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Original Scientific Paper

Determination of four perfluorinated compounds (PFAS) in muscle and liver tissues of common pheasants (*Phasianus colchicus*) from two locations in northern Serbia

Ognjen Krnjaja[1], Aleksandar Bajčić[1], Branka Borović[1], Milenko Babić[1], Damjan Gavrilović[1], Saša Janković[1] and Aleksandar Popović[2]*

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ABSTRACT

This study examined and established the presence of perfluoroalkyl substances (PFAS) (perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), and perfluorohexanesulfonic acid (PFHxS)) in the muscle and liver tissues of common pheasants (Phasianus colchicus) collected from two locations in northern Serbia. Breast muscle and liver samples from twenty-five pheasants were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Samples were collected during the 2024–2025 hunting season. Higher concentrations of PFAS were detected in liver (40.0% of all examined samples) compared to breast muscle tissue (8.0% of all examined samples). Total PFAS concentrations in pheasant liver ranged from less than the limit of quantification (<LOQ) to 5.774 µg/kg, while only concentrations detected in muscle tissue were 0.123 µg/kg and 0.171 µg/kg. The most commonly found of the four examined compounds was PFOS, which was followed by PFNA. The aim of this research was to determine the levels of four PFAS in game pheasant tissue collected from two locations in Northern Serbia and compare the results with those similar or different game birds from other countries or regions. According to available literature, this is the first study to examine the presence of PFAS in pheasant tissues in Serbia.

1. Introduction

Perfluoroalkyl substances (PFAS) have been widely used in many industries over the past eighty years. Primarily these compounds are utilised in the paper and electronics industries, cosmetics, household products, fire extinguishers, and general consumer goods. Due to the strong C-F bond, PFAS can be resistant to photochemical, oxidative, and microbial degradation. PFAS are divided into those with a shorter chain (less than 8 carbon atoms) and those with a long chain (more than 8 carbon atoms). The length of the chain affects their toxicity, since PFAS with a smaller number of carbon atoms in the chain remain in the human body for several days or months, while those with a longer chain can remain in the human body for several years. (*Felder et al.,* 2023). PFAS do not accumulate in adipose tissue, but primary in serum, liver, and kidney (*Death et al.,* 2021). These compounds are connected with hepatotoxicity, neurotoxicity, fertility problems, impacts on the development of foetuses and children, and weakened immune response to vaccines (*Mikolajczyk et. al.,* 2024).

Four PFAS compounds, i.e., perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorohexanesulfonic acid (PFHxS), and per-

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fluorooctanesulfonic acid (PFOS), have been classified as toxic by the European Food Safety Authority (*EFSA,* 2020). The maximum allowable concentrations of PFAS in meat and offal of game animals are set by the European Commission Regulation 915/2023 (*European Commission,* 2023) and are: for meat, PFOS — 5.0 µg/kg, PFOA — 3.5 µg/kg,PFNA — 1.5 µg/kg,PFHxS — 0.6 µg/kg and the sum of all four PFAS — 9.0 µg/kg; for offal, PFOS — 50.0 µg/kg, PFOA — 25.0 µg/kg, PFNA — 45.0 µg/kg, PFHxS — 3.0 µg/kg and the sum of all four PFAS — 50.0 µg/kg. These values are also set in the legal infrastructure of Serbia (*Official Gazette of the Republic of Serbia,* 2024).

Over the last two decades, many studies have been conducted on the presence of PFAS compounds in game animals, mainly waterfowl birds (*Sinclair et al.,* 2006*; Sharp et al.,* 2021*; Szabo et al.,* 2022*; Dayan et al.,* 2025) and large mammals (*Kowalczyk et al.,* 2018; *Stahl et al.,* 2012; *Felder et al.,* 2023; *Schröder et al.,* 2024; *Arioli et al.,* 2019). The common pheasant (*Phasianus colchicus*) is one of the most important and widespread game species in Serbia, which makes it a good choice for biomonitoring of PFAS compounds. The greatest distribution of the common pheasant is in the northern, lowland, and agricultural regions of Vojvodina, in the north of Serbia, from where the samples in the current study were taken. Pheasant habitats are near rivers, agricultural fields, and on the edges of forests. The animal's diet includes seeds, cereals, fruits, insects, and small invertebrates (*Popović et al.,* 2019).

In Serbia, the presence of PFAS compounds was investigated in samples of surface water, sediment, and soil in the basin of large rivers in northern Serbia (*Beškoski et al.,* 2013; *Buljovčić et al.,* 2022). A study that examined the concentration of PFAS compounds in wild boar muscle tissue was conducted on animals from central regions of Serbia (*Krnjaja et al.,* 2025).

The aim of this research was to determine the levels of four different types of PFAS (PFOA, PFNA, PFOS, and PFHxS) in game pheasant tissue collected from two different agricultural regions of Vojvodina (Northern Serbia) and compare the results with those similar or different game birds from other countries or regions. According to available literature, this is the first study in Serbia to determine the presence of PFAS compounds in tissues of common pheasant using liquid mass chromatography LC-MS/MS.

2.Materials and Methods

2.1 Sample collection

Breast muscles and livers were collected from 25 pheasants during one hunting season (autumn-winter 2024–25) from two locations in Vojvodina, in northern Serbia. Twelve birds were collected from the hunting ground DUNAV-SREM, in the municipality of Stara Pazova, Srem district (44.9850° N 20.1608° E), and thirteen were collected from the hunting ground BRZAVA, in the municipality of Plandište, South Banat district (45.2272° N 21.1217° E). Pheasants were collected within the framework of the national residue monitoring program in Serbia, and no animals were killed specifically for this research. Sex, weight, and age were measured by authorised hunters. All animals

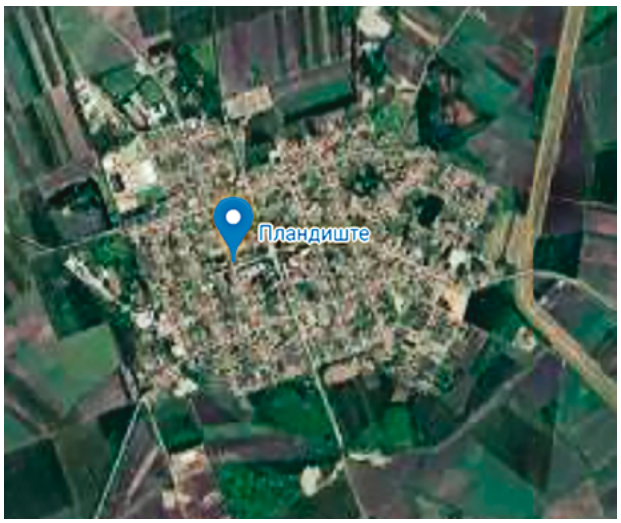

Figure 1. Plandište sampling location
Source: Google Maps, accessed 22 April 2026.

Figure 2. Stara Pazova sampling location
Source: Google Maps, accessed 22 April 2026.

examined in this study were males aged 2 to 3 years, weighing 1.2 to 1.5 kg. After sampling, the tissue samples were placed in polypropylene tubes and stored at −18 °C, and were thawed at 4 °C, and homogenised in a blender one day before analysis by LC-MS/MS.

2.2 Reagents and Standards

The solvents and chemicals used during this study were HPLC grade. Acetonitrile (Honeywell, Germany), methanol (Honeywell, Germany), water (Honeywell, Germany), ammonium acetate (Sigma Aldrich, Germany) and a QuEChERS kit containing 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate dibasic sesquihydrate (SCDS), and 0.5 g sodium citrate tribasic dihydrate (SCTD) (Phenomenex, USA) were used. The PFAS standards, containing the four PFAS compounds examined in this study, as well as internal standards containing their ^{13}C-labeled analogues, were manufactured by Wellington Laboratories Inc., Canada. Working standards were prepared by diluting the stock standards in methanol.

2.3 Sample Preparation and Instrumental Analysis

After adding internal standards, each sample was additionally homogenised with acetonitrile on an UltraTurrax machine. The homogenised material was then centrifuged and the supernatant separated. QuEChERS salts were added to the supernatant, after which the mixture was centrifuged again. The resulting supernatant was evaporated to dryness under a steam of nitrogen, and the dry residue was dissolved again in acetonitrile. After vortexing, the dissolved residue was transferred to a polypropylene cuvette and centrifuged again to obtain the supernatant that was subjected to HPLC analysis. Pheasant tissue samples, in which no PFAS were previously found, were used as blank samples, and after spiking with standard, as calibration materials and the spiked control (QC spike). Calibration range was from 0.1 to 5 μg/kg. The limit of quantification (LOQ) was determined during the validation process and was 0.1 μg/kg for each compound.

For analysis and detection, we used a HPLC with mass detector LCMS 8050 (Shimadzu, Japan). The HPLC system was equipped with a 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. HPLC analysis included 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. Multiple reaction monitoring (MRM) mode was used for quantification. Ion transitions for MS/MS are presented in Table 1.

2.4 Quality Control

Quality control materials (QC) Fish/T0687QC and Dried Egg/T06142QC were purchased from the Food Analysis Performance Assessment Scheme (FAPAS, UK). During the instrumental analysis, the results obtained for the QC materials were within the range specified in the manufacturer's certificate. Another control was conducted with blank samples spiked with calibration standards (QC spike). Recovery values were in the acceptable range of 80–120%.

3. Results and Discussion

The concentrations of PFAS, as well as the number of pheasant tissues in which PFAS were detected in birds from the two sampling locations, are shown in Table 2.

Table 1. Optimised multiple reaction monitoring (MRM) transitions for target PFAS compounds and corresponding isotopically labelled internal standards.

Analyte	Internal Standard	Precursor Ion (m/z)	Quantifier Product Ion (m/z)	Qualifier Product Ion (m/z)
PFOS	[$^{13}C_8$]PFOS	499.0	80.0	99.0/169.0
PFOA	[$^{13}C_8$]PFOA	413.0	369.0	169.0
PFNA	[$^{13}C_9$]PFNA	463.0	419.0	219.0
PFHxS	[$^{13}C_3$]PFHxS	399.0	80.0	99.0

Table 2. Concentrations of PFAS (μg/kg) registered in tissue samples of pheasants from the two sampling locations

Sampling location	Tissue	N	N (%)	Range
Plandište	Liver	13	4 (30.8%)	0.431–1.268
	Muscle	13	nr	-
Stara Pazova	Liver	12	6 (50.0%)	0.115–5.774
	Muscle	12	2 (16.7%)	0.123–0.171
Total	Liver	25	10 (40.0%)	/
	Muscle	25	2 (8.0%)	/

n — number of examined samples; N (%) — number and percentage of samples in which PFAS registered; nr — not registered, so below the LOQ (0.1 μg/kg)

Table 3. Concentrations of PFAS compounds (μg/kg) in pheasant liver samples from the two sampling locations

Sampling location	Sample ID	PFOS	PFOA	PFNA	PFHxS	Range (total)
Plandište	1	0.847	nr	0.421	nr	0.431–1.268
	2	0.779	nr	nr	nr	
	3	nr	nr	nr	nr	
	4	nr	nr	nr	nr	
	5	nr	nr	nr	nr	
	6	nr	nr	nr	nr	
	7	nr	nr	nr	nr	
	8	nr	nr	nr	nr	
	9	nr	nr	nr	nr	
	10	nr	nr	nr	nr	
	11	0.431	nr	nr	nr	
	12	0.448	nr	0.314	nr	
	13	nr	nr	nr	nr	
Stara Pazova	1	nr	nr	nr	nr	0.115–5.774
	2	nr	nr	nr	nr	
	3	nr	nr	nr	nr	
	4	nr	nr	nr	nr	
	5	nr	nr	nr	nr	
	6	nr	nr	nr	nr	
	7	0.253	nr	nr	nr	
	8	nr	nr	0.115	nr	
	9	0.915	nr	0.906	nr	
	10	nr	nr	0.167	nr	
	11	0.999	nr	0.320	nr	
	12	2.580	nr	3.194	nr	

nr — not registered, so below the LOQ (0.1 μg/kg)

PFAS were detected more often in the pheasants liver than in breast muscle. The PFAS detection frequency was higher at the Stara Pazova location (50%) than at the Plandište location (30.8%). In samples in which PFAS were detected, total PFAS concentrations in liver ranged from 0.431 µg/kg to 1.268 µg/kg in birds from Plandište, and from 0.115 µg/kg to 5.774 µg/kg in those from Stara Pazova. In breast muscle samples, PFAS were detected in only two samples from the Stara Pazova location with concentrations of 0.123 µg/kg and 0.171 µg/kg. Higher concentrations and more frequent occurrence of PFAS in liver, compared with those in muscle, could be explained by the affinity of PFAS for organs that are metabolically active and rich in proteins.

Because of the greater distribution of PFAS in pheasant liver, these results are given separately in Table 3.

Table 3 shows that PFOA and PFHxS were not detected in any of the collected liver samples. PFNA was detected in the livers of two birds from the Plandište location at concentrations of 0.421 µg/kg and 0.314 µg/kg. At Stara Pazova, the frequency was higher, and PFNA was detected in the livers of five pheasants in concentrations from 0.115 µg/kg to 3.194 µg/kg. PFOS was detected in four birds from the Plandište location in concentrations from 0.431 µg/kg to 0.847 µg/kg, and in four birds from the Stara Pazova location with concentrations from 0.253 µg/kg to 2.580 µg/kg. Graphical presentations of the obtained results for PFNA and PFOS from the singular sampling locations are given in Figure 3 and 4.

Due to the large number of samples in which the concentrations of PFAS compounds were below the LOQ, it was not possible to make any significant statistical comparison between the two locations or between breast muscle and liver samples.

Compared to similar studies that examined the concentration of PFAS compounds in the tissues of wild birds, this study shows lower values. In a study that examined waterfowl in the Northeast Atlantic flyway, concentrations of PFOS in breast muscle ranged from LOQ to 46.6 µg/kg, and of PFNA were up to a maximum of 4.06 µg/kg (*Dayan et al., 2025*). PFOS and PFNA were also the most common PFAS found in livers of ducks from isolated areas in Australia (*Szabo et al., 2022*). PFOS was found in 30% of duck livers with a maximum concentration upto 5.3 µg/kg, while PFNA was found in 22% of the livers with a maximum concentration of 0.16 µg/kg (*Szabo et al., 2022*). Also, in Victoria,

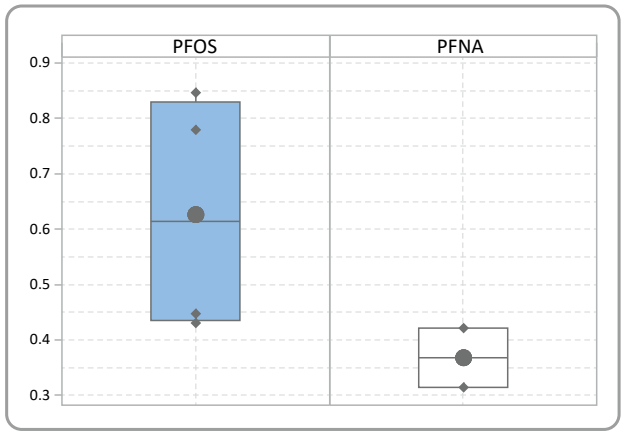

Figure 3. Box plot of registered per-and polyfluoroalkyl substances in the liver of pheasants from the Plandište sampling location

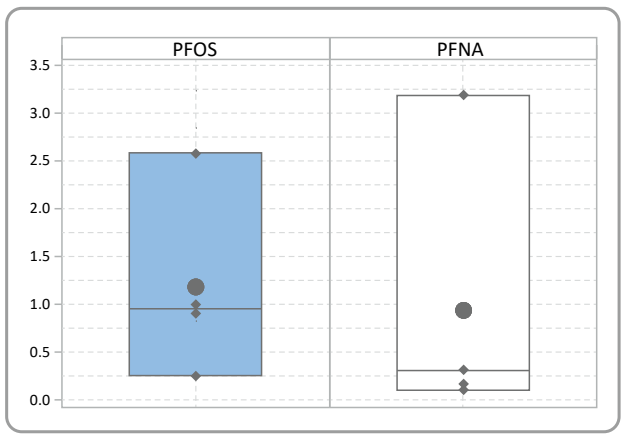

Figure 4. Box plot of registered per-and polyfluoroalkyl substances in liver of pheasants from the Stara Pazova sampling location

Australia, the maximum concentration of PFOS was 33 µg/kg in wild duck breast muscle, and 340 µg/kg in wild duck liver (*Sharp et al., 2021*). In animals from the Niagara River (United States), the concentration of PFOS in mallard livers ranged from 28 µg/kg to 425 µg/kg (*Sinclair et al., 2006*).

4. Conclusion

This study shows results of examination of four PFAS compounds (PFOS, PFOA, PFNA, PFHxS) in the liver and breast muscle of common pheasant from two locations in northern Serbia. Higher concentrations in pheasant liver compared to breast muscle were likely due to the well-known bioaccumulation of PFAS in protein rich and metabolically active organs. The higher prevalence of PFAS in the pheasants from Stara Pazova suggests possible local contamination. Although in a large number of sam-

ples the values were below the detection limits, this study shows that PFAS compounds are present in tissues of common pheasants. Because pheasant is an important game species in Serbia, and these wild birds are consumed by humans, these results indicate the need for further testing and continued biomonitoring of PFAS, and a larger number of samples from different locations in Serbia.

