition to enriched and conventional cages, there are floor, free-range, and organic systems for raising laying hens (*Nielsen et al.,* 2023).

The aim of the study was to investigate the opinions of residents in Zrenjanin municipality regarding their preferences when purchasing eggs, particularly in relation to organoleptic characteristics and different housing systems for laying hens. Additionally, this study aimed to examine consumers' perceptions of the health effects of egg consumption, with the goal of providing recommendations and guidelines for future research and consumer education.

2. Materials and methods

An anonymous and voluntary online questionnaire was conducted, active for approximately one month, from 31st of July to 4th of September 2024. A total of 69 respondents completed the survey.

2.1. Survey structure

The questionnaire consisted of 63 questions, out of which first four questions were related to sociodemographic information of the respondents, including sex, age, residential location, and professional qualifications.

The remaining 59 questions included 29 multiple-choice questions, each with at least three options and the possibility for additional written comments. These questions adressed consumer preferences when purchasing eggs, perceptions of the health benefits of eggs, potential exposure of humans to veterinary drug residues, and the impact of different laying hen production systems on egg quality and safety. This section also included statements rated on a 5-point Likert scale (1 = do not agree at all; 5 = completely agree), divided into three parts. The first part addressed the organoleptic characteristics of eggs, the second part focused on the consumption of eggs and their impact on human health, and the third part examined the influence on hen housing systems on egg quality and integrity.

The following seven questions asked respondents to rate from 0 (unimportant) to 5 (extremely important) how relevant certain factors are for egg consumption in their daily diet.

The final two open-ended questions allowed respondents to provide comments on egg safety and production processes, including the housing conditions of laying hens.

2.2. Statistical analysis

The data collected through the survey were analyzed using R software (version 4.1.2) (*R Core Team*, 2021). Analysis began with descriptive statistics (*Mutavdžić and Nikolić Đorić*, 2018), followed by non-parametric tests, including the Mann-Whitney U and Kruskal-Wallis tests. When significant differences between groups were found, Dunn's post-hoc test was applied to identify specific group differences.

3. Results

3.1. Sociodemographic profile of respondents

Sociodemographic characteristics are presented in Table 1. Most respondents were female (59.4%), and across all participants, the largest age group was

Table 1. Sociodemographic characteristics of respondents

Variable	Categories	Frequency	Percentage
Gender	Male	28	40.6%
	Female	41	59.4%
Age	≤30	22	31.9%
	31-45	25	36.2%
	46-60	16	23.2%
	>61	6	8.7%
Professional qualification	High school	37	53.6%
	Academic studies	16	23.2%
	Integrated academic studies	5	7.2%
	Master's/PhD	11	15.9%
Place of residence	Urban area	48	69.6%
	Rural area	21	30.4%

between 31 and 45 years (36.2%). Regarding professional qualifications, more than half had completed high school (53.6%). Most respondents lived in urban areas (69.6%).

3.2. Consumer perceptions of egg quality and health

Respondents' perceptions of the organoleptic characteristics of eggs, as well as their perceptions regarding egg consumption and its impact on human health, are summarized in Table 2. Statements were rated on a 5-point Likert scale (1 = do not agree at all; 5 = completely agree).

3.3. Factors influencing egg purchase decisions

Respondents rated the importance of several factors when purchasing eggs on a scale from 0 (not important at all) to 5 (extremely important). The results are summarized in Table 3.

4. Discussion

This study explored consumer perceptions of egg quality, health impacts, and purchasing behavior in Zrenjanin municipality. The findings provide insight into preferences regarding organoleptic properties, health-related beliefs, safety considerations, and factors influencing purchasing decisions.

Table 2. Respondents' agreement with statements about organoleptic characteristics of eggs

Variable	$\bar{X}$	SD
Raw eggs are more nutritionally rich/better for the diet than boiled eggs (V1)	2.710	1.112
Dark-shelled eggs are more nutritionally rich/better to eat (V2)	2.304	1.075
Eggs with darker yolks are more delicious (V3)	3.174	1.392
By washing eggs, there is a risk that harmful pathogens are pushed from the shell into the inside of the egg (V4)	2.667	1.431
By placing the eggs in a container of water, the freshness of the eggs can be checked (V5)	3.565	1.355
The taste of eggs depends on the diet of the laying hen (V6)	4.304	0.928
The taste of eggs depends on the way the laying hen is kept (V7)	3.841	1.195
Daily consumption of eggs is good for human health (V8)	3.464	1.219
Excessive egg consumption increases the risk of heart disease (V9)	2.855	1.364
The use of eggs contributes to an increase in muscle mass (V10)	3.841	1.023
Consuming a large amount of eggs has/would have a negative effect on health (V11)	2.870	1.434
Eggs are an irreplaceable source of nutrients and vitamins (V12)	3.667	1.107
Eating eggs directly affects the increase in blood cholesterol (V13)	2.710	1.261
The way eggs are prepared (boiled, raw, baked) greatly affects human health (V14)	3.377	1.237

Note: V = variable number from the questionnaire.

Table 3. Importance of factors when buying eggs (0-5 scale)

Factor	Mean	Median	Min-Max
Price	2.83	3	0-5
Package	2.87	3	0-5
Supporting the local economy	4.23	5	0-5
Hygiene of workers	4.46	5	2-5
Shell hardness	3.13	3	0-5
Free-range eggs	3.61	3	0-5
Housing systems of hens	3.75	4	0-5

Note: Factors were rated on a 0-5 scale, where 0 = not important at all and 5 = extremely important.

According to the survey results, most respondents purchased eggs once a week and consumed them two or more times per week. Eggs were most commonly bought in supermarkets, and the majority of respondents were female. Consumers generally valued external characteristics of eggs, such as shell cleanliness, quality and color, while internal parameters, such as yolk quality, were less decisive when choosing eggs. Similar findings have been reported by *Mitrović* (2022), highlighting consumer sensitivity to shell color. Moreover, comparable results were observed by *Tolimir et al.* (2017), who reported that consumers in Belgrade placed particuar importance on external egg characteristics, with shell color among the most influential factors in purchasing decisions.

Respondents largely believed that the taste of eggs is influenced by both the hen's diet ($\bar{X}$ = 4.304) and housing system ($\bar{X}$ = 3.841). This corresponds with consumer preferences observed in studies where free-range eggs were rated higher in flavor and acceptability (*Anderson*, 2011). The relatively low agreement ($\bar{X}$ = 2.304) that dark-shelled eggs are nutritionally superior reflects common consumer misconceptions, as a study by *Roberts* (2004) confirmed that shell color does not influence nutritional content and is primarily breed-dependent. Respondents' mixed views ($\bar{X}$ =2.667 ± 1.431) on whether washing eggs could push pathogens inward echo scientific findings that washing can remove the protective cuticle, potentially increasing contamination risk (*Board and Tranter*, 1995; *Hutchison et al*., 2004). Collectively, these results underscore that consumer perceptions align with evidence on sensory qualities and risk factors and highlight the importance of accurate education regarding egg handling and nutrition.

One of the greatest food safety issues related to table eggs is contamination with *Salmonella* spp., which can occur through vertical transmission via the reproductive tract or horizontally through the shell after laying (*Vidaković Knežević et al*., 2017; *Holt*, 2021). In our survey, 36.2% of respondents associated soiling of eggs with pathogen transmission, whereas nearly one-third (30.4%) were unfamiliar with the term "egg safety". Similar knowledge gaps among consumers have been reported elsewhere, highlighting the need for targeted education on microbial hazards (*EFSA*, 2021). Trust in institutional control was relatively high, as 71% of respondents expressed confidence in *Salmonella* monitoring, while 63.8% placed primary responsibility for egg safety on producers.

Animal welfare was also a relevant concern: 52.2% of respondents emphasized the importance of compliance with animal welfare legislation, and 30.4% valued information on housing conditions. Notably, 36.2% believed that animal welfare and egg safety are directly related. This reflects the dual challenge of balancing welfare and biosecurity. Free-range housing is associated with benefits such as improved skeletal health (*Tuyttens et al*., 2008), but also with higher risks of parasitic and bacterial infections (*Fossum et al*., 2009). Consumer preferences for welfare-friendly systems have been documented across Europe, although misconceptions regarding food safety in such systems remain widespread (*Harvey and Hubbard*, 2013).

In terms of nutritional perceptions, 34.8% of respondents agreed that eggs contribute to muscle mass, while 30.4% recognized them as an irreplaceable source of nutrients and vitamins. These beliefs are supported by biochemical evidence showing that eggs contain bioactive components, such as phospholipids, lutein, zeaxanthin, and high-quality proteins, that have roles in immune function and the prevention of chronic disease (*Sanlier and Üstün*, 2021; *Miranda et al.*, 2015). Importantly, the majority of respondents rejected the idea that egg consumption substantially increases cholesterol or cardiovascular risk (23.2%). Current scientific consensus supports this perception: recent meta-analyses indicate that moderate egg consumption does not significantly raise blood cholesterol levels or cardiovascular risk in healthy populations (*Alexander et al*., 2016; *Drouin-Chartier et al*., 2020). This position is reflected in the Dietary Guidelines for Americans, which state that one egg per day can be included in a healthy diet without adverse cardiovascular effects (*Phillips*, 2021).

5. Conclusion

This study showed that consumers in the Zrenjanin municipality placed greater importance on production conditions and animal welfare than on the physical characteristics of eggs. While respondents generally perceive eggs as a healthy and safe food, notable knowledge gaps remain, particularly regarding the concept of egg safety and microbiological risks. Hygiene during handling was recognized as a critical factor influencing consumer trust. Furthermore, free-range systems were perceived as the most

desirable future direction for egg production, reflecting a preference for welfare-friendly farming practices. Overall, these findings highlight the need for continued consumer education and transparent communication in order to align perceptions with scientific evidence and support sustainable egg production.

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Original Scientific Paper

Characterization of imported olive oil: contribution to efficient verification of declared quality and oil type

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ABSTRACT

The feasibility of applying basic chemical parameters and fatty acid analysis for 26 samples of imported olive oil, coupled with statistical multivariate methods, to confirm the declared quality and type of oil is described. The research included the determination of peroxide value, iodine value, saponification value, free fatty acids expressed as oleic acid, moisture and volatile matter, as well as fatty acid content according to the requirements of the olive oil regulation. Of the 26 olive oil samples, declarations stated 19 were extra virgin, 5 were virgin, while one sample each of pure olive oil and olive pomace oil were tested as control samples. The results showed that, on such a limited number of samples and quality parameters, principal component analysis (PCA) cannot be successfully applied, while linear discriminant analysis (LDA) gave satisfactory results when all tested parameters were included.

1. Introduction

Olive oil (OO) makes up only a small portion of the total consumption of vegetable oils today, but it has tremendous economic importance for producing countries while its nutritional value and peculiar sensory attributes are important for consumers worldwide (*Caporaso and Boscou*, 2021; *Rosati et al*., 2014). Studies have shown that OO has beneficial influence in reducing the risk of coronary heart disease, cancer, inflammation, diabetes, Alzheimer's disease, and metabolic disorders (*Malheiro et al*., 2014; *Beltrán et al.*, 2016, *Rousos et al*, 2025). These properties have their origins in the unique composition of triacylglycerols and fatty acids (FAs) in OO, its richness in monounsaturated FAs, especially oleic acid (*Malheiro et al*, 2014; *Lanca and Ninfali*, 2020), its low levels of polyunsaturated FAs, and its high levels of natural antioxidants. The characteristic composition of OO is responsible for its resistance to rancidity and its antioxidant properties (*Beltran et al*., 2016; *Tsimidou*, 2016; *Mastralexi and Tsimidou*, 2021).

National authorities have legislated olive oil standards and grades (*Official Gazette SCG*, 2004), following the groundwork laid by major OO regulating authorities, such as Codex Alimentarius (*Codex Stand*, 2024), International Olive Council (*IOC*, 2021), European Union (*EU*, 2022), United States Department of Agriculture (*USDA*, 2010) and Australian authorities (*Primefact* 231, 2006). The highest quality OO, so-called virgin olive oil (VOO), is obtained mechanically at monitored temperature to avoid changes in the composition of the oil and loss of quality. VOO for consumption is divided, according to the method of production

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and quality, into extra virgin (EVOO) and common VOO. The price of OO is proportional to the quality. The cheaper grades of OO are pure OO (PuOO) and olive pomace oil (PoOO). There are known cases of cheaper oils being over-declared as higher quality or of their blending in order to achieve economic benefit.

OO is expected to meet some basic physical and chemical quality standards which differentiate it from other oils. Therefore, some of the basic quality parameters should be met, such as peroxide and iodine values, free FA content and FA composition of OO, as are regulated in the legislation concerning OO (*Official Gazette SCG*, 2004; *Codex Stand*, 2024; *IOC*, 2021; *EU*, 2022; *USDA*, 2010; *Primefact 231*, 2006). Data on sterol composition, wax content, trans FA content, UV absorption and triacylglycerol composition are generally used only to determine the adulteration of OO.

One purpose of this research was to determine whether it is possible to confirm the declared quality grade using multivariate analysis on the basic quality parameters of OO. Also, another aim was to determine the significance of the parameters that are essential for OO's grade categorization.

2. Materials and methods

2.1. Chemicals

All standard chemicals and reagents were purchased from Merck KGaA, Darmstadt, Germany. Ultrapure water, ≥18 MΩ, was ELGA DV-25 and ELGA Ultrapure (LabWater, Lane End, High Wycombe, UK).

2.2. Samples

Samples were part of the regular controls of food quality parameters of OO according to regulatory requests (*Official Gazette SCG*, 2004), obtained from retail and from importers. The research included total of 26 samples, mostly virgin olive oils (19 EVOO and 5 VOO). Two samples, one each of PuOO and PoOO, were analysed for the purpose of verifying statistical results. The samples were labelled in the laboratory according to the corresponding declarations on the OOs.

2.3. Chemical parameter determinations

Peroxide value (PV), iodine value (IV), saponification value (SV), free FA (FFA) content, and moisture and volatile content (CW) were determined according to their respective ISO reference methods (SRPS EN ISO 3960:2017, SRPS EN ISO 660:2021, SRPS EN ISO 3961:2019, SRPS EN ISO 662:2017, SRPS EN ISO 3657:2023). Analyses were carried out following the requirements of national regulation for OO.

2.4. GC chromatography

FA determination was performed according to the reference method (ISO 5509:2000). Oils were converted to FA methyl esters (FAMEs) as described by Spirić et al. (2010). FAMEs were determined by capillary gas chromatography on a Shimadzu 2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with the flame ionization detector and a capillary HP-88 column 100 m × 0.25 mm × 0.20 μm (J&W Scientific, Folsom, California, USA). The chromatographic peaks in the samples were identified by comparing relative retention times of FAME peaks with peaks in Supelco 37 Component FAME mix standard (Supelco, Bellefonte, Pennsylvania, USA).

2.5. Statistical analysis

Principal component analysis (PCA) and linear discriminant analysis (LDA) of FAs and chemical analysis of OOs were performed using the JMP Statistical Discovery 10 software (SAS Institute, Cary, North Carolina, USA). Descriptive statistics and preparation of data for multivariate statistical analysis were performed in MS Office 2016 Excel.

3. Results and discussion

Results of chemical parameters (IV, PV, FFA, CW and SV) and FA determination of OO samples are presented in Table 1.

Data shown in Table 1 were further subjected to multivariate statistical analysis in an attempt to determine whether it was possible to group the samples into the declared OO quality types, based on the results obtained for chemical parameters and FA composition.

Graphical representation of the PCA results of the data from Table 1 is presented in Figure 1. The first two principal components (PC1 and PC2) accounted for less than 50% of the total data variance (33% and 15%, respectively). The confidence ellipses (at a 95% confidence interval) showed complete overlap of the

Table 1. Fatty acid content and chemical parameters of the olive oils analysed

Label*	C14:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0	C24:0	IV	PV	FFA	CW	SV
E	0.05	12.74	0.70	0.04	0.06	2.73	74.29	6.54	0.79	0.37	0.24	0.12	0.06	89.55	2.56	0.40	0.17	191.03
E	0.04	17.84	1.65	0.10	0.20	2.74	60.92	13.55	0.68	0.31	0.23	0.09	0.10	89.88	1.62	0.33	0.16	192.54
E	0.05	13.19	0.77	0.01	0.01	2.75	74.55	7.21	0.66	0.34	0.21	0.10	0.05	89.79	6.18	0.59	0.09	190.92
E	0.01	11.83	0.72	0.04	0.06	2.73	76.83	6.22	0.44	0.37	0.35	0.13	0.05	78.48	5.13	0.30	0.10	188.08
E	0.01	10.82	0.71	0.01	0.01	2.45	76.81	5.57	0.42	0.41	0.50	0.10	0.01	84.84	5.71	0.70	0.11	187.97
E	0.01	10.71	0.50	0.01	0.01	2.30	78.91	5.35	0.72	0.36	0.01	0.09	0.01	90.26	7.84	0.78	0.17	191.55
E	0.02	10.77	0.57	0.02	0.02	1.91	79.54	4.47	0.77	0.36	0.02	0.08	0.02	80.45	9.82	0.69	0.11	195.54
E	0.02	11.78	0.62	0.02	0.02	2.36	78.09	5.25	0.82	0.39	0.02	0.10	0.02	89.35	4.80	0.99	0.17	185.94
E	0.03	11.49	0.51	0.03	0.05	1.85	77.95	6.10	0.49	0.28	0.19	0.09	0.03	80.06	3.13	0.54	0.07	189.24
E	0.01	13.44	0.88	0.08	0.13	2.18	71.70	9.22	0.60	0.32	0.22	0.10	0.04	75.19	9.83	0.96	0.13	186.96
E	0.02	11.22	0.64	0.07	0.11	2.37	75.21	7.98	0.63	0.07	0.02	0.09	0.04	81.09	6.04	0.72	0.17	193.70
E	0.05	14.28	1.02	0.06	0.10	2.37	69.19	10.92	0.61	0.30	0.02	0.08	0.04	81.96	9.22	0.78	0.16	195.41
E	0.04	11.29	0.50	0.04	0.04	2.27	78.24	5.51	0.45	0.37	0.02	0.11	0.04	83.00	9.72	0.96	0.15	186.18
E	0.02	11.80	0.67	0.05	0.09	3.11	75.71	6.89	0.65	0.47	0.02	0.17	0.02	82.09	9.97	0.85	0.16	189.27
E	0.02	11.66	0.56	0.03	0.02	2.37	78.30	6.09	0.21	0.36	0.01	0.11	0.04	77.24	7.42	0.96	0.13	190.07
E	0.02	10.42	0.44	0.03	0.02	2.09	78.03	7.32	0.43	0.29	0.02	0.08	0.04	93.94	9.60	0.80	0.14	192.48
E	0.02	9.96	0.52	0.06	0.07	3.29	80.00	4.79	0.43	0.37	0.04	0.10	0.03	80.36	7.31	0.54	0.11	186.34
E	0.02	9.69	0.46	0.05	0.09	2.07	81.06	5.18	0.46	0.32	0.02	0.10	0.03	84.31	1.45	0.17	0.10	188.28
E	0.02	10.55	0.49	0.04	0.06	2.28	79.74	5.78	0.47	0.35	0.02	0.10	0.03	82.02	7.64	0.60	0.18	186.99
V	0.01	12.98	1.18	0.10	0.19	2.37	70.96	10.62	0.83	0.33	0.01	0.11	0.01	80.06	8.96	0.30	0.10	187.04
V	0.02	11.58	0.60	0.03	0.05	2.21	78.27	5.93	0.56	0.33	0.31	0.10	0.04	84.94	9.12	0.93	0.18	185.39
V	0.05	11.93	0.57	0.05	0.06	2.57	77.97	4.84	0.53	0.36	0.21	0.11	0.04	81.09	7.54	0.43	0.09	185.52
V	0.02	11.33	0.52	0.03	0.04	2.20	79.95	4.56	0.45	0.32	0.02	0.10	0.04	81.32	9.80	0.98	0.17	188.98
V	0.03	13.67	1.01	0.08	0.16	1.97	73.41	8.48	0.41	0.31	0.02	0.09	0.03	80.52	5.49	0.37	0.07	187.94
O	0.02	11.85	0.61	0.05	0.08	2.60	75.49	7.66	0.41	0.30	0.02	0.08	0.03	93.26	3.71	0.52	0.12	194.43
P	0.05	12.37	0.80	0.09	0.04	2.94	70.59	10.76	0.71	0.46	0.02	0.18	0.08	83.51	5.52	0.40	0.09	182.11

* Olive oil label abbreviations correspond as follows: E – extra virgin olive oil, O – pure olive oil, P – olive pomace oil and V – virgin olive oil. IV, iodine value; PV, peroxide value; FFA, free fatty acid value; CW, moisture and volatile content; SV, saponification value.

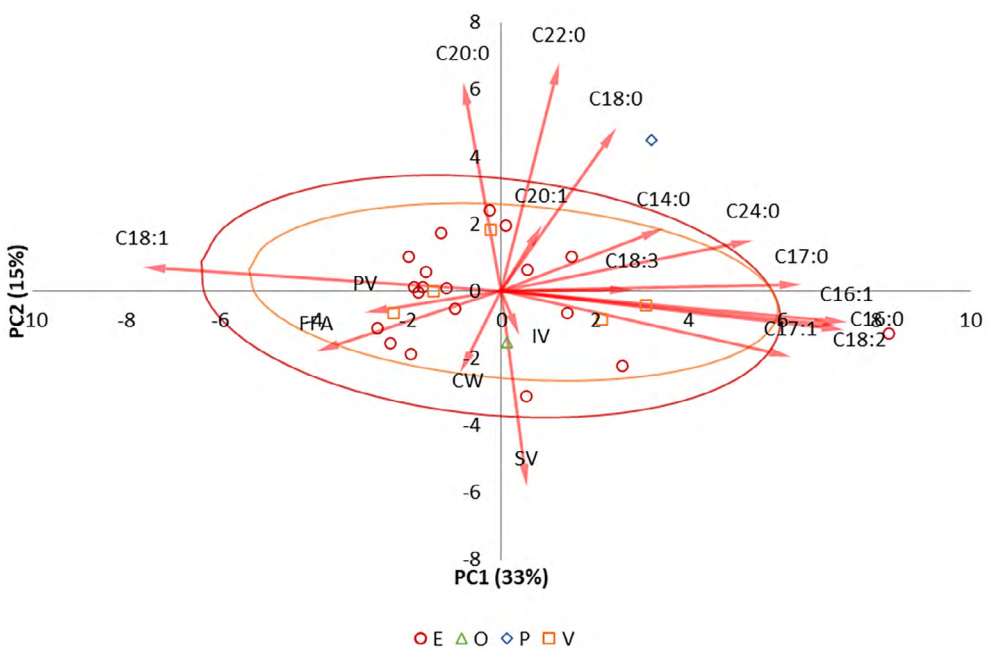

Figure 1. PCA of the examined olive oils' fatty acid content and chemical parameters

data for EVOO and VOO. The control PuOO sample was also included in the confidence ellipses, while the PoOO sample was completely separated from the other groups. The general conclusion is that PCA cannot be applied to determine OO types with the given dataset. The exclusion of PoOO was, in the first place, due to its greater content of saturated FA. The lack of separation is most likely a consequence of comparing the analytical results for samples originating from different geographical climates, produced from different olive cultivars, and with variations in the method and quality of production. All these factors likely contribute to variability in the data that cannot be directly attributed to the analysed parameters.