Određivanje četiri perfluorovana jedinjenja (PFAS) u mišićnom tkivu i jetri fazana (*Phasianus colchicus*) sa dve lokacije u severnoj Srbiji

Ognjen Krnjaja, Aleksandar Bajčić, Branka Borović, Milenko Babić, Damjan Gavrilović, Saša Janković i Aleksandar Popović

INFORMACIJE O RADU

Ključne reči:
PFAS
Biomonitoring
Phasianus colchicus
LC-MS/MS
Lovna divljač
Srbija

APSTRAKT

Ova studija je ispitala i utvrdila prisustvo perfluoroalkilnih supstanci (PFAS) (perfluorooktansulfonska kiselina (PFOS), perfluorooktanoinska kiselina (PFOA), perfluorononanoinska kiselina (PFNA) i perfluoroheksansulfonska kiselina (PFHxS)) u mišićnom tkivu i jetri fazana (Phasianus colchicus) prikupljenih sa dve lokacije u severnoj Srbiji. Uzorci grudnog mišića i jetre dvadeset pet fazana analizirani su tečnom hromatografijom i tandemskom masenom spektrometrijom (LC-MS/MS). Uzorci su prikupljeni tokom lovne sezone 2024–25. Veće koncentracije PFASa su otkrivene u uzorcima jetre (40,0% svih ispitivanih uzoraka) u poređenju sa mišićnim tkivom (8,0% svih ispitivanih uzoraka). Ukupne koncentracije PFAS u uzorcima jetre kretale su se od <LOQ do 5,774 µg/kg, dok su jedinje koncentracije detektovane u mišićnom tkivu bile 0,123 µg/kg i 0,171 µg/kg. Najčešće detektovano jedinjenje bio je PFOS, a posle njega PFNA. Cilj ove studije je da se pokaže potencijal fazana kao bioindikatora kontaminacije PFAS. Prema dostupnoj litaraturi, ovo je prva studija koja je ispitala prisustvo PFAS u tkivima u Srbiji.

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Original Scientific Paper

Quantifying heat intensity during thermal processing of smoked pork loin using p value kinetics

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ABSTRACT

Smoked pork loin is a high-quality meat product available on the domestic and European markets. During production, it is subjected to a combination of smoking and roasting, while controlling the pasteurisation process remains a challenge. This smoked meat product is sold either packaged or unpackaged, and in retail, it is directly sliced in front of the consumer. During the thermal processing of smoked pork loin, food business operators typically validate temperature as a parameter for assessing process efficacy, with the introduction of heat continuing until 70 °C is reached at the thermal centre of the product, after which cooling begins. By using a thermocouple in the verification process of heat treatment, it was shown that the required temperature was achieved in all parts of the chamber. The initial temperature of the raw material, before the initiation of thermal processing, was 6.86–8.18 °C; the process itself lasted 6 hours and 20 minutes; and a temperature of 70 °C was achieved at all verification points (locations in the thermal processing chamber). The placement of probes significantly affected the measurement results, determining the pasteurisation value (P value), which ranged from 53.8 to 640.42 minutes. The results confirm the safety of the product, with probes placed in the peripheral parts of the product showing significantly greater P values of 478.16–640.42 minutes, compared to probes placed in the central region at 186.29–205.49 minutes, and in the thermal centre at 53.8–93.08 minutes. The validation of thermal processing for smoked meat products should be established and implemented through P values (a minimum of 40 minutes is required), rather than through temperature, as is currently the case in the meat processing industry in Serbia.

1. Introduction

Smoked pork loin is a popular meat product made from the finest, lean cuts of the pork loin; it has a pale red colour, a mildly salty taste, and an elongated shape. In accordance with national legislation (*Official Gazette RS*, 50/2019 and 34/2023), smoked meat products are produced from cuts of meat — with or without attached bones, skin, subcutaneous fat, and residual meat on vertebral bones after manual deboning — as well as from added ingredients. These products are smoked and heat-treated at pasteurisation temperatures. The production of smoked pork loin generally follows the standard procedure for smoked, pasteurised meat products: first, the pieces are dried at 60–70 °C with 30–40% relative humidity for 30–120 minutes (depending on size), then smoked at 65–75 °C with 50–70% relative humidity until the desired color is reached (*Polak et al*., 2020). Literature on pork quality perception is inconsistent, especially concerning sensory and intrinsic attributes and consumer familiarity; flavour, tenderness, and juiciness are the primary sensory factors shaping overall perceptions of pork loin quality (*Millán, Á. et Retamosa*, M., 2025). Validating sensory evaluation methods is essential to ensure that sensory science is based on objective data rather

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than subjective judgments (*Petrovic et al*., 2024). We must also take into account that sensory measurements are subjective judgments rather than objective physical measurements, making quality attributes harder difficult to define and assess (*Nute*, 2010). Heat treatment significantly affects the properties of pork loin (tenderness/soft texture, juiciness, and salty notes), which primarily shape perceived its quality (*Watanabe et al*., 2019). Pasteurisation-range heat treatment (typically 55–75 °C) effectively reduces microbes in pork products, but also alters sensory traits, like tenderness and drip loss. Around 65 °C has been reported to best balance sensory quality preservation with safety (*Moeller et al*., 2010). Pasteurisation heats the product to a precise temperature for a set time to destroy harmful microbes-bacteria, parasites, and viruses, while largely preserving its flavour, texture, and nutrients. In meat processing, this step is essential for food safety and for prolonging the product's shelf life (*Banu*, 2023).

The pasteurisation value (P value) is calculated from the lethality observed at the product's coldest point during heat treatment. Pasteurisation is validated by tracking F_{70} values against the heat-resistant microorganism *Enterococcus faecium*, using temperature data exceeding 55 °C at the geometric centre of the meat product (*Vuković*, 2012). The P value limit is determined by measuring how long it takes to reduce a specified microorganism's count by one log cycle, using an assumed average contamination level of 10^6 cfu per gram, multiplying this by the product's mass, and reporting the result in minutes (*Rašeta et al.*, 2021). The P value (analogous to the F value but using 70 °C as reference and z = 10 °C) is increasingly cited as a more product-relevant metric for moderate heat treatments than the classic F_0 (121.1 °C, z = 10 °C). The z value is the temperature increase required for a tenfold (1 log) reduction in a microorganism's decimal reduction time (D value) during pasteurisation (*Holdsworth and Simpson*, 2016). Incorporating P values into the optimisation process for a pasteurised meat product verifies that the pasteurisation intensity is adequate and that the product will remain safe under the specified storage conditions. This approach also allows heat-treatment levels to be compared across different products. The food business operator can then monitor and manage every stage of thermal processing, maintaining tight control throughout and guaranteeing the safety of the meat product, regardless of location inside the pasteurisation chamber (*Rašeta et al*., 2025).

In many instances, taste is the biggest driver of consumer satisfaction with meat products, often outweighing factors like tenderness or juiciness (*Garmyn*, 2020). While juiciness and tenderness play a role, they are only supporting elements in determining overall consumer approval (*Lees et al*., 2024). In smoked meats, visual appeal and scent shape provide the first impressions and set expectations for quality, while the distinctive smoky hue and aroma are highly prized (*Abdullah et al*., 2022). However, the meat industry faces difficulties in the heat treatment of meat products, frequently leading to overheating as food safety concerns take precedence. This overheating can result in considerable cooking losses and considerable variability between products. Consequently, there is significant potential to enhance heat treatment methods to prevent unnecessary heating. Thermal pasteurisation plays an essential role in the meat sector, enabling the creation of premium sausage products that can enjoy a prolonged shelf life when stored properly at designated refrigeration temperatures. Pasteurisation of smoked pork loin with temperature management is essential to secure and maintain product safety.

For meat products that undergo pasteurisation heat treatment, the complex thermal process includes not only heating but also steps like drying, smoking, and cooling until the temperature in the thermal centre drops below 55 °C. This overall heat burden results in the peripheral parts of the product being subjected to a very intense thermal regime. Nonetheless, to validate pasteurisation, food business operators in Serbia typically apply a critical limit temperature of 70 °C in the thermal centre of the product as a critical control point (CCP) within the food-safety management system (*Official Gazette RS*, 50/2019, 34/2023). This exposes the product to significant stress during processing and leads to substantial energy consumption, high thermal treatment loads, and sensory deterioration of the product. When achieving a temperature of 70 °C at all points in the chamber in the thermal centre of the product, the P values have already exceeded the set limit for pasteurised products (a minimum of 40 minutes).

However, both domestic (Official Gazette RS, 50/2019, 34/2023) and European (*EC Regulation*, 2004) legislation also allows pasteurisation to be assessed by means of the P value, which must be at least 40 minutes, while the temperature at the thermal centre of the product must be no lower than 65 °C, provided the heat-treatment time is sufficiently long. The same legislation also stipulates that pasteurisation must be validated and controlled as a CCP under the hazard analysis and critical control points (HACCP) system to ensure safety and consistent quality. A fully executed heat-treatment process, apart from its

primary function of ensuring the safety of the final product through the preservative effect of heat, also prevents irreversible discoloration and maintains hygiene standards throughout. The most direct way to validate pasteurisation is to place thermocouple probes in the product's thermal centre at different positions within the heating chamber. By positioning probes outside the thermal centre, one can assess the heat load in the product's peripheral areas while still targeting a core temperature of 70 °C. These peripheral sections, particularly in meat products such as smoked pork loin that is subjected to prolonged heating, experience excessive thermal exposure, which could lower their nutritional value and drive-up energy costs.

To examine the approach to ensuring the CCP through direct measurement of achieving 70 °C at the thermal centre of smoked pork loin, as was documented in the food safety assurance system and designated as a critical control limit by the expert team of the food business entity, temperature measurements inside the smoked pork loin were also taken throughout the pasteurisation process. This meat product was specifically selected for the study because it has a thermodynamically homogeneous matrix, and differences in temperature primarily arise from the product zones (thermal centre, central zone, and periphery) wherein the thermocouple sensor is placed. The primary objective of this work was to present the thermal burden of the product, since the validation of pasteurisation is set only to achieve 70 °C in thermal centre of the product. Also, by using statistical analysis methods, we generated a proposal for optimising the pasteurisation process of smoked pork loin at 65 °C, while ensuring a minimum P value of 40 minutes in the product's thermal centre.

2. Materials and Methods

2.1 Pork Loins

During operations, the butchers adhered to all requirements in the prerequisite programs of the food safety assurance system (good hygienic and manufacturing practices, standard operating procedures, and sanitation standard operating procedures). The procedure for cutting and processing pork was carried out in the meat cutting room at temperatures up to 12 °C, in accordance with national (*Official Gazette RS*, 25/2011, 27/2014 and 86/2023) and European (*EC Regulation*, 2004) regulations. To produce pork loin, pork carcasses were chilled to a core temperature below 4 °C, split lengthwise, and the middle section (which included the loin) was separated from the shoulder and ham. The loin, identified as the muscle running along the back of the pig, from the shoulder to the hip, was separated from the belly by cutting along the natural seam between the two muscles. The bones were removed using a boning knife, and the tenderloin was removed. Using the size of the *spinalis dorsi* as an anatomical landmark, each loin was transacted near the 7th rib. A thick slice was then cut immediately posterior to the 7th rib, and subcutaneous fat, connective tissue was removed.

2.2 Producing smoked pork loins

Boneless loins were taken from the fabrication line about 24 hours post-mortem. Properly shaped pork loins were wet-brined and then mechanically processed in a tumbler. Afterwards, they were tied with twine and hung on the trolley rods. The prepared pieces were left in the ante-room and, according to the first-in–first-out (FIFO) principle, were sent for pasteurisation-level thermal treatment in the Maurer chamber (Figure 1) that accommodated two carts filled with product, with a total weight of 431 kg (Figure 2). Weight was equally distributed between two carts.

2.3 Thermal processing — pasteurisation

The food business operator wanted to validate pasteurisation using a temperature of 70 °C in the thermal centre of the smoked pork loin as part of its implemented HACCP-based food safety assurance system. The validation of pasteurisation was performed during regular production. Pasteurisation of the smoked pork loin product was carried out in the Maurer chamber in three stages: drying for 100 minutes at a chamber temperature of 70 °C; smoking for 30 minutes at a chamber temperature of 70 °C, and; cooking at a chamber temperature of 80 °C until the pasteurisation value required by the regulations (*Official Gazette RS*, 50/2019 and 34/2023) was reached. The entire thermal treatment of the smoked pork loin in the Maurer chamber lasted 380 minutes. After that time, the smoked pork loins were removed from the chamber and left to cool to 55°C (considered the end-point of thermal processing). Since the product was not packaged and had been exposed to smoke during the production process, it was not spray-cooled with water to lower the product temperature.

2.4 Validation of pasteurisation

Measurements were obtained using the thermal validation system Ellab (E-Val Pro, serial number 411982, validated software — US FDA, 21 CFR part 11, GMP, ver. 4.6.1.0), and the technical report was prepared using the Ellab ValSuite software, version 5.2.015. Thermoelements with compensating cables were utilised, and temperatures were recorded at one-minute intervals. All used probes were 20 cm long, with the measuring point located at the tip. During the measurements, probes were strictly calibrated to the designated thermocouple channels without damaging the probes' rubber sheaths. A total of 7 probes were used (thermocouple channels: 1, 2, 4, 6, 7, 8, 9) to record temperature and P value every minute (Figure 3). To completely assess the product's thermal load, probes were placed in the thermal centre of the product (probes 7 and 9), central zone in the region within 2 cm from the centre (probes 1, 6, and 8), and at the periphery of the pork loins, within 4 cm from the thermal centre (probes 2 and 4). A schematic layout of probe positions in one smoked pork loin is presented relative to the product's cross-section (Figure 4). The temperature monitoring was finished when 70 °C was reached at all measurement points.

Figure 1. Maurer pasteurisation chamber with one door, which can accommodate two carts

Figure 2. Pork loins prior to pasteurisation during placement of the tied pieces onto the carts

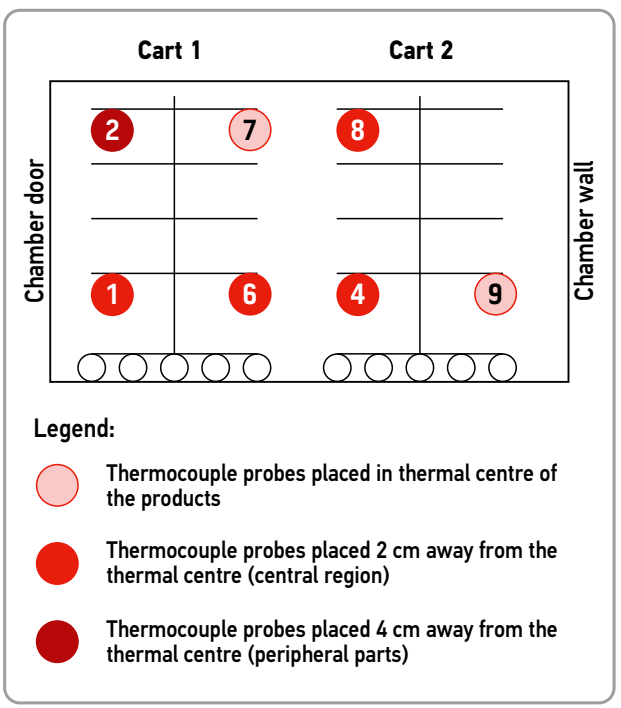

Figure 3. Positions of the probes installed in the Maurer chamber on carts, viewed from the side

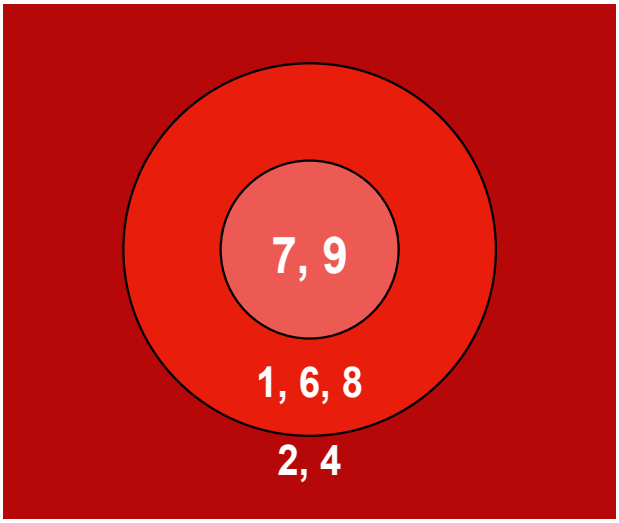

Figure 4. Probe position in a smoked pork loin in different heat regions of the product, seen from cross-section

2.5 Statistical modelling

Statistical modelling using multiple linear regression (MLR) was used to determine P value dependency of pasteurisation time and temperature. Models developed using full factorial design for screening the influence of the two parameters was applied. JMP Statistical Discovery 10 (SAS Institute Inc., USA https://www.jmp.com) was used for statistical analysis and presentation of results. MS Office 2016 Excel software was applied to prepare data for further analysis.

3. Results and Discussion

To validate the thermal processing of smoked pork loin in the Maurer chamber, the temperature was recorded in real time every minute at various points within the product, observed transversely. The temperatures recorded in the products are presented in Figure 5, while P values are shown in Figure 6.

A full factorial design was used to model the pasteurisation of smoked pork loin and predict the time (minutes) required to achieve a specific P value at a given temperature (°C), with results shown in Table 1. The model was assessed by multiple channels (1, 2, 4, 6, 7, 8, and 9) and locations within the pork loin. R^2 values ranged from 0.92–0.95 across all channels, i.e., the models explained 92–95% of the variance in the pasteurisation time. These high R^2 values suggested

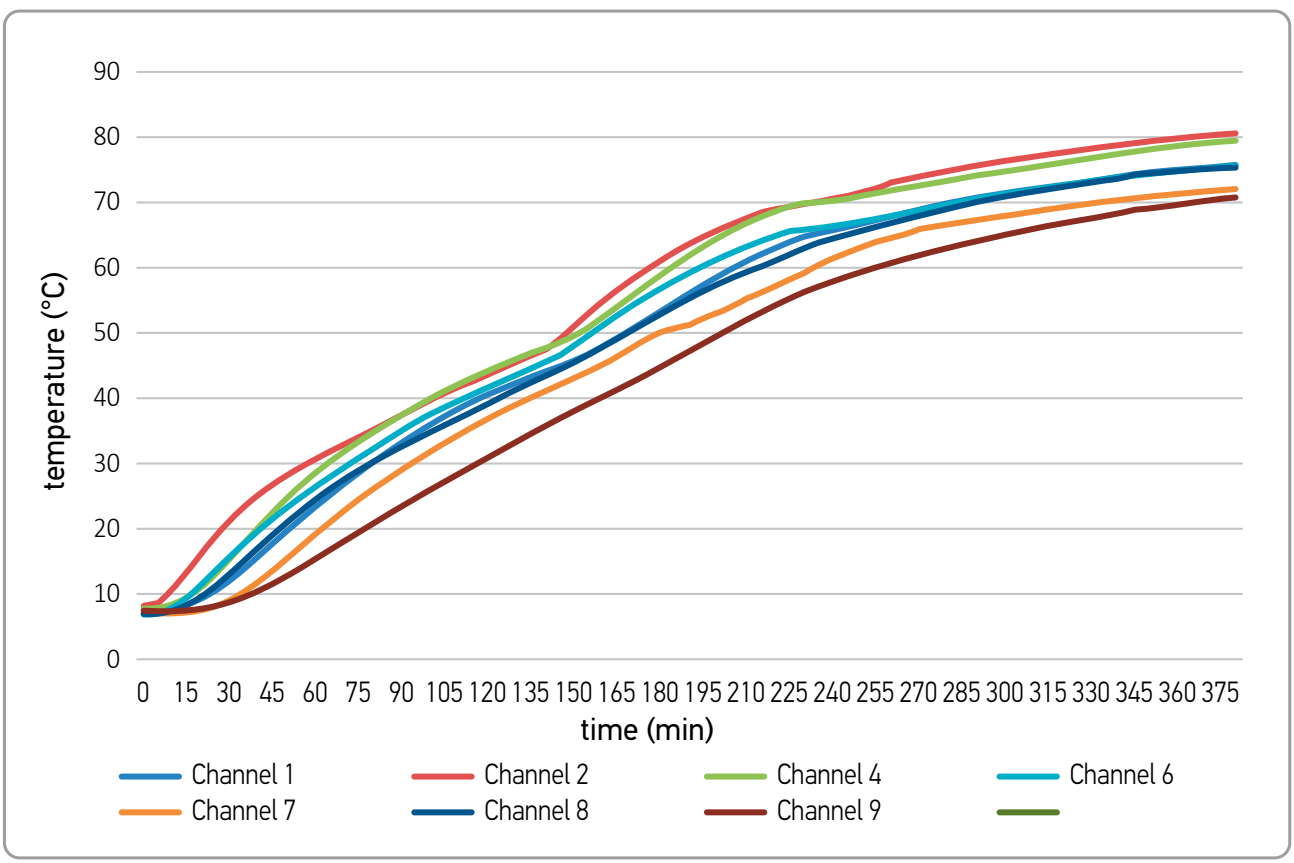

Figure 5. Temperatures achieved in the thermal centre of the smoked pork loin (probes 7 and 9), in central region (probes 1, 6, and 8) and in the peripheral region (probes 2 and 4).

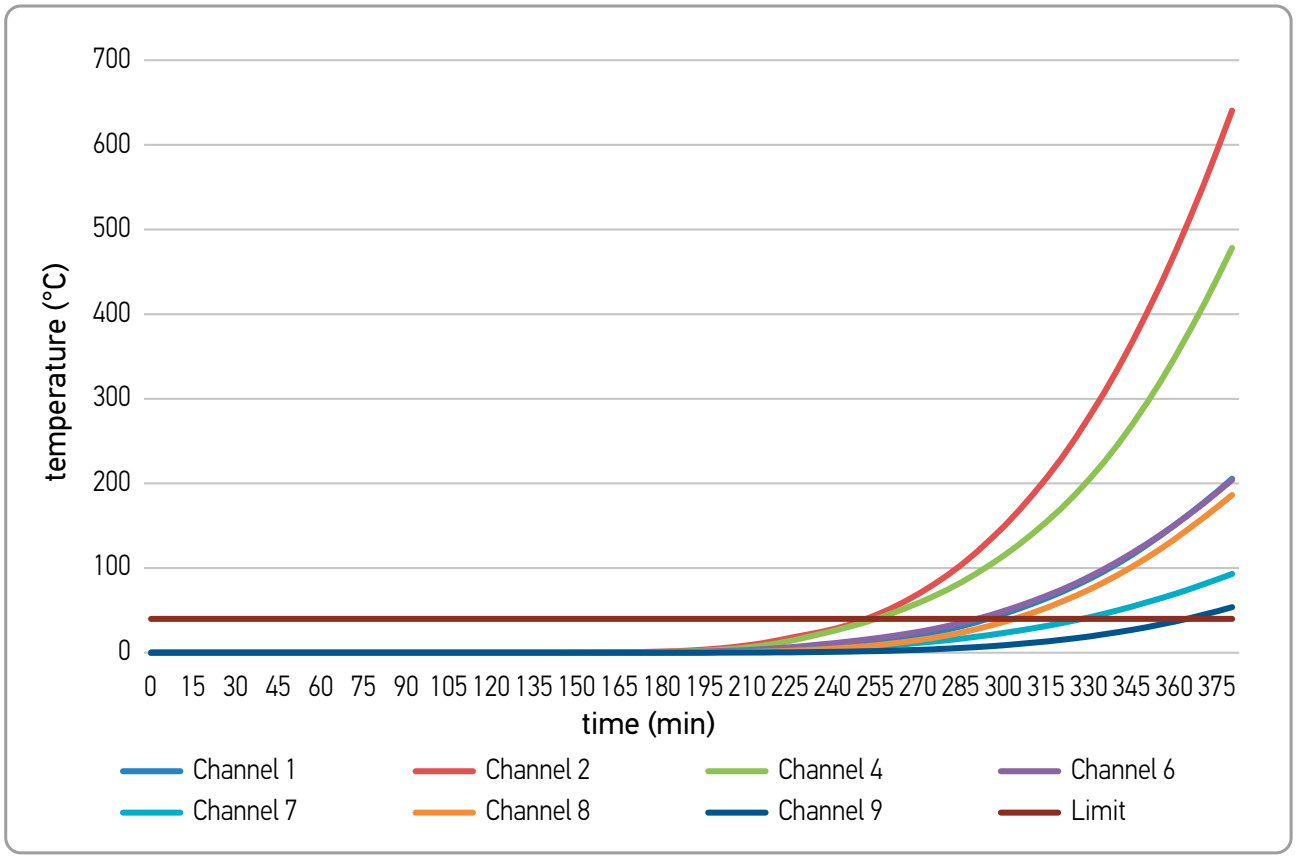

Figure 6. P values achieved in the thermal centre of the product (probes 7 and 9), in the central region (probes 1, 6, and 8) and in the peripheral region (probes 2 and 4) of the smoked pork loin

a very good fit of the model to the data. Time and temperature were strong predictors of pasteurisation effectiveness in the smoked pork loin, while the models captured most of the data variability.

Differences in intercept values across channels reflect variations in the initial microbial load, or physical location. The intercept value is the predicted pasteurisation time when both time and temperature are zero, and serves as baseline value for the model (Figure 7). The intercept values differed across the channels (from 7.06 to 436.4), and were statistically significantly different ($p < 0.0001$), except channel 9, which was significantly different at $p < 0.05$.

Longer processes have higher pasteurisation values, while the magnitude of coefficients indicates the sensitivity of the pasteurisation process to changes in time. The coefficients for the time were all positive and significant ($p < 0.0001$), ranging from 0.5–5.59, which shows that when the time increased, the predicted pasteurisation time also increased. The coefficients for temperature were all negative and significant ($p < 0.0001$), ranging from −2.21 to −24.3. This indicates

Table 1. Prediction model determination coefficients (R^2), intercept, and parameter coefficient values

Parameter	Channel						
	1	2	4	6	7	8	9
R^2	0.94	0.92	0.92	0.93	0.95	0.95	0.92
Intercept	143.46*	436.40*	311.55*	171.12*	41.34*	198.60*	7.06**
Time (min)	2.24*	5.59*	4.24*	2.08*	0.97*	2.65*	0.50*
Temperature (°C)	−10.18*	−24.30*	−18.32*	−9.78*	−4.35*	−12.78*	−2.21*
Time*Temperature	−0.011*	−0.021**	−0.021**	−0.013*	−0.004*	−0.016*	$4.3 \cdot 10^{-5}$***

Asterisks denotes following statistical significance: * $p < 0.0001$, ** $p < 0.05$ and *** no significance for confidence interval of 95% ($p = 0.05$).

that when temperature increased, the predicted pasteurisation time decreased. This is aligned with the understanding that higher temperatures require shorter processing times to achieve the same level of pasteurisation. Large negative coefficients suggest a strong inverse relationship between temperature and pasteurisation time. The coefficients for the time/temperature interaction were mostly negative and significant ($p < 0.0001$ or $p < 0.05$), except for channel 9, which was not significant. The values were close to zero. The significant negative interaction suggests that the effect of temperature on pasteurisation time depends on the processing time, and vice versa. The small values, close to zero, indicate the interaction has a very small effect.

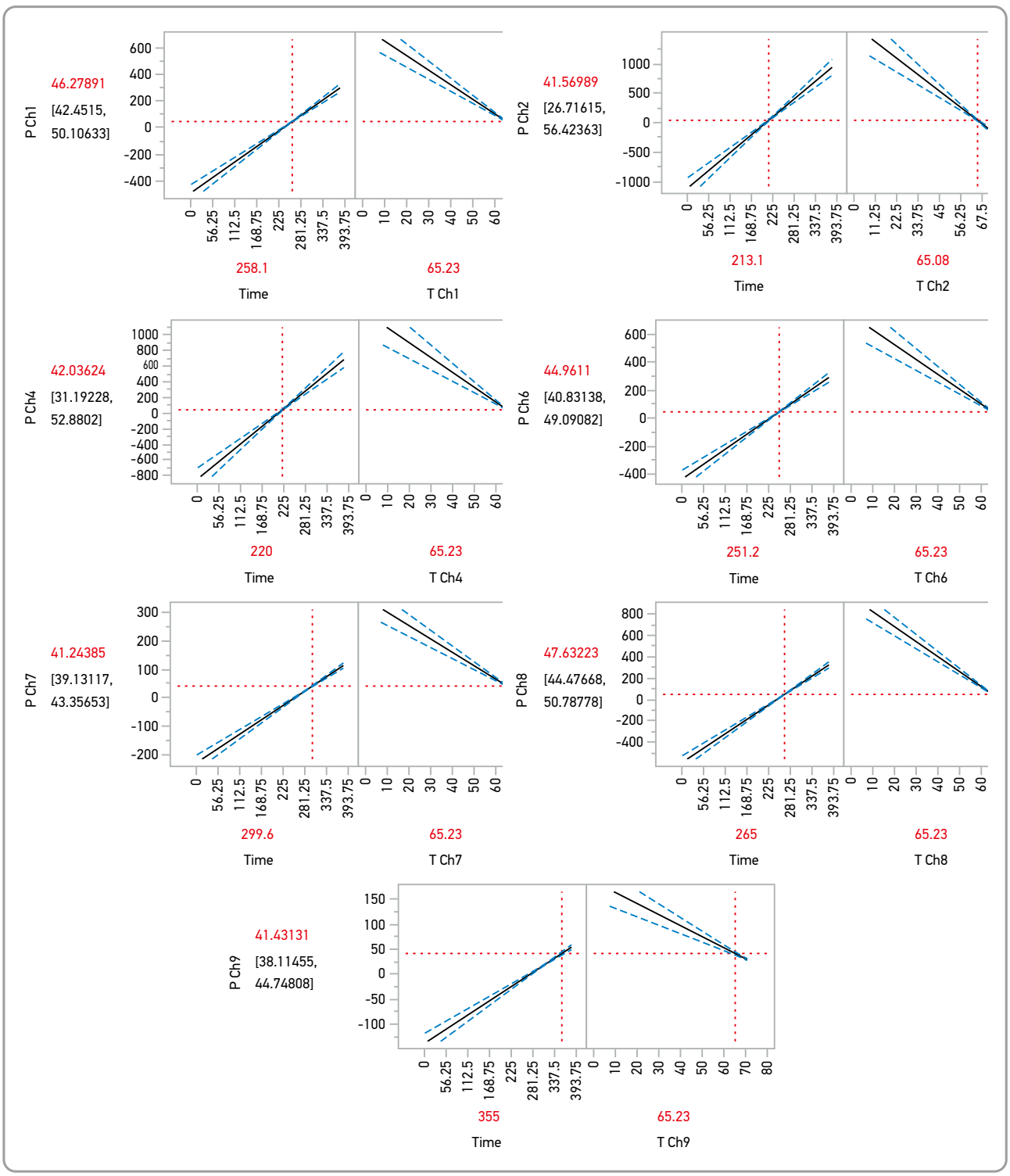

Figure 7. Prediction of pasteurisation time necessary to achieve a P0 value >40 at approximately 65oC

Sensory qualities like flavour, tenderness, and juiciness are the main drivers of consumer acceptance for pork loin products, including smoked products; pasteurisation and cooking parameters directly affect these attributes (*Millan and Retamosa*, 2025). The primary task during production of smoked pork loin by the food business operator is to obtain a safe product (*Official Gazette RS*, 41/2009 and 17/2019) within the specified shelf life (*Official Gazette RS*, 30/2024) and storage conditions of 0 °C to 4 °C (*Official Gazette RS*, 50/2019 and 34/2023). The shelf life of the final smoked pork loin, as a ready-to-eat (RTE) product, is primarily determined by the preservative effect of heat during pasteurisation. Aldehydes from smoke coagulate proteins and alter the meat's texture, and combined with phenols, organic acids, and alcohols, have limited antimicrobial effects, mainly on the product surface (*Vuković*, 2012). However, any preservative effect of smoke on the surface gradually diminishes due to evaporation, diffusion into deeper layers, and reactions with protein components. Smoke does not prevent the growth and reproduction of microorganisms. Applying steam pasteurisation to commercially slaughtered beef carcasses at 82.2 °C for 6.5 seconds markedly lowered the bacterial levels, and when the time-temperature settings were fine-tuned, the sensory quality was within acceptable limits (*Nutsch et al*., 1998). However, further pasteurisation process optimisation is necessary, as although smoking is highly effective for preserving food, it also contaminates products with carcinogenic polycyclic aromatic hydrocarbons (PAHs), levels of which climb steadily as temperatures increase from 55 °C to 95 °C and smoking times lengthen from 2 to 9 hours (*Racovita et al*., 2020). Including the P value in the optimisation process verifies that the required degree of pasteurisation has been reached and assures product safety under the specified storage conditions (*Rašeta et al.*, 2021).

It is necessary to compare the reported results with existing thermal profiles to determine the accuracy of the measuring instruments in the Maurer chamber. Once this is done, the food business operator will then be able to oversee the entire thermal processing process, maintaining comprehensive control and ensuring the safety of smoked pork loin throughout the entire thermal treatment.

Thermal processing of the smoked pork loin was optimised using MLR statistical modelling to ensure the required P value of > 40 minutes was reached. This was done for the coldest point in the studied Maurer chamber (channel 9), at a media temperature of 65 °C, over a total pasteurisation time of 355 minutes—which included the active heating phase and the cooling phase until 55 °C was reached in the thermal centre of the product (Figure 7). The MLR model allowed us to examine the possibilities for optimising the pasteurisation process. At the moment of completing the active heating, the probe in channel 9 recorded a P value of 53.8 for 380 minutes. However, we also needed to take into account the time required for the cooling phase to 55 °C, which, according to records from the food business operator, required an additional 120 minutes. Therefore, the total time reduction was the sum of the difference in duration between the regular process (70 °C is reached in the thermal centre, taking 375 minutes), and the optimised process of 355 minutes, plus the product cooling time. Mathematically, this was a reduction in the pasteurisation time of smoked pork loin of 20 + 120 = 140 minutes. This reduction in pasteurisation time would be highly meaningful in terms of time savings and energy consumption.

The circulation routes within the chamber are designed to create uniform temperature fields, which is essential for achieving consistent heat penetration from the surface to the thermal centre of the meat product. The positioning of products within the chamber significantly affects their temperature profiles during processing (*Feyissa and Frosch*, 2023). For this reason, the P value in the product placed in the thermal centre located at the upper position (channel 7) was greatest, at 93.08 minutes. Considering that the channel 7 probe was located in the thermal centre of the product, we assessed the safety of the pasteurisation process. Using the MLR model, the entire pasteurisation process lasted 303 minutes at the channel 7 location. Generally, though, the approach to ensuring the safety of the entire process should be oriented towards the coldest location in the Maurer chamber, which in our case was the product measured by channel 9, corresponding to a total pasteurisation duration of 355 minutes. The defined time refers to the processing in the chamber until 65 °C is reached in the thermal centre of the smoked pork loin, encompassing the total time of active heating, plus cooling time for the product's thermal centre to reach 55 °C.