The results of LDA of chemical parameters and FA content are shown graphically in Figure 2. The first and second canonical functions (canonical 1 and canonical 2) were used for grouping data and obtaining scoring coefficients. Both canonical functions explained 94.6% of the total data variance. Canonical function correlations were 0.984 for canonical 1 and 0.923 for canonical 2. There were no misclassified samples in the analysis (19 EVOO, 5 VOO, 1 PuOO and 1 PoOO). From Figure 2, it can be seen that the groups are completely separated from each other without overlapping. From the LDA results, it can be concluded that chemical parameters have less effect than FAs on the separation of OO groups by type, but they cannot be omitted. The separation of EVOO from VOO was most influenced by differences in the content of the FAs oleic (C18:1n-9), palmitic (C16:0) and palmitoleic (C16:1) acids. LDA of OO by type based only on FA content produced a satisfactory separation, as can be seen in Figure 3. In addition, though, LDA results derived only from FA content had misclassified samples.

In other studies, PCA and LDA were effective in determining the origin and/or type of OOs from individual olive cultivars (*Fuentes et al*., 2018, *Zhang et al*., 2024, *Blasi et al*., 2019, *Roussos et al*., 2025, *Revelou et al*., 2021). Different olive cultivars were effectively grouped according to origin based on FA composition and physicochemical parameters by these statistical methods (*Fuentes et al*., 2018, *Zhang et al*., 2024, *Blasi et al*., 2019, *Revelou et al.,* 2021). The methods were applied to monovarietal OOs (*Fuentes et al*., 2018, *Zhang et al*., 2024, *Blasi et al*., 2019, *Roussos et al*., 2025, *Revelou et al*., 2021).

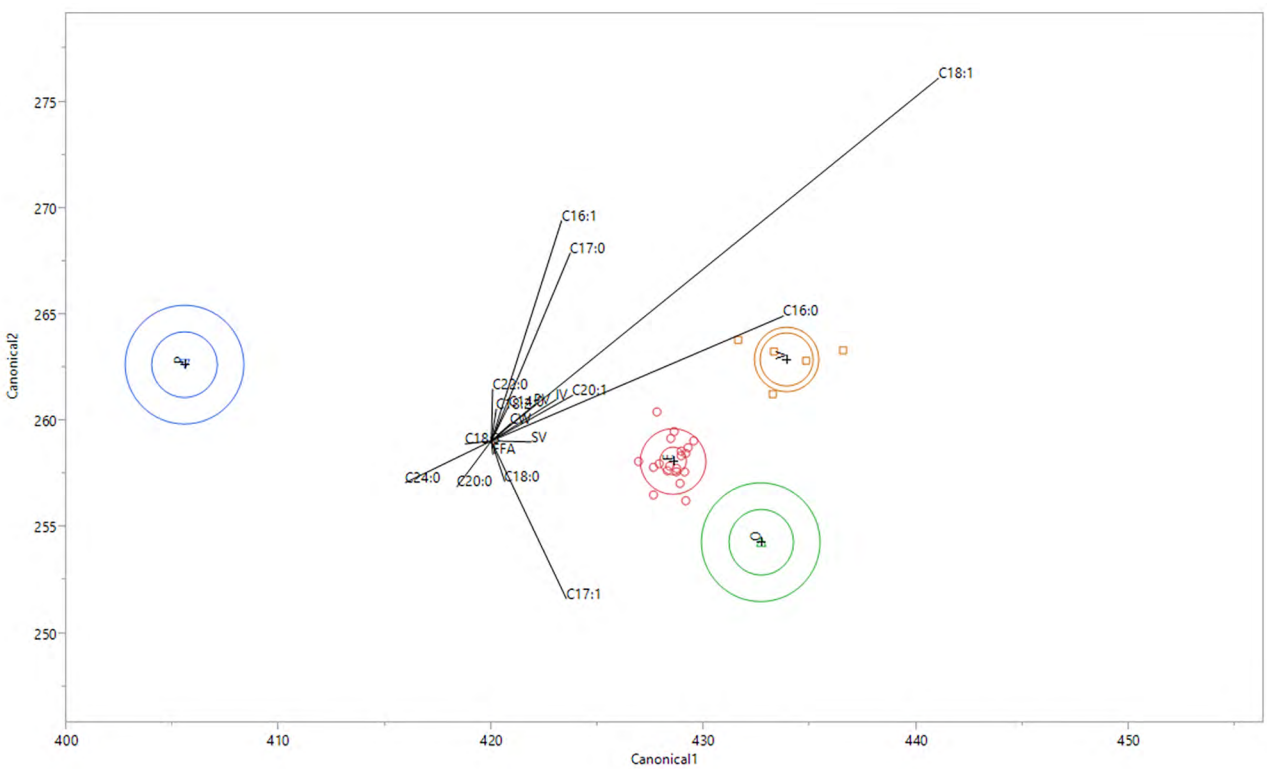

Figure 2. LDA of the examined olive oils' fatty acid content and chemical parameters. Inner circles denoted the 50% contour plot for each group, and outer circles represent the 95% confidence level for each mean

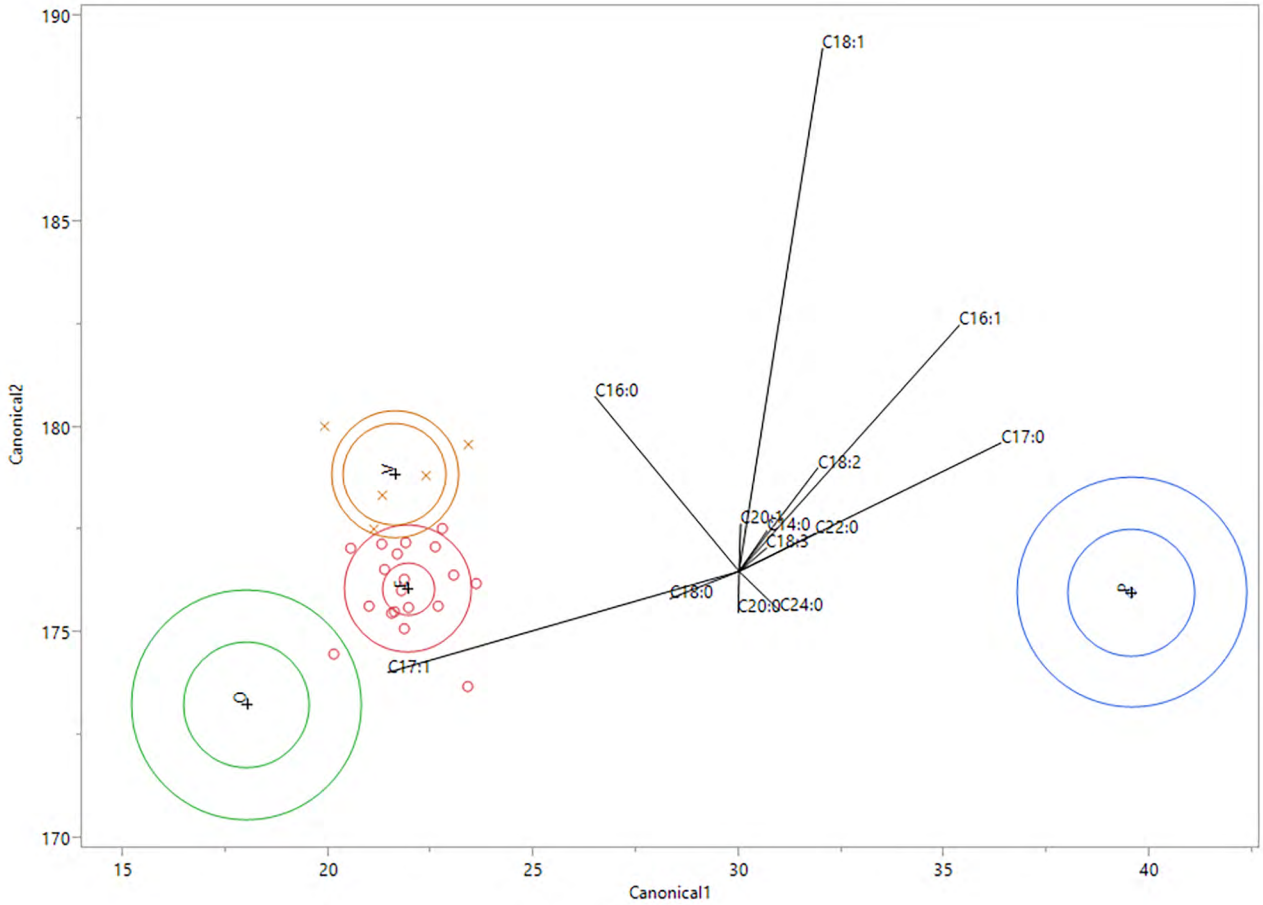

Figure 3. LDA of olive oils based only on fatty acid content. Inner circles denoted the 50% contour plot for each group, and outer circles represent the 95% confidence level for each mean

4. Conclusion

The results of the OO import control based on the requirements of the current regulations are not sufficient to confirm the declared type of OO. This is especially true for EVOO and VOO. However, the results of regular controls can be improved and applied to verify the declared quality and type of OO by processing them with multivariate statistical analysis methods. Although the presented results showed that PCA cannot be successfully applied, LDA proved to be completely satisfactory, even on the small number of OO samples included in the research. Further improvement of the LDA method must be carried out by more extensive validation using a larger number of samples and by including other parameters for testing the quality and adulteration of OO, such as potassium levels, waxes, sterols, etc. This augmentation of analytical techniques would ensure more reliable verification of imported OO, more efficient implementation of legal regulations and better protection of the interests of the domestic market and consumers, from both health and economic aspects.

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Original Scientific Paper

Effect of pre-fermented Swiss chard extract on the color stability of cured-cooked meat products

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Swiss chard extract

ABSTRACT

In response to growing consumer concerns over the safety of synthetic antioxidants and preservatives (e.g., nitrates and nitrites) in meat products, this study explores the feasibility of using Swiss chard extract (SCE) as a natural alternative for sodium nitrite in cured-cooked meat products. Three formulations of cured-cooked meat products were manufactured in a Serbian meat plant: a control sample (C) containing 95 ppm sodium nitrite, and two experimental batches with 3% (T3) and 6% (T6) SCE , respectively, without added nitrites. Instrumental color evaluation was conducted using the *CIE L*a*b** system immediately after production and during 60 days of refrigerated storage. The extract-treated meat products exhibited color values comparable to the nitrite-containing control, with only slight differences in lightness and redness. These findings demonstrate that SCE can maintain color stability in cured-cooked meat products, highlighting its potential as a clean-label substitute for synthetic nitrites.

1. Introduction

Nitrites have been widely used in the meat industry due to their multifunctional technological effects: the stabilization of cured meat color, inhibition of pathogenic microorganisms (especially *Clostridium botulinum*), and antioxidative protection (*Honikel*, 2008, *Shakil et al*., 2022, *Zhang et al*, 2023). However, over the past decade, increased attention has been drawn to the potential health risks associated with nitrite intake. It is well known that nitrites, in the presence of secondary amines formed during processing or digestion, can lead to the formation of carcinogenic *N*-nitroso compounds (*Vuković*, 2020, *Šojić et al*., 2020, *Yhang et al*., 2023). As a result, regulatory frameworks have imposed stricter limits on the use of nitrites. Simultaneously, growing consumer demand for clean-label products has driven research into natural ingredients that can replace synthetic additives while preserving product safety and quality (*Melios et al*., 2024, *Xie et al*., 2023). Leafy vegetables rich in nitrates, such as celery, spinach, beetroot, and Swiss chard (*Beta vulgaris* subsp. cicla) are increasingly being considered as viable natural alternatives in cured meat systems (*Jin et al*., 2018, *Iammarino et al*., 2022, *Luetic et al*., 2023). These plant extracts, in their interactions with

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nitrate-reducing bacteria (e.g., *Staphylococcus carnosus*), can generate nitrites *in situ*, replicating the effect of traditional curing agents (*Kim et al*., 2019).

Moreover, these vegetables are sources of bioactive compounds, such as betalain pigments, vitamin C, and polyphenols, which could improve the color stability and oxidative resistance of meat products. Among leafy vegetables, Swiss chard is especially notable for its elevated nitrate concentrations, which have been reported to range from 200 to 1000 mg/kg fresh weight, depending on variety, growing conditions, and harvest season. Swiss chard extract (SCE) also contains flavonoids (2.4–3.0 mg/g), betacyanins, and ascorbic acid, which may support color retention, and antioxidative protection in cured meat products without synthetic nitrites (*Iammarino et al.*, 2022).

The aim of this study was to evaluate the potential of pre-fermented SCE as a natural alternative to sodium nitrite in cured-cooked meat products. The experiment focused on instrumental color evaluation (*CIEL**, *CIEa**, *CIEb**, and *ΔE* values) over a defined storage period, comparing two concentrations of SCE (3% and 6%) with a conventional nitrite-containing control.

2. Materials and methods

2.1. Preparation of SCE

For the purposes of this study, a commercial plant-based extract derived from Swiss chard (MC-NRB®, Meat and Cracks, Germany) was used in combination with a nitrate-reducing starter culture (M-CULTURE® NRC, Meat and Cracks, Germany). Two batches of the extract were prepared one day prior to use, following the manufacturer's instructions. In the first preparation (R1), 600 g of MC-NRB® and 150 g of M-CULTURE® NRC were mixed with 6 L of cold potable water in a clean plastic container until a homogeneous solution was achieved. In the second preparation (R2), 1200 g of MC-NRB® and 300 g of M-CULTURE® NRC were dissolved in 12 L of cold potable water. Both containers were covered and incubated at 25 °C for 24 hours to allow enzymatic reduction of nitrate to nitrite. After pre-fermentation, the solutions R1 and R2 were stored under refrigeration (4 ± 1 °C) and used as natural curing agents in meat formulations at levels of 3% and 6%, respectively.

2.2. Preparation of cured-cooked meat products

Pasteurized canned meat products in the type of cooked ham were produced in an industrial meat processing facility (Zlatiborac LLC., Mačkat, Serbia) based on the formulations shown in Table 1. Three batches of 200 kg each were prepared: a control batch (C) and two treatment batches (T3 and T6). All batches were produced on the same day from pork ham of identical origin and quality. For the control batch (C), the curing brine contained 1.9% nitrite curing salt, resulting in approximately 95 mg/kg of sodium nitrite in the final product. In contrast, brines for the T3 and T6 batches were prepared with the same concentration (1.9%) of regular table salt,

Table 1. Composition of cured-cooked meat formulations (%)

Ingredient	C (Control)	T3 (3% R1)	T6 (6% R2)
Pork ham meat	72.00	72.00	72.00
Water	23.95	20.95	17.95
Table salt	–	1.90	1.90
Nitrite curing salt	1.90	–	–
Phosphate blend	0.50	0.50	0.50
Starch	1.00	1.00	1.00
Sugar	0.20	0.20	0.20
Carrageenan	0.40	0.40	0.40
Sodium isoascorbate	0.05	0.05	0.05
Extract R1 (fermented)	–	3.00	–
Extract R2 (fermented)	–	–	6.00

–, indicates the absence of the respective ingredient in the formulation.

without added nitrite. Brines were prepared using a brine mixer (Metalquimia, Spain), which was thoroughly cleaned and sanitized between each preparation. The injection sequence followed the order T3 → T6 → C, with the injector cleaned between batches to prevent cross-contamination. Following brine injection and mechanical tenderization, the meat batters from each batch were transferred into vacuum tumblers. Prior to tumbling, batch T3 was supplemented with fermented extract R1 (3%), and batch T6 with extract R2 (6%). Tumbling was performed for 2 hours under vacuum, after which the meat batters were stuffed into polyamide casings and rested at 4 ± 1 °C for 24 hours. Thermal processing was carried out in the same chamber for all batches until a core temperature of 72 °C was reached. The products were then cooled and stabilized at 4 ± 1 °C for 48 hours.

2.3. Instrumental color determination

The color of cured-cooked meat pieces was measured on the surface of the fresh product for each tested variant individually, with a total of 12 measurements per sample. Color was determined using a Minolta Chroma Meter CR-400 (Minolta Co., Ltd., Osaka, Japan) under D65 illumination, with a standard 2° observer angle and an 8 mm aperture on the measuring head. Prior to measurement, the instrument was warmed up in accordance with the manufacturer's instructions and calibrated using the standard procedure. Color characteristics were expressed in the *CIE* system (*CIE*, 1976), through: *CIEL** value, indicating lightness (black–white axis), and *CIEa** value, indicating the proportion of red color (red–green spectrum). The total color difference (*ΔE*) between treatment samples (T) and the control sample (C) on day 0 was calculated as follows (*Šojić et al*., 2020):

$$\Delta E = \sqrt[2]{(L_T^* - L_C^*)^2 + (a_T^* - a_C^*)^2 + (b_T^* - b_C^*)^2};$$

2.4. Statistical Analysis

The experiment was designed as a 3 × 5 factorial with three treatments (C, T3, T6) and five storage times (0, 15, 30, 45, and 60 days) as fixed factors. Color parameters (*CIEL**, *CIEa**, *CIEb**, *ΔE*) were analyzed by two-way analysis of variance (ANOVA), and differences were tested at the 5% significance level ($P < 0.05$) using Fisher's Least Significant Difference (LSD) test for post hoc comparisons. Statistical analyses were conducted using STATISTICA software, version 12.0 (StatSoft Inc., Tulsa, OK, USA).

3. Results and discussion

Color is one of the most important quality indicators of meat and meat products (*Škaljac*, 2014). Instrumental color measurements are presented in Table 2. At the beginning of storage, lightness (*CIEL**) values ranged from 73.77 in treatment T6 to 75.46 in the control sample (C). No statistically significant differences ($P > 0.05$) were observed between the control and the treatment groups (T3 and T6). During the 60-day storage period, a general trend of decreasing *CIEL** values was observed in all three formulations (C, T3, and T6). However, at the end of storage, the differences in *CIEL** remained statistically insignificant ($P > 0.05$), indicating that the addition of SCE did not adversely affect the lightness of the product during storage.

At the beginning of the storage period, the yellowness component (*CIEb**) ranged from 6.83 in treatment T6 to 8.38 in the control sample (C). Statistically significant differences ($P < 0.05$) were observed between T6 and the other two samples (K and T3). However, throughout the 60-day storage period, *CIEb** values remained stable and did not change significantly over time ($P > 0.05$). At the end of the storage, *CIEb** values in both T3 and T6 were significantly higher than in the control ($P < 0.05$), likely due to the presence of anthocyanins and other pigment compounds derived from Swiss chard and their interactions with meat proteins and brine components during curing and thermal treatment (*Kim et al*., 2019).

The total color difference (*ΔE*) values, calculated relative to the control on day 0, ranged from 2.03 (C – day 15) to 5.82 (T6 – day 60). According to the scale proposed by *Pogorzelska et al*. (2018), these differences correspond to "noticeable" to "distinct" visual changes. On day 0, *ΔE* values were 2.39 for T3 and 3.57 for T6, suggesting only very slight to noticeable differences compared to the control. Similar findings were reported by *Sindelar et al*. (2007), who investigated the use of various concentrations of celery extract as a total replacement for nitrites in cooked ham.

With regard to the two most important color parameters (*CIEL** and *CIEa**), the use of SCE at optimal concentrations appears to produce effects comparable to conventional sodium nitrite (*Kim et*

Table 2. Instrumental color parameters of cured-cooked meat products

Time (day)	CIEL* value		
	C	T3	T6
0	75.46 ± 1.52^{Aa}	74.38 ± 1.20^{Aa}	73.77 ± 2.62^{Aa}
15	75.46 ± 1.88^{Aa}	73.26 ± 2.38^{Ba}	73.79 ± 1.66^{Aba}
30	71.05 ± 1.05^{Ab}	71.54 ± 0.96^{Ab}	70.73 ± 2.05^{Ab}
45	72.36 ± 1.44^{Ab}	71.37 ± 1.81^{ABb}	70.71 ± 1.30^{Bb}
60	71.38 ± 1.07^{Ab}	71.09 ± 1.28^{Ab}	70.29 ± 2.24^{Ab}

Time (day)	CIEa* value		
	C	T3	T6
0	10.37 ± 0.87^{Aa}	10.13 ± 0.42^{Aab}	9.78 ± 1.25^{Aa}
15	10.39 ± 1.26^{Aba}	10.71 ± 0.88^{Aa}	9.76 ± 0.61^{Ba}
30	10.98 ± 0.87^{Aa}	10.02 ± 0.55^{Bb}	10.07 ± 0.94^{Ba}
45	11.17 ± 0.72^{Aa}	10.81 ± 0.88^{Aa}	10.26 ± 1.78^{Aa}
60	10.28 ± 0.66^{Aa}	10.29 ± 0.71^{Aab}	10.35 ± 1.06^{Aa}

Time (day)	CIEb* value		
	C	T3	T6
0	6.83 ± 0.98^{Bb}	7.52 ± 0.58^{Ba}	8.38 ± 0.98^{Aa}
15	7.35 ± 0.59^{Ba}	7.46 ± 0.47^{Ba}	8.59 ± 1.00^{Aa}
30	6.37 ± 0.59^{Cb}	7.07 ± 0.42^{Ba}	8.34 ± 0.57^{Aa}
45	6.73 ± 0.40^{Bb}	7.23 ± 0.46^{Ba}	8.01 ± 0.78^{Aa}
60	6.28 ± 0.68^{Bb}	7.57 ± 0.61^{Aa}	7.89 ± 0.85^{Aa}

Time (day)	ΔE value		
	C	T3	T6
0	-	2.39 ± 1.35^{Ab}	3.57 ± 1.29^{Ab}
15	2.03 ± 0.85^{Ab}	3.29 ± 2.07^{Aab}	3.51 ± 1.52^{Ab}
30	4.78 ± 1.40^{Aa}	4.13 ± 1.27^{Aa}	5.37 ± 2.05^{Aa}
45	3.85 ± 1.33^{Ba}	4.38 ± 1.52^{ABa}	5.54 ± 2.06^{Aa}
60	4.39 ± 1.83^{Aa}	4.81 ± 2.03^{Aa}	5.82 ± 1.80^{Aa}

Values are presented as means ($\bar{X} \pm SD$). Values with different uppercase letters (A–C) within the same row differ significantly ($P < 0.05$); values with different lowercase letters (a–c) within the same column differ significantly ($P < 0.05$).

al., 2019; *Schopfer et al*., 2022). *Kim et al*. (2019) also observed that increasing concentrations of SCE led to enhanced redness, likely due to higher levels of residual nitrite in cooked meat samples. The most pronounced changes were observed on day 60, mainly due to a reduction in lightness (*CIEL**) for all three meat products. Despite these trends, the *ΔE* differences between treatments and the control were not statistically significant at any time point ($P > 0.05$). Taken together, the results demonstrate that the addition of 3% fermented SCE maintains color properties close to the conventional nitrite-treated product. This supports its potential use as a natural alternative to synthetic curing agents—specifically sodium nitrite—in the production of cured-cooked meat products such as cooked ham, without compromising visual appearance at any stage of the storage period.

4. Conclusion

The results of this study demonstrate that the application of 3% pre-fermented SCE can effectively maintain the color stability of cured-cooked meat products, such as cooked ham, with no significant differences in lightness (*CIEL**) and redness (*CIEa**) compared to the nitrite-treated control. The slightly elevated yellowness (*CIEb**) observed in meat products containing SCE is attributed to natural pigments present in the extract. Overall, total color differences (*ΔE*) remained within acceptable sensory thresholds, supporting the feasibility of using SCE as a natural alternative to nitrite in the development of clean-label meat products.