The minimum temperature at which pasteurisation begins in the thermal centre of a meat product generally starts around 55 °C, but the process depends on both temperature and holding time to achieve microbial lethality (*Baldwin*, 2012; *Hwang et al*., 2019). Research indicates that the processes of curing and smoking pork loin, followed by heat treatment to internal temperatures of approximately 65–70 °C for periods ranging from 30 seconds to several minutes, affect the texture, purge loss, and shelf life of the product (*Granados*, 1984). A temperature of 65° C is high enough to destroy most microorganisms, including bacteria, viruses, and fungi, thus extending the shelf life of the products and reducing the risk of infections (*Azizpour et al.*, 2024). Pasteurisation does not kill spores (*Cockey and Tatro*, 1974), nor all vegetative forms of bacteria. However, it is a thermal treatment sufficient to ensure limited shelf life of meat products in the presence of brine ingredients and under cold chain storage conditions (*Olušk*i, 1973).

To assess the intensity of pasteurisation in the smoked pork loin, probes were also placed in the central zone 2 cm from the thermal centre (channels 1, 6 and 8). P values obtained ranged from 186.29 to 205.49 minutes. Greater values were obtained for products located on the lower parts of the carts, which was consistent with the chamber's circular heat circulation during operation. Two probes (channels 2 and 4) were placed in peripheral parts of the product (4 cm from the thermal centre), and they achieved P values ranging from 478.16 to 640.42 minutes. These results indicate a significant difference in the intensity of thermal treatment at the product periphery compared with the thermal centre.

The results of this study should serve as a basis for further optimisation of the pasteurisation process, with the mandatory assurance of the CCP through P values instead of temperature, which is the current practice among food business operators in Serbia. Additionally, sensory evaluation of the smoked pork loin was not conducted, so this study limitation should be addressed in the future. The topic is relevant, the methodology is sound, and the findings have practical implications for the meat processing industry. The use of P values for pasteurisation validation is a novel approach in Serbia that warrants further investigation and dissemination.

4. Conclusion

This study was conducted on smoked pork loin commercially produced by a food business operator, and for which a critical limit was set of 70 °C in the thermal centre, exposing the product to significant stress during processing. Overly stringent thermal treatment leads substantial and unnecessary energy consumption, a high thermal treatment load, and results in sensory deterioration of the product. While achieving a temperature of 70 °C at all points in the chamber in the thermal centre of the product, the P values had already exceeded the minimum limit for pasteurised products, i.e., 40 minutes. Those long P values were added to by the cooling process (the hold until the temperature in the thermal centre drops below 55 °C).

During validation at a core temperature of 70 °C, the pasteurisation value (P value) in the thermal centre ranged from 53.8 to 93.08 minutes, depending on the product's location on the chamber carts (these values did not account for the fact that the P value continued to rise during cooling). At the same time, our monitoring of the two more peripheral layers in the product also showed very high readings (that will also increase while the product cools): the intermediate central zone reached P values of 186.29–205.49 minutes, and the outermost peripheral layer, 478.16–640.42 minutes.

These results indicate that the pasteurisation of smoked pork loin should be validated primarily by the P value — which must be at least 40 minutes for a pasteurised product — rather than by temperature alone. Relying solely on temperature exposes the product to an overly long pasteurisation process, likely resulting in diminished final quality and greater yield losses.

Theoretically for the studied smoked pork loin, the pasteurisation process could be optimised. The suggested pasteurisation regime suggested by the authors is 355 minutes of complete thermal treatment (heating and cooling), with 65 °C achieved in thermal centre of the product. According to the applied MLR statistical model, this would still achieve a P value > 40 min. This suggested lower-temperature pasteurisation regime will expose the product to significantly lower temperatures for shorter times than currently is the case, which likely would positively affect the biological value, the presence of nutrients, and the degree of degradation of the additives used in the production of these sausages.

Kvantifikacija intenziteta toplote tokom termalne obrade dimljene svinjske pečenice korišćenjem p-vrednosti

Mladen Rašeta, Radivoj Petronijević, Mirjana Lukić, Boris Mrdović, Jelena Jovanović, Elmin Tarić i Becskei Zsolt

INFORMACIJE O RADU

Ključne reči:
Pasterizacija
Validacija
Barene kobasice
Toplotna obrada
Termokapl

APSTRAKT

Dimljena svinjska pečenica je visokokvalitetan mesni proizvod dostupan na domaćem i evropskom tržištu. Tokom proizvodnje izlaže se kombinaciji dimljenja i pečenja, pri čemu kontrola postupka pasterizacije ostaje izazov. Ovaj dimljeni proizvod od mesa se prodaje upakovan ili neupakovan, i u prodaji se pred potrošačem direktno narezuje.. Tokom termičke obrade dimljene svinjske pečenice, subjekti u poslovanju hranom, obično validuju temperaturu kao parametar za procenu efikasnosti procesa, pri čemu se uvođenje topolote nastavlja sve dok se ne dostigne 70 °C u termalnom centru proizvoda, nakon čega se započinje sa hlađenjem.. Korišćenjem termokapla u procesu verifikacije postupka toplotne obrade, pokazano je da je zahtevana temperatura postignuta u svim delovima komore. Početna temperatura sirovine, pre inicijacije toplotne obrade, iznosila je 6,86–8,18 °C; dok je sam postupak trajao 6 sati i 20 minuta; gubitak težine tokom termalne obrade bio je 15,8–18,3%; a temperatura od 70 °C postignuta je na svim tačkama provere. Postavljanje sondi značajno je uticalo na rezultate merenja, određena je vrednost pasterizacije (p-vrednost), koja se kreće od 53,8 do 640,42 minuta. Rezultati potvrđuju bezbednost proizvoda, pri čemu su sonde postavljene u perifernim delovima proizvoda pokazale znatno više P vrednosti 478,16–640,42 minuta, u odnosu na sonde koje su postavljene u središnjim delovima 186,29–205,49 minuta, do sondi koje su postavljene u termalnom centru proizvoda 53,8–93,08 minuta, Verifikacija termalne obrade za dimljene mesne proizvode trebala bi biti uspostavljena i implementirana kroz p-vrednosti (potreban minimum je 40 minuta), umesto kroz temperaturu, kao što je to trenutno slučaj u industriji prerade mesa u našoj zemlji.

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Original Scientific Paper

Novel Two-Step Sous-Vide Technique: An Innovative Strategy to Preserve Chicken Breast Quality and Freshness

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ABSTRACT

This study investigated the effects of single-step versus double-step sous-vide treatments on chicken breast quality under varying storage durations (0, 7, 14, and 21 days) and temperatures (4 °C and 10 °C). Chicken pectoralis major muscles were vacuum-sealed and cooked either at 60 °C for 120 min (single-step) or using a two-phase profile (50 °C followed by 60 °C) with equal total cooking times. Quality parameters including moisture content, cooking loss, colour (CIELAB), lipid oxidation (thiobarbituric acid reactive substances; TBARS), and odour acceptability were evaluated. The double-step method yielded significantly higher moisture retention and lower cooking loss than the single-step method, likely due to less muscle fibre shrinkage and improved protein solubility. Storage at 4 °C maintained superior colour stability, lower lipid oxidation, and better odour scores compared to 10 °C. By Day 21 at 10 °C, TBARS values exceeded the sensory threshold, resulting in off-odours and reduced acceptability. From a sustainability perspective, the double-step sous-vide approach can contribute to reduced food waste by extending shelf life and maintaining product quality over longer distribution periods. This sous-vide controlled heating profile may also improve energy efficiency compared to prolonged single-step high-temperature cooking. Aligning advanced cooking methods with optimal storage conditions thus supports more sustainable poultry production.

1. Introduction

Global demand for poultry meat has been steadily rising, largely owing to its health benefits and lower cost relative to other meat types. Between 2000 and 2020, worldwide consumption of meat, eggs, and milk saw an overall increase of 23–30%, with growth of 2–6% in developed countries and up to 60% in developing nations (*Food and Agriculture Organization of the United Nations*, 2021). Poultry production is expected to expand most prominently in Indonesia, Russia, Brazil, Japan, and India (*Uzundumlu & Dilli*, 2023). This surge in popularity can be attributed to poultry's high protein content, complete amino acid composition, and relatively greater levels of polyunsaturated fatty acids (PUFAs) compared to other meat sources (*Marangoni et al*., 2015). Concurrently, consumer behaviour has shifted: around 80% of poultry products are now offered as processed or cut-up portions, a striking departure from eight decades ago when most poultry were sold whole (*Barbut*, 2020). This transformation in production and consumption patterns also increases the urgency for sustainable processing solutions, as higher demand

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and more complex supply chains can amplify food waste and environmental impacts if product quality deteriorates before reaching consumers. Consumer acceptance is also influenced by sensory quality and sustainability-oriented assurance practices, making quality preservation essential for strengthening trust and reducing waste (*Ramadhanti & Tamimi,* 2025; *Tamimi* et al., 2025).

The growing demand for poultry-based ready-to-eat food products has spurred the development of processing methods that preserve both sensory quality and nutritional value. However, older adults may eat less poultry due to chewing and swallowing issues associated with various oral impairments (*Hong et al.,* 2015; *Rémond et al.,* 2007). This issue is particularly relevant to chicken breast muscles, which are often tougher and crumblier than leg muscles (*Hong et al.,* 2015; *Zhang et al.,* 2022). Therefore, the food industry needs to develop innovative poultry-based ready-to-eat products that not only improve texture and maintain nutritional value but also respond to broader sustainability concerns in poultry processing. Such efforts are increasingly important for enhancing resource efficiency, preserving product quality, extending shelf life, and minimizing waste across the poultry sector.

One increasingly popular approach is sous-vide cooking, which employs low-temperature, long-time (LTLT) processing to retain the meat's natural moisture, nutritional content, and flavour (*Zavadlav et al*., 2020). Sous-vide involves placing food in vacuum-sealed bags and cooking it at precisely controlled temperatures between 55–70 °C, thereby enhancing meat characteristics and minimizing moisture loss (*Gil et al*., 2022). Unlike conventional methods, sous-vide utilizes a sealed environment that mitigates oxidation and flavour deterioration while also reducing the risk of recontamination during storage (*Baldwin*, 2012). From a sustainability perspective, its controlled heating process can be more energy-efficient than traditional cooking, while the extended shelf life helps prevent premature disposal of products.

Although sous-vide cooking is typically conducted in a single step, a novel two-phase approach — referred to as the double-step method — has recently garnered attention. This technique has been reported to enhance meat texture by reducing shear force while increasing tenderness and chewiness compared to the single-step method (*Ismail et al*., 2019). In addition, it lowers cooking loss, improves oxidative stability, and boosts total protein solubility, thus contributing to an overall higher-quality final product (*Bıyıklı et al*., 2020; *Christensen et al*., 2011; *Karpińska-Tymoszczyk et al*., 2020). Notably, endogenous proteolytic enzymes in meat exhibit peak activity at 40–50 °C, leading to increased desmin degradation and, consequently, improved tenderness (*Akoğlu et al*., 2018). These improvements in moisture retention and oxidative stability have the added advantage of extending product usability during distribution, which supports waste reduction goals in the poultry industry (*Afidah et al.,* 2025).

Food safety remains a critical consideration in sous-vide processing. A study has shown that maintaining sous-vide treatments at 50 °C or 60 °C for 120 minutes achieves the necessary pasteurization standard, characterized by a three-log reduction of *Enterococcus faecalis* (*Hasani et al.*, 2023). Additionally, chilling plays a vital role in preserving chemical, sensory, and nutritional properties while mitigating microbial and enzymatic degradation (*Fernandes et al.*, 2016). Ensuring optimal storage conditions is also essential to preventing deteriorations in meat quality, encompassing reductions in moisture, texture, colour, and aroma (*Barbut & Leishman*, 2022). While sous-vide technology offers potential benefits in enhancing the quality and shelf life of poultry meat, challenges remain regarding post-cooking storage conditions. One study demonstrated that sous-vide products stored at 3 °C maintained minimal microbial growth and retained their sensory attributes for up to five weeks. However, products stored at higher temperatures showed unacceptable microbial growth after three weeks (*Nyati*, 2000). These findings underscore that proper integration of cooking and storage strategies is crucial, not only for quality retention but also for maximizing sustainability gains by reducing spoilage-related waste.

This study aims to investigate how differing storage durations (0, 7, 14, and 21 days) and temperatures (4 °C and 10 °C) affect the quality of sous-vide-treated chicken breast meat. Key parameters under examination include moisture content, cooking loss, colour, lipid oxidation, and odour acceptability. The results are expected to yield valuable insights into optimal storage conditions for sous-vide poultry products and to advance food technology by enhancing both the quality and shelf life of poultry products in the global market.

2. Materials and Methods

2.1 Experimental design

The objective of this study was to examine the effect of applying a two-step temperature approach with differing time ratios to sous-vide cooking of chicken breasts, as well as the impact of storage duration and temperature. Chicken breast samples were either cooked at a single temperature of 60 °C or subjected to two temperature steps (50 °C as the first step and 60 °C as the final step) under different time ratios but the same total cooking time (see Table 1). A two-way completely randomized design was employed, with three replicates for each sous-vide treatment. Chicken pectoralis major muscles (24 hours post-mortem, pH24 = 5.8 ± 0.12) were sourced from a slaughterhouse in Budapest, Hungary. The breasts were trimmed of excess fat and connective tissue, then cut into 2 cm-thick portions weighing approximately 105 ± 10 g each. The samples were vacuum-sealed (Multivac C100, MULTIVAC Sepp Haggenmüller SE & Co. KG, Germany) in PA/PE bags (200 × 250 mm²) prior to cooking.

In this investigation, sous-vide cooking was conducted using two thermostatic water baths (Laboratory Water Bath LP507/01). A Type-T thermocouple, inserted into the centre of the chicken breast samples, was employed to monitor the internal temperature throughout the cooking process. Following recommendations by the BC Centre for Disease Control and Prevention (2016), the cooked chicken breasts were chilled in ice water (1 °C) and subsequently stored in a cold environment (2 °C) for six hours to reduce their temperature below 4 °C (*BC Centre for Disease Control*, 2016). Rapid cooling in ice water helps to inhibit bacterial growth, while also halting the cooking process and preventing the poultry from becoming overcooked and dry.

The stability of sous-vide-cooked chicken breast samples, subjected to either the one-step or two-step temperature treatments, was assessed during storage at chilled (4 ± 0.5 °C) or mildly chilled (10 ± 0.5 °C) temperatures for a maximum of 21 days, with continuous temperature monitoring. On days 0, 7, 14, and 21, samples were collected to evaluate moisture content, cooking loss, colour, lipid oxidation, and aroma acceptability.

2.2 Moisture content and cooking loss

The standard AOAC International 950.46 method (2005) was utilized in triplicate to determine the moisture content, i.e., the proportion of water present in a material, expressed as a percentage of the dry weight relative to the wet weight, of chicken breasts (*AOAC*, 2002). Approximately 4 grams of the cooked chicken breast were weighed and then dehydrated in an air-forced oven (Labor Müszeripari Müvek, Hungary) at 105 °C for sixteen hours. For the cooking loss, sometimes called weight loss or drip loss, and denoting the mass of food lost during cooking, the initial weight of chicken breast samples was determined. Then after cooking treatment the chicken breast sample was weighed after removing the water content from its packaging.. The moisture content (%) and cooking loss (%) were calculated using equations (1) and (2).

$$\text{Moisture content (\%)} = (\text{dried sample weight}/\text{raw sample weight}) * 100 \quad (1)$$

$$\text{Cooking loss (\%)} = (\text{initial weight-final weight})/\text{initial weight} \times 100 \quad (2)$$

2.3 Colour measureme nt

The colour characteristics of the chicken breasts were evaluated using the CIELAB grading system. After calibrating the instrument, each sample's lightness (L*), redness (a*), and yellowness (b*) were measured with a Konica Minolta Sensing CR-410 colourimeter. Five parallel measurements were taken per sample, with three samples in total.

The L* axis, which ranges from 0 (black) to 100 (white), reflects the sample's lightness or darkness. Positive a* values indicate red, while negative values indicate green. Positive b* values indicate

Table 1. Sous-vide treatment of the cooked chicken breast groups

Group	Time at 50°C (min)	Time at 60 °C (min)	Treatment Time Ratio 50 °C/60 °C	Total Treatment Time (min)
T1	0	120	0:1	120
T2	40	80	1:2	120
T3	60	60	1:1	120

yellow, while negative values indicate blue. The total colour difference (ΔE) was then calculated using Equation (3).

$$\Delta E = \sqrt{(\Delta L*)^2 + (\Delta a*)^{2+} (\Delta b*)^2} \quad (3)$$

2.4 Lipid oxidation

A slightly modified version of the thiobarbituric acid reactive substances (TBARS) method described by *Dias et al.* (2013) was used to measure lipid oxidation in chicken breast samples (*Dias et al.*, 2013). First, a 5-g portion of chicken breast was homogenized for 2 minutes with 20 mL of 5% trichloroacetic acid (TCA) and 0.5 mL of the antioxidant, butylated hydroxytoluene (BHT), using a Digital Ultra-Turrax homogenizer. The mixture was then centrifuged for 10 minutes, and the supernatant was filtered through No. 1 filter paper before being adjusted to 25 mL in volumetric flasks using 5% TCA. Next, 2 mL of the filtrate was combined with 2 mL of 0.08% thiobarbituric acid (TBA) in glass tubes, which were heated at 95 °C in a water bath for 30 minutes. After heating, the tubes were cooled and vortexed, and the absorbance was measured at 532 nm with a U 2900 UV-visible spectrophotometer (Hitachi Ltd., Tokyo, Japan) against a blank mixture of 5% TCA and 0.08% TBA. Finally, TBARS values, expressed as milligrams of malondialdehyde per kilogram (mg MDA/kg) of meat, were calculated from the average of triplicate measurements.

2.5 Odour acceptability

Under aseptic conditions, each sample was opened and its odour assessed according to the method described earlier (*Youssef et al.*, 2014). A five-point scale was used to rate odour acceptability, where 1 = acceptable, 2 = slightly acceptable, 3 = neither acceptable nor unacceptable, 4 = slightly unacceptable, and 5 = unacceptable. Five panellists conducted the evaluation.

2.6 Statistical analysis

The statistical analyses were conducted using IBM SPSS (version 27.0). Descriptive statistics and analysis of variance (ANOVA) were utilized to examine the effects of treatment, storage duration, and storage temperature on parameters such as moisture content, cooking loss, colour characteristics (L*, a*, b*, and ΔE), TBARS, and odour acceptability. Tukey's post hoc test was then applied to determine significant differences among groups. Standard deviations were displayed as error bars in bar charts created with Microsoft Excel 2021.

3. Results and Discussion

3.1 Moisture content and cooking loss

The moisture content results are presented in Figure 1, whereas cooking loss findings are shown in Figure 2.

According to the ANOVA and post-hoc Tukey's test, each treatment (T1, T2, and T3) differed significantly in moisture content (%) and cooking loss (%) ($p < 0.05$). Moreover, the double-step treatments (T3 and T2) had substantially lower cooking loss (%) and higher moisture content (%) than the single-step treatment (T1). These findings align with previous research on pork loin, turkey, and chicken breast, which showed that as cooking

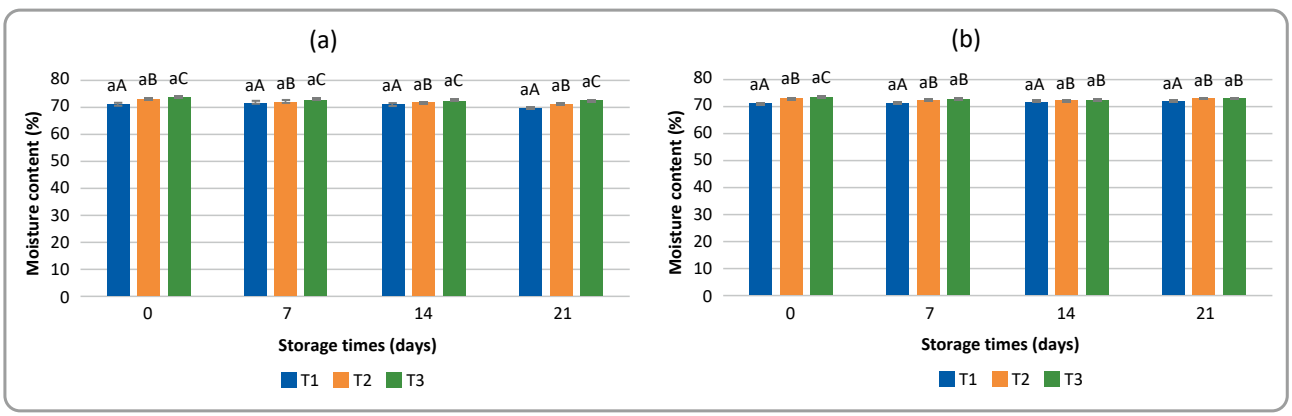

Figure 1. Moisture content (%) of sous-vide chicken breasts stored at (a) 4 °C and (b) 10 °C for up to 21 days, using T1 (60 °C/120 min), T2 (50 °C/40 min + 60 °C/80 min), and T3 (50 °C/60 min + 60 °C/60 min). With $p < 0.05$, lowercase letters (a, b, c) denote significant differences across storage days within the same treatment, while uppercase letters (A, B, C) denote significant differences among treatments within the same storage day.

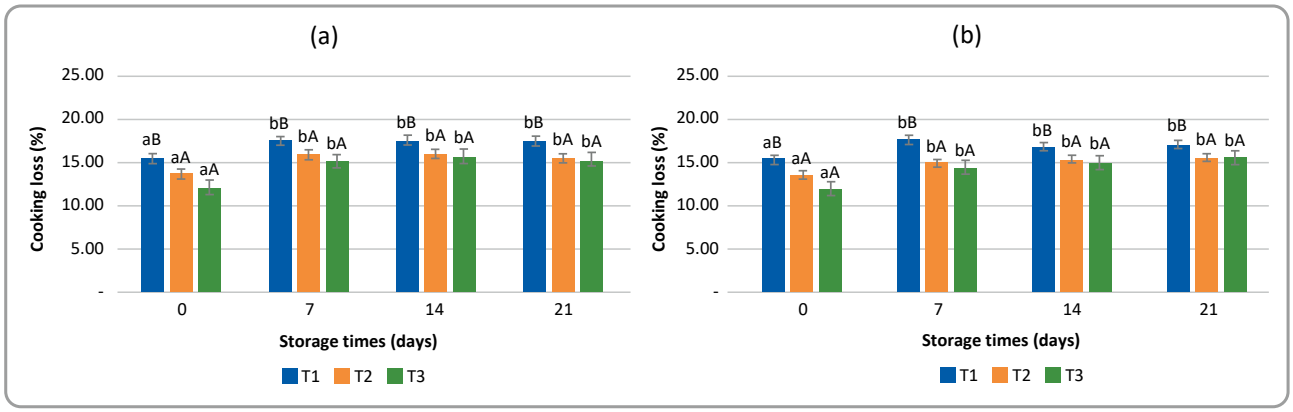

Figure 2. Cooking loss (%) of sous-vide chicken breasts stored at (a) 4 °C and (b) 10 °C for up to 21 days, using T1 (60 °C/120 min), T2 (50 °C/40 min + 60 °C/80 min), and T3 (50 °C/60 min + 60 °C/60 min). With $p < 0.05$, lowercase letters (a, b, c) denote significant differences across storage days within the same treatment, while uppercase letters (A, B, C) denote significant differences among treatments within the same storage day.

temperature increased, cooking loss rose while moisture content decreased (*Bıyıklı et al*., 2020; *Hasani et al*., 2022; *Hwang et al*., 2019). The moisture content of meat depends on the degree of thermal protein denaturation, as higher temperatures cause muscle fibres to shrink and expel water (*Offer et al*., 1984). Additionally, during cooking, collagen becomes soluble and forms a gel, increasing the meat's water content, which can lead to cooking loss when water and gelatinous material are expelled (*Zielbauer et al*., 2016). While moisture loss can concentrate nutrients, it may also result in the loss of heat-sensitive nutrients and liquids containing specific nutrients (*Ayub & Ahmad*, 2019). Moisture content plays a pivotal role in industries such as food processing, as it affects product quality, shelf life, and safety.

There was no significant difference in cooking loss (%) based on storage temperature ($p = 0.542$). However, a significant difference emerged in moisture content (%) ($p = 0.016$), with samples stored at 4 °C maintaining higher moisture content than those stored at 10 °C. While moisture content (%) did not differ significantly ($p > 0.05$) over the storage period, cooking loss (%) did ($p < 0.05$) at both 4 °C and 10 °C. The temperatures in both storage conditions varied notably between Day 0 and Days 7, 14, and 21. Previous work has shown that storage duration can influence chicken breast weight loss, with a significant increase observed after Day 9 (*Kaewthong et al*., 2019). During storage, meat can lose weight through evaporation — when water vapor escapes the surface — and through microbial activity, which may break down muscle fibres and cause moisture loss. Consequently, cooking loss can rise as the meat dries out. Storing meat at low temperatures (0–4 °C) can enhance water-holding capacity and minimize thawing-cooking loss (*Fernandes et al*., 2016). Furthermore, both storage temperature and duration — as well as microbial growth —c an affect the ability of meat myofibrils to retain water (*Zhang et al*., 2016).

3.2 Colour measurement

The colour properties of sous vide treated chicken breasts during chilled storage for 21 days are presented in Figure 3, 4, and 5.

Figure 3 shows the lightness (L*) values of the chicken breast samples. On Day 0, ANOVA with post hoc Tukey's test revealed significant differences ($p < 0.05$) among treatments, where single-step sous-vide (T1) exhibited higher L* values than the double-step methods (T2 and T3), likely due to myoglobin changes during denaturation at elevated temperatures (*Christensen et al.*, 2011). No significant differences ($p > 0.05$) were observed on Day 21 for samples stored at 4 °C after 14 days, nor from Day 7 to Day 21 for samples stored at 10 °C across all treatments (*Silva-Buzanello et al*., 2019). In terms of storage duration, significant differences ($p < 0.05$) began on Day 7 at 4 °C and Day 14 at 10 °C, with L* values decreasing over time, although storage temperature did not show a significant difference ($p = 0.054$) (*Pizato et al*., 2015). Additionally, there is a known correlation between cooking loss and lightness, suggesting multiple factors can affect the L* value of cooked chicken breast (*Saláková et al*., 2009).

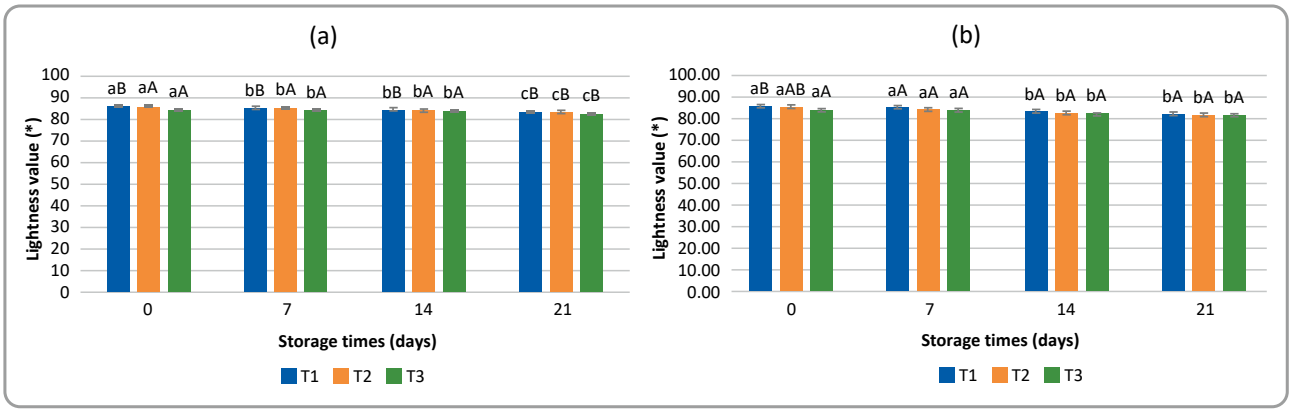

Figure 3. The lightness value (L*) of sous-vide chicken breasts stored at (a) 4 °C and (b) 10 °C for up to 21 days, using T1 (60 °C/120 min), T2 (50 °C/40 min + 60 °C/80 min), and T3 (50 °C/60 min + 60 °C/60 min). With $p < 0.05$, lowercase letters (a, b, c) denote significant differences across storage days within the same treatment, while uppercase letters (A, B, C) denote significant differences among treatments within the same storage day.

The redness (a*) measurements (Figure 4) indicate that on Day 0, T3 had a significantly higher redness value ($p < 0.05$) than T2 and T1, likely because T3 was cooked at 60 °C for a shorter time and 50 °C for a longer time (*Hunt et al.*, 1999). Myoglobin, which influences redness, begins denaturing between 55 °C and 65 °C, and is fully denatured at 80 °C (*Geileskey et al.*, 1998). These differences persisted until Day 14 at 4 °C and Day 7 at 10 °C, after which no significant differences ($p > 0.05$) were observed (*Hong et al.*, 2015). Notably, pink colouration may concern consumers who view it as undercooked or unsafe, and a pink threshold of a* = 3.8 has been established, which all samples in this study remained below (*Kaewthong et al.*, 2019). Significant differences ($p < 0.05$) in redness appeared during storage at 10 °C on Days 14 and 21, when the redness values declined (*Kaewthong et al.*, 2019). In contrast, no significant changes ($p > 0.05$) were seen for samples stored at 4 °C. Storage temperature also significantly influenced redness ($p = 0.003$), with samples at 4 °C exhibiting higher values than those at 10 °C. Similar to lightness, the reduction in redness is attributed to microbial activity that alters meat colour (*Pizato et al.*, 2015).

According to the ANOVA and Tukey's post-hoc test, a significant difference ($p < 0.05$) in yellowness (b*) was observed between the single-step sous-vide (T1) and double-step sous-vide (T2 and T3) treatments, with T2 and T3 recording higher b* values (*Hasani et al.*, 2022). This outcome is attributed to the reduced exposure of meat to 60 °C in the two-step methods, potentially leading to increased metmyoglobin concentrations (*Ayub & Ahmad*, 2019). Regarding storage temperature, there was no significant difference ($p = 0.309$) between 4 °C and 10 °C. However, for storage duration, a significant difference ($p <$

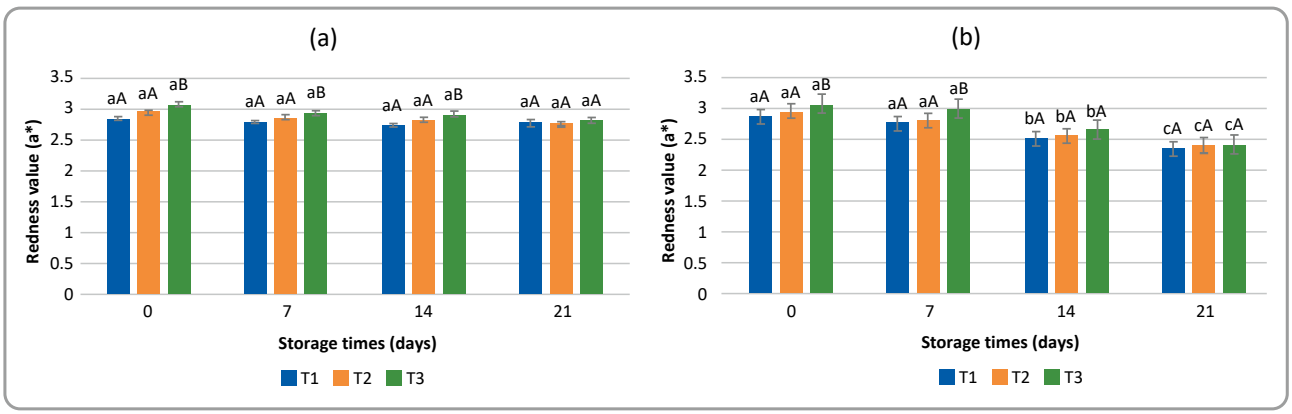

Figure 4. The redness value (a*) of sous-vide chicken breasts stored at (a) 4 °C and (b) 10 °C for up to 21 days, using T1 (60 °C/120 min), T2 (50 °C/40 min + 60 °C/80 min), and T3 (50 °C/60 min + 60 °C/60 min). With $p < 0.05$, lowercase letters (a, b, c) denote significant differences across storage days within the same treatment, while uppercase letters (A, B, C) denote significant differences among treatments within the same storage day.

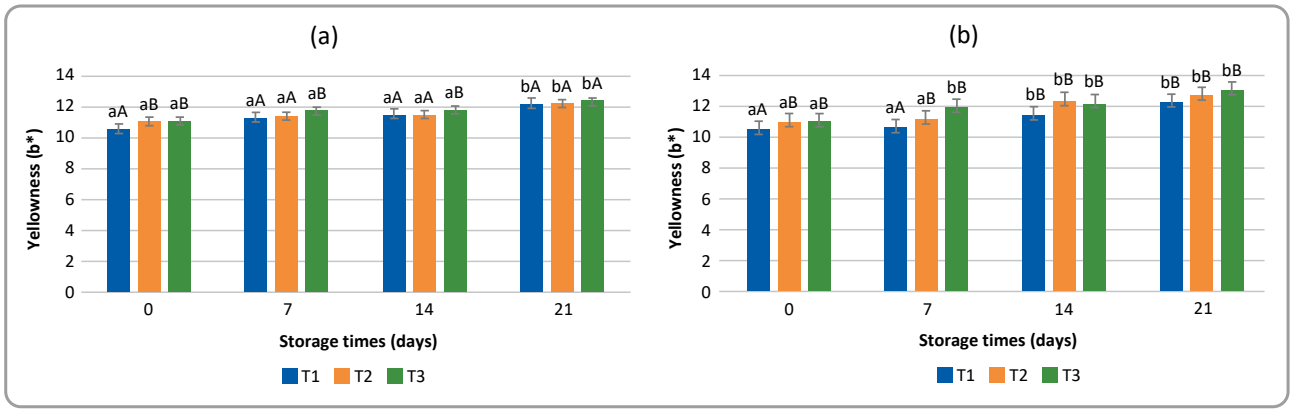

Figure 5. The yellowness value (b*) of sous-vide chicken breasts stored at (a) 4 °C and (b) 10 °C for up to 21 days, using T1 (60 °C/120 min), T2 (50 °C/40 min + 60 °C/80 min), and T3 (50 °C/60 min + 60 °C/60 min). With $p < 0.05$, lowercase letters (a, b, c) denote significant differences across storage days within the same treatment, while uppercase letters (A, B, C) denote significant differences among treatments within the same storage day.

0.05) emerged: yellowness began increasing on Day 21 at 4 °C and on Day 14 at 10 °C, which aligns with previous findings (*Pizato et al*., 2015). Discolouration during storage generally results from pigment oxidation in muscle tissue, the stability of which depends on species and muscle biochemistry (*Genot*, 2003).

Tables 2 and 3 show the total colour differences (ΔE) for chicken breasts stored at 4 °C and 10 °C. When E exceeds 3.5, a clear colour difference is perceived in meat products (*Tomasevic et al*., 2019). The comparison of storage temperatures reveals that category IV (indicating a clearly perceptible colour

Table 2. Total colour difference (ΔE) between chicken breasts cooked with different sous-vide treatments and stored for different durations at 4 °C.