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Original Scientific Paper

Coccidiostat residues in withdrawal feed for broilers – implications of cross-contamination in feed production lines

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ABSTRACT

This study investigated the presence of residues of eleven coccidiostats in unmedicated feed intended for broilers in the withdrawal phase, with the aim of assessing potential cross-contamination during feed production. Between 2023 and 2024, a total of 341 feed samples were analysed, using validated LC-MS/MS methodology, for amprolium, diclazuril, halofuginone, lasalocid, salinomycin, maduramycin, monensin, narasin, nicarbazin, robenidine, semduramycin. Detected concentrations ranged from below the limit of quantification to 85.0 mg/kg. Non-compliant samples were identified for diclazuril (2/243), lasalocid (1/328), salinomycin (12/329), maduramycin (11/324), monensin (6/324), narasin (6/315), nicarbazin (2/327), and robenidine (7/333). The highest detected concentrations per analyte were: diclazuril 0.133 mg/kg, lasalocid 1.33 mg/kg, salinomycin 85.0 mg/kg, maduramycin 0.834 mg/kg, monensin 25.30 mg/kg, narasin 58.15 mg/kg, nicarbazin 33.70 mg/kg, and robenidine 35.2 mg/kg. Amprolium, halofuginone, semduramycin were not detected in any feed sample. Overall, 42 out of 341 feed samples (12.3%) exceeded the maximum permitted levels, indicating insufficient cleaning of production lines and inadequate separation between medicated and non-medicated feed. The occurrence of such residues in feed intended for broilers in the withdrawal phase poses a risk of non-compliant residues in edible tissues, highlighting the need for stricter hygiene protocols, improved quality control in feed mills, and continuous monitoring to safeguard food safety and public health.

1. Introduction

Coccidiosis is a parasitic disease caused by protozoa of the genus *Eimeria*, affecting poultry worldwide, particularly broilers in intensive production systems. The disease remains a major threat to animal health and welfare, often causing significant economic losses due to reduced productivity, increased mortality, and treatment costs (*Bampidis et al*., 2022; *EFSA*, 2021). Preventive use of coccidiostats in feed is still considered indispensable for maintaining flock health and production efficiency (*EFSA*, 2008; *EFSA*, 2021).

In the European Union and Serbia, coccidiostats are authorized as feed additives for poultry

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and include ionophores (lasalocid, monensin, salinomycin, maduramycin, narasin, semduramycin) and synthetic compounds (amprolium, diclazuril, nicarbazin, decoquinate, robenidine, halofuginone). These substances are administered during early and mid-production phases, but are withdrawn several days before slaughter to avoid residues in edible tissues. During the withdrawal phase, animals should be fed non-medicated feed; however, cross-contamination can occur if medicated and non-medicated feeds are produced sequentially without adequate cleaning of production lines. This so-called unavoidable carry-over may result in detectable coccidiostat residues in non-target feed and subsequently in animal products (*Mortier et al*., 2005; *Stolker et al.*, 2007; *Galarini et al*., 2021).

To manage this risk, the European Union has established maximum permissible levels of unavoidable carry-over in non-target feed, set at 1% of the authorized concentration in target feed (*Directive 2021/808/EC*). Serbia has harmonized its national legislation with these requirements (*Official Gazette RS, Nos. 4/2010, 113/2012, 27/2014, 54/2017*). Table 1 presents the current maximum levels of unavoidable carry-over for the 12 coccidiostats included in this study.

The presence of coccidiostats in withdrawal feed above these maximum levels increases the likelihood of residues in edible tissues, potentially leading to non-compliance with established maximum residue limits (MRLs) in meat and other animal products. This risk is particularly relevant for ionophores, such as lasalocid, monensin, narasin and nicarbazin, which have relatively high carry-over limits and can persist in production lines if flushing procedures are inadequate. Continuous monitoring and strict feed mill hygiene protocols are, therefore, essential to ensure compliance with food safety regulations and protect public health.

Table 1. Maximum levels of unavoidable carry-over (1%) of coccidiostats in non-target feed (mg/kg) (Source: Commission Regulation (EU) 2020/499)

	Maximum level (mg/kg)
Amprolium	n/a (no EU carry-over limit set, national rules may apply)
Diclazuril	0.03
Halofuginone	0.03
Lasalocid	1.25
Salinomycin	0.70
Maduramycin	0.05
Monensin	1.25
Narasin	2.10
Nicarbazin (DNC)	3.75
Robenidine	0.70
Semduramycin	0.25

2. Materials and methods

Between 2023 and 2024, 341 withdrawal feed samples for broilers were collected from various feed mills, including rinsing batches used when switching from medicated to unmedicated feed. Eleven coccidiostats were analysed: amprolium (AMP), diclazuril (DICL), halofuginone (HAL), lasalocid (LAS), salinomycin (SAL), maduramycin (MAD), monensin (MON), narasin (NAR), nicarbazin (DNC), robenidine (ROBN), semduramycin (SEM). Sample preparation involved homogenization, extraction of 5.0 g feed with 25 mL acetonitrile, shaking for 60 min, and filtration through a 0.22 µm filter. LC-MS/MS analysis was performed on a Shimadzu triple quadrupole system with a Kinetex C18 column (100 × 2.1 mm, 2.6 µm) using gradient elution (water/acetonitrile with 0.1% formic acid) at 0.55 mL/min. Electrospray ionization was applied in positive or negative mode, depending on the analyte. Quantification was done using matrix-matched calibration at four levels. The method was validated according to Commission Implementing Regulation (EU) 2021/808 (*European Commission*, 2021), achieving recoveries between 80–110% and LOQs of 0.005–0.02 mg/kg. Internal quality control included fortified blank feed samples in each batch.

3. Results

The analysis of 341 feed samples labelled as withdrawal feed for broilers revealed the presence of residues from 8 of the 11 investigated coccidiostats. Amprolium, halofuginone and semduramycin were not detected in any feed sample. Non-compliant results (above the maximum permissible levels for unavoidable carry-over) were recorded for diclazuril, lasalocid, salinomycin, maduramycin, monensin, narasin, nicarbazin, and robenidine. Overall, 42 out of 341 feed samples (12.3%) exceeded the legal limits. Salinomycin showed the highest proportion of non-compliant feed samples (3.6%), followed by

Table 2. Concentration of coccidiostats in withdrawal feed for broilers (2023–2024)

Coccidiostat	n (samples)	Min–Max (mg/kg)	Positive* n (%)	Non-compliant** n (%)
Amprolium (AMP)	84	<LOQ – n.d.	0 (0.0)	0 (0.0)
Diclazuril (DICL)	243	<LOQ – 0.133	6 (2.47)	2 (0.82)
Halofuginone (HAL)	85	<LOQ – n.d.	0 (0.0)	0 (0.0)
Lasalocid (LAS)	328	<LOQ – 1.33	27 (8.23)	1 (0.30)
Salinomycin (SAL)	329	<LOQ – 85.0	197 (59.88)	12 (3.65)
Maduramycin (MAD)	324	<LOQ – 0.834	56 (17.28)	11 (3.40)
Monensin (MON)	324	<LOQ – 25.30	124 (38.27)	6 (1.85)
Narasin (NAR)	315	<LOQ – 58.15	95 (30.16)	6 (1.90)
Nicarbazin (DNC)	327	<LOQ – 33.70	98 (29.97)	2 (0.61)
Robenidine (ROBN)	333	<LOQ – 35.20	65 (19.52)	7 (2.10)
Semduramycin (SEM)	83	<LOQ – n.d.	0 (0.0)	0 (0.0)

*Positive: detected residues above LOQ but below maximum permissible carry-over level.

**Non-compliant: detected residues above maximum permissible carry-over level as defined in Regulation (EU) No. 574/2011 (*European Commission*, 2011).

maduramycin (3.4%) and robenidine (2.1%). The highest detected concentration was 85.0 mg/kg for salinomycin, corresponding to more than 100 times the allowable carry-over limit. Such elevated concentrations strongly indicate inadequate cleaning protocols between the production of medicated and unmedicated feed batches.

The majority of feed samples with detectable residues were below the maximum permissible levels, yet a significant portion (approximately 80%) contained more than one coccidiostat, typically in low concentrations. This finding aligns with previous studies (*Mortier et al*., 2005; *Galarini et al*., 2021) showing that cross-contamination in feed mills often involves multiple analytes, reflecting sequential production of different medicated feeds without adequate line flushing. The detection of high concentrations (e.g., 85.0 mg/kg salinomycin, 58.15 mg/kg narasin, 35.2 mg/kg robenidine) in withdrawal feed raise concern over possible residues in edible tissues, particularly when withdrawal periods are not respected. These results highlight the importance of strict hygiene protocols, physical separation of production lines, and targeted monitoring of high-risk compounds, such as salinomycin, narasin, and robenidine.

4. Discussion

The detection of residues from multiple coccidiostats in withdrawal feed for broilers, including concentrations exceeding the maximum permissible levels for unavoidable carry-over, indicates that while most producers follow regulatory requirements, some hygiene gaps persist within feed manufacturing processes. The overall non-compliance rate of 12.3% observed in this study is relatively low considering the total number of samples, but still highlights that cleaning procedures between medicated and non-medicated batches are not always fully effective. Similar challenges have been reported in some EU countries, where non-compliance rates typically range between 6–9% (*Mortier et al*., 2005; *Roila et al*., 2019; *Annunziata et al*., 2020), indicating that the current findings are consistent with trends observed across Europe.

Salinomycin was the most frequently detected analyte, present in 59.9% of feed samples, with the highest individual concentration (85.0 mg/kg) and non-compliance rate (3.65%). Maduramycin and robenidine also showed notable non-compliance rates (3.40% and 2.10%, respectively). The frequent detection of multiple coccidiostats in the same feed sample, usually at low levels, likely reflects sequential production of different medicated feeds without complete line clearance. While these residues do not necessarily indicate intentional misuse, they underline the need for consistent application of hazard analysis and critical control point (HACCP) principles to prevent cross-contamination at all stages.

From a food safety perspective, the presence of coccidiostats above regulatory limits in withdrawal feed is concerning, because this feed should be completely free of these compounds to ensure compliance with MRLs in meat. Although the majority of feed samples were compliant, the occurrence of exceedances—particularly at high concentrations—confirms that risks remain if preventive measures fail. Maintaining strong HACCP-based monitoring, regular verification of cleaning protocols, and staff training across the feed production chain will help sustain compliance levels and further reduce contamination risks. The feed producers generally adhered to established legal and safety standards, with non-compliance rates similar to those reported in EU countries. However, continuous improvement in good manufacturing practice (GMP), good hygiene practice (GHP), and HACCP implementation is essential to ensure that carry-over remains within permissible limits, thereby safeguarding food safety and consumer trust.

5. Conclusion

This study showed that 87.7% of withdrawal feed samples for broilers complied with legal carry-over limits for coccidiostats, while 12.3% exceeded these thresholds. Although most feed was compliant, high concentrations in some non-compliant feed samples confirm that preventive measures are not always fully effective. Strengthening hygiene protocols, quality control, and HACCP implementation across the feed production and distribution chain is essential to ensure residue-free withdrawal feed, protect food safety, and maintain consumer trust.

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Original Scientific Paper

Levels of Cu, Zn, Fe and Mn in livers of different species of animals in Serbia

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ABSTRACT

Liver samples (n=67) of chicken, pork, beef and lamb were analyzed for levels of Cu, Zn, Fe and Mn. Livers were gathered from different meat processing facilities within the Serbian National Residue Monitoring Program in 2024. Analysis of micro elements was performed by inductively coupled plasma mass spectrometry (ICP-MS). Our data show that the mean level of Cu was significantly higher in lamb liver, while Zn and Fe were significantly higher in pork liver. The mean Mn levels determined in the liver samples were not statistically different between the animal species. The distribution of the elements in liver samples was studied by applying principal component analysis (PCA). The differing mineral status in livers of different species of animals implies differences in their nutritional benefits for the human diet.

1. Introduction

Animal liver is a major storage organ both for trace elements and heavy metals. It can accumulate higher concentrations of toxic elements than most other foods and may pose health risk to humans (*Potthast,* 1993). Some heavy metals and metalloids are always present in livers and are often essential to health, even though they are toxic at high concentrations (*Nriagu* et al., 2009; *Counotte at al.,* 2019). The levels of trace elements in animals' livers is of interest for the proper functioning of humans and animals (*Kicińska et al.,* 2019; *Klich et al.,* 2023). Inadequate intake of these elements may be harmful to animal health and meat production potential (*Suttle,* 2010). Micronutrients, such as Fe, Zn, Mn, B, Cu, Mo, Cl and Ni, play an important role in human body tissues. They are mostly involved in the functioning of enzymes, as cofactors or metal activators. Their deficiencies result in serious health and nutritional consequences in humans, causing dietary micronutrient malnutrition (*Guan et al.,* 2024; *Xu et al.,* 2025).

Scientific information about Cu, Zn, Fe and Mn levels in livers of different species of animals in Serbia is limited (*Tomovic et al.,* 2011; *Nikolic et al.,* 2017; *Subotic et al.,* 2017). The aim of this study was to determine and compare the distribution of Cu, Zn, Fe and Mn in livers of chicken, pork, beef and lamb which were gathered from different meat processing facilities within the Serbian National Residue Monitoring Program in 2024.

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2. Materials and methods

2.1. Sample collection and preparation

A total of 69 liver samples of chicken (n=21), pork (n=23), beef (n=17) and lamb (n=6) were examined. Liver samples were acquired during regular slaughter and analyzed within the Serbian National Residue Monitoring in 2024. After collection, livers were labelled and stored in polyethylene bags and frozen at -18 °C. Frozen livers were thawed at 4 °C and homogenized, then approximately 0.5 g (wet weight) of sample was mineralized by adding 5 mL of nitric acid (67–70%, TraceMetal grade, Fisher Chemical, Belgium) and 5 mL deionized water, purity of 0.067 µS/cm, produced by a Purelab DV35 water purification system (ELGA, Buckinghamshire, UK). Microwave assisted digestion was performed in a MARS 6 iWawe Microwave Digestion System (CEM Technology, USA). After cooling at room temperature, the digests were quantitatively transferred into polypropylene volumetric flasks and diluted to 100 mL with deionized water.

2.2. Quality assurance

Inductively coupled plasma mass spectrometry (ICP-MS), (iCap Q mass spectrometer, Thermo Scientific, Bremen, Germany), was used for analysis of the ^{63}Cu, ^{66}Zn, ^{57}Fe and ^{55}Mn isotopes. The quality of the analytical process was verified by analysis of the certified reference material NIST 1577c (bovine liver, Gaithersburg, MD, USA). Reference material was prepared in the same way as test samples using microwave digestion. The obtained concentrations were in the range of certified values.

2.3. Statistical analysis

Statistical analysis of experimental data was performed using software Statistica 10.0 (StatSoft Inc., Tulsa, OK, USA). One-way analysis of variance (ANOVA) and Tukey's HSD test for comparison of means were used to analyze differences in the elements' levels in livers from different animal species. Principal component analysis (PCA) was used to group the observed samples and to discover the possible correlations among the element levels.

3. Results

3.1. Contents of micro elements

Contents of Cu, Zn, Fe and Mn measured in the analyzed chicken, pork, beef and lamb livers, expressed as mean±standard deviation, are presented in Table 1. Different superscripts within the same row indicate significant differences of mean element levels according to Tukey's HSD test ($p < 0.05$).

3.2. Principal component analysis (PCA)

PCA was applied to the correlation matrix, which included the four parameters of four sample groups of livers from the different animal species (*Hammer et al., 2001*). PCA was applied to group the observed the possible correlations among the measured Cu, Zn, Fe and Mn levels and the animal species (chicken, pork, beef and lamb liver) (Figure 1).

Table 1. Content of Cu, Zn, Fe and Mn (mg/kg; mean±standard deviation) in chicken, pork, beef and lamb livers.

Element	Chicken liver, n=21	Pork liver, n=23	Beef liver, n=17	Lamb liver, n=6
Cu	4.25^{a}±1.74	8.70^{a}±4.51	58.12^{b}±26.43	121.74^{c}±46.89
Zn	39.47^{a}±12.71	77.39^{b}±21.21	40.35^{a}±7.97	44.49^{a}±5.96
Fe	118.12^{a}±27.37	233.38^{b}±89.07	60.36^{a}±37.60	66.26^{a}±18.43
Mn	3.05^{a}±0.65	3.56^{a}±0.77	3.01^{a}±0.41	3.04^{a}±1.37

$^{a-c}$ *Values within the same row with different superscripts are significantly different ($p<0.05$).*

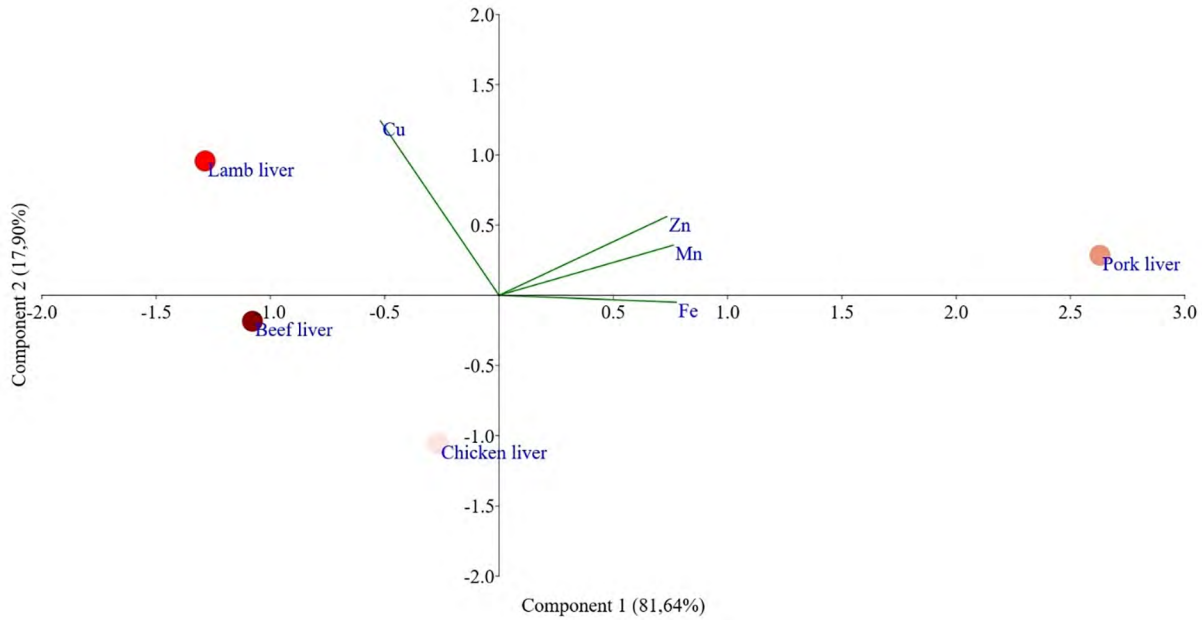

Figure 1. Bi-plot of principle component analysis (PCA) of Cu, Zn, Fe and Mn levels in livers from different animal species.

4. Discussion

4.1. Micro elements

The highest mean level of Cu was quantified in lamb liver (121.74 mg/kg) compared with significantly lower levels present in chicken, pork and beef liver. Levels of Cu were significantly higher in beef liver compared to chicken and pork liver. The mean Cu levels in beef (58.12 mg/kg) and pork (8.70 mg/kg) liver were similar to the average level reported by *Nriagu et al.* (2009) (63.9 mg/kg) and *Sola et al.* (1997) (6.1 mg/kg), respectively.

The highest mean Zn (77.39 mg/kg) and mean Fe (233.38 mg/kg) levels were established in pork liver, and they were significantly higher compared to other analyzed liver samples. The obtained levels were lower than those established in our previous investigation i.e., in livers of intensive and extensive pigs from Serbia, except for the Zn level in livers of extensive pigs (54.27 mg/kg) (*Nikolic et al.*, 2017). The mean levels of Zn and Fe in pork liver in the current study were similar to that reported by *López-Alonso et al.,* (2007) (they reported Zn – 81.3 mg/kg; Fe – 195 mg/kg).

The mean Mn levels (3.01-3.56 mg/kg) determined in liver samples were not statistically different between the four food animal species. Similar mean Mn levels were found by *Nikolic et al.*, (2017) and *López-Alonso et al.,* (2007) in pig liver, but our results for pork and chicken liver were higher than those reported by *Sola et al.* (1997) (they reported Mn – 1.2 mg/kg, pig liver; Mn – 0.8 mg/kg, duck liver).

4.2. PCA analyses

The first two components (PC1 and PC2) in the PCA resulting from the examination of the levels of Cu, Zn, Fe and Mn in liver from different animal species accounted for 99.54% of the total variance (PC1 81.64%, PC2 17.90%). The influence of different parameters that described the examined liver samples can be evaluated from Figure 1. The examined livers from the different animal species are located on different sides of Figure 1. Pork liver, in which the highest Zn, Mn and Fe levels were observed, is located on the upper right side of the figure. Lamb, beef and chicken liver are located on the opposite side of the figure. Lamb liver, in which the highest mean Cu level was observed in comparison with other livers, is located highest up on the upper left side (Figure 1).

5. Conclusion

This study aimed to provide information on levels of Cu, Zn, Fe and Mn in livers from different animal species (chicken, pork, beef and lamb) collected during the Serbian National Residue Monitoring Program in 2024. The concentration of Cu was significantly higher in lamb liver, while concentrations

of Zn and Fe were significantly higher in pork liver. While most other authors researching this topic have published element levels in pork liver, this work highlights the importance of analyzing livers from different species of animals. Moreover, the differences seen in micro element levels imply differences in the benefits of the livers from domestic animal species for the human diet.

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Review Paper

Sustainable nanofiber-based strategies for improving quality and safety in meat systems

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ABSTRACT

Nanofiber technology has emerged as a sustainable strategy to improve the quality and safety of meat systems. With their high surface area, tunable porosity, and encapsulation efficiency, nanofibers enable applications in active packaging, intelligent sensing, and functional reformulation. Biopolymer-based nanofiber films, particularly from plant (pea and mungbean) proteins, enhance oxygen and moisture barriers, extend shelf life, and provide mechanical stability. When combined with natural antimicrobials or antioxidants, they allow controlled release systems that reduce microbial growth and lipid oxidation without compromising sensory attributes. Moreover, intelligent nanofiber packaging contributes to real-time spoilage detection, while functional reformulation supports phosphate and nitrite replacement. Recent advances highlight protein isolates as promising materials for nanofiber production, aligning with sustainability and circular economy principles. Although challenges remain in scalability, cost, and regulatory approval, nanofibers represent a versatile platform for developing clean-label and eco-friendly meat systems.

1. Introduction

Meat and meat products are highly perishable due to their nutrient-rich composition, high water activity, and vulnerability to microbial spoilage and oxidative deterioration. These factors not only shorten shelf life but also compromise quality and safety. Conventional preservation methods rely heavily on synthetic additives such as phosphates, nitrites, and antioxidants, which have been effective but are increasingly criticized due to potential health risks and rising consumer demand for clean-label and sustainable alternatives. In parallel, concerns over non-biodegradable packaging materials further drive the search for innovative and eco-friendly solutions (*Mills*, 2014; *Amna et al*., 2015).

Nanofiber technology has emerged as a promising strategy to address these challenges. Owing to their high surface-to-volume ratio, tunable porosity, and ability to encapsulate and release bioactive compounds in a controlled manner, nanofibers enable multifunctional applications in meat systems (*Lim*, 2017). They have been shown to enhance oxidative stability, inhibit microbial growth, improve texture, and extend shelf life, while also serving as carriers for natural antimicrobials and antioxidants (*Arkoun et al*., 2017; *Liu et al*., 2022). In addition, nanofiber-based films can improve barrier properties of packaging, function as freshness indicators, and contribute to the reformulation of low-additive meat products (*Jiang et al*., 2024).

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From a sustainability perspective, the utilization of renewable biopolymers, such as proteins and polysaccharides, in nanofiber production reduces reliance on fossil-derived polymers and aligns with consumer expectations for environmentally friendly processing. Collectively, these advances highlight nanofibers as versatile tools capable of simultaneously improving quality, safety, and sustainability in modern meat systems.

2. Production strategies for nanofibers

Nanofibers can be produced by various methods, such as phase separation, ball-milling, homogenization, self-assembly, freeze-drying, electrospinning, and other spinning techniques, including air and force spinning (*Henriksson et al*., 2007; *Piras et al*., 2019; *Delgado-Aguilar et al.*, 2015). Electrospinning remains the most versatile and extensively applied technique in food systems. Applying a high-voltage electric field to polymer solutions or melts generates ultrafine fibers with high surface area and interconnected pore structures (*Ji et al*., 2024). Electrospinning enables precise control over fiber morphology and the incorporation of bioactive compounds, such as antioxidants, antimicrobials, and colorants. Advanced variants—including coaxial, needleless, and multi-jet electrospinning—enhance encapsulation efficiency and scalability, though equipment cost and throughput remain challenges (*Zahmatkeshan et al*., 2019; *Sofi et al*., 2020). Other strategies, such as self-assembly, interfacial polymerization, and freeze-drying, offer opportunities for designing functional nanostructures, but are less established for food applications due to technical and economic constraints (*Wei et al*., 2012). Overall, mechanical methods are advantageous for bulk nanofiber production, whereas electrospinning provides unmatched versatility for developing functional nanofibers tailored for preservation, active packaging, and reformulation of meat products (*Jiang et al*., 2014).

3. Functional applications in meat systems

3.1 Control of lipid oxidation

Lipid oxidation is one of the most critical factors leading to meat quality deterioration, resulting in rancidity, discoloration, and nutrient loss (*Serdaroğlu et al*., 2024). Nanofiber-based systems can effectively encapsulate and gradually release natural antioxidants, thereby prolonging oxidative stability during storage (*Ansarifar et al*., 2022). Electrospun nanofibers loaded with compounds, such as α-tocopherol, polyphenols, and essential oils, have demonstrated significant reductions in thiobarbituric acid reactive substance (TBARS) and peroxide values in beef, chicken, and fish products (*Aytac et al*., 2017; *Khaledian et al*., 2019; *Lin et al*., 2019). In addition, the intrinsic antioxidant potential of whey protein isolate-based nanofibers further supports their potential as clean-label strategies to delay oxidative rancidity (*Carvalho et al*., 2018).

3.2 Antimicrobial effects

Microbial spoilage remains a major concern for meat safety and shelf life. Nanofibers incorporated with antimicrobial agents—including essential oils, organic acids, polyphenols, or bacteriocins—exhibit strong inhibitory effects against foodborne pathogens, such as *E. coli, Salmonella* spp., and *Listeria monocytogenes*. Electrospinning allows efficient encapsulation of these compounds, preventing premature volatilization or deactivation and ensuring sustained release during storage. Chitosan- and gelatin-based nanofibers, in particular, have been shown to extend the microbial stability of fresh meat and poultry products while maintaining sensory attributes (*Arkoun et al*., 2017; *Lin et al*., 2018b; *Liu et al*., 2022).

3.3 Functional reformulation and additive replacement

Beyond preservation, nanofibers contribute to the functional reformulation of meat products by improving water-holding capacity, emulsification, and textural properties (*Jiang et al*., 2024). This makes them attractive as natural alternatives to conventional additives such as phosphates and nitrites. For instance, nanofibers incorporating cinnamic aldehyde, thyme oil, and plant or animal protein sources have shown potential to replace nitrites while maintaining color and microbial safety (*Aytac et al*., 2017; *Ceylan et al*., 2022; *Dogan et al*., 2022). Recent studies investigating nanofiber-based applications in meat systems are summarized in Table 1.

3.4 Sensory quality and consumer acceptance

The integration of nanofibers into meat systems should not compromise sensory quality. Studies indicate that antioxidant- and antimicrobial-loaded nanofibers enhance juiciness, preserve color

stability, and reduce off-flavors associated with lipid oxidation (*Dogan et al*., 2022; *Yilmaz et al*., 2022). Moreover, the use of biopolymer-based nanofibers aligns with consumer expectations for sustainability, naturality, and clean-label production, thereby improving market acceptance (*Jiang et al*., 2014).

Table 1. Applications of Nanofiber-Based Systems in Meat Products

Nanofiber Type	Active Components/ Biological Activity	Meat Product	Findings	References
Chitosan and Poly(ethylene oxide) Nanofibers	Chitosan	Fresh beef slices	Increased bioavailability of chitosan; antibacterial effects against *E. coli*, *L. innocua*, *S. aureus*, and *S.* Typhimurium	*Arkoun et al*. (2017)
Gelatin, Chitosan, and 3-Phenyllactic Acid (GCP) Nanofibers	Chitosan and PLA	Chicken breast	Controlled release of antibacterial agents; enhanced antibacterial effect against *Staphylococcus aureus* and *Escherichia coli*	*Liu et al.* (2022)
Gelatin, Glycerin, and ε-Polylysine Nanofiber-Coated Wraps	ε-Polylysine	Beef	Improved mechanical properties; antibacterial effect against *Listeria monocytogenes*	*Lin et al.* (2018a)
ε-PL and Chitosan Electrospun Nanofibers	ε-PL, Chitosan	Fresh chicken slices	Reduced growth of *S.* Typhimurium and *S. enteritidis*; use of nanofiber-coated aluminum foil	*Lin et al.* (2018b)
Polyurethane Nanofibers	Olive oil (VOO) and zinc oxide (ZnO)	Fresh meat	Inhibition of *Staphylococcus aureus* and *Salmonella*; potential replacement for PVC film	*Anma et al*. (2015)
Chitosan Nanofibers	Tea tree oil	Chicken meat	Increased bioavailability and sustained release; enhanced antibacterial effect against *Salmonella*	*Cui et al*. (2018)
Glycyrrhiza Polysaccharide Nanofibers	Tea tree oil, encapsulated with gliadin nanoparticles	Chicken and pork meat	Efficient encapsulation and release of tea tree oil; reduction of *Salmonella* Typhimurium	*Cai et al*. (2021)
PVA Nanofibers	Black seed oil and curcumin	Salmon fillet	Sous vide (SV) cooking with nanofiber-coated fillet; reduced bacterial growth (TMAB and TPB), lowered TBA values	*Ceylan et al*. (2022)
Chitosan Nanofibers	Chrysanthemum essential oil	Fresh beef slices	Slow release of essential oil; long-term antibacterial effects; reduced TBARS values	*Lin et al*. (2019)
Polyvinylpyrrolidone Nanofibers	Lavender essential oil	Minced meat	Slow release of essential oil; reduction of aerobic mesophilic bacteria, psychrotrophic bacteria, yeast, and molds	*Dogan et al*. (2022)
Gelatin Nanofibers	Eugenol	Beef samples	Reduced bacterial growth (TMAB and TPB) and TBA; improved organoleptic and textural properties	*Yilmaz et al*. (2022)
Chitosan Nanofibers	Liquid smoke	Sea bass fillet	Slow and sustained release of liquid smoke; reduction of TPB, YM, and TMAB	*Ceylan et al*. (2017)
Cellulose Nanofibers	Ginger essential oil and stearic acid	Ready-to-cook chicken meat	Reduced growth of lactic acid bacteria, psychrotrophic bacteria, yeast, and molds; lowered peroxide values	*Khaledian et al*. (2019)
PLA Nanofibers	α-Tocopherol and cyclodextrin complex	Beef samples	Improved solubility and preservation of α-TC; reduced TBARS values	*Aytac et al*. (2017)
Cellulose Nanofibers	Anthocyanin	Meat and fish	Increased hydration peroxide RSA	*Hazarika et al*. (2023)

4. Conclusion

Nanofiber technology represents a sustainable and multifunctional approach for enhancing the quality and safety of meat systems. By enabling controlled release of natural antimicrobials and antioxidants, nanofibers can effectively reduce microbial growth, delay lipid oxidation, and maintain desirable sensory properties. Their application extends beyond preservation, providing opportunities for functional reformulation, such as phosphate and nitrite replacement, in line with clean-label expectations. Moreover, the utilization of plant-derived proteins for nanofiber production aligns with sustainability goals and circular economy principles. Despite existing challenges related to large-scale production, cost-efficiency, and regulatory frameworks, nanofibers offer significant potential as next-generation solutions that integrate active, intelligent, and eco-friendly functions into meat systems. Their future adoption in industrial practice will contribute to healthier, safer, and more sustainable meat products.

5. Future perspectives

Although nanofiber technologies are highly promising for meat preservation and reformulation, several barriers hinder their large-scale adoption. Key challenges include scaling up electrospinning processes, managing production costs, and ensuring compliance with food safety regulations. Future research should prioritize the development of multifunctional nanofibers with combined antioxidant, antimicrobial, and sensing capabilities, enabling both protection and real-time monitoring of meat quality and safety. Furthermore, the utilization of renewable biopolymers, such as plant-derived proteins and polysaccharides, not only supports sustainability, but also adds value to agricultural by-products, aligning with clean-label and circular economy principles (*Singh et al*., 2023).

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Original Scientific Paper

First report on ovine toxoplasmosis in Bosnia and Herzegovina a case study from Srednja-Bosna Canton

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ABSTRACT

Toxoplasma gondii is a major foodborne zoonotic parasite, with sheep meat identified as a high-risk transmission vehicle to humans. This preliminary study aimed to determine the seroprevalence of *T. gondii* in sheep intended for human consumption in Bosnia and Herzegovina. Liver samples from 250 sheep were collected at slaughterhouse located in Canton Sarajevo and tested for anti-*T. gondii* IgG antibodies using an indirect ELISA on meat juice. An exceptionally high seroprevalence of 98.8% was detected, one of the highest rates reported globally. The near-universal exposure suggests widespread environmental contamination and intense infection pressure in the studied region. Given the strong epidemiological link between undercooked mutton and human toxoplasmosis, these findings indicate a significant public health risk. Effective mitigation should focus on consumer education about safe meat preparation and the adoption of integrated One Health strategies to reduce transmission at the animal-human interface.

1. Introduction

Toxoplasma gondii is a globally distributed zoonotic protozoan parasite (*Buxton*, 1990) whose transmission pathways, life cycle, and persistence in the external environment render it highly relevant in the context of food safety (*Belluco et al.*, 2016). Toxoplasmosis is a significant public health concern and is classified among the most important foodborne diseases, with *T. gondii* ranked as the fourth most important foodborne parasite worldwide and the second in Europe. Human infection is primarily acquired through oral ingestion of infective stages: oocysts from contaminated sources, such as water, soil, or unwashed vegetables, or tissue cysts present in raw or undercooked meat from infected animals (*Bouwknegt et al.*, 2018; *FAO/WHO*, 2014). In Europe, it is estimated that 30–63% of human toxoplasmosis cases are attributable to the consumption of undercooked meat (*Thomas et al.*, 2022). Among different meat types, sheep (lamb) meat has been identified as posing a particularly high risk of transmission (*Marin-Garcia et al.,* 2022).

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Sheep are highly susceptible to *T. gondii* infection and frequently develop lifelong latent infections with tissue cyst formation, particularly within muscle (*Condoleo et al*., 2023; *Innes et al.*, 2009). Global serological surveys have revealed a high prevalence of infection in sheep, with a meta-analysis reporting a mean seroprevalence of 33.9% (*Ahaduzzaman & Hasan*, 2022). Considerable variability exists between regions, influenced by climatic and husbandry factors; under favourable conditions (e.g., warm, humid climates with high environmental oocyst contamination), flock-level prevalence can exceed 90% (*Cenci-Goga et al*., 2013). In the Balkans, particularly high values have been documented—for example, a seroprevalence of 84.5% in Serbian sheep, among the highest recorded globally (*Klun et al*., 2006). Multiple epidemiological studies have implicated lamb/mutton consumption as a major risk factor for human toxoplasmosis. Case-control studies in Norway and France demonstrated a significant association between the consumption of undercooked lamb and the disease; therefore, mutton is considered the highest-risk meat type—exceeding pork, poultry, and beef—due to its high tissue cyst burden (*Thomas et al*., 2022). Similarly, a U.S. study found that consumption of "rare" lamb markedly increased infection risk (*Thomas et al*., 2022). Consequently, toxoplasmosis is recognised as a major foodborne zoonosis, with mutton serving as an important transmission vehicle to humans.

In Bosnia and Herzegovina, no published data are available regarding *T. gondii* occurrence in sheep meat. However, international literature consistently demonstrates that sheep are frequently infected throughout their lifespan, and their meat can harbour infectious cysts that pose a public health hazard. In this context, a preliminary investigation was undertaken to detect anti-*Toxoplasma gondii* antibodies in sheep livers collected at the slaughter line from animals intended for human consumption.

2. Materials and methods

2.1 Sampling

Sampling was carried out in slaughterhouse in Canton Sarajevo, with all animals originating from five farms located in Srednja-Bosna Canton. From each farm, 50 liver samples were taken from sheep older than 1 year of age during slaughter in June 2025, while the meat of slaughtered animals, after veterinary inspection, was placed on the market for human consumption. A total of 250 livers from sheep were sampled and immediately delivered to the laboratory and frozen at -20°C. Before the start of the analysis, the livers were thawed in the refrigerator, after which the meat juice was extracted and used for further analysis.

2.2 Laboratory analysis

A total of 250 meat juice samples underwent analysis using a commercial indirect ELISA kit for the detection of anti-*Toxoplasma gondii* IgG antibodies (ID Screen® Toxoplasmosis Indirect Multi-species, Innovative Diagnostics, Grabels, France), following manufacturer-provided guidelines. All meat juice samples were diluted at a 1:2 ratio as recommended by the manufacturer. Optical density (OD) readings were obtained using an ELISA plate reader (Epoch,BioTek, USA) set to 450 nm wavelength with a reference filter at 620 nm. As per the manufacturer's specifications, the sensitivity and specificity of this ELISA are 98% and 95%, respectively.

3. Results and discussion

The present study revealed an extremely high seroprevalence of *Toxoplasma gondii* in sheep from central Bosnia and Herzegovina, with 247 out of 250 animals (98.8%) testing positive for IgG antibodies. This seropositivity is among the highest ever reported for ovine toxoplasmosis. By comparison, a recent global meta-analysis estimated an average seroprevalence of only about 33.9% in sheep worldwide (*Ahaduzzaman & Hasan*, 2022). Even within the Balkans, a region already known for elevated *T. gondii* rates, our findings stand out. For example, a large survey in neighbouring Serbia found 84.5% of sheep seropositive, one of the highest national rates documented to date (*Klun et al*., 2006). Our nearly universal seropositivity exceeds even that benchmark, suggesting that exposure to *T. gondii* in the sampled population was essentially ubiquitous.

Several factors could explain this remarkable prevalence. All sampled animals originated from Srednja-Bosna Canton, an area whose environmental and husbandry conditions likely facilitate intense transmission. Sheep in this region are typically raised in free-range or extensive systems, allowing frequent contact with soil, water, and feed that can be contaminated by oocysts shed from cats (the definitive hosts). In general, flocks managed in open

environments with high cat presence tend to show higher infection rates (*Condoleo et al*., 2023). Climatic conditions also play a role: *T. gondii* oocysts survive best in mild, moist environments, leading to especially high sheep infection levels in certain climates (*Cenci-Goga et al*., 2013). The Bosnian farms in this study could offer a similarly conducive setting, given the nearly 99% exposure rate observed. The fact that only one liver was definitively negative (with two inconclusive) suggests that almost every sheep brought to slaughter had been infected with *T. gondii* at some point in its life. This is consistent with the notion that sheep are highly susceptible to *T. gondii* and often acquire lifelong infections on pasture (*Condoleo et al*., 2023). It also aligns with prior reports that sheep are frequent carriers of *T. gondii* cysts compared to other livestock, like cattle (*Thomas et al*., 2022). The only available study from Bosnia and Herzegovina to date was recently conducted in pigs (*Muftić et al*., 2025), reporting an animal-level seroprevalence of 13.3% and farm-level seropositivity of 44.8%. These findings confirm the occurrence of *T. gondii* infection among domestic livestock in the country.

It is important to acknowledge that our detection method – an ELISA on meat juice – indicates exposure (presence of antibodies) rather than direct confirmation of viable parasites in tissues. However, abundant serological exposure could imply a high likelihood that infective tissue cysts are present in the meat. Previous studies have shown a good correlation between seropositivity and the finding of cysts or DNA in sheep tissues (*Marín-García et al*., 2022). For instance, one survey in France recovered live *T. gondii* from about one-third of seropositive lambs under 1 year old (*Thomas et al*., 2022). Our results provide the first substantive data on ovine toxoplasmosis in Bosnia and Herzegovina, and they suggest that infection is widespread to an extraordinary degree in at least the studied region. This finding closes an information gap for the country and aligns it with observations from other high-prevalence areas. It underscores that Bosnia, like many parts of Europe, harbours a substantial reservoir of *T. gondii* in the sheep population, which has important implications for food safety.

These results carry a clear public health message: consumers in Bosnia and Herzegovina (and by extension, the wider Balkans) should avoid eating undercooked sheep meat. If a large proportion of regional mutton is infected (as our data indicate), it could be a major contributor to the overall burden of disease. Fortunately, the risk can be greatly mitigated by proper food handling. Cooking meat to safe internal temperatures (≥67 °C for whole cuts of lamb, as recommended by health agencies) will reliably inactivate *T. gondii* cysts (*EFSA*, 2007). Freezing meat at sub-zero temperatures for an adequate duration is also known to kill the parasite (*Dubey et al.,* 1990; *Dubey*, 2004). Compared with areas where rare ovine meat is favoured, in countries or communities where mutton is traditionally eaten well-done, like in Bosnia and Herzegovina, the incidence of toxoplasmosis from meat is much lower (*Cenci-Goga et al.*, 2013). In contrast, cultural practices of consuming raw or lightly cooked meats can greatly increase infection risk. Notably, a case-control study in the United States identified rare lamb consumption as one of the most significant risk factors for acute toxoplasmosis (*Thomas et al*., 2022). Similarly, outbreaks and epidemiological investigations in Europe have repeatedly linked undercooked lamb to toxoplasmosis cases in humans (*Thomas et al.*, 2022). In our context, any local customs of tasting raw meat during preparation or dishes involving undercooked lamb (such as certain traditional sausages or liver dishes) would pose a serious hazard and should be discouraged. Vulnerable populations, pregnant women in particular, must be made aware of these risks. Even a single exposure to raw or undercooked infected mutton during pregnancy can lead to severe consequences (congenital toxoplasmosis) (*Rostami et al*., 2020). Prior research has shown that pregnant women who consume raw/undercooked sheep products have a significantly higher likelihood of seroconversion (*Chung & Tirabassi*, 2021). Therefore, our findings should prompt the strengthening of educational campaigns on safe meat preparation and perhaps targeted advice during prenatal care in Bosnia and Herzegovina. Another implication is the need for vigilance in the meat production and inspection chain. Unlike many bacterial or viral pathogens, *T. gondii* cannot be detected by routine meat inspection – the cysts are microscopic and cause no overt changes in the meat's appearance. All the sheep in this study were slaughtered under veterinary supervision and passed routine *ante-mortem* and *post-mortem* checks (since they were destined for human consumption). The fact that virtually all were seropositive underscores that *T. gondii* contamination is invisible to standard meat hygiene protocols. This suggests that additional measures might be warranted if one aims to reduce toxoplasmosis risk. For example, farms could implement control strategies, such as limiting cat access

to sheep feed and water sources, controlling rodent populations (to reduce intermediate hosts that cats prey on), and possibly using vaccines where available. According to official statistics, a total of 64,718 sheep were slaughtered in Bosnia and Herzegovina during 2024, of which 54,511 were lambs and 9,207 were older animals (*BHAS*, 2025). Our livers were collected exclusively from the latter category, which is the primary source of mutton available for human consumption. Given that this group is directly relevant to consumer exposure, the exceptionally high seroprevalence observed in our study underscores the potential public health importance of mutton consumption in the country.

This preliminary investigation has several limitations that should be considered when interpreting the results. First, our sampling was geographically limited to one region and one point in time. While the results strongly indicate a high prevalence in that area, they may not capture the situation in other parts of Bosnia and Herzegovina. It is possible that regions with different climates, farming practices, or degrees of cat exposure could have lower (or higher) *T. gondii* prevalence in small ruminants. Second, as noted, we relied on an indirect ELISA test on meat juice to infer infection status. This method detects antibodies that diffuse from the tissue into fluid during freezing/thawing; it has the advantage of using slaughterhouse samples without requiring blood collection. The single negative result could represent a truly uninfected animal, probably from an atypical management system, or seroconversion that has not occurred yet. It could also theoretically be a false negative (given the kit specificity ~95%, a few false results are expected). Without confirmatory tests (such as PCR or bioassay for parasite presence), we must assume the serology reflects true exposure, but we did not directly prove that each seropositive meat contained viable parasites. In future studies, it would be valuable to complement serology with molecular detection (e.g. PCR on tissue samples) to confirm the presence of *T. gondii* DNA in the meat. Such data could quantify how heavily infected the tissues are (parasite load), not just whether exposure occurred. Additionally, genotyping any detected parasites could reveal which strains circulate in Bosnian sheep – information relevant to understanding virulence and potential human health outcomes.

Despite these limitations, our study provides a crucial first look at *T. gondii* in the meat production chain in Bosnia and Herzegovina. The findings raise important questions and directions for further research. One priority is to investigate risk factors at the farm level: for example, does infection correlate with flock size, presence of cats, feeding practices, or geographical features (altitude, rainfall, etc.)? Identifying key risk factors could help in designing interventions to reduce transmission on farms. Another worthwhile avenue is to assess the public health impact more directly – for instance, by measuring *T. gondii* seroprevalence in the human population of the region and examining if people who frequently consume home-raised mutton have higher infection rates. This could solidify the causal link between the high infection pressure in sheep and actual human toxoplasmosis cases locally.

4. Conclusion

This preliminary study revealed an exceptionally high seroprevalence (98.8%) of *Toxoplasma gondii* IgG antibodies in sheep livers collected (in Srednja Bosna Canton) at slaughter in Bosnia and Herzegovina. This is among the highest prevalence levels reported globally and suggests that environmental contamination and animal exposure to *T. gondii* in the region are extensive. While this suggests extensive exposure of sheep to *T. gondii* in the region, serology alone does not confirm the presence of viable tissue cysts. Future studies using molecular and biological methods are, therefore, essential to determine the occurrence of viable parasites in edible tissues and to evaluate the accuracy of serology as a surveillance tool. Given the well-documented risk of human infection from consuming undercooked mutton, these results indicate a potentially significant public health concern. Additionally, broader epidemiological surveillance across multiple regions and seasons, coupled with human seroprevalence data, is needed to fully understand the transmission dynamics and zoonotic risk. Until such data are available, these findings should be interpreted with caution, while still highlighting the importance of safe meat preparation and the value of a One Health approach to toxoplasmosis prevention.