Treatment		T1				T2				T3			
	Storage duration (days)	0	7	14	21	0	7	14	21	0	7	14	21
T1	0		I	II	III	I	II	III	III	II	III	III	IV
	7	0.99		I	II	I	I	II	III	I	II	II	III
	14	1.74	0.81		II	II	I	I	II	I	I	II	III
	21	3.01	2.05	1.27		III	II	I	I	II	I	I	II
T2	0	0.65	0.38	1.15	2.39		I	II	III	I	I	III	I
	7	1.17	0.20	0.68	1.89	0.53		II	III	I	II	II	III
	14	2.37	1.51	0.72	0.78	1.81	1.38		I	I	I	I	II
	21	3.18	2.23	1.44	0.19	2.57	2.07	0.90		II	I	I	I
T3	0	1.69	0.97	0.56	1.49	1.18	0.89	0.76	1.64		I	II	III
	7	2.29	1.37	0.60	0.74	1.69	1.22	0.32	0.90	0.77		I	II
	14	2.73	1.84	1.05	0.47	2.15	1.69	0.39	0.56	1.81	0.49		II
	21	3.95	3.03	2.23	1.02	3.36	2.88	1.59	0.83	2.32	1.67	1.22	

T1: 60 ◦C/120 min; T2: 50 ◦C /40 min and 60 ◦C/80 min; T3: 50 ◦C/60 min and 60 ◦C/60 min. The total colour difference (ΔE) categories using Roman numerals, where I ($0 < \Delta E < 1$) indicates no difference, II ($1 < \Delta E < 2$) indicates only experienced observers can detect a difference, III ($2 < \Delta E < 3.5$) indicates inexperienced observers can detect a difference, and IV ($3.5 < \Delta E < 5$) indicates a clear difference in colour is detectable.

Table 3. Total colour difference (ΔE) between chicken breasts cooked with different sous-vide treatments and stored for different durations at 10 °C.

Treatment		T1				T2				T3			
	Storage duration (days)	0	7	14	21	0	7	14	21	0	7	14	21
T1	0		I	III	IV	I	II	IV	IV	II	II	IV	IV
	7	0.78		II	III	I	I	II	IV	I	II	III	IV
	14	2.51	1.76		II	II	I	II	III	I	I	II	III
	21	4.12	3.38	1.62		IV	III	I	I	III	II	I	I
T2	0	0.65	0.54	1.98	3.56		II	III	IV	II	II	IV	IV
	7	1.60	0.87	0.93	2.54	1.06		III	III	I	I	III	III
	14	3.67	2.96	1.24	0.58	3.08	2.10		I	III	II	I	II
	21	4.60	3.88	2.12	0.54	4.02	3.02	0.94		III	III	I	I
T3	0	1.69	0.97	1.00	2.55	1.18	0.33	2.13	3.03		II	III	III
	7	2.48	1.84	0.73	1.82	1.86	0.99	1.29	2.22	1.03		II	III
	14	4.21	3.47	1.73	0.35	3.65	2.62	0.72	0.58	2.93	1.88		I
	21	4.93	4.22	2.48	0.92	4.34	3.36	1.26	0.39	3.37	2.52	0.93	

T1: 60 ◦C/120 min; T2: 50 ◦C /40 min and 60 ◦C/80 min; T3: 50 ◦C/60 min and 60 ◦C/60 min. The total colour difference (ΔE) categories using Roman numerals, where I ($0 < \Delta E < 1$) indicates no difference, II ($1 < \Delta E < 2$) indicates only experienced observers can detect a difference, III ($2 < \Delta E < 3.5$) indicates inexperienced observers can detect a difference, and IV ($3.5 < \Delta E < 5$) indicates a clear difference in colour is detectable.

difference) was observed more strongly at 4 °C than at 10 °C. The most pronounced colour difference was noted between Day 0 and Day 21 of storage. These results indicate that both storage temperature and duration influenced the colour characteristics of the sous-vide treatments.

3.3 Lipid Oxidation

Figure 6 present the findings of the TBARS analysis, used to assess lipid oxidation in sous-vide chicken breast, by measuring the concentration of secondary oxidation compounds.

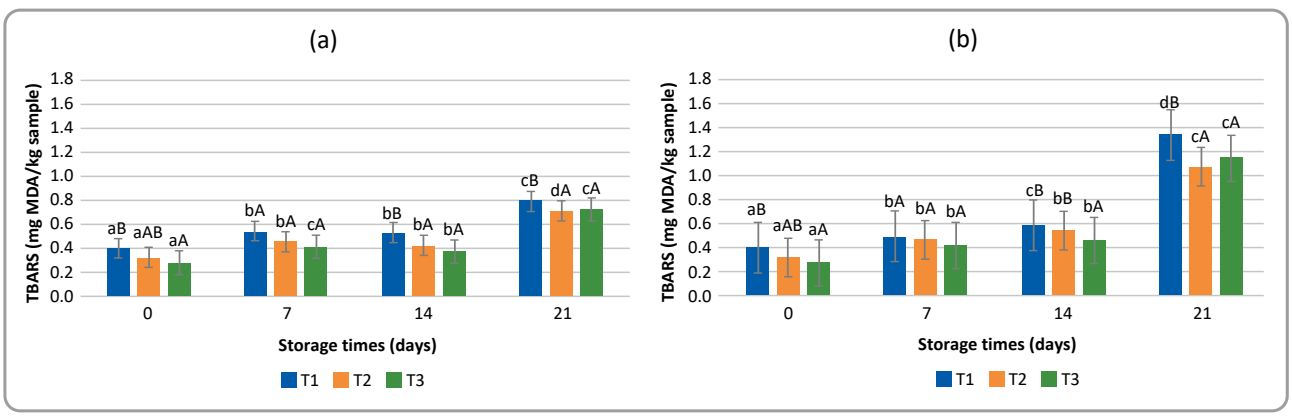

Figure 6. TBARS (mg MDA/kg sample) of sous-vide chicken breasts stored at (a) 4 °C and (b) 10 °C for up to 21 days, using T1 (60 °C/120 min), T2 (50 °C/40 min + 60 °C/80 min), and T3 (50 °C/60 min + 60 °C/60 min). With $p < 0.05$, lowercase letters (a, b, c) denote significant differences across storage days within the same treatment, while uppercase letters (A, B, C) denote significant differences among treatments within the same storage day.

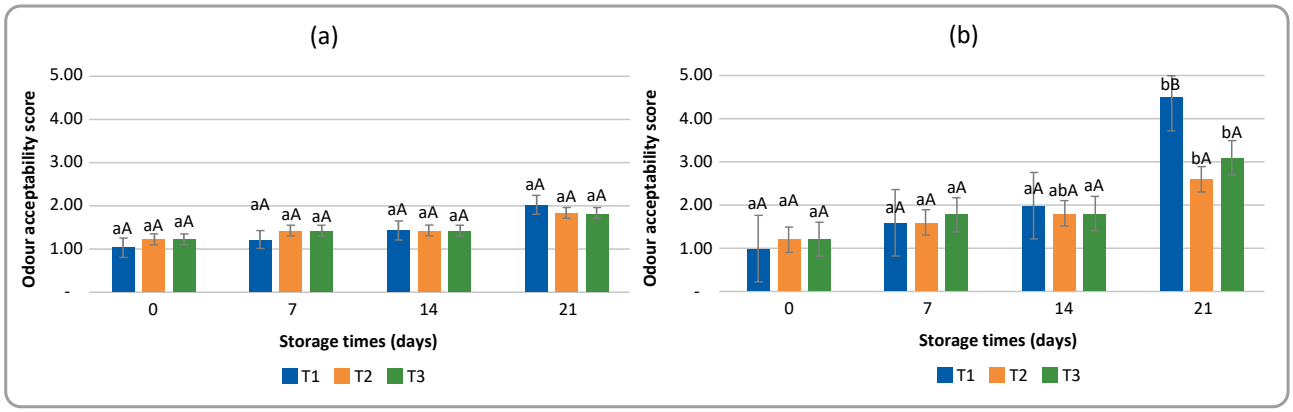

Figure 7. Odour acceptability score of sous-vide chicken breasts stored at (a) 4 °C and (b) 10 °C for up to 21 days, using T1 (60 °C/120 min), T2 (50 °C/40 min + 60 °C/80 min), and T3 (50 °C/60 min + 60 °C/60 min). With $p < 0.05$, lowercase letters (a, b, c) denote significant differences across storage days within the same treatment, while uppercase letters (A, B, C) denote significant differences among treatments within the same storage day.

ANOVA and post-hoc Tukey's analysis showed that on Day 0, the single-step treatment (T1) differed significantly ($p < 0.05$) from the double-step treatments (T2 and T3), with the double-step treatments exhibiting lower values (*Hasani et al.*, 2022; *Karpińska-Tymoszczyk et al.*, 2020). This difference may be attributed to higher cooking temperatures causing increased protein denaturation and the release of iron ions, which have potent pro-oxidant effects (*Ganhão et al.*, 2011). Additionally, cooking meat can accelerate lipid oxidation while deactivating antioxidant compounds or enzymes (*Hong et al.*, 2015). Regarding storage temperature, there was a significant difference ($p = 0.044$) between 4 °C and 10 °C, with higher values observed at 10 °C. Lipid oxidation also rose significantly over time at both temperatures ($p < 0.05$), possibly because early oxidation forms hydroperoxides that convert into secondary products, like ketones and aldehydes. Changes in fatty acid and amino acid compositions persisted until the fourth week, underscoring the influence of storage temperature and duration on lipid oxidation (Liu et al., 2019). TBARS values quantify specifically malonaldehyde, which significantly contributes to aroma degradation in stored meat products.

3.4 Odour Acceptability

The results of odour acceptability scoring are shown in Figures 7. This standardized assessment facilitated an objective evaluation of the chicken's odour, enabling conclusions about the product's sensory characteristics and potential consumer appeal.

According to the ANOVA and post-hoc Tukey's test, no significant differences ($p > 0.05$) were observed among the three treatments (T1, T2, and T3), and all remained acceptable until Day 14 of storage. However, on Day 21 at 10 °C, an unpleasant odour was detected ($p < 0.05$), possibly resulting from the formation of secondary lipid oxidation products exceeding the sensory threshold limit of 1 mg MDA/kg sample (*Akoğlu et al.*, 2018).

4. Conclusion

This study highlights the effectiveness of both single-step (60 °C/120 min) and double-step (50 °C/60 °C) sous-vide cooking methods in producing high-quality chicken breast, with the two-step treatments exhibiting notably lower cooking loss and higher moisture content. Over a 21-day storage period, the product's colour, lipid oxidation, and odour acceptability were influenced by storage temperature (4 °C vs. 10 °C) and duration. While lipid oxidation and discolouration increased at the higher storage temperature, 4 °C generally helped maintain better sensory attributes. Nevertheless, by Day 21 at 10 °C, even the double-step treatments showed diminished odour acceptability, likely due to secondary lipid oxidation exceeding sensorial thresholds. Overall, the findings underscore the potential benefits of double-step sous-vide processing and emphasize the importance of controlling storage temperature and duration to preserve product quality and extend shelf life.

Nova dvostepena sous-vide tehnika: Inovativna strategija za očuvanje kvaliteta i svežine pilećih grudi

Endrit Hasani, Masagus Haidir Tamimi, István Dalmadi i György Kenesei

INFORMACIJE O RADU

Ključne reči:
Pileće grudi
Dvostepeni sous-vide postupak
Rok trajanja
Smanjenje otpada hrane
Održiva prerada živinskog mesa

APSTRAKT

Ova studija ispitivala je efekte jednostepenog i dvostepenog sous-vide tretmana na kvalitet pilećih grudi tokom različitih perioda skladištenja (0, 7, 14 i 21 dan) i pri različitim temperaturama skladištenja (4 °C i 10 °C). Mišići pectoralis major pilića vakuumski su pakovani i termički obrađeni ili na 60 °C tokom 120 minuta (jednostepeni postupak) ili primenom dvostepenog temperaturnog profila (50 °C, zatim 60 °C) uz jednako ukupno vreme termičke obrade. Analizirani su parametri kvaliteta uključujući sadržaj vlage, gubitak mase tokom kuvanja, boju (CIELAB), lipidnu oksidaciju (supstance reaktivne na tiobarbiturnu kiselinu; TBARS) i prihvatljivost mirisa. Dvostepeni postupak pokazao je značajno bolje zadržavanje vlage i manji gubitak mase tokom kuvanja u poređenju sa jednostepenim postupkom, verovatno usled manjeg skupljanja mišićnih vlakana i bolje rastvorljivosti proteina. Skladištenje na 4 °C omogućilo je bolju stabilnost boje, niži nivo lipidne oksidacije i bolje ocene mirisa u poređenju sa skladištenjem na 10 °C. Do 21. dana skladištenja na 10 °C, vrednosti TBARS-a premašile su senzorni prag, što je dovelo do pojave neprijatnih mirisa i smanjene prihvatljivosti proizvoda. Sa aspekta održivosti, dvostepeni sous-vide pristup može doprineti smanjenju otpada hrane produženjem roka trajanja i očuvanjem kvaliteta proizvoda tokom dužeg perioda distribucije. Ovakav kontrolisani temperaturni profil sous-vide obrade može takođe poboljšati energetsku efikasnost u poređenju sa produženim jednostepenim kuvanjem na visokoj temperaturi. Usklađivanje naprednih metoda termičke obrade sa optimalnim uslovima skladištenja na taj način podržava održiviju proizvodnju živinskog mesa.

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Original Scientific Paper

Salt reduction possibility in marinated chicken meat

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ABSTRACT

In this study, the possibility of NaCl reduction and its partial replacement with KCl in marinated, grilled chicken thighs was investigated. In the control group, 20 g of NaCl per kg of meat was added to the marinade, while in experimental group 1, that amount of NaCl was reduced by 25%. In experimental group 2, one-third of the NaCl was replaced with KCl; in group 3, one-half (both related to the control group); and in group 4, one-third of the NaCl was replaced with KCl, as related to group 1. Sensory properties, the sodium content and the sodium/potassium ratio were analysed for the grilled chicken thighs. Sensory evaluation was performed by ten trained assessors using numerical-descriptive scales. Sensory evaluation showed the mean intensity of saltiness score was statistically significantly lower ($p < 0.1$) in the chicken thighs from experimental group 4 compared with the control group and experimental groups 1 and 2. The mean overall acceptability score of the chicken thighs from experimental group 3 was lower than the scores of the control and groups 1 and 2 ($p < 0.01$), as well as lower than the score of group 4 ($p < 0.05$).

1. Introduction

Salt intake in modern human nutrition significantly exceeds physiological needs, which is why excessive sodium intake is now causing serious health problems. With the development of technology, salt no longer has a primary role in food preservation; instead, salt consumption has increased alongside the growth of industrial food production.

Salt has been identified as the most significant cause of essential hypertension, which is one of the main risk factors for the development of cardiovascular and kidney diseases (*O'Donnell et al.,* 2012; *Laranjo et al,* 2017*)*. Excessive salt intake leads to an increased risk of heart attack (*Perry & Beevers,* 1992), indirectly contributes to weight gain *(Feng et al.,* 2010), and increases the risk of *Helicobacter pylori* infection and the development of stomach cancer (*Tsugane et al.,* 2004).

The sodium requirement for basal metabolism in humans is 2,000 mg per day. However, the mean daily sodium intake is much higher, ranging from 3,500 to 5,000 mg per day, which corresponds to 9–12 g of table salt consumed. The American Heart Association recommends that individuals with high blood pressure limit their daily sodium intake to no more than 1,500 mg, and people with heart disease to no more than 1,000 mg per day. The intake of table salt depends not only on physiological needs but also on habits acquired in early childhood and dietary traditions. Approximately 20% of the total daily salt consumed through food originates from meat products (*Wirth,* 1991).

The salt content in meat products primarily depends on technologically justified amounts and their impact on perceived saltiness. Meat products' salt content can be reduced by lowering the amount of added sodium chloride (NaCl) (*Sofos,* 1983),

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by substituting part of the NaCl with other salts (*Sofos*, 1983; *Guàrdia et al.,* 2006), by using flavour enhancers and masking agents (*Desmond,* 2006), or through a combination of these approaches (*Sofos,* 1983). Potassium chloride (KCl) is the most commonly used substitute for NaCl; however, according to numerous studies, complete substitution is not possible, as it leads to an increase in bitter taste and a reduction in perceived saltiness (*Lilić et al.* 2012; *Soglia* 2014; *Lilić et al.* 2016; *Borović et al.* 2021).

In recent decades, production and consumption of chicken meat have been increasing, which can be attributed to several factors; rapid feed conversion compared to other animal species, favourable health characteristics (low fat content and high protein proportion), pleasant sensory qualities, affordable price, and a short rearing period, i.e., a fast reproductive cycle. To enhance its sensory properties, chicken meat is often marinated. Marinades are flavoured mixtures of spices, NaCl and other ingredients used to enhance the flavour, texture, colour, and tenderness of meat and meat products (*Lyon et al.* 2005; *Gómez et al.* 2019; *Latoch et al.* 2020; *Lopes et al.* 2022; *Rahman et al*. 2023).

The aim of this study was to investigate the possibility of reducing NaCl and substituting it with KCl in marinated chicken thighs, as well as the impact on the sensory properties of the grilled meat product.

2. Materials and Methods

Within the study, five experimental groups were formed—marinated boneless chicken thighs with different amounts of added NaCl, with the aim of reducing the total amount of NaCl, i.e., sodium. The marinade was prepared from the following ingredients: sunflower oil, garlic, lemon juice, mustard, acacia honey, NaCl, and KCl, in the amounts shown in Table 1. In the control group (C), only NaCl was added to the marinade, at 20 g per kilogram of thigh meat, while in group 1, 15 g of NaCl were added per kilogram of meat. In group 2, one-third of the NaCl was substituted with KCl relative to the control group; in group 3, half of the NaCl was substituted with KCl relative to the control group; and in group 4, one-third of the NaCl was substituted with KCl relative to group 1. Marination lasted for 24 hours, after which the marinated chicken thighs were thermally processed by grilling on an electric grill with non-stick ribbed plates and were evaluated immediately after heat treatment by 10 trained assessors.