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Review Paper

Post-harvest intervention of chicken meat effected by myopathy: potential of enzymatic marination in quality improvement

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ABSTRACT

Over the past decade, the incidence of myopathies in broiler chickens, such as wooden breast myopathy (WB), white striping (WS), and spaghetti meat (SM), has significantly increased in intensive poultry production systems worldwide. These conditions negatively affect the sensory, nutritional, and technological quality of meat, resulting in substantial economic losses. In Serbia, although no official prevalence data exist, empirical observations suggest a rising frequency of these defects. One promising approach to improve the quality of affected meat involves marination using natural proteolytic enzymes (bromelain, papain, ficin). This review presents current knowledge on broiler myopathies, their impact on meat quality, and use of exogenous enzymes in meat processing. Additional focus is given to marination parameter optimization, emerging technologies, and strategic recommendations for industry and research.

1. Introduction

Modern poultry production has undergone a remarkable transformation over the past 50 years, driven by advances in genetics, nutrition, and management practices. These changes have enabled broiler chickens to reach market weight in nearly half the time compared to traditional breeds, with a marked increase in breast muscle yield. While these achievements have contributed to meeting the growing global demand for affordable animal protein, they have also led to unintended consequences related to muscle tissue health (*Korver*, 2023).

One of the most concerning outcomes is the rising prevalence of growth-related myopathies affecting the *Pectoralis major* muscle, such as wooden breast myopathy (WB), white striping (WS), and spaghetti meat (SM) (*Giampietro-Ganeco et al.*, 2024). These conditions compromise the organoleptic and functional properties of meat, leading to consumer rejection, reduced processing yields, and significant economic losses across the supply chain. Industry estimates from North America alone suggest that losses due to downgraded chicken meat affected by myopathy may exceed US$1 billion annually.

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From a sustainability perspective, the inability to efficiently utilize affected meat exacerbates food waste and undermines production efficiency. Given the increasing pressure to create circular and resource-efficient food systems, the poultry sector must develop innovative, science-based strategies to mitigate quality defects while preserving product value (*Barbut*, 2024). The integration of biotechnological methods, such as enzymatic marination, can provide a viable and scalable solution. Enzymes offer the potential to improve texture and sensory attributes of downgraded meat, thus enabling its reintegration into high-quality processed products.

2. Prevalence and contributing factors of broiler breast myopathies

The three most prevalent breast myopathies in broilers are WS, WB, and SM. Studies show that WS can affect up to 96% of birds in moderate forms, and WB in more than 10%, especially in birds with high live weights. Histologically, these conditions are characterized by degeneration and necrosis of muscle fibers, infiltration of fat and connective tissue, and inflammatory responses. Globally, the prevalence of these myopathies varies notably depending on region, production system, and genetic lines used. In North America (the USA and Canada), the prevalence of WS is 40–96% and WB 10–25%. In Europe, WS occurs in 20–60% of cases and WB in 5–15%. In Asia (China, Thailand), the prevalence of WS is 20–70%, and WB is lower. In South America and Oceania, it is similar to that in North America (*Che et al*., 2022). For Serbia, there are no published data on WB myopathy.

Factors that influence the development of the myopathies are: Intensive genetic selection for fast growth and breast muscle yield, pushing the physiological limits of muscle development; Metabolic and oxidative stress resulting from rapid muscle growth that outpaces vascularization and repair capacity; Nutritional imbalances that may exacerbate muscle damage; Management practices, including housing conditions and handling during transport and slaughter (*Barbut et al*., 2024). For instance, a landmark study synthesizing data from multiple countries suggested that incidences of WB and WS are negligible or absent in slower-growing breeds or traditional production systems, but can reach epidemic proportions in commercial fast-growing lines bred for maximum breast yield (*Petracci et al*., 2019).

3. Use of exogenous enzymes in meat processing

Exogenous enzymes such as bromelain (from pineapple), papain (from papaya), and ficin (from fig latex) have been widely studied for their ability to improve meat tenderness by hydrolyzing muscle proteins and connective tissue. In addition to the aforementioned enzymes, bacterial enzymes are also used as exoenzymes, namely *Bacillus* proteases (*B. subtilis*) and aspartic proteases (*Aspergillus oryzae*). These five exoenzymes are generally recognized as safe (GRAS) for use in the meat industry (*Baltić et al*., 2021). Optimization is critical to balancing enzymatic tenderization without compromising structural integrity. Literature suggests using bromelain concentrations in marinade between 0.1–0.3%, papain at 0.05–0.2%, and ficin at similar ranges, with marination times from 30 minutes to 2 hours at 4–8°C.

Bromelain and papain are highly water-soluble (*Boonkerd and Wantha*, 2024). Bromelain's stability and proteolytic activity are significantly influenced by both pH and temperature conditions. The optimal pH for bromelain is generally between 3 and 7, with some sources citing a slightly narrower range of 5.5–8.0, or even 6.5–7.5. Beyond this range, its activity declines, although both acidic and basic forms preserve substantial enzymatic activity, down to pH 4.0. Stem bromelain operates within a pH range of 4–10, although its activity decreases above pH 10 (optimal values of 4–8), whereas fruit bromelain functions properly at pH values of 3–8. Its stability is also pH-dependent; it remains stable at pH 5.0 over extended time periods and across all temperatures, likely due to the proximity to the pH of fresh pineapple fruit. However, its activity and specific activity continue to rise up to pH 7.0 (*Varila et al*., 2021). Papain's stability and activity are significantly influenced by both temperature and pH, with optimal conditions varying based on the context. Generally, papain exhibits stability over a broad temperature range, with some studies indicating activity from 10 to 90°C, while others suggest an optimal temperature between 40 and 80°C. However, the optimal temperature for papain activity is often reported at around 60°C, with the enzyme retaining its activity at higher temperatures, but experiencing decreased stability over time. Papain retains activity at higher temperatures, but its stability decreases over time (*Baralola et al*., 2023). For example, at 80°C, the enzyme functions properly, but may rapidly lose its activity within an hour at

90°C. Papain's activity is also highly pH-dependent, with optimal performance often reported between pH 6 and 7, although a range from 3.0 to 9.0 is also cited, depending on the substrate (*Nascomento et al*., 2024). Some studies specify optimal pH ranges around 5.0–5.5, while others report it to be around 7.5 (*Kusumasari et al*., 2024). Papain's stability significantly decreases under strong acidic conditions and extreme pH levels, with better stability at neutral pH values. Immobilized papain can retain more activity at pH 12 than free papain, while pH levels of 5.5–6.5 enhance enzyme activity while suppressing bacterial growth. Overexposure can lead to overly soft texture, requiring strict process control, especially in industrial applications. Ficin is a cysteine protease (occurring in four forms) extracted from the trees *Ficus carica* and *Ficus glabrata*, and it is used in the food industry. In dairy processing, it is used to hydrolyze milk proteins intended for infant and elderly nutrition in order to reduce the incidence of allergic reactions. It is also used in cheese production, particularly when protein content is high, as in camel milk. It is additionally used for meat tenderization. According to recent studies, it is frequently used in human medicine as an oral antiseptic for chronic inflammations of the oral cavity and for the prevention of dental caries. It is also used as a cosmetic agent for skin diseases. It has antitumor activity. The optimal pH for the proteolytic activity of ficin is between 5 and 8, and the optimal temperature is 37-55°C (*Aider*, 2021).

4. Optimization of marination parameters

In chicken breasts affected by myopathies, enzyme-assisted marination can significantly reduce meat toughness, improve water retention, and enhance sensory properties such as succulence and palatability. For instance, *Tasoniero et al*. (2016) observed a significant reduction in Warner-Bratzler shear force from 22.8 N to 17.2 N after bromelain treatment, with parallel improvements in color and juiciness. Enzymatic marination also led to reduced cooking loss and improved consumer acceptability of WB-affected meat. Proteolytic enzymes belong to the class of hydrolases and can be classified in a variety of ways. Considering their chemical mechanism of catalysis, proteolytic enzymes are divided into serine proteinases, aspartic proteinases, threonine peptidases, metalloproteinases, and cysteine proteinases—the latter category includes bromelain, ficin, and papain (*Obaha and Novinec*, 2023). Furthermore, proteolytic enzymes are categorized by their mode of action into exopeptidases and endopeptidases (proteinases). Combining these two categories, an enzyme can be, for example, a serine endopeptidase or a serine exopeptidase, etc. (*Kieliszek et al*., 2021). Bromelain, ficin, and papain are cysteine endopeptidases. More specifically, bromelain cleaves peptide bonds between aromatic, basic, and hydrophobic residues (*Kumar et al*., 2023), ficin cleaves bonds in proteins like casein or gelatin, and papain has a broad specificity (*Vatic et al., 2020*). Lately, applying exogenous enzymes from plant sources, such as papain, bromelain, and ficin, has been suggested for meat cuts, through the methods of injection, marination, or infusion in order to reduce meat hardness (*Maqsood et al*., 2018). Bromelain and papain are cysteine proteases with a broad, often indiscriminate, proteolytic activity and can degrade both myofibrillar and connective tissue proteins (*Fernández-Lucas et al*. 2017). Furthermore, the microstructural changes that result from exposure to these proteases can also result in the release of cell content/endogenous enzymes, which would likely enhance proteolysis (*Barekat & Soltanizadeh*, 2017). Thus, the observed changes in myofibrillar spacing and increased fragmentation are probably associated with decreased muscle tenderness and improved texture quality. Valorizing WB myopathy with enzyme-based interventions can enhance texture and yield, reduce food waste, and create new product lines such as marinated fillets, shredded poultry, or nuggets. Studies have reported up to 20% improvements in water-holding capacity and consumer acceptance. From an economic perspective, converting downgraded meat into high-value products supports profitability and sustainability goals.

Future research is needed on enzyme formulations and delivery systems (e.g., encapsulation). Long-term strategies include genetic selection for robustness and pre-harvest interventions to reduce myopathy incidence.

5. Conclusion

Myopathies in broiler chickens challenge meat quality and economic viability. Enzymatic marination offers a practical and science-based solution for valorizing affected meat. Future directions include optimizing process conditions, developing safety protocols, and scaling up industrial trials. National classification of myopathic meat could aid regulatory clarity and support quality-based processing

incentives. Together, these efforts can support sustainable poultry production in Serbia and beyond. Novel enzyme formulations and delivery means should be investigated. Genetic selection of poultry for robustness and pre-harvest interventions could lower myopathy incidence.

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Original Scientific Paper

Performance evaluation of the LC-MS/MS method for determination of chlorates in plant-origin foodstuffs

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ABSTRACT

Chlorate (ClO_3^-) is a monovalent inorganic anion obtained by deprotonation of chloric acid. Its widespread historic use (as an herbicide) and numerous present applications result in notable findings of chlorate residues in food commodities. The aim of this paper is to present validation data confirming the fitness for purpose of the modified "QuPPe" method for LC-MS/MS determination of chlorate in plant-origin foodstuffs with high water content. The validation study was conducted according to SANTE 11312/2021 in the form of a full initial validation on three matrices representative of the leafy vegetable group.

The results of the key validation parameters demonstrate the fitness for purpose of the proposed method. Recovery values (96.5-105.3%), repeatability (4.1-16.2%), intermediate precision (3.9-16.4%) and expanded measurement uncertainty (12.6-32.1%) are in compliance with the criteria laid down in the SANTE document. Use of a graphitized carbon column for the separation of chlorate introduces the necessary step of frequent preconditioning with planar compounds e.g., chlorophyll. However, this process does not result in significant delays or extended run times if carried out regularly.

1. Introduction

Chlorate (ClO_3^-) is a monovalent inorganic anion obtained by deprotonation of chloric acid. Due to its strong oxidizing properties, it was used as a herbicide for many decades, mainly in the form of its sodium salt, $NaClO_3$ (*EFSA*, 2015). In 2008, it was banned as a pesticide in the EU according to Commission Decision No 2008/865/EC (*European Commission*, 2008).

However, usage of this anion goes beyond the scope of plant protection products since chlorate is a common disinfectant of drinking water and of water that is used for production and washing of plant-origin foodstuffs. Chlorate is also extensively used in other applications, e.g., production of chlorine-based biocides (active chlorine, chlorine dioxide, sodium hypochlorite, calcium hypochlorite) and the bleaching of paper. It should be noted that use of chlorate in herbicide formulations outside the EU is still relevant.

As a result of this cumulative process, findings of chlorate residues in food are common, and since the ban of plant protection products containing chlorate was carried out without setting a maximum

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residue level (MRL) in foodstuffs under Regulation (EC) No 396/2005 (*European Commission*, 2005), the default MRL of 0.01 mg/kg is applied. The World Health Organization (WHO) has established a guideline level of 0.7 mg/L for chlorate in drinking water (*EFSA*, 2015), and in the EU, a regulatory level of 0.25 mg/L in drinking water is set by Directive (EU) 2020/2184 (*European Commission*, 2020b). Having in mind the MRL of 0.01 mg/kg in food, the majority of tested foodstuffs would be deemed non-compliant with the legal limits. Positive samples were reported in significant numbers since 2014, according to the EFSA Scientific Opinion, and multiple studies were conducted in order to establish an MRL for chlorate residues in foodstuffs. Therefore, the EU has adopted Commission Regulation 2020/749 (*European Commission*, 2020a) amending Annex III to Regulation (EC) No 396/2005 (*European Commission*, 2005), which set the MRL for chlorate in or on some products.

Besides acute toxicity, potential chronic health risks associated with the presence of chlorate anion in food and drinking water are related to the thyroid gland and (in the case of chronic exposure) can cause bone marrow hyperplasia (*EFSA*, 2015). Chlorate anion is chemically similar to perchlorate anion, exhibiting thyrotoxic activity due to its strong oxidizing properties (*EFSA*, 2015, *CVUAS*, 2014). The presence of this substance in the body can also cause damage to red blood cells (methemoglobinaemia and haemolysis) (*Steffen and Wetzel*, 1993).

Having in mind the ubiquitous and often inevitable presence of chlorates in the food chain, as well as their harmful effects, it is necessary to develop reliable and accurate analytical methods for their quantitative determination in various food commodities. Analytical methodology for measurement of chlorates is historically based on spectrophotometric/colorimetric techniques. Together with ion chromatography with conductometric detection, these methods are suitable for "clean" samples (e.g., water), while for complex matrices, such as plant-origin food, liquid chromatography coupled with mass spectrometric detection (LC-MS/MS) is a technique of choice for sensitive and accurate determination of chlorate anion (*EFSA*, 2015).

The European Reference Laboratory for Single Pesticide Residue Methods (EURL-SRM), have developed and proposed the modification of their "QuPPe" method (Quick method for the Analysis of Residues of Highly Polar Pesticides), extending it to analysis of (among other substances) chlorate (*Anastassiades et al*, 2021). For the less complex matrices, the method is based on extraction and dilution steps only (in acidic methanol-water mixture). Another specific issue in chromatographic analysis of chlorate is that low mass anions cannot be separated on traditional reverse-phase phenyl LC columns used for the majority of other pesticides. In the case of chlorate, the method recommends porous graphitized carbon columns or hydrophilic interaction liquid chromatography (HILIC).

The aim of this paper is to present the validation data confirming the fitness for purpose of the modified QuPPe method for LC-MS/MS determination of chlorate in plant-origin foodstuffs with high water content. This type of foodstuff was selected because chlorate findings are frequent in prepacked and pre-washed lettuces, endives and mixed salads, since water used for washing can be previously treated with hypochlorite. Validation was performed according to the Sante protocol "Analytical quality control and method validation procedures for pesticide residues analysis in food and feed" (*SANTE 11312/2021*, 2021).

2. Materials and methods

Standard solution (1000 mg/L) of chlorate was purchased from Sigma (St. Louis, USA). Water and methanol (HPLC grade) were purchased from Honeywell (Charlotte, USA). Formic acid (≥98.0 %) was supplied by Merck (Darmstadt, Germany). The LC-MS/MS system consisted of Shimadzu (Kyoto, Japan) components: two LC-40D xs UHPLC pumps connected in binary gradient mode, DGU-405 degassing unit, SIL-40C xs autosampler, CTO-40C column oven, SCL-40 system controller and LCMS 8045 triple-quadrupole mass spectrometer. A LC column Thermo Scientific (Waltham, USA) Hypercarb™, 100 × 2.1 mm, 5µm particle size was used for separation of chlorates.

The QuPPe method (*Anastassiades et al*., 2021) provides detailed description of sample preparation. An aliquot of 10 g of a previously homogenized sample (lettuce, endive, mix salad) was extracted with 1% formic acid in methanol in 50 mL polypropylene centrifuge tube. After vigorous shaking, tubes were centrifuged for five minutes at 3500 g. This extract was filtered through 0.2 µm PTFE syringe filter directly into the HPLC vial, and 5 µL of extract was injected into LC-MS/MS system.

Mobile phases consisted of water containing 1% acetic acid and 5% methanol (Solvent A) and

1% acetic acid in methanol (Solvent B). Flow rate was 0.35 mL/min, and the gradient elution program was as follows: 0.0 min: 10 % A; 0.0–2.5 min, linear gradient up to 100 % A, hold for 3 min and back to initial conditions. Total run time was 8 minutes. The mass spectrometer was set to negative electrospray ionisation and operated in MRM mode. Transitions for chlorate were 83>67 and 85>69 with collision energies of -25 and -28 V respectively.

Validation was performed on three matrices (lettuce, endive and a mix of lettuce, rucola and baby spinach). According to the *SANTE 11312/2021* (2021) protocol, the following sample scheme was used per matrix: calibration standards (corresponding to chlorate concentrations of 0.025, 0.050, 0.5 and 1 mg/kg); reagent blank; blank sample; five spiked samples at 0.05 mg/kg; five spiked samples at 0.5 mg/kg; calibration standards. The chosen concentrations are below the MRL for chlorate in Commission Regulation (EU) 2020/749 (*European Commission*, 2020a) which is set to 0.7 mg/kg for the whole group (leaf vegetables, herbs and edible flowers).

3. Results and discussion

The results shown in Table 1 indicate that all key investigated parameters were in compliance with the requirements of *SANTE/11312/2021* (2021) (Recovery 70–120 %; RSD_{rep} < 20 %; RSD_{ip} <20%). The interpretation of the raw data showed that other parameters show satisfactory performance as well. The specificity of the method was proven by the absence of interferences at the retention times of interest in blank samples. The ion ratio was consistent and in all samples, was within the acceptable tolerances (± 30% (relative) of average of calibration standards from same sequence). The obtained results were in accordance with findings from other authors (*Huertas-Perez et al*, 2022, *Constantinou et al*, 2019).

Retention times were also consistently within specified times (± 0.1 min), providing suitable conditioning of the chromatographic columns had preceded the run. In general, porous graphitized carbon columns, such as Hypercarb™, have to be prepared (primed) in a specific way prior to analysis in order to enhance the chromatographic behaviour of the

Table 1. Validation data obtained for the investigated matrices according to the most significant criteria laid down in *SANTE 11312* (2021).

Matrix	Fortification level (g/kg)	Trueness (%)	Repeatability (RSD_{rep} %)	Intermediate precision (RSD_{ip} %)	Expanded uncertainty (%)
Lettuce	0.05	105.3	4.1	8.3	20.7
	0.5	110.0	16.2	14.9	32.1
Endive	0.05	100.6	11.6	13.8	28.6
	0.5	96.5	13.7	16.4	27.1
Mixed salad	0.05	101.6	6.1	3.9	12.6
	0.5	97.3	12.5	9.7	19.3

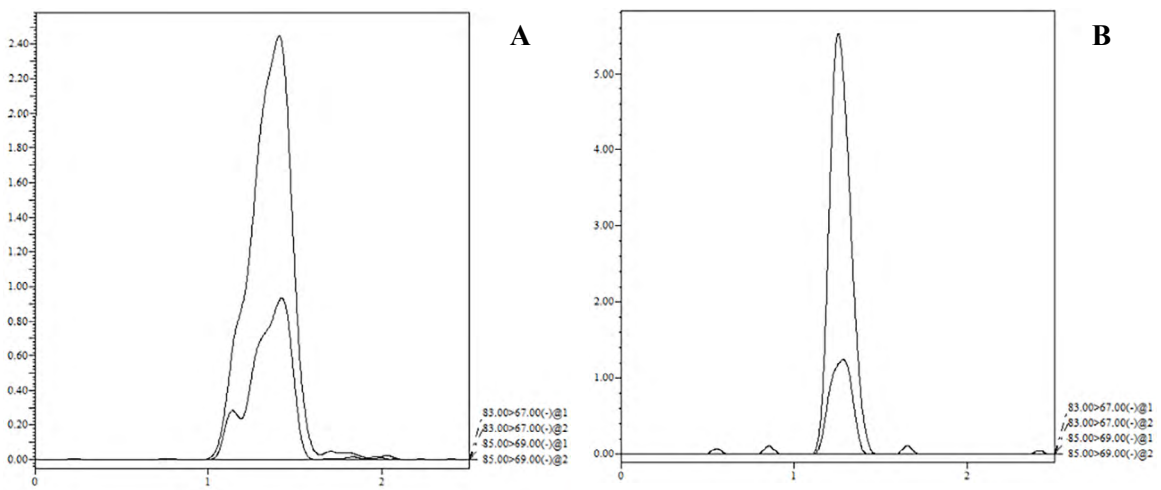

Figure 1. Peak shape before (A) and after (B) priming with 50 injections of spinach extract

column. The purpose of priming is to mask the active sites on the carbon with planar molecules, such as chlorophyll (*Anastassiades et al*., 2017). This is accomplished by injecting 50-100 extracts of spinach blank (with no chlorate) upon receiving a new column, and least 5 injections before and after each test batch. Spinach extracts are prepared according to the QuPPe method. Without this step, Hypercarb™ columns will exhibit shifted retention times and uneven peak shapes. Figure 1 illustrates the chlorate peak before and after treatment of the column. This applies to all porous graphitized carbon columns.

4. Conclusion

On the basis of the obtained results, it can be concluded that the QuPPe method provides an efficient means for the monitoring and control of chlorate levels in plant-origin leafy foods at the levels laid down in the relevant EU regulation. Additional steps are needed to ensure retention time consistency and peak shape when using graphitized carbon LC columns; however, this has no significant effect on overall analysis time if performed regularly.

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Original Scientific Paper

Assessment of Mercury intake through fish consumption among different population groups in Serbia

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ABSTRACT

Fish are the major sources of healthful long-chain omega-3 fats and are also rich in other nutrients. On the other hand, fish contain many harmful substances which can be transferred through the food chain with undesirable effects on human health. Mercury is one of the toxic elements that does not have an essential biological function but can cause harmful effects on the nervous system, primarily in children. Having in mind the toxicity of mercury and the fact that fish is the main source of mercury in human diets, the aim of this study was to assess mercury intake by fish consumption in different Serbian population groups. In the period 2023-2024, 723 samples of different fish types were analysed by inductively coupled plasma mass spectrometry (ICP MS). The highest concentration of mercury was found in tuna – maximum 0.969 mg kg^{-1}, mean 0.274 mg kg^{-1}. The fish with the lowest mercury content was trout, with a mean of 0.009 mg kg^{-1}. The obtained results were used to calculate the weekly intake of mercury/methylmercury in three population groups in Serbia – toddlers, adults and pregnant women. The intake of methylmercury in the adult population and in pregnant women was almost the same – 0.067 and 0.065 µg kg^{-1} body weight per week, respectively, while it was twice as high in toddlers. Generally, mercury intake through fish consumption in Serbia is very low, so far from tolerable weekly intakes, and this gives an opportunity to increase the share of fish in the Serbian diet in order to achieve positive effects from fish consumption.

1. Introduction

Fish is a very important food in the human diet because it is rich in essential nutrients, primarily omega-3 fatty acids, vitamins and minerals, which have a beneficial effect on the cardiovascular system, brain, vision and immune system. However, considering the pollution of aquatic ecosystems, fish also contain toxic substances, such as persistent organohalogen compounds and heavy metals. Among the heavy metals, the presence of mercury (Hg) is mainly associated with fish.

Mercury is found in nature as elemental, and in inorganic and organic compounds. Mercury and mercury compounds were used in the production of plastic materials and caustic soda, in thermometers and electrical switches, and as fungicides, and today, Hg is still used in dental amalgam. Of the organic compounds in the environment, methylmercury (MeHg) is the most common and most toxic (*ATSDR,*

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1999). The International Organization for Research on Cancer (IARC) classifies MeHg in group 2B, i.e., as a possible human carcinogen, while elemental Hg and inorganic Hg compounds are not classified as carcinogens (*IARC*, 1993). Methylation of Hg is the result of microbial activity and is of primary importance for the circulation of Hg in the environment and for Hg toxicity, because methylation results in a stable liposoluble compound with good resorptive properties that passes through the blood-brain and placental barriers (*Ersoy and Çelik*, 2010; *Merritt and Amirbahman*, 2009; *Saei-Dehkordi et al.*, 2010). Thanks to accumulation in aquatic organisms and their predators, MeHg levels in these animals reach several times higher than those in water.

The dominant intake of Hg in humans is through food, and over 90% of ingested Hg comes from fish and fish products. Methylmercury is very well resorbed from the digestive tract (90%), while the resorption of inorganic Hg compounds is very low, only about 8%. The half-life of Hg in humans is 70 days, and it is excreted in saliva, urine, faeces and milk. The toxic effect of Hg is caused by it binding to sulfhydryl and other groups of proteins and by changing their structure and function (*ATSDR*, 1999). Much information about the toxicity of MeHg was obtained after the accidents in Iraq (*Bakir et al.*, 1973) and the Japanese cities of Minamata and Niigata (*Irukayama et al.*, 1977). Inorganic compounds of Hg and elemental Hg have low toxicity due to poor resorption. The exceptions are $HgCl_2$ and Hg vapor, which can be absorbed through the lungs and enter the bloodstream directly. Unlike Hg^{2+}, which is predominantly nephrotoxic and accumulates in the kidneys, organic Hg is neurotoxic. Methylmercury damages neurons and leads to diffuse encephalopathy causing sensory, motor and cognitive disorders. Given that Hg passes through the placenta, a teratogenic effect can occur. Genotoxicity in the form of chromosomal aberrations caused by the binding of Hg to sulfhydryl groups on RNA and DNA has also been proven (*ATSDR*, 1999; *WHO*, 1990). It is important to note that MeHg can cause harmful effects on the nervous system, primarily in children, even at relatively low levels of exposure (*Davidson et al.*, 1995; *Grandjean et al.*, 1997).

According to the available data, the intake of fish in Serbia is relatively small. Carp, trout, hake, mackerel, sardine, sprat and tuna are the most commonly consumed fish species. The aim of this work was to evaluate the intake of Hg through consumed fish in different population groups of Serbia (toddlers, adults, pregnant women), based on the examination of the content of Hg in fish and the estimated intake of fish.

2. Materials and methods

A total of 723 samples of fish and fish products from the Serbian market were tested in the period 2023-2024 for Hg concentration. The following types of fish were tested: hake (n= 253), mackerel (n= 88), sprat (n= 28), tuna (n= 34), salmon (n= 72), sea bass (n= 30), sea bream (n= 33), carp (n= 26), trout (n= 48), as well as canned sardine (n= 19) and canned tuna (n= 92).

After homogenization, the samples were prepared by microwave digestion using nitric acid (67% Trace Metal Grade, Fisher Scientific, Bishop, UK). The determination of the ^{202}Hg isotope was performed using inductively coupled plasma mass spectrometry (ICP-MS) on an iCap Qc instrument (Thermo Scientific, Bremen, Germany), in the kinetic energy discrimination (KED) mode. Basic operating conditions of the instrument were: RF power (1550 W); cooling gas flow (14 L/min); nebulizer flow (1 L/min); collision gas flow (1 mL/min); dwell time (10 ms). Standard stock solution containing 1000 mg/L of Hg was purchased from CPAchem (Bogomilovo, Bulgaria). This solution was used to prepare standards for five-point calibration curves (including zero). A multi-element internal standard (6Li, 45Sc, 71Ga, 89Y and 209B) was used to correct the results due to fluctuations in the system itself. A reference material (NIST 1577c - beef liver, Gaithersburg, Maryland, USA) was used for quality control of the assay.

Descriptive statistics data analysis was performed using Microsoft Office® Excel.

3. Results and discussion

Levels of Hg in different fish and fish products from the Serbian market are shown in Table 1. Results are presented as mean ± standard deviation (sdev).

The Hg content was not exceeded the maximum permitted amounts prescribed by the current Rulebook of the Republic of Serbia (*Serbia*, 2025) in the analysed samples. As expected, considering the fish size, age and diet, the highest concentration of Hg was found in tuna – a maximum of 0.969 μg g^{-1}, mean of 0.274 μg g^{-1}. The fish with the lowest Hg content was trout, with a mean of 0.009 μg g^{-1}.

Table 1. Concentration of Hg in fish and fish products on the market in Serbia

Fish/ fish products	Number of samples	min Hg, μg g^{-1}	max Hg, μg g^{-1}	mean Hg ± sdev, μg g^{-1}
Hake	253	0.002	0.222	0.032 ± 0.027
Mackerel	88	0.009	0.128	0.031 ± 0.020
Sprat	28	0.008	0.049	0.019 ± 0.014
Tuna	34	0.017	0.969	0.274 ± 0.240
Salmon	72	0.001	0.043	0.017 ± 0.009
Sea bass	30	0.001	0.077	0.038 ± 0.015
Sea bream	33	0.014	0.089	0.036 ± 0.020
Carp	26	0.003	0.042	0.011 ± 0.010
Trout	48	0.002	0.023	0.009 ± 0.005
Caned sardines	19	0.005	0.105	0.034 ± 0.028
Canned tuna	92	0.008	0.804	0.077 ± 0.108

The obtained data are comparable to the results available in the professional literature. Some deviations can be explained by variations in the examined fish (species and subspecies), and the different origins and ages of the fish (*Brodziak-Dopierała and Fischer*, 2023; *U.S. Food and Drug Administration*, 2012; *Nina Bilandžić et al., 2017, FRIDA, 2025*.)

Table 2 shows the estimated weekly intake of Hg in three population groups in Serbia based on the obtained concentrations of Hg in fish and fish products and the estimated intake of individual fish (*EFSA Database*, 2024). As Hg in fish is mainly found in the form of MeHg, i.e., up to 100% is in this form (*Norwegian Scientific Committee for Food and Environment*, 2019), we corrected the values obtained for Hg intake by a factor of 1.075 (atomic mass of Hg – 200.56 g mol^{-1}, molecular mass of MeHg – 215.63 g mol^{-1}) and obtained the weekly intake of MeHg. The EFSA Scientific Panel on Contaminants in the Food Chain (CONTAM) has established a tolerable weekly intake (TWI) of 1.3 μg kg^{-1} body weight (bw) for MeHg and 4 μg kg^{-1} bw for inorganic Hg (EFSA, 2012).

Based on the results, it can be concluded that the intake of MeHg in the adult population and in pregnant women is almost the same, while it is twice as high in children. Nevertheless, the calculated hazard index (HI = weekly intake of MeHg/TWI) for the population of children is low, just 0.10, which leads us to the conclusion that there is no unacceptable risk of consuming fish from the aspect of toxic effects of ingested Hg, i.e., the risk is acceptable. The HIs obtained in studies around the world also show a low risk, and its value is primarily determined by the food consumed, i.e., the prevalence of fish in the dietary habits of certain countries. As Serbia belongs to the countries with a low proportion of fish in the diet, it is understandable that the intake of Hg is low, but on the other hand, the benefit from the positive nutritional characteristics of fish as a food is also low.

From the available literature data, it can be concluded that Hg intake is correlated with the share of fish in the diet. Significantly higher amounts of Hg intake were obtained in countries where the diet mostly is based on fish or in locations with a highly polluted environment. Thus, the population of the

Table 2. The estimated weekly intake of Hg/MeHg for three population groups in Serbia

Population group	Mean fish intake, g kg^{-1} bw per day	Hg intake, μg kg^{-1} bw per week	MeHg intake, μg kg^{-1} bw per week
Toddlers	0.564	0.115	0.124
Adults	0.226	0.062	0.067
Pregnant women	0.233	0.061	0.065

bw - body weight

Faroe Islands consumes an average 3.6 μg kg^{-1} bw of Me per week (*Weihe et al.*, 1996), while the estimated intake of Hg in Greenland is as much as 846 μg Hg per week, which for a person weighing 70 kg would represent an intake of 12.1 μg kg^{-1} bw per week or 9.3 times the tolerable intake (*Johansen et al.*, 2000).

A study of Hg intake in Portugal showed that the population aged 17 to 74 years averaged 1.25 μg kg^{-1} bw/week, while the intake of women aged 18 to 45 years was slightly lower – 1.13 μg kg^{-1} bw/week (*Vasco et al.*, 2025). *Lee et al.* (2006) estimated that the weekly intake for a South Korean adult with an average bw of 55 kg is 0.21 μg kg^{-1} bw. The average Hg daily intake of children in the Jinhu area of China was 0.14 μg kg^{-1} bw, which in relation to TWI represents 74% of the recommended safe intake level (*Sun et al.*, 2011). In the adult population of France, the estimated intake of 0.895 μg kg^{-1} bw was lower than the tolerable value (*Leblanc et al.*, 2005), but at the same time, higher than the estimated intake in Serbia in our study. Low values of Hg intake were obtained by *Puklova et al.* (2010) in the Czech Republic. They estimated that the Hg weekly food intake averages only 0.08 μg/kg bw. The authors explain such a low value by the small proportion of fish in the diet of the Czech population. Also, higher values of the estimated intake of Hg in Serbia were obtained in a study in which 20 European countries participated in the period 2004-2011, when levels were, for the adult population 0.24 μg kg^{-1} bw, and for children 0.32 μg kg^{-1} bw (*EFSA*, 2012).

4. Conclusion

This study aimed to evaluate the intake of Hg/MeHg through the consumption of fish and fish products among different population groups in Serbia. Compared to tolerable values, there is no unacceptable risk of harmful effects of Hg ingested through fish. The HI for all three population groups is far below 1, with a maximum of 0.10 for children. There is, therefore, an opportunity to increase the share of fish in the diet in order to achieve positive effects, both nutritional and health, without significantly increasing the risk of the toxic effect of Hg as an undesirable component of fish flesh.

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Original Scientific Paper

Longitudinal microbiological evaluation of hygiene practices in retail food shops

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ABSTRACT

This study presents a comprehensive microbiological assessment of process hygiene in 49 retail food shops over four years (January 2021 to December 2024). A total of 613 swabs from food contact surfaces and 303 swabs from food handlers' hands were analyzed to evaluate compliance with hygiene standards. Results indicated a gradual improvement in hygiene practices, with non-compliant food contact surfaces decreasing from 6.42% in 2022 to 3.30% in 2024, attributed to enhanced training and sanitation protocols. Additionally, 2.97% of food handlers' hands were non-compliant according to swab counts, primarily due to elevated aerobic colony counts, with non-compliance rates of 5.45% in 2021, 7.27% in 2022, and a decrease to 1.94% in 2024. The findings underscore the importance of continuous monitoring and targeted interventions to mitigate microbial contamination risks. Despite progress, challenges remain in maintaining optimal hygiene standards, emphasizing the need for ongoing training and adherence to hygiene protocols within the retail food sector to protect public health and prevent foodborne illnesses. Future research should focus on the long-term efficacy of training programs and sanitation practices to establish best practices for the industry.

1. Introduction

Maintaining rigorous process hygiene in retail food establishments is paramount for safeguarding public health, preventing foodborne illnesses, and ensuring compliance with stringent food safety regulations (*Rani et al*., 2023). This study addresses the critical need for continuous monitoring and evaluation of microbiological parameters on food contact surfaces and food handlers' hands, which are primary vectors for cross-contamination (*Kebede & Getu*, 2023). Such assessments are crucial because inadequate hygiene practices, including improper handwashing and contaminated surfaces, significantly contribute to the microbial load in food preparation environments, thereby elevating the risk of foodborne disease outbreaks (*Josefsen et al.*, 2011; *Fallahizadeh et al*., 2025). Furthermore, understanding the prevalent microbial loads on these surfaces and hands allows for the targeted implementation of effective sanitation protocols and hygiene training programs, which are essential for mitigating food safety risks (*Josefsen et al*., 2011). This comprehensive investigation provides critical insights into the efficacy of current hygiene protocols within

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diverse retail settings, highlighting areas that require enhanced intervention to achieve optimal food safety standards. This research, therefore, aims to systematically evaluate the microbiological status of food contact surfaces and food handlers' hands in retail shops over four years, providing a robust dataset for assessing hygiene compliance and identifying potential areas for improvement. This longitudinal study utilized a systematic sampling approach to gather comprehensive data, which is essential for understanding the ecological persistence of microorganisms in retail environments (*Josefsen et al*., 2011; *Janecko et al*., 2023).

2. Materials and methods

During four years (from January 2021 to December 2024), an assessment of process hygiene was carried out in 49 retail shops. Overall, a total of 613 swabs of food contact surfaces and 303 swabs of food handlers' hands were investigated for microbiological parameters of process hygiene (Table 1).

2.1. Swab samples

Swabs from the food contact surfaces and food handlers' hands were taken after cleaning, washing and disinfection procedures. Sampling was conducted according to the standard method *SRPS EN ISO 18593* (2018). On the sampling day, swabs were transported to the laboratory in a hand-held refrigerator and analyzed within 24 hours. All samples were analyzed in an accredited laboratory according to *SRPS ISO/IEC 17025* (2006).

Table 1. Number of swabs sampled each year

Year	Food contact surfaces	Food handlers' hands
2021	112	55
2022	109	55
2023	180	90
2024	212	103
Total	**613**	**303**

2.2. Microbiological examinations

Swabs from the food contact surfaces and food handlers' hands were tested for aerobic colony count (ACC) according to *SRPS EN ISO 4833-1* (2014) and *Enterobacteriaceae* (ENT) in line with *SRPS EN ISO 21528-2* (2017). Results of the microbiological analyses were expressed as the number of bacteria per cm^2 (CFU/cm^2) and number of bacteria per swab (CFU/swab), for swabs taken from the food contact surfaces and food handlers' hands, respectively.

2.3. Evaluation of microbiological results

Evaluation of the microbiological contamination results was carried out in accordance with the limit values set by the self-control plans of the food business operators (Table 2).

Table 2. Microbiological criteria in the self-control plans of the food business operators

Microorganism group	Porcelain, glass, smooth metal food contact surfaces	Plastic, wood, and stone food contact surfaces	Food handlers' hands
Aerobic colony count	≤ 10 CFU/cm^2	≤ 30 CFU/cm^2	≤ 2000 CFU/swab
Enterobacteriaceae	≤ 1 CFU/cm^2	≤ 1 CFU/cm^2	≤ 10 CFU/swab

3. Results and discussion

Over a comprehensive four-year period, spanning from January 2021 to December 2024, a detailed analysis was conducted across 49 retail shops in Serbia, focusing on the examination of 613 swabs collected from various food contact surfaces. These surfaces included critical items such as cutting boards, slicing machines, knives, refrigerator doors, and metal working surfaces, all of which play a pivotal role in food safety and hygiene practices. In 2021, 112 swabs were examined, followed by 109, 180, and 212 swabs in 2022, 2023, and 2024, respectively, as detailed in Table 1. This progressive increase in the number of swabs examined over the years indicates a growing commitment to monitoring and ensuring food safety standards within these retail environments.

The results of the analysis revealed that 3.59% (22 out of 613) of the food contact surfaces failed to meet the compliance criteria established in the self-control plans mandated for food business operators. Notably, the rate of noncompliance varied across the years: in 2022, the noncompliance rate was 6.42%, which then decreased to 4.44% in 2023, and further dropped to 3.30% in 2024, as illustrated in Table 3. These findings align closely with previous studies conducted by *Legnani et al.* (2004), *Garayoa et al.* (2014), and *Golić et al.* (2019). However, a stark contrast is observed in the research conducted by *Moračanin et al*. (2017), which reported a significantly higher noncompliance rate of 41.96%. The progressive reduction in the percentage of non-compliant swabs from 2022 to 2024 suggests that enhanced training initiatives for employees on the correct implementation of sanitation procedures are crucial for effective good hygiene practices (GHP) and hazard analysis critical control point (HACCP) protocols. This emphasis on training is essential for fostering a culture of food safety and compliance within retail operations.

Throughout the four-year study, the primary factor contributing to the noncompliant results for food contact surfaces was identified as an unacceptable aerobic colony count (ACC). The data showed that in 2022, a staggering 100% of noncompliant swabs were attributed to elevated ACC levels, followed by 75% in 2023 and 85.71% in 2024. This trend underscores the importance of continuous monitoring and the need for stringent sanitation practices to mitigate microbial contamination risks in food handling environments.

During the four years (from January 2021 to December 2024), in the same retail shops, 303 swabs from food handlers' hands were examined. In 2021 and 2022, 55 hand swabs were examined, in 2023, 90, and in 2024, 103 hand swabs (Table 1).

The microbiological examination of these swabs revealed that 2.97% (9 of 303) of the swabs failed to meet the compliance criteria outlined in the self-control plans mandated for food business operators. The data indicated a reasonably consistent level of hygiene among food handlers across the four years, with non-compliance rates reported as follows: 5.45% in 2021, 7.27% in 2022, although a notable decrease to 0% and 1.94% in 2023 and 2024, respectively, as presented in Table 4. A critical observation from the findings was the prevalence of elevated aerobic colony counts, which accounted for 77.78% of the non-compliant results across all years studied. This trend aligns with previous research conducted by *Jovanović et al.* (2021) and *Ivanović et al.* (2013), which reported similar hygiene challenges among food handlers. In contrast, a study by *Rašeta et al.* (2012) indicated a significantly higher rate of non-compliance, with percentages reaching as high as 30.0%, highlighting the

Table 3. Microbiological status of the food contact surfaces

Year	Number of swabs	Noncompliant		Finding	Frequency		Finding	Frequency	
		n	%		n	%		N	%
2021	112	0	/	ACC	/	/	ACC + ENT	/	/
2022	109	7	6.42	ACC	7	100.00	ACC + ENT	/	/
2023	180	8	4.44	ACC	6	75.00	ACC + ENT	2	25.00
2024	212	7	3.30	ACC	6	85.71	ACC + ENT	1	14.29

ACC – aerobic colony count; ENT – *Enterobacteriaceae* count.

Table 4. Microbiological status of the swabs from food handlers' hands

Year	Number of swabs	Noncompliant		Finding	Frequency		Finding	Frequency	
		n	%		N	%		n	%
2021	55	3	5.45	ACC	3	100.00	ACC + ENT	/	/
2022	55	4	7.27	ACC	3	75.00	ACC + ENT	1	25.00
2023	90	0	/	ACC	/	/	ACC + ENT	/	/
2024	103	2	1.94	ACC	1	50.00	ACC + ENT	1	50.00

ACC – aerobic colony count; ENT – *Enterobacteriaceae* count.

ongoing concerns regarding microbial contamination in food handling environments.

These findings emphasize the need for ongoing training and adherence to hygiene protocols among food handlers to minimize the risk of microbial contamination and ensure the safety of food products served to consumers.

4. Conclusion

The comprehensive microbiological assessment conducted over four years in 49 retail food shops reveals significant insights into the hygiene practices related to food contact surfaces and food handlers' hands. The study shows a gradual improvement in compliance with hygiene standards, particularly noted by the reduction in non-compliant food contact surfaces, which decreased from 6.42% in 2022 to 3.30% in 2024. This suggests that enhanced training and implementation of sanitation protocols among food handlers are effective in mitigating microbial contamination risks.

However, the presence of non-compliant results, particularly related to elevated aerobic colony counts, underscores the ongoing challenges faced in maintaining optimal hygiene standards. The findings emphasize the critical importance of continuous monitoring and evaluation of hygiene practices in retail food establishments. Addressing the identified areas of concern through targeted interventions and training programs is essential for further reducing microbial loads and ensuring food safety compliance.

Overall, this study highlights the necessity for sustained efforts in improving hygiene practices within the retail food sector, which is vital for protecting public health and preventing foodborne illnesses. Future research should focus on exploring the long-term impact of training programs and sanitation protocols on hygiene compliance to establish best practices for the industry.

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Original Scientific Paper

Determining the source of contamination with MOSH and MOAH in the production of finely chopped cooked sausages

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ABSTRACT

Testing for the presence of mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH) hydrocarbon fractions was carried out on finished products at a food business entity. On this occasion, the presence of the MOSH fraction beyond the limits of the customer's internal standard (2 mg/kg) was determined in finely chopped cooked sausages at the end of their shelf life; levels were 48.2±9.37 mg/kg in sausage packed in polyamide casing and 8.41±1.64 mg/kg in sausage packed in collagen casing. The meat batter was tested systematically and in a targeted manner, successively in the production steps of the finished product, showing that aromatic hydrocarbon levels were: after passing through the crusher (0.92±0.18 mg/kg), microcutter (1.63±0.32 mg/kg), cutter (1.14±0.22), and filler (1.19±0.23 mg/kg) machines. The raw materials used in the product were also tested (1.00±0.19 mg/kg), as were the casings (28408.82±5525.51 mg/kg polyamide casing and 25947.15±5046.72 mg/kg collagen casing) into which the final product was filled. The obtained test results determined the casings were the source of contamination.

1. Introduction

Over the past twenty years, the presence of mineral oil hydrocarbons (MOH) in foods has become a growing concern due to potential health risks. These oils are commonly used throughout the food supply chain—during harvesting, production, storage, and transportation—and are sometimes included as food additives (*Grob,* 2018). These compounds were suggested as important contaminants of the human body, with possible routes of contamination including air inhalation, food intake, and dermal absorption (*Lachenmeier et al*, 2017). MOH are a wide range of products deriving from petroleum distillation fractions (*Purcaro et. al.*, 2024), containing intricate mixtures of isomers and which are classified into two main categories: mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH). Edible oils typically contain higher concentrations of MOH compared to most other food products (*Brühl*, 2022). Mineral oils (such as paraffinum liquidum or white oil), which consist of MOSH and MOAH, are widely applied in various consumer products such as medicines and cosmetics (*Weber et. al.,* 2018).

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Current evidence indicates that contamination can occur at various points throughout the entire production process. MOH are commonly utilized in food contact materials and food additives. They consist of complex mixtures, such as straight and branched open-chain alkanes (paraffins), primarily alkylated cycloalkanes (naphthenes), and can be generally categorized as MOSH and MOAH (*Biedermann and Grob, 2009*).