2.1. Sensory evaluation

In a laboratory equipped in accordance with the SRPS EN ISO 8589:2015 (*Serbia*, 2015) standard, marinated, grilled chicken thighs from the experimental groups were sensory evaluated by ten trained assessors. The cooked chicken thighs were presented to the assessors in individual booths, while optimal working conditions were maintained in terms of temperature, air humidity, and lighting (*Baltić,* 1994). The following sensory attributes were evaluated: acceptability of colour on the surface and on the cut section, consistency, acceptability of saltiness, intensity of saltiness, intensity of bitter taste, and the overall acceptability of the product.

2.2. Determination of sodium and potassium content

Aliquots of approximately 0.5 g of homogenized chicken thigh were transferred into Teflon vessels, and 5 mL of nitric acid (p.a., Sigma) and 1.5 mL of hydrogen peroxide (30%, p.a., Merck) were added. The microwave digestion program consisted of three steps: 5 min from room temperature to 180 °C, 10 min hold at 180 °C, and 20 min venting. After cooling at

Table 1. Product composition (g)

Group	Chicken thighs, boneless	Sodium chloride	Potassium chloride	Lemon juice	Honey	Mustard	Garlic	Oil
C	1000	20		22	15	4.5	2	90
1	1000	15		22	15	4.5	2	90
2	1000	13.3	6.7	22	15	4.5	2	90
3	1000	10	10	22	15	4.5	2	90
4	1000	10	5	22	15	4.5	2	90

room temperature, the digested solutions were quantitatively transferred into disposable flasks and diluted to 100 mL with deionized water (Elga). The analysis was performed using inductively coupled plasma mass spectrometry (ICP-MS). Measurements were carried out using an iCap Q (Thermo Scientific, Bremen, Germany), equipped with a collision cell and operating in kinetic energy discrimination (KED) mode. The isotopes ^{23}Na and ^{39}K were measured. Torch position, ion optics, and detector settings were adjusted daily using a tuning solution (Thermo Scientific Tune B) to optimize measurements and minimize possible interferences. For quantitative analysis, a five-point calibration curve (including zero) was constructed for each isotope in the concentration range of 0.1–2.0 mg/L. An additional line of the peristaltic pump was used for online introduction of a multi-element internal standard (^{6}Li, ^{45}Sc – 10 ng/mL; ^{71}Ga, ^{89}Y, ^{209}Bi – 2 ng/mL), covering a wide mass range. Concentrations of each measured isotope were corrected for response factors of both higher and lower mass internal standards by interpolation. Mean scores and analytical results are presented below.

3. Results and Discussion

The results of the sensory evaluation of marinated chicken thighs are presented in Table 2. Samples from all examined groups were sensorially acceptable. Grilled chicken thighs from all groups had uniform colour acceptability and consistency, with no statistically significant differences between the scores ($p > 0.05$).

The presence of a bitter taste was detected only in group 3 chicken thighs. The intensity of the bitter taste differed significantly between the control and group 3, as well as between experimental groups 1 and 3 ($p < 0.01$). Bitter taste intensity also significantly differed between groups 2 and 3, as well as between groups 3 and 4 ($p < 0.05$). The obtained results are in agreement with the studies of many authors (*Desmond* 2006; *Ruuseunen & Puolanne* 2005*, Zanardi et al.* 2010), who stated that an increase in the KCl content in foods can result in bitter and metallic tastes.

The intensity of saltiness was directly influenced by the amount of added NaCl. The highest saltiness intensity (4.70 ± 0.90), observed in the control group, was statistically significantly higher ($p < 0.01$) that that of experimental groups 1, 3, and 4 (3.40 ± 0.66; 3.35 ± 0.10; and 2.80 ± 0.75, respectively). Statistically significant differences ($p < 0.01$) in saltiness intensity were also observed between experimental groups 2 and 4 (4.10 ± 0.70 and 2.80 ± 0.75).

The highest scores for saltiness acceptability were achieved by the control and experimental groups 1 and 2 (4.40 ± 0.92, 4.10 ± 0.54, and 4.30 ± 0.64, respectively), with no statistically significant differences between these groups ($p > 0.05$). However, compared with the control, in group 2, in which one-third of the NaCl was replaced by KCl, there was no significant change in saltiness, although a bitter taste was noted. Lower saltiness acceptability was determined in group 4 chicken thighs (mean of 3.15 ± 1.34), which was significantly lower than that observed in the control and experimental group 2 ($p < 0.05$). The least acceptable salty taste, due to the notable bitter taste, was observed in group 3 chicken thighs, in which one-half of the NaCl had been replaced with KCl—assessors scored this group as 2.70 ± 0.60, which was significantly lower ($p < 0.01$) than the scores for the control and experimental groups 1 and 2.

The scores for overall acceptability were very similar to those for saltiness acceptability, with no statistically significant differences ($p > 0.05$) between the mean scores of the control, and experimental groups 1, 2, and 4. The mean overall acceptability score only of the group 3 chicken thighs was significantly lower than those for the control and experimental groups 1 and 2 ($p < 0.01$), as well as lower than that for group 4 ($p < 0.05$).

Despite statistically significant differences in the intensity of saltiness, saltiness acceptability, and the appearance of a bitter taste, all evaluated chicken thighs were sensorially acceptable.

Similar studies were conducted by *Lee et al.* (2012), who used only NaCl and KCl in different ratios for marinating chicken breasts and found that substitution of up to 50% of the NaCl with KCl is possible without inducing significant changes in the sensory characteristics of the product. *Cavalho et al.* (2013) investigated the possibility of substituting NaCl with KCl at levels of 25% and 50% in marinated beef and chicken. For marination, in addition to sodium and potassium salts, they used a mixture of garlic, anise, turmeric, chili, ground pepper, oregano, and thyme. They also found that substitution of up to 50% of the NaCl with KCl was possible.

The results obtained in the current study were partially in accordance with the findings of *Lee et al.* (2012) and *Cavalho et al.* (2013), who showed that in marinated chicken fillet, up to 30% of the NaCl can be substituted with KCl without significant changes in sensory characteristics. In our research though, substituting 50% of the NaCl with KCl

Table 2. Sensory analyses of marinated, grilled chicken thighs, n=10

	Group				
	C	**1**	**2**	**3**	**4**
Saltiness acceptability					
X±SD	$4.40\pm0.92^{x,a}$	4.10 ± 0.54^{x}	$4.30\pm0.64^{x,a}$	2.70 ± 0.60^{y}	3.15 ± 1.34^{b}
Coefficient of variation	20,83	13.13	14.89	22.22	42.62
Range of variation	2.00–5.00	3.00–5.00	3.00–5.00	2.00–3.50	1.00–5.00
Saltiness intensity					
X±SD	4.70 ± 0.90^{x}	3.40 ± 0.66^{y}	4.10 ± 0.70^{x}	3.35 ± 1.00^{y}	2.80 ± 0.75^{y}
Coefficient of variation	19.15	19.51	17.07	29,89	26.73
Range of variation	2.00–5.00	2.00–4.00	3.00–5.00	2.00–5.00	2.00–4.00
Bitterness intensity					
X±SD	1.20 ± 0.60^{x}	1.20 ± 0.60^{x}	1.80 ± 0.60^{a}	$2.75\pm0.64^{y,b}$	1.70 ± 0.90^{a}
Coefficient of variation	50,00	50.00	33.33	23.35	52.94
Range of variation	1.00–3.00	1.00–3.00	1.00–3.00	2.00–3.50	1.00–4.00
Overall acceptability					
X±SD	4.40 ± 0.92^{x}	4.55 ± 0.47^{x}	4.20 ± 0.40^{x}	$3.20\pm0.33^{y,a}$	4.15 ± 0.55^{b}
Coefficient of variation	20.83	10.37	9.52	10.36	13.25
Range of variation	2.00–5.00	4.0–5.00	4.00–5.00	3.00–4.00	3.00–5.00

Legend: a, b Statistically significant difference ($p < 0.05$); x, y Statistically significant difference ($p < 0.01$)

led to changes in sensory characteristics, primarily in saltiness and the appearance of a bitter taste, that were in accordance with reports by *Ruusunen & Poulanne* (2005), *Desmond* (2006), and *Petracci et al.* (2013).

The sodium and potassium contents and their ratios in the grilled chicken thighs are presented in Table 3. The highest sodium content was determined in the control group, corresponding to the highest amount of added NaCl, while the lowest was found in experimental groups 2 and 4, in which the amount of NaCl was half that of the control.

The highest potassium content was found in group 3, in which 50% of the NaCl was replaced with KCl, followed by group 2, in which one-third of the NaCl was replaced with KCl. The sodium-to-potassium ratio was the highest in the control chicken thighs and the lowest in group 2 chicken thighs.

The recommended daily intake of sodium (*Dietary Guidelines for Americans*, 2005) is 2,300 mg. The recommended dietary intake of potassium is 4,700 mg per day for adolescents, 3,000 mg per day for children aged 1–3 years, 3,800 mg per day for children aged 4–8 years, and 4,500 mg per day

Table 3. Sodium and potassium content and the sodium/potassium ratio in grilled chicken thighs after marination

Group	Sodium, mg/100 g	Potassium, mg/100 g	Sodium/Potassium Ratio
C	1820	440	4.14
1	1190	430	2.77
2	1067	800	1.33
3	960	1150	0.83
4	960	890	1.07

for children aged 9–13 years. The optimal sodium-to-potassium ratio for healthy adults is 0.49, along with a recommendation to consume fruits and vegetables rich in potassium. In this study, the best sodium-to-potassium ratio was achieved in the chicken thighs in which 50% of the NaCl was replaced with KCl, but even this ratio was still considerably higher than the recommended ratio. A slightly higher ratio was observed in the chicken thighs from the other experimental groups, corresponding to the amount of added NaCl.

4. Conclusion

Reducing the salt content in the marinade by replacing NaCl with KCl did not affect the colour or consistency of marinated, grilled chicken thighs. Although differences were observed among the examined groups in terms of saltiness intensity, saltiness acceptability, and the occurrence of a bitter taste, all chicken thighs were nevertheless considered acceptable with regard to their sensory properties. The sensory evaluation scores for overall acceptability were very similar to those for saltiness acceptability, and no statistically significant differences were observed among the mean scores of the control and experimental groups 1, 2, and 4. In contrast, the mean overall acceptability score of the group 3 chicken thighs was significantly lower than those of the control and experimental groups 1 and 2, as well as lower than that of experimental group 4.

The production of marinated chicken thighs with reduced NaCl/sodium content, and without significant sensory changes, is possible by reducing the amount of NaCl added as well as by partially replacing some of the NaCl with KCl, achieving more favourable sodium-potassium ratios according to health recommendations.

Mogućnost redukcije sadržaja soli u mariniranom pilećem mesu

Branka Borović, Vesna Đorđević, Slobodan Lilić, Ivana Branković Lazić, Ognjen Krnjaja, Aleksandar Bajčić i Nikola Betić

INFORMACIJE O RADU

Ključne reči:
Pileći batak
Marinada
Redukcija sadržaja soli

APSTRAKT

U ovoj studiji ispitivana je mogućnost smanjenja sadržaja natrijum-hlorida (NaCl) i njegove delimične zamene kalijum-hloridom (KCl) u mariniranim pilećim batacima sa karabatakom. Analizirane su senzorne osobine, kao i sadržaj natrijuma i odnos natrijuma i kalijuma. Senzorna ocena izvršena je od strane deset obučenih ocenjivača primenom numeričko-deskriptivnih skala. U kontrolnoj grupi u marinadu je dodato 20 g NaCl po kilogramu mesa, dok je u prvoj eksperimentalnoj grupi količina NaCl smanjena za 25%. U drugoj grupi jedna trećina natrijum-hlorida zamenjena je kalijum-hloridom, a u trećoj grupi jedna polovina (u oba slučaja u odnosu na kontrolnu grupu). U četvrtoj grupi jedna trećina natrijum-hlorida zamenjena je kalijum-hloridom u odnosu na prvu grupu. Intenzitet slanosti bio je statistički značajno niži ($p < 0,1$) kod uzoraka četvrte eksperimentalne grupe u odnosu na kontrolnu, prvu i drugu grupu. Ocena ukupne prihvatljivosti uzoraka iz treće eksperimentalne grupe bila je niža od ocena kontrolne, prve i druge grupe ($p < 0,01$), kao i od ocene četvrte grupe ($p < 0,05$).

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Original Scientific Paper

New autochthonous bacterial isolates: probiotic potential and microplastic biodegradation performance

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ABSTRACT

Contemporary research prioritizes bacterial consortia that combine probiotic efficacy with environmental detoxification. From this screening, two novel autochthonous isolates — *Hafnia alvei* UUNT_MP41 and *Hafnia paralvei* UUNT_MP29 — were selected for their robust probiotic phenotypes and potential to catalyze microplastic biodegradation. Whole genome sequencing (WGS) identified critical functional genetic elements, the tesA and lipR genes, both esterase homologs (of PpEst and EstC9) that have been implicated in polymer breakdown. These findings validate the candidacy of *Hafnia alvei* UUNT_MP41 and *Hafnia paralvei* UUNT_MP29 as next-generation, dual-function probiotics, establishing a new paradigm for biotic formulations that offer simultaneous therapeutic and ecological benefits.

1. Introduction

Modern biotechnology is increasingly focused on discovering novel microbial strains that offer a dual benefit: therapeutic potential as probiotics and robust capabilities for environmental bioremediation. The gastrointestinal tract (GIT) of aquatic species, such hinge in global food supplies, the stability and composition of the fish gut microbiome directly influence disease mitigation and optimal growth (*De Schryver and Vadstein,* 2014; *Li et al.,* 2017). Consequently, isolating beneficial autochthonous strains, such as those derived from the common carp (*Cyprinus carpio*), is paramount for formulating effective probiotic supplements (*Kamada et al.,* 2013). Simultaneously, the planet contends with a growing environmental catastrophe caused by plastic accumulation. Microplastics (MPs), defined as fragments under 5 mm, are pervasive contaminants in all ecosystems, particularly in aquatic environments (*Andrady,* 2011). Their consumption by marine and freshwater fauna leads to trophic transfer through the food web, creating serious public health concerns (*Mato et al.,* 2001*; Browne et al.,* 2008). Given that conventional physical and chemical cleanup methods are often inefficient and ecologically detrimental, there is a compelling need for sustainable, biological alternatives, notably microbial degradation (*Othman et al.,* 2021). This convergence of environmental and health crises has spurred the development of dual-function microorganisms designed to provide both host benefits and ecological utility. Accordingly, this investigation aimed to exploit the rich microbial diversity of the *C. carpio* intestine to characterize two distinct *Hafnia* isolates. These strains were selected based on their robust probiotic attributes and genomic evidence specifically, the detection of genes homologous to known esterases (*PpEst,*

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EstC9) suggesting capacity for polymer breakdown. Our investigation thoroughly details the genomic and functional profile of these isolates, providing essential data for establishing a pioneering class of biotic formulations with simultaneous therapeutic and ecological relevance (*Savini et al*., 2008).

2. Materials and Methods

2.1. Identification and Isolation of Indigenous Microbiota from the Cyprinus carpio Digestive Tract

Bacterial strains were previously isolated from *C. carpio* specimens obtained at the DTD fish market in Serbia (*Dragacevic et al,* 2025). including probiotic strains, is a crucial task in today's world to expand the genetic data pool and identify new genes. In this study, we investigated the gut microbiota of one industrial species, Cyprinus carpio, and identified representatives of various microbial genera, including Citrobacter, Serratia, Bacillus, Enterococcus, and Kocuria. Notably, we discovered two strains of Hafnia with potentially probiotic properties. We conducted next-generation sequencing (NGSTotal genomic DNA, extracted with the GeneJet Purification Kit (Thermo Scientific), was quantified using a NanoDrop 2000c and verified for integrity through gel electrophoresis. Sequencing was carried out on an Illumina 2 × 250 bp platform. Phylogenetic analysis was subsequently conducted using the Type (Strain) Genome Server (TYGS) (*Meier-Kolthoff & Göker,* 2019; *Meier-Kolthoff et al.,* 2022). To ensure rigorous technical reproducibility, genomic DNA was extracted in triplicate for each strain. The samples then underwent 16S rRNA gene sequencing to confirm identity, followed by comprehensive whole-genome next-generation sequencing (NGS) for in-depth genomic profiling (*Martín and Langella*, 2019).

The three-dimensional structures of selected *Hafnia*-derived enzyme candidates were predicted using AlphaFold2 implemented through ColabFold (*Mirdita et al*., 2022).

To explore their biodegradation potential, a local sequence similarity search was performed using BLASTp (*Altschul et al*., 1990). Annotated proteins from the *Hafnia* genomes were queried against the Plastic Microbial Biodegradation Database (PMBD) (*Gan and Zhang*, 2019). Domain prediction was conducted using the InterPro database (*Blum et al*., 2025).

3. Results

From the gut of healthy *C. carpio*, 53 bacterial strains were isolated and identified through 16S rRNA sequencing.

Among the 53 strains, six genera were identified in the GIT of *C. carpio*. Out of the 46 initially isolated strains, 46 were prioritized for investigation, while seven isolates — specifically those belonging to the genera *Citrobacter* (n=4) and *Serratia* (n=3) — were excluded from further downstream analysis. These 46 microorganisms were selected based on their potential as next-generation probiotics (NGPs), a field of growing significance in contemporary biotechnology (*Lalowski and Zielińska*, 2024).