Up to now, there has been no comprehensive study on MOH risk assessment for the Serbian population. Globally, there is a number of toxicological and risk assessment studies on MOHs (*JECFA*, 2002). Animal research indicates that MOSH can cause deposits and inflammatory responses in the liver of specific rat strains. In 2012, the European Food Safety Authority (EFSA) identified MOAH as containing carcinogenic compounds that may accumulate in human tissues and potentially lead to the formation of microgranulomas (*EFSA*, 2012). In accordance with Codex Alimentarius Committee on Contaminants in Foods—Foresight on emerging issues in food and feed safety relevant to contaminants (*Codex Alimentarius*, 2024), MOAH with three or more aromatic rings are associated with genotoxicity and carcinogenicity, and a 1 mg/kg limit has been set for foods with a high fat/oil content (> 4% fat/oil to ≤50% fat/oil). Detecting MOH in food presents significant challenges and demands a thorough, multifaceted analytical approach to address the complex issues involved (*Sdrigotti et. al*., 2021).

2. Materials and methods

Sample testing was performed in accordance with the EU standard (*European Commission*, 2017). This European Standard outlines a highly efficient method for determining saturated and aromatic hydrocarbons (from C10 to C50) in vegetable fats, oils, and food products based on vegetable oils (it is not intended for other matrices), validated through laboratory testing. The gold standard method for MOSH-MOAH analysis (*Weber et. al.*, 2018) utilizes online-coupled high performance liquid chromatography-gas chromatography-flame ionization detection (HPLC-GC-FID) analysis. It can also be employed to examine MOSH and MOAH. The method has been evaluated in an laboratory study involving both naturally contaminated and spiked samples of vegetable oils, mayonnaise, and margarine, with MOSH concentrations ranging from 4 mg/kg to 197 mg/kg, and MOAH from 2 mg/kg to 51 mg/kg. Based on the study results, the method is suitable for detecting MOSH and MOAH concentrations above 10 mg/kg. If natural sources interfere, the mineral origin of MOSH and MOAH can be confirmed by examining the pattern using GC-MS.

Compositional information on MOH is primarily from gas chromatography (GC). GC of MOSH results in a pattern of unresolved peaks of unidentified components (even at the highest separation efficiency available) with n-alkanes and some predominant iso- and cycloalkanes on top. GC of MOAH results in a pattern of unresolved peaks with hardly any distinct peak on top. (*EFSA*, 2012).

3. Results

After receiving unsatisfactory test results for the presence of MOSH and MOAH in ready-to-eat (RTE) finely chopped cooked sausages, our laboratory instigated an investigation at the customer's request.

It is noticeable from Figure 1 that the limit of 2 mg/kg, i.e., the customer's internal standard for MOSH, was exceeded several times in the examined sausages. The most commonly occurring fractions were C20-C35 hydrocarbons.

Tests of the meat batter from different production stages showed that all sausage samples examined contained ΣMOSH/MOAH at levels below the permitted limit (Figure 2). However, the results of testing the content of MOSH/MOAH showed that the sum of these hydrocarbons in the casings significantly exceeded the limit of 2 mg/kg. The most abundant fractions were C16-C35. Indeed, the industrial casings harboured extremely high levels of contamination (polyamide casing 28408.82±5525.51 mg/kg and collagen casing 25947.15±5046 mg/kg). After filling the product batter into casing, during the sausage shelf life, there was an increase in the concentration of MOSH in the finely chopped cooked sausages, to 48.2+9.37 mg/kg (for sausages packed in polyamide casing) and to 8.41+1.64 mg/kg (for those packed in collagen casing). The obvious source of the MOSH in the meat product was due to migration of the compounds from the contaminated casing.

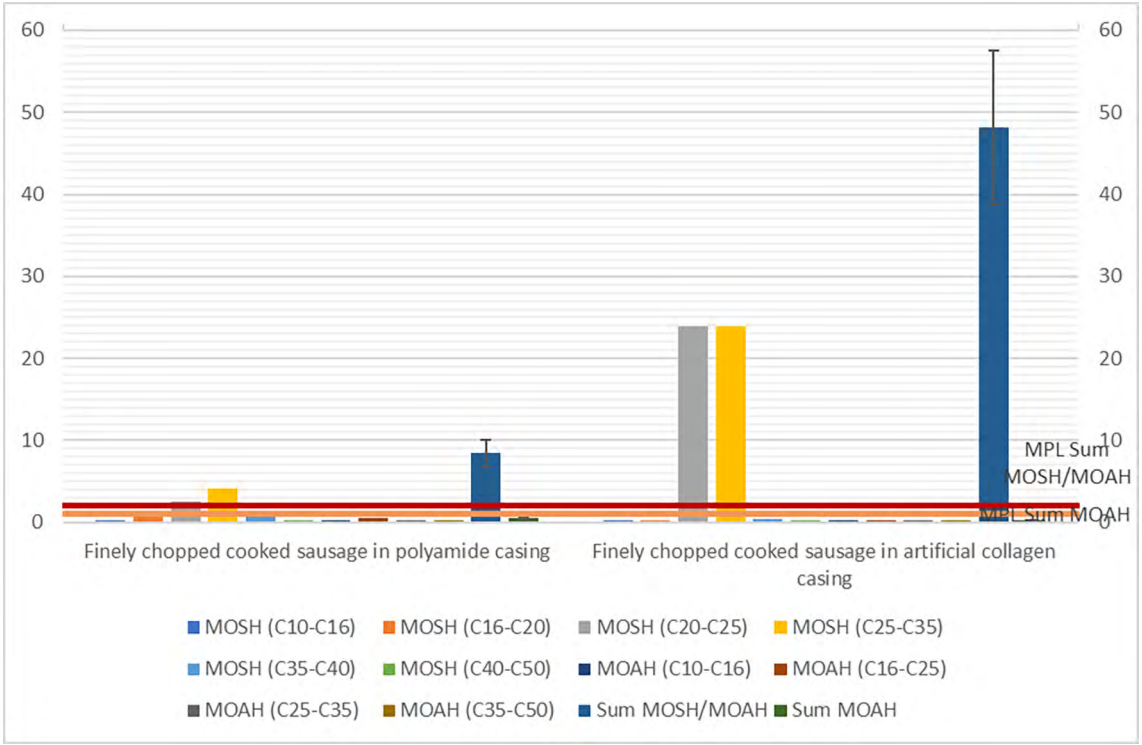

Figure 1. MOSH and MOAH measured in finely chopped cooked sausages packed in polyamide and in artificial collagen casings

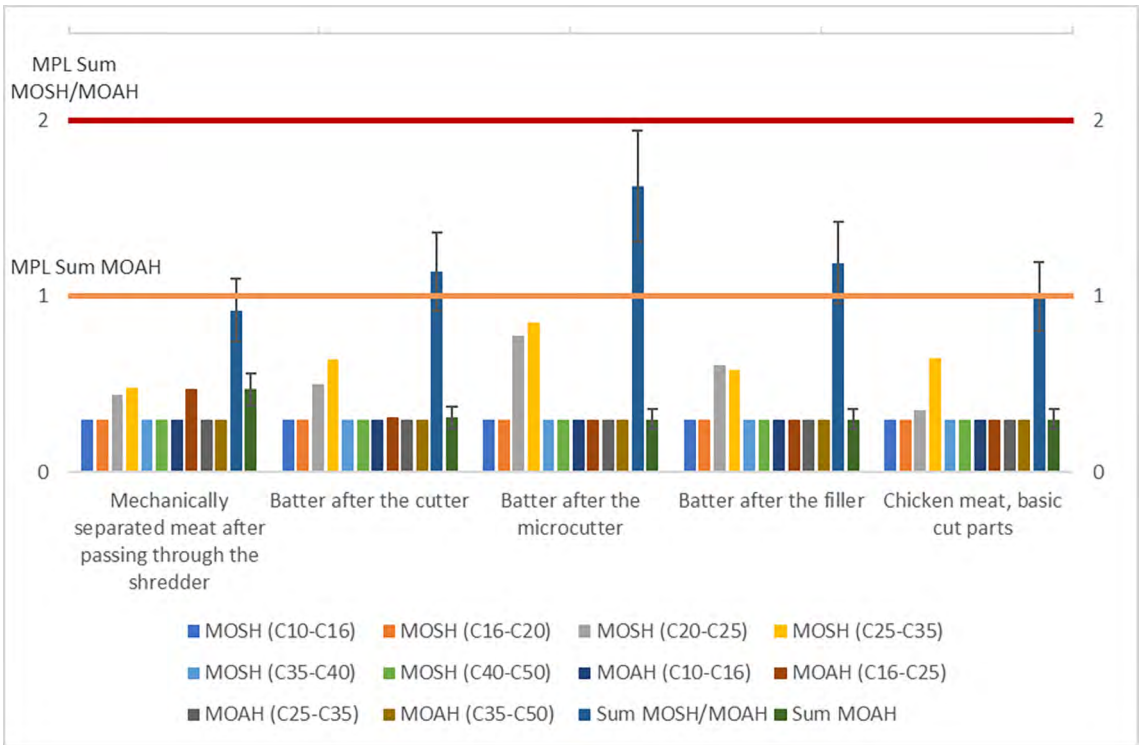

Figure 2. MOSH and MOAH measured at different production stages in the meat batter for the finely chopped cooked sausages

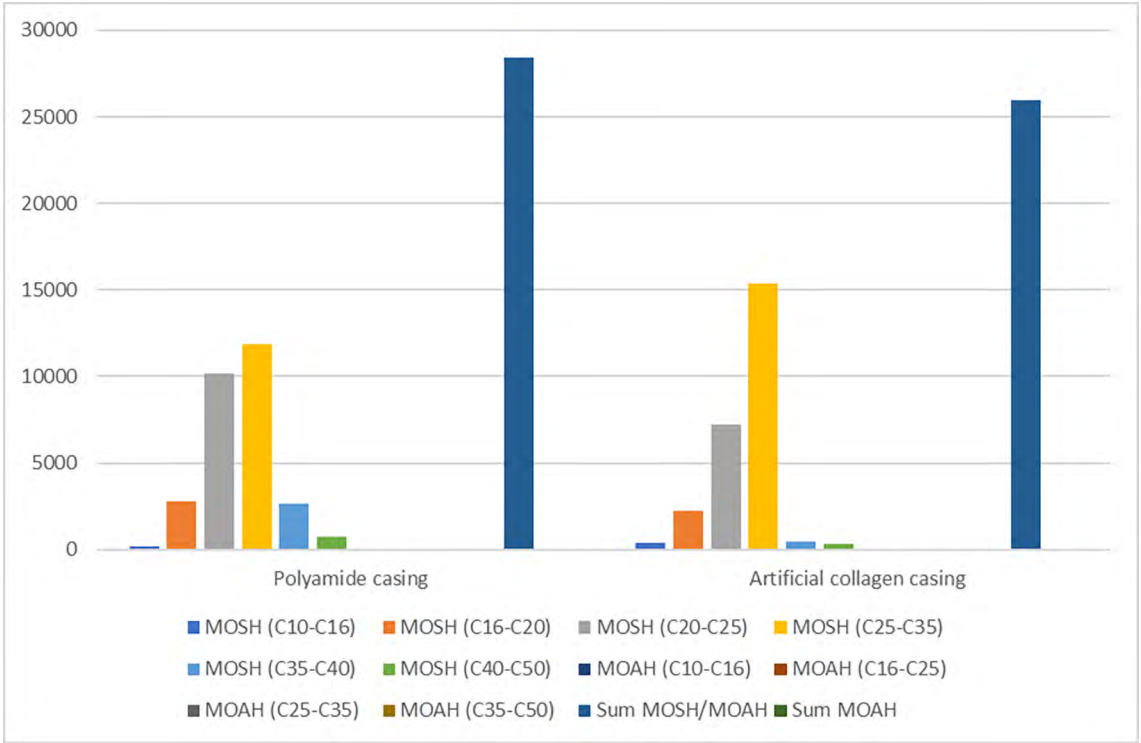

Figure 3. MOSH and MOAH measured in sausage casings (polyamide and artificial collagen)

4. Discussion

MOH are environmental and processing contaminants composed of a complex mixture of liposoluble compounds of petrogenic origin, containing between 10 and 50 carbon atoms (*EFSA*, 2023). Consumers are exposed to a range of MOH through food intake. According to EFSA's Scientific Opinion, MOAH may be carcinogenic and mutagenic (*EFSA*, 2012). Across the different age classes, the three food groups with the highest average contribution to the mean dietary exposure to MOSH were: grains and grain-based products; milk and dairy products and; animal and vegetable fats and oils and primary derivatives thereof (*EFSA*, 2023). Major sources of MOH in food are food packaging and additives, processing aids, and lubricants (*EFSA*, 2012). Another study found the highest contamination levels, measured in two beef rib samples, probably occurred during slaughter or handling prior to commercialization (*Albendea and Purcaro*, 2025).

There have been, and still are, discussions about the reliability of the results, particularly for measurements at low concentrations. The LC-GC-FID method can be considered as standard and reliable, validated by collaborative tests. Nonetheless, sometimes there large differences in results from different laboratories. There are several potential reasons for this: blank and cross-contamination due to the ubiquitous presence of MOH; interference removal is a critical step at the lower limit of quantitation and; chromatogram interpretation needs experience. Reduction of uncertainty and analyst interpretation is fundamental to guarantee more reliable results (*Purcaro*, 2024)

Nuclear magnetic resonance (NMR) spectroscopy has recently been suggested for MOSH-MOAH analysis, especially with the possibility of detecting only the toxicologically relevant aromatic rings (*Weber et. al*., 2018). Moreover, carcinogenic, genotoxic, and mutagenic effects have been associated with compounds with three to seven aromatic rings (*EFSA*, 2012, 2023). For this subfraction, a surrogate reference point of 0.49 mg/kg body weight (b.w.) per day, calculated from data on eight polycyclic aromatic hydrocarbons, was considered (*EFSA*, 2023). Some of the compounds, such as benzene hydrocarbons, may be of potential health hazard, which specifically includes carcinogenic effects (*Lachenmeier et al*., 2017).

In cosmetics, MOSH and MOAH occurred in 27 authentic samples, with contents in the range of 90-109 mg/kg and 0.02-1.10 mg/kg, respectively (*Lachenmeier et. al*., 2017).

In a survey in China, levels of MOAH and MOSH in food for infants and young children

were in the range of 0.50-1.34 mg/kg for MOAH, and 1.00-6.04 mg/kg for MOSH (*Zhu et al.*, 2019). MOSH from C16 to C35 may accumulate and cause microgranulomas in several tissues including lymph nodes, spleen and liver (*EFSA*, 2012). A mixture of highly branched alkanes and alkylated cycloalkanes was found to accumulate in different human tissues as liver or adipose tissue (*Isola et. al*., 2023).

The highest dietary exposure to MOSH was estimated for the young population, with lower bound–upper bound (LB–UB) means and 95th percentiles of 0.085–0.126 and 0.157–0.212 mg/kg bw per day, respectively. Considering a margin of exposure approach, EFSA concluded that the present dietary exposure to MOSH does not raise concern for human health for all age classes (*EFSA*, xxxx). Foodborne MOAH with three or more non- or simple-alkylated, aromatic rings may be mutagenic and carcinogenic, and therefore of potential concern. Revision of the existing acceptable daily intake for some food grade MOSH is warranted on the basis of new toxicological information (*EFSA*, 2012).

MOSH is generally considered of no concern at the concentration found, although accumulate in human body, while genotoxicity of MOAH with ≥3 aromatic rings is confirmed, and in the absence of reliable toxicity data, the dietary exposure to 1–2 ring MOAH might raise a concern (*Purcaro et. al.*, 2024). The no-observed-adverse-effect level for induction of liver microgranulomas by the most potent MOSH, 19 mg/kg b.w. per day, was used as a reference point for calculating margins of exposure (*EFSA*, 2012).

5. Conclusion

Generation of further data for the refinement of the risk assessment of MOSH and MOAH presence in food is needed. Current dietary exposure towards to MOSH for all age classes raises no concern for human health.

Our work revealed extremely high levels of MOSH contamination in the industrial casings used to package the sausages. Moreover, during product storage, the MOSH increased in the finely chopped cooked sausages to levels that greatly exceeded the producer's internal standard; we hypothesize this was due to migration from the contaminated casing.

Based on the levels of MOH in the tested finely chopped cooked sausages, this product was aligned with the Codex Alimentarius advice, according to which the concentration of the MOAH fraction in food with more than 4% fat should not exceed 1 mg/kg. However, the sausage product was not aligned with the manufacturer's specification, according to which the concentration of the MOSH fraction should not exceed 2 mg/kg.

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Original Scientific Paper

Acrylamide in potato snacks products on the Serbian market: levels and variability

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ABSTRACT

The occurrence and levels of acrylamide (AA) in commercially available potato snack products from the Serbian market were investigated. A total of 205 samples, including potato crisps, snacks, crackers and other potato products from potato dough, were tested using a modified QuEChERS extraction method followed by LC-MS/MS. Statistical processing of the data allowed the assessment of AA distribution, compliance with regulatory limits, and identification of samples exceeding the permitted maximum of 750 µg/kg. AA concentrations varied widely, ranging from 133.4 to 2816.0 µg/kg, with 36 samples (17.56%) above the legal threshold. The variability reflects differences in raw materials, recipe formulations, and thermal processing conditions. The results highlight the need for ongoing monitoring and implementation of mitigation strategies to reduce dietary exposure to acrylamide.

1. Introduction

Food intended for human consumption can serve as a pathway for diverse biological, chemical, or physical hazards that may negatively impact health. Contamination may be primary occurring during production, processing, treatment, preparation, and distribution or secondary, resulting from inadequate storage practices. Consumption of contaminated food has been linked to various illnesses in humans (*Benjo et al.*, 2011). Acrylamide (AA) is a low-molecular-weight, highly water-soluble organic compound that forms in carbohydrate-rich foods subjected to high-temperature processing (typically above 120 °C) under low-moisture conditions. The predominant formation mechanism is the Maillard reaction between free asparagine and reducing sugars, resulting in AA presence in thermally treated products such as French fries, potato chips, bread, biscuits, and coffee (*EFSA CONTAM Panel*, 2015; *Xu et al.*, 2014; *Fan et al.*, 2017; *Yaylayan et al.*, 2003). Toxicological studies in animal models have demonstrated that AA is carcinogenic, inducing tumours in multiple organ systems following oral or other exposure routes (Klaunig, 2008). Repeated-dose experiments across species have revealed neurotoxicity including hind-limb paralysis, impaired motor coordination, and histopathological lesions in peripheral and central nervous tissues and body-weight loss (*EFSA CONTAM Panel*, 2015). In mice, additional adverse effects include testicular degeneration, reduced sperm counts, ovarian cysts, epithelial hyperplasia in various tissues,

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inflammatory responses, and cataract development (*EFSA CONTAM Panel*, 2015). AA induces tumours across both sexes in rodents: in rats, common sites include mammary gland adenomas, fibroadenomas, thyroid follicular-cell tumours, and mesotheliomas in specific strains; in mice, tumour sites encompass Harderian gland tissue, mammary glands, lung adenomas and adenocarcinomas, ovarian granulosa-cell tumours, skin sarcomas, and squamous neoplasms of the stomach and forestomach (*EFSA CONTAM Panel*, 2015). Globally, cancer remains a leading cause of death, and dietary and environmental factors significantly influence risk (*Boskovic & Baltic*, 2016). AA is classified as neurotoxic and is considered probably carcinogenic and genotoxic to humans, based on mechanistic and animal evidence (*IARC*, 1994; *JECFA*, 2005; *EFSA*, 2012; *IARC*, 2014; *Adani et al.*, 2020). Since its detection in food, monitoring efforts worldwide have consistently identified potential public health concerns (*Claeys et al.*, 2016). In Serbia, food-safety governance includes inspection of imported goods, mandatory self-control by domestic producers, and surveillance by inspection authorities. Maximum allowable levels of acrylamide in categories such as including potato crisps, snacks, crackers and other potato products from potato dough are delineated in the Rulebook on Maximum Concentrations of Certain Contaminants in Food (*Official Gazette RS*, 73/2024, 90/2024, 47/2025 and 61/2025. Despite mitigation strategies in industrial and culinary settings, recent evidence suggests that certain items especially coffee, bakery goods, and fried potato products continue to contain notable levels of acrylamide, highlighting the importance of ongoing monitoring and risk evaluation (*Zhu et al.*, 2022; *EFSA*, 2023). The objective of this study was to assess the occurrence and levels of acrylamide in selected food groups where its formation is expected due to technological processing.

2. Materials and methods

2.1. Samples

In the period May 2022 to October 2023, 205 samples of potato snack products were tested. The study was conducted on commercially available potato snack products purchased from various retail outlets across the Serbian market. Samples were collected covering different types of potato snack products such as potato crisps, snacks, crackers and other potato products from potato dough. All samples were stored in original packaging at room temperature until analysis.

2.2. Analytical procedure

Acrylamide (AA) levels in the samples were determined using a modified QuEChERS extraction method followed by LC-MS/MS analysis, as described by *Fadwa* (2012) and *Surma et al.* (2017). Briefly, approximately 5 g of homogenized sample was extracted with water and acetonitrile, followed by clean-up with Bond Elut QuEChERS AOAC kit components. The extract was then analyzed by LC-MS/MS using multiple reaction monitoring (MRM) mode for acrylamide quantification with a limit of quantification (LOQ) of 50 µg/kg and a limit of detection (LOD) of 25 µg/kg. LC-MS/MS system and software (LabSolutions) used for determination of presence of AA were produced by Shimadzu, Kyoto, Japan. All chemicals used were of analytical grade and were used as received without any further purification.

2.3. Data analysis

The results analysis and graphical presentation of their distribution was performed using Microsoft Office Excel 2016.

3. Results and discussion

Table 1 presents the key statistical parameters of acrylamide (AA) levels in tested potato snack products. All 205 samples showed detectable AA concentrations, ranging from 133.4 to 2816.0 µg/kg. The mean concentration was 657.0 µg/kg, with a median of 582.3 µg/kg and a standard deviation of 364.1 µg/kg. When considering the Serbian regulatory limit of 750 µg/kg and accounting for measurement uncertainty, 36 samples (17.56%) exceeded the permitted maximum (Figure 1). The large variability in AA levels among potato snack products can be explained by differences in raw materials, recipe formulations, and processing conditions. Thermal treatment, particularly frying and baking at high temperatures with low moisture, significantly influences AA formation through Maillard reactions involving asparagine and reducing sugars (*Xu et al.*, 2014; *Fan et al.*, 2017; *Yaylayan et al.*, 2003). Products with higher sugar or asparagine content and more intense thermal treatment generally contained higher AA concentrations. The mean and median

AA levels observed in Serbian snack products are comparable with those reported in European studies. *EFSA* (2015) reported average levels of 677.5 µg/kg in potato crisps and 444.5 µg/kg in other potato snack products, indicating that Serbian products fall within the expected European range. Similarly, *Claeys et al.* (2016) documented substantial variation in AA content across Belgian snack products, highlighting the influence of production parameters and the necessity for ongoing monitoring. 17.56% of the potato snack product samples exceeded the regulatory limit, indicating that while most products comply with safety regulations, a notable fraction could contribute to dietary exposure to AA, a compound recognized for its neurotoxic and potential carcinogenic effects (*EFSA CONTAM Panel*, 2015; *IARC*, 2014). This observation underlines the importance of implementing mitigation strategies, including recipe optimization and modification of processing conditions, to reduce AA formation. The high variability suggests batch-specific and manufacturer-specific factors strongly affect AA levels. Therefore, statistical evaluation is essential to identify critical control points and implement targeted interventions to protect consumer health. These findings demonstrate that continuous surveillance and adherence to good manufacturing practices are necessary to maintain AA levels within safe limits. In summary, AA content in Serbian potato snack products shows considerable variability, with a significant number of samples exceeding regulatory limits. The results align with European data, reinforcing the need for systematic monitoring, improved production practices, and possible reformulation to minimize consumer exposure to this potentially harmful compound. Future studies should consider a wider range of snack products, explore seasonal and batch-to-batch differences, and assess the effectiveness of AA reduction strategies. These findings emphasize the need for continuous surveillance and implementation of mitigation strategies, in line with the most recent *EFSA* (2023) recommendations, which highlight benchmark levels as evolving tools to further reduce dietary exposure to acrylamide across the EU (*EFSA*, 2023; *Zhu et al.*, 2022).

Table 1. Descriptive statistics of acrylamide (AA) concentrations in potato snack products

Parameter	Value
Minimum	133.4 µg/kg
Maximum	2816.0 µg/kg
Mean	657.0 µg/kg
Median	582.3 µg/kg
Standard deviation	364.1 µg/kg
Numbers of samples (n)	205

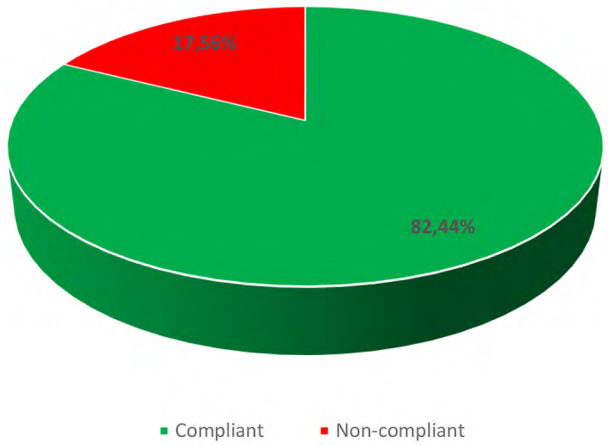

Figure 1. Percentage of potato snack product samples complying and not complying with the regulatory limit of acrylamide

4. Conclusion

Acrylamide is an important safety-related parameter in snack products. The results of this study show that acrylamide concentrations in commercially available potato snack products in the Serbian market are highly variable, with some samples exceeding the maximum regulatory limit of 750 µg/kg. Such variation likely reflects differences in raw materials, processing methods, and cooking conditions specific to each product type and manufacturer. While a significant proportion of the tested potato snack products complied with regulatory standards, a substantial number of samples surpassed the allowed threshold, highlighting potential health risks associated with dietary acrylamide intake. Statistical evaluation, including the consideration of measurement uncertainties, helped to classify borderline samples correctly. Outlier results indicate that both production practices and raw material variability contribute to acrylamide formation. These findings underscore the importance of ongoing monitoring and the adoption of mitigation strategies during processing. Careful control of manufacturing conditions, combined with regular testing, is essential to minimize acrylamide exposure in consumers. This approach also supports compliance with regulatory limits and encourages safer snack product formulation.

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Original Scientific Paper

Application of infrared ocular thermography for welfare and meat quality assessment of slaughter pigs

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ABSTRACT

Infrared ocular thermography is a non-invasive tool used in the pork industry to quickly assess animal stress and its impact on meat quality. The aim of this study was to investigate the association between the infrared ocular temperature and blood indicators and meat quality traits in slaughter pigs. The study was conducted on 60 market-weight pigs (average live weight of about 110 kg and 6 months old) of the same genetics. Infrared ocular thermography images were obtained immediately after stunning. Blood samples were collected at exsanguination to determine glucose level and oxidative stress biomarkers, including advanced oxidation protein products (AOPP), ceruloplasmin, reduced glutathione (GSH), total antioxidant capacity (TAC), total oxidant status (TOS) and the oxidative stress index (OSI). Meat quality traits, including pH, temperature, colour (L^*, a^* and b^* values), water-holding capacity (drip, thawing and cooking loss) and pork quality classes, were measured. Pigs with infrared ocular temperatures above 32 ºC had higher plasma concentrations of GSH and TOS. Pork obtained from the same group of pigs had higher cooking loss and a^* value, along with tendency towards higher L^* and b^* values. Furthermore, pigs with infrared ocular temperatures above 32 ºC had higher tendency towards development of pale, firm and non-exudative meat. These results indicate that elevated ocular temperature is linked to oxidative stress and changes in meat quality, suggesting a connection between pre-slaughter stress and post-mortem meat characteristics. In conclusion, infrared ocular thermography has potential as rapid, non-invasive tool for assessing pig welfare and predicting pork quality.

1. Introduction

The meat industry and consumers are becoming increasingly critical regarding how food is produced, with key concerns related to economic, animal welfare and meat safety and quality (*Sanchez et al*., 2022, *Svoboda et al*., 2024). Traditional methods for assessing animal welfare (behaviour observation, biochemical stress indicators and resource-based indicators) and meat quality (sensory analysis, chemical composition and technological properties) are often invasive/destructive, costly, inaccurate, labor-intensive and time-consuming, making them unsuitable for industrial use (*Sanchez et al*., 2022; *Svoboda et al*., 2024). Therefore, there is a need for novel and advanced methods that are non-invasive/non-destructive, fast, accurate and adaptable to industry conditions (*Sanchez et al*., 2022, *Svoboda et al*., 2024).

Infrared thermography is a non-invasive technique that enables body temperature measurement

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without direct contact with the animal (*Weschenfelder et al*., 2013). Because skeletal muscle temperature is associated with early postmortem pH decline, thermography can detect elevated temperatures in pigs resulting from poor pre-slaughter handling, thereby providing a tool to predict variations in pork quality (*Bressan et al*., 1992; *Weschenfelder et al*., 2013). However, surface temperature measurements may be less accurate due to interference from dirt, hair or water (*Banhazi and Black*, 2009; *Weschenfelder*, 2013).

The brain, as the major source of metabolically produced heat and the central regulator of body temperature, represents the site of core temperature. Consequently, ocular temperature, due to its close proximity to the brain, serves as a reliable indicator of core temperature (*Tan et al.*, 2009; *Kessel et al*., 2010; *Johnson et al*., 2011, *Weschenfelder*, 2013). Because ocular blood flow is closely regulated by sympathetic activity, even mild stress responses can be detected through changes in ocular temperature (*Weschenfelder et al*., 2013). Accordingly, infrared ocular thermography is regarded as a promising non-invasive tool for assessing physiological responses and pork quality variations (*Weschenfelder et al*., 2013). However, available scientific literature lacks data on the physiological range of ocular temperature and threshold values that could indicate stress and, consequently, compromised welfare and deterioration in pork quality. Considering the aforementioned facts, the aim of this study was to investigate the association between the infrared ocular temperature and blood indicators and meat quality traits in slaughter pigs.

2. Materials and methods

2.1. Ethical approval

The experimental procedures used throughout this study were approved by the Local Ethics Committee on Animal Experimentation of the Faculty of Veterinary Medicine, University of Belgrade, Serbia (Approval No: 01-159 of 14 February 2025).

2.2. Experimental pigs and pre- and post-slaughter conditions

The study was conducted on 60 market-weight pigs (average live weight of about 110 kg and 6 months old) of the same genetics ([Yorkshire × Landrace] sows sired with Pietrain boars) across three groups (Group 1=11 pigs; Group 2=19 pigs; Group 3=30 pigs). Pigs originated from two commercial farms (Farm A – Group 1 and 2; Farm B – Group 3), where they were housed under similar conditions. All pigs were subjected to the same pre-slaughter procedures (loading, transport, unloading and lairage). Pig slaughter and carcasses processing were performed at the two commercial slaughterhouses in compliance with the standard industry-accepted practices.

2.3. Capture of infrared thermography images

Ocular images of each pig were captured immediately after stunning, while the animals were suspended by their hind legs and just prior to exsanguination, using a handheld infrared camera (Testo 875, Testo Ltd, United Kingdom) operated by the same trained technician. The images were captured from the right side of each pig at a distance of one meter, with focus on the eye. All thermography measurements were performed under controlled environmental conditions, with stable ambient temperature and relative humidity, to ensure accurate and consistent results. Thermograms were exported to a computer and processed using the appropriate software (IRSoft, Testo Ltd, United Kingdom) to obtain parameters of the recorded region. The examined region (eye) was outlined, and the maximum temperature of the marked area was calculated. The analysis and marking of the region of interest were performed by the same trained evaluator. A representative ocular thermographic image from taken on a pig in the present investigation is provided in Figure 1.

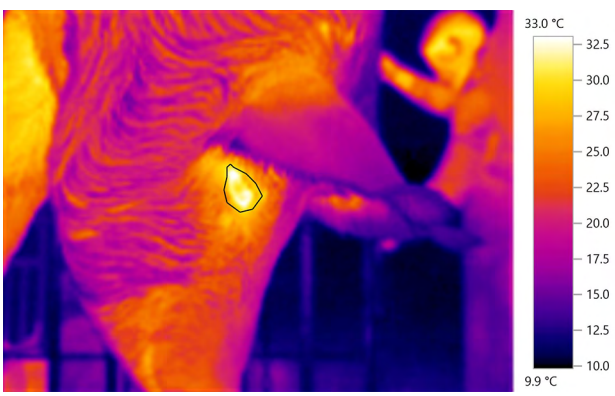

Figure 1. Determination of infrared ocular temperature in pigs after stunning

2.4. Determination of blood glucose concentration at exsanguination

Whole-blood glucose concentration was measured on the slaughter line within 60 seconds of exsanguination using a handheld device (Accu-Chek® Performa, Roche Diagnostics, Mannheim, Germany). To assess oxidative stress indicators, whole blood samples collected in 10 mL EDTA vacutainers during exsanguination were transported on ice packs (maintained at 3 ± 1 °C) inside a portable cooler box. Immediately upon arrival at the laboratory (180 minutes after sampling), whole blood samples were centrifuged at 3,000 rpm for 10 minutes. Plasma aliquots were then transferred into uniquely labelled tubes and immediately frozen at −80 °C pending analysis. Plasma ceruloplasmin concentration was determined based on its p-phenylenediamine (PPD) oxidase activity, according to the protocol of Hussein et al. (2019). Reduced glutathione (GSH) concentration in plasma was analyzed using a modified Ellman's method (*Jollow et al*., 1974), with 5,5'-dithio-bis-(2-nitrobezoic) acid (DTNB). Protein oxidation in plasma was evaluated by quantifying advanced oxidation protein products (AOPP), according to the method described by *Witko-Sarsat et al*. (1996). Total antioxidant capacity (TAC) in plasma was determined using the method of Erel (2004), with 2,2+-azinobis(3-ethylbenzothiazoline-6-sulfonic-acid) (ABTS). Total oxidative status (TOS) was determined according to *Erel* (2005), based on the oxidation of a ferrous ion-o-dianisidine complex to ferric ion by oxidants present in the sample. The oxidative stress index (OSI) was calculated as the ratio between TOS and TAC values.

2.5. Determination of pork quality traits

Sampling and determination of pork quality traits were performed on the carcass left side. Meat pH and temperature measurements were conducted in the chilling room 45 minutes and 24 hours postmortem using a portable pH meter (Testo 205, Testo AG, Lenzkirch, Germany) with an accuracy of ± 0.01, by insertion into *musculus longissimus lumborum* (at the level of the 3rd to 5th lumbar vertebrae) and *musculus semimembranosus* (central region).

Samples used for determination of colour and water-holding capacity traits were collected 24 hours postmortem by excising a portion of the musculature between the 3rd and 5th lumbar vertebrae, approximately 2.5 cm thick. Pork colour was subjectively assessed by three experienced technicians using the reference standards established by the *National Pork Producers Council* (2000), with scores ranging from 1 (pale) to 6 (dark). Objective evaluation of pork colour traits (*L*a*b**; CIELab, 1996) was performed using a portable colorimeter (NR110, 3NH Technology Co., Ltd, Shenzhen, China) equipped with a 4-mm aperture, 2° viewing angle, and D65 illuminant. Measurements were taken at nine randomly selected points on the lateral and medial surfaces of the longissimus lumborum muscle chops to obtain a representative average value (*Hunt et al*., 1991). Three methods (drip loss, thawing loss and cooking loss) were used for determination of water-holding capacity traits, as described in *Honikel* (1998) and *Klauke et al.* (2013).

Pork quality classes were defined based on pH_{24h}, drip loss variations and *L** (lightness) values, according to the criteria of *Correa et al.* (2007): (i) pale, soft, and exudative (PSE) meat; (ii) moderately pale, soft, and exudative (moderate PSE) meat; (iii) pale, firm, and non-exudative (PFN) meat; (iv) red, soft, and exudative (RSE) meat; (v) red, firm, and non-exudative (RFN) meat; (vi) moderately dark, firm, and dry (moderate DFD) meat; and (vii) dark, firm, and dry (DFD) meat.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 9.5.1, GraphPad Software, San Diego, CA, USA). In relation to infrared ocular temperature, pigs were divided into two groups: (1) lower temperature – pigs with an infrared ocular temperature ≤32 °C (n=25); (2) higher temperature – pigs with an infrared ocular temperature >32 °C (n=35). The 32 °C threshold was established based on preliminary data, which showed that ocular temperatures above this value were consistently associated with increased stress indicators and changes in meat quality. The student's t-test was performed to detect significant differences in blood indicators and meat quality traits between two groups. The data were presented as means along with standard deviations. Pearson correlations (r_p) were used to evaluate the associations between infrared ocular temperature and blood indicators and meat quality traits. Correlation coefficients were interpreted according to *Taylor* (1990) as weak ($|r_p| < 0.35$), moderate ($0.36 \leq |r_p| < 0.67$) and strong ($|r_p| \geq 0.68$). Statistical significance was set at $P < 0.05$, while $0.05 < P < 0.10$ was considered indicative of a tendency.

Table 1. The effect of infrared ocular temperature on oxidative stress parameters in pigs (n=60)

Infrared ocular temperature	Lower (≤32 °C)	Higher (>32 °C)	P - value	Significance
Number of pigs	25	35		
Glucose (mmol/L)	7.25 ± 1.37	7.64 ± 2.58	0.489	ns
AOPP (µM/L)	93.02 ± 13.61	92.30 ± 11.65	0.827	ns
Ceruloplasmin (mg/dL)	30.87 ± 11.41	29.31 ± 11.84	0.611	ns
GSH (µmol/L)	0.82 ±0.35	0.53 ± 0.32	0.050	*
TAC (mmol/L)	0.60 ± 0.29	0.68 ± 0.42	0.420	ns
TOS (µmol/L)	90.42 ± 34.48	110.30 ± 70.45	0.039	*
Oxidative stress index	0.25 ± 0.26	0.33 ± 0.35	0.380	ns

* Statistical significance at $P<0.05$; t: tendency ($0.05 < P < 0.10$); ns: not statistically significant ($P>0.05$)

Table 2. The effect of infrared ocular temperature on pork meat quality (n=60)

Infrared ocular temperature	Lower (≤32 °C)	Higher (>32 °C)	P - value	Significance
Number of pigs	25	35		
Physiciochemical parameters				
Musculus longissimus lumborum				
pH_{45min}	6.44 ± 0.25	6.38 ± 0.23	0.348	ns
T_{45min} (°C)	34.44 ± 1.78	33.76 ± 1.25	0.089	t
pH_{24h}	5.67 ± 0.26	5.64 ± 0.21	0.533	ns
T_{24h} (°C)	5.99 ± 1.03	5.08 ± 1.13	0.003	*
Musculus semimembranosus				
pH_{45min}	6.56 ± 0.19	6.56 ± 0.23	0.967	ns
T_{45min} (°C)	34.17 ± 2.38	33.26 ± 2.47	0.159	ns
pH_{24h}	5.52 ± 0.15	5.57 ± 0.22	0.313	ns
T_{24h} (°C)	6.24 ± 1.00	5.49 ± 1.10	0.008	*
Water-holding capacity indicators (%)				
Drip loss	1.96 ± 1.08	1.92 ± 0.80	0.867	ns
Thawing loss	2.26 ± 1.17	3.30 ± 3.76	0.187	ns
Cooking loss	17.87 ± 4.71	20.56 ± 5.23	0.049	*
Meat color indicators				
*L** value	47.69 ± 2.98	48.95 ± 2.80	0.099	t
*a** value	5.41 ± 2.33	4.26 ± 1.50	0.022	*
*b** value	6.03 ± 0.90	6.63 ± 1.53	0.062	t
Sensory color score	2.40 ± 0.69	2.27 ±0.58	0.437	ns
Meat quality classes				
Moderately PSE meat	4.00	8.57	0.484	ns
RFN	60.00	68.57	0.493	ns
PFN	16.00	31.43	0.074	t
Moderately DFD	20.00	11.43	0.359	ns

* Statistical significance at $P<0.05$; t: tendency ($0.05 < P < 0.10$); ns: not statistically significant ($P>0.05$)

Table 3. Relationships between infrared ocular temperature and blood indicators and meat quality traits in slaughter pigs (n=60)

	Infrared ocular temperature (°C)		
	r_p	*P* - value	Strength
Blood indicators			
Glucose (mmol/L)	0.057	0.663	-
AOPP (µM/L)	0.143	0.274	-
Cerulopasmin (mg/dL)	-0.174	0.184	-
GSH (µmol/L)	-0.062	0.637	-
TAC (mmol/L)	0.009	0.942	-
TOS (µmol/L)	0.084	0.521	-
Oxidative stress index	0.116	0.379	-
Physicochemical parameters			
Musculus longissimus lumborum			
pH_{45min}	-0.251*	0.050	weak
T_{45min} (°C)	-0.439*	0.001	moderate
pH_{24h}	0.069	0.599	-
T_{24h} (°C)	-0.472*	0.001	moderate
Musculus semimembranosus			
pH_{45min}	0.109	0.409	-
T_{45min} (°C)	-0.311*	0.016	weak
pH_{24h}	0.246	0.058	-
T_{24h} (°C)	-0.418	0.001	moderate
Water-holding capacity trait (%)			
Drip loss	0.131	0.326	-
Thawing loss	0.030	0.817	-
Cooking loss	0.347*	0.007	weak
Meat colour traits			
*L** value	0.248*	0.050	weak
*a** value	-0.572*	0.001	moderate
*b** value	0.539*	0.001	moderate
Sensory color score	-0.150	0.253	-

Level of significance: * $P < 0.05$.

3. Results

3.1. Effects of the infrared ocular temperature on blood indicators and meat quality traits of slaughter pigs

Effects of the infrared ocular temperature on blood indicators and meat quality traits are shown in Tables 1 and 2. Infrared ocular temperature significantly affected plasma concentrations of GSH (*P*=0.050) and TOS (*P*=0.039).

Infrared ocular temperature significantly affected cooking loss (*P*=0.049) and the *a** value (*P*=0.022). Additionally, pigs with higher infrared ocular temperature (>32 ºC) exhibited an increased tendency towards higher *L** (*P*=0.099) and *b** (*P*=0.062) values and towards a higher occurrence of PFN meat (*P*=0.074).

3.2. Pearson correlations between infrared ocular temperature and blood indicators and meat quality traits in slaughter pigs

Pearson correlations between infrared ocular temperature and blood indicators and meat quality traits in slaughter pigs are shown in Table 3. Infrared ocular temperature was weakly negatively correlated with pH_{45min} in *musculus longissimus lumborum* (P=0.050) and T_{45min} in *musculus semimembranosus* (P=0.016). Furthermore, a weak negative correlation was found between infrared ocular temperature and cooking loss (P=0.007) and L^* value (P=0.050). In addition, infrared ocular temperature moderately negatively correlated with T_{45min} (P=0.001) and T_{24h} (P=0.001) in *musculus longissimus lumborum*. A moderate negative correlation was found between infrared ocular temperature and a^* value (P=0.001), but moderate positive correlation between infrared ocular temperature and b^* value (P=0.001).

4. Discussion

Infrared ocular temperature is a non-invasive method for assessing body temperature in pigs, offering an alternative to rectal measurements (*Weschfelder et al*., 2013; *Yanmaz*, 2020; *Ferreira et al*., 2024). In this study, pigs with ocular temperatures above 32 ºC had lower plasma concentration of GSH and a higher plasma concentration of TOS (Table 1), indicating exposure to stressful conditions prior to slaughter (*Bernatoniene and Kopustinskiene*, 2018). As GSH serves as a major cellular antioxidant that neutralizes free radicals (*Bernatoniene and Kopustinskiene*, 2018), these results indicate activation of oxidative stress.

In the present investigation, meat obtained from pigs with ocular temperatures above 32 ºC had lower water-holding capacity (higher cooking loss) and paler colour (lower a^* value) (Table 2). Furthermore, the same group of pigs had higher tendency towards increased L^* and b^* values and development of PFN meat. Nevertheless, with L^* values ranging from 42 to 50, the meat remained within the RFN class, which is considered acceptable to consumers (*Correa et al*., 2007). Accordingly, the findings of this study indicate that pigs with higher infrared ocular temperatures (>32 °C) experienced only mild stress, resulting in slower postmortem metabolism, partial protein denaturation and an increased risk of PFN meat (*Čobanović et al*., 2021). As supporting evidence of these findings, no associations were observed between infrared ocular temperature and the plasma concentrations of glucose, AOPP, ceruloplasmin, GSH, TOS, TAC or OSI; nevertheless, weak to moderate correlations were established between infrared ocular temperatures and meat physicochemical (pH and temperature 45 minutes postmortem) and colour traits (a^* value) (Table 3).

The negative correlation with the a^* value indicates a more yellowish and less reddish meat colour, potentially associated with a faster pH decline and activation of oxidative stress, leading to reduced pigment stability in pork (*Ereke*, 2022). Consistent with the present study, *Weschfelder et al.* (2013) reported that higher infrared ocular temperatures were associated with lower meat pH 60 minutes postmortem in the *musculus longissimus dorsi* and *musculus semimembranosus*, as well as with higher meat temperatures 60 minutes postmortem and increased drip loss in the longissimus dorsi muscle. Although correlation coefficients were low, this was attributed to the limited variability in meat quality within the study. The obtained results suggest that elevated infrared ocular and skeletal muscle temperatures at the time of slaughter can influence the extent and rate of pork acidification, potentially contributing to meat quality defects (*Weschefelder et al*., 2013). Furthermore, *Gariepy et al.* (1989) reported that higher infrared skin temperatures were associated with the occurrence of defects and variations in pork quality.

5. Conclusion

The results of this study showed that the use of infrared ocular temperature has potential to serve as a tool for rapid, non-invasive tool for pig welfare assessment and prediction of pork quality variations. However, although a degree of association between infrared ocular temperature and certain blood indicators and meat quality traits was established, the correlation coefficients were relatively low. This may be attributed either to the limited variability in pork quality or to the insufficient accuracy of the infrared thermography equipment used. Therefore, to more reliably validate the use of infrared ocular temperature for monitoring pig welfare and variations in pork quality, further research should be conducted under diverse pre-slaughter conditions, with larger sample sizes and including pigs of various genotypes.

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