Based on taxonomic classification via Kraken analysis, strain UUNT_MP29 was identified as *Hafnia paralvei*, while strain UUNT_MP41 showed the highest sequence homology with *Hafnia alvei*. Given their potential as NGPs, both strains underwent rigorous genomic characterization. The resulting assembled genomes were deposited in the NCBI database (*Wood et al.,* 2019*; Sayers et al.,* 2022) under BioProject accessions PRJNA1185451 (UUNT_MP41) and PRJNA1185452 (UUNT_MP29), with corresponding BioSample entries SAMN44705350 and SAMN44705351.

Despite only moderate sequence identity, the enzyme structures predicted by AlphaFold2 showed strong conformational similarity to known PBAT-depolymerases. These results indicated that the enzymes are structurally equivalent (fold-equivalent) to previously characterized PBAT-degrading esterases, thereby identifying the two *Hafnia* strains as potentially bifunctional microorganisms suitable for both probiotic applications and bioremediation of biodegradable plastics.

Analysis of the biodegradation potential of annotated *Hafnia* proteins identified two particularly promising candidates: TesA and LipR. Both sequences displayed significant homology to well-characterized plastic-degrading enzymes, namely PpEst (UniProt: AMW89397.1) from *Pseudomonas pseudoalcaligenes* and EstC9 (UniProt: AOR05752.1) from a *Sphagnum*-associated microbiome, both of which have been experimentally validated for PBAT hydrolysis (*Wallace et al*., 2017; *Müller et al*., 2017).

Sequence comparison revealed that TesA exhibits very high identity (>86%) across the two different *Hafnia* strains and other *Hafnia* in the database., while its similarity to the *P. pseudoalcaligenes* ortholog remains moderate (~45%). Domain prediction

confirmed the presence of an SGNH hydrolase-type esterase domain (IPR013830) in TesA and an alpha/beta hydrolase fold-3 domain in LipR.

Taken together, the sequence homology, conserved catalytic domains, and high structural similarity to validated PBAT-depolymerases strongly support the potential of TesA and LipR from *Hafnia* strains to be involved in PBAT biodegradation.

4. Discussion

A central finding was the identification of TesA and LipR orthologs putatively involved in the depolymerization of PBAT-type plastics. The discovery of these biodegradation pathways in gut-associated *Hafnia* strains is particularly significant, as recent literature increasingly recognizes the teleost gastrointestinal tract as a "hotspot" for specialized enzymes capable of degrading recalcitrant microplastic polymers (*Talwar et al*., 2018). The coexistence of these degradative capabilities within bacteria that can withstand the harsh conditions of the GIT positions these isolates as potential "mobile bioremediation units" (*Kutralam-Muniasamy et al.*, 2023).

Moreover, the physiological relevance of these bacteria likely extends beyond aquaculture. The gastrointestinal environment of freshwater fish shares fundamental functional similarities with the human GIT, including comparable enzymatic niches and high microbial density (*Rawls et al*., 2006). The metabolic robustness of these *Hafnia* strains under such conditions suggests broad adaptability across diverse vertebrate digestive systems. By maintaining metabolic activity during transit, these strains could reduce microplastic bioaccumulation in the host while simultaneously providing probiotic benefits (*Yadav et al*., 2025). These findings establish a compelling paradigm for developing multi-functional biotic formulations that address concurrent clinical and ecological challenges through targeted genomic selection.

4.1 Future Perspectives and Strategic Directions

The identification of *Hafnia* strains with plastic-degrading potential opens several high-impact research avenues. A key trend in this field is the "safe-by-design" approach enabled by precision genome editing. Although environmental bacteria isolates often carry complex genetic profiles, the application of CRISPR-Cas9 or base-editing technologies could selectively remove undesirable elements, such as antibiotic resistance genes, thereby creating safe microbial chassis for bioremediation or probiotic use without contributing to the global resistome crisis (*Dudeja et al*., 2025).

Future deployment of suitable bacteria should be evaluated within the One Health framework. Investigations should focus on how plastic-degrading probiotics can reduce microplastic bioavailability across the food chain. By mitigating polymer accumulation at the primary consumer level in aquaculture, these bacteria could protect both ecosystem health and human consumers from trophic transfer of contaminants (*Bora et al*., 2024).

Future efforts will focus on rigorous safety-by-design assessments, including detailed functional validation of the biodegradative loci and targeted removal or inactivation of any antibiotic resistance determinants. By advancing from in silico genomic insights to in vivo and field-based evaluations in aquaculture settings, these *Hafnia* strains hold the potential to pioneer a new class of multifunctional probiotics that actively contribute to both animal welfare and global microplastic bioremediation strategies.

5. Conclusion

In summary, this research successfully identified 53 bacterial strains from the gut of healthy *Cyprinus carpio*, pinpointing two specific strains — *Hafnia paralvei* (UUNT_MP29) and *Hafnia alvei* (UUNT_MP41) — as particularly promising for biotechnological use. Beyond their classification as next-generation probiotics (NGPs), these strains exhibit a remarkable secondary potential: the ability to degrade biodegradable plastics. Through genomic analysis and AlphaFold2 structural prediction, we identified two key enzymes, TesA and LipR, which share high conformational similarity and conserved catalytic domains with validated PBAT-depolymerases. Despite moderate sequence identity to known orthologs, the structural equivalence suggests these enzymes are functional candidates for plastic hydrolysis. These findings conclude that *Hafnia* strains from the common carp are bifunctional microorganisms capable of both enhancing aquatic host health and contributing to environmental bioremediation. This dual-purpose potential opens new avenues for integrating sustainable aquaculture with the management of plastic pollution, marking a significant step forward in contemporary biotechnology.

Izolacija i karakterizacija novih autohtonih sojeva: probiotički potencijal i svojstva biodegradacije mikroplastike

Mina Popović, Darya Tsibulskaya, Nevenka Rajić, Milan Kojić, Lazar Milojević, Aleksandar Nikšić i Luka Dragačević

INFORMACIJE O RADU

Ključne reči:
Probiotici sledeće generacije
Biodegradacija PBAT-a
Sekvenciranje celog genoma

APSTRAKT

Savremena istraživanja daju prioritet bakterijskim konzorcijumima koji kombinuju probiotičku efikasnost sa detoksikacijom životne sredine. U okviru ove studije izdvojena su dva nova autohtona izolata — Hafnia alvei UUNT_MP41 i Hafnia paralvei UUNT_MP29 — zbog njihovih snažnih probiotičkih fenotipova i potencijala da katalizuju biodegradaciju mikroplastike. Evaluaciju je potvrdilo snažno antimikrobno antagonistčko dejstvo protiv patogena. Sekvenciranje celog genoma (WGS) identifikovalo je ključne funkcionalne genetske elemente: gen ClpB — povezan sa probiotičkim karakteristikama i gene tesA i lipR — homologe esteraza (PpEst i EstC9) koji su umešani u razgradnju polimera. Ključno je da ovi sojevi omogućavaju degradaciju mikroplastike u uslovima koji oponašaju ljudski gastrointestinalni trakt. Ovi nalazi potvrđuju njihovu kandidaturu kao probiotika sledeće generacije sa dvostrukom funkcijom, uspostavljajući novu paradigmu za biotičke formulacije koje istovremeno nude terapijsku i ekološku korist.

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Original Scientific Paper

Implementation of an on-site allergen control plan based on risk assessment according to International Food Standard (IFS) guidelines

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ABSTRACT

This paper outlines the requirements, methodologies, and practical considerations for conducting allergen risk assessments in compliance with IFS standards. IFS Food Version 8, effective January 2024, provides a comprehensive and standardized framework for risk assessment, process control, and compliance in food manufacturing. Compliance with IFS Food Version 8 requires a systematic and risk-based approach aligned with global food safety standards. By following the described requirements and methodologies, food manufacturers can ensure compliance, protect consumer health, and maintain the highest food safety levels across the supply chain. Validation of an established allergen control system in a food business operator's production premises was conducted by taking 100 swabs from surfaces with potential allergen contamination; no positive results were obtained, which demonstrated the functionality of the implemented control system. The monitoring results from swabs and the evaluation of allergen presence and contamination risks served as the basis for developing the food business operator's allergen control plan as an integral part of its self-monitoring plan.

1. Introduction

Rising customer expectations and multiple food safety incidents have driven a surge in the adoption of quality assurance schemes (*Schulze et al.*, 2008). The implementation of food safety management systems must be grounded in the principles of food safety and security at a global level (*Lee et al.*, 2023). International Organization for Standardization (ISO) standards serve as the starting point for quality standards in the meat production and processing industry. Previous studies have primarily examined the motivations behind companies implementing ISO 9001 and how different sectors evaluate the standard (*Calisir et al.*, 2001). Certification schemes are designed to ensure product specifications are met and production processes remain consistent. The adoption of these standards is debated both theoretically and practically; consequently, many firms join not by choice but because major buyers, like large processors or retailers, compel them to (*Walgenbach*, 2007).

The most prominent sector-specific quality assurance schemes are Global Good Agricultural Practices (GLOBALGAP), the British Retail Consortium (BRC) global standard and the International Food Standard (IFS). Complete removal of risk from food cannot be guaranteed; rather, food safety

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depends on stringent controls throughout the food chain to lower public health hazards, reflecting a multifaceted, interdisciplinary approach (*Pacala et al*., 2024). Standards share common objectives, so their requirements overlap and show substantial similarity; major differences appear at the audit level, with differing scopes, criteria and categorizations. Requirements are organized into mandatory and recommended sections, allowing organizations to implement changes progressively. IFS, Food Safety System Certification (FSSC) and International Organization for Standardization (ISO) follow the High Level Structure (HLS), a shared framework that eases integration of multiple management systems for monitoring, corrective actions, and audits (*Bomba et Susol*, 2020).

Risk assessment is central to food safety management, enabling improved identification, evaluation, and control of hazards across the food production chain. IFS Food Version 8, released April 2023 and effective from 1 January 2024, offers a detailed framework for performing risk assessment in food manufacturing (*Kiwa*, 2023; *Elpress*, 2023). IFS Food Version 8 emphasizes a risk-based approach to food safety, aligning with global standards, such as *Codex Alimentarius*, ISO 22003-2, and Global Food Safety Initiative (GFSI) benchmarking requirements (*IFS Certification*, 2023). IFS standard requires organizations to: conduct a hazard analysis for each step in the production process; determine critical control points (CCPs) and other control measures; establish validated critical limits for each CCP; develop a monitoring system for each CCP; implement corrective actions and; validate the HACCP plan (*IFS Certification*, 2023a, *SafetyCulture* 2023).

Food allergy is an increasing worldwide public-health issue. Undeclared allergenic ingredients or trace contamination during food processing present significant health risks to sensitized individuals (*Janković et al*., 2024). IFS Food Version 8 states that for every raw material, a risk assessment must be conducted to determine which allergens must be declared, including accidental or technically unavoidable cross-contamination with legally declared allergens and trace amounts (*IFS*, 2023). Allergen cross-contamination happens when a food or ingredient that contains an allergen is accidentally mixed into another food that is not meant to include that allergenic component (*Codex Alimentarius*, 2020). All needed relevant information must be available for the country(ies) where the finished products will be sold, and be documented and up-to-date for all raw materials. Food business operators (FBOs) must:

- Maintain a continuously updated inventory of all on-site raw materials containing allergens, including any blends and formulations that incorporate allergenic raw materials (*IFS*, 2023a);
- Implement and sustain risk-based controls from incoming goods through dispatch to minimize possible allergen cross-contamination;
- Consider cross-contamination risks at least for: environment, transport, storage raw materials, personnel (including contractors and visitors);
- Track and monitor the effectiveness of the measures put in place.

In accordance with IFS requirements, all potential allergenic risks must be assessed, covering food-contact and packaging materials and the workplace, in order to evaluate their probability and severity, specify targeted control measures, and apply risk-based controls from receipt through to dispatch to reduce allergen cross-contamination (*IFS*, 2023a). Performing a risk analysis regarding allergen presence in meat products in accordance with IFS and *Codex Alimentarius* involves several critical steps. Firstly, the scope of the risk analysis must be defined (type of meat and meat product and population at risk, e.g., consumers with specific allergies), and then, possible routes of cross contamination (environment, transport, storage, raw materials, personnel including contractors and visitors) are considered.

The current version of IFS Food Version 8 emphasizes the need to validate the absence of allergens at every step of the production process. This specific requirement is novel and must be considered during each product's risk assessment. At that time, a specific control measure should be prescribed for each identified potential hazard (*IFS*, 2023a). IFS Food Version 8, Section 4.19 specifically addresses the prevention of risks posed by the presence of allergens in production. The main risk is cross-contamination, which can originate from the work environment, transportation, storage, raw materials, and personnel in the production facility, including both employees and external visitors. Therefore, allergen risk management requires specifically defining sampling locations that will indicate cross-contamination and, within the FBO's self-control plan,

conducting systematic examinations through swab testing for allergens. The obtained monitoring results and checklist evaluation results of potential allergen presence in an FBO's production premises is then used to develop a sampling plan for allergen presence in the production areas, as part of the self-monitoring (HACCP) plan.

Checklist grades are used by auditors during evaluations to gauge how well a facility or process complies with the IFS standards, and they help in identifying areas needing improvement to ensure ongoing compliance and safety. In accordance with IFS Food Version 8, a grade B is considered a deviation (non-critical non-conformity) and requires corrective actions. This approach introduces additional pressure on FBO to achieve the highest possible level of safety and control in producing their products.

While the IFS provides detailed guidance including templates, decision trees, cleaning validation protocols, and analytical testing frameworks (*IFS*, 2023), translating these requirements into consistent practice across organizations presents ongoing challenges. The requirement for a process-oriented HACCP-based risk assessment demands technical expertise and familiarity with quantitative assessment methodologies that not all food producers possess (*Allergenen Consultancy*, 2018). Furthermore, the requirement to evaluate and confirm safety protocols necessitates that businesses perform laboratory testing and internal reviews. These activities impose resource requirements that can fluctuate greatly based on the scale of the company and the intricacy of its manufacturing processes. Clarity on assessment goals is vital; companies must differentiate between the process-oriented assessment required by the IFS to determine cross-contamination prevention and the Voluntary Incidental Trace Allergen Labelling (VITAL) based risk assessments some organizations use for labeling decisions (*Holzhauser et al.*, 2020; *FACTS*, 2022; *Eurofins*, 2025). The key takeaway is that the primary function of the IFS allergen risk assessment is to identify and manage operational dangers, not to provide justification for precautionary labeling. A critical element of IFS 4.19.1 compliance involves maintaining current allergen master lists encompassing all allergenic ingredients and blends used in production (*IFS*, 2023). Meeting this standard requires rigorous supplier oversight to ensure accurate ingredient labeling and to identify manufacturing cross-contact risks. Although IFS provides checklists, companies must actively engage with suppliers to secure the specific allergen data necessary for a complete risk assessment (*ECA Academy*, 2020; *Health Canada*, 2025). As it is stated in IFS White Paper (*IFS*, 2025), where the summary of existing guidance documents regarding allergen control are summarized, environmental monitoring and testing for allergen residues provide objective evidence that cleaning protocols are effective and that segregation measures prevent unintended allergen presence. This data-driven approach to verification supports compliance demonstration and identifies areas requiring corrective action. The convergence of IFS Food Version 8 and subsequent versions toward GFSI-benchmarked requirements reflects broader international harmonization efforts. Risk assessment is an ongoing process that requires continuous improvement. Organizations should regularly review and update their risk assessments to reflect changes in production processes, regulatory requirements, and emerging risks (*Safety Forward*, 2025)

Food allergies, conditions in which the immune system reacts to specific foods, are a growing concern worldwide in terms of food safety. They pose significant public and individual health challenges. Although they impact a small segment of the population, the reactions can be extremely severe or even life-threatening. Additionally, it is clear that food allergies can drastically reduce quality of life for sufferers, a situation that could be improved with a consistent strategy for managing allergens throughout the food production and distribution processes (*Codex Alimentarius*, 2020). Although IFS standard states that in cases of accidental presence, the labeling of legally declared allergens and traces must be based on a risk analysis (*IFS*, 2023a), the zero tolerance policy as part of legal obligation forces FBOs to increase the level of security and safety regarding the presence of undeclared allergens in final products. The zero tolerance policy regarding undeclared allergens in final products is primarily addressed in the Food Safety Modernization Act (*FSMA*, 2011) in the United States. This act emphasizes the importance of preventive controls to ensure food safety, including allergen management. Additionally, in the European Union, Regulation (EU) No. 1169/2011 on the provision of food information to consumers mandates clear labeling of allergens and outlines the legal responsibilities of food businesses to avoid cross-contamination and ensure that allergenic ingredients are declared. These regulations

collectively establish a framework to which food producers must adhere, reinforcing the principle of zero tolerance for undeclared allergens in food products. In order to handle allergen risk especially in a young population, FSMA advises an individual approach to create voluntary guidelines to assist in developing plans for managing the risks of food allergies and anaphylaxis in schools and early childhood education programs (*FSMA*; 2011). These guidelines sholud be accessible to local educational agencies, schools, early childhood education programs, and other interested parties.

The labelling of food allergens on pre-packaged products is crucial for safeguarding individuals with food allergies, especially since there is no clinical treatment to prevent these reactions at present. Where accidental or technically unavoidable cross-contamination could introduce traces of these allergens, that must be indicated and based on a risk assessment, taking into account allergen cross-contamination from raw materials processed on-site. Finished products containing allergens that require declarations must be declared in accordance with legal requirements.

While recent advances in immunotherapy for food allergens have demonstrated encouraging outcomes, the only effective way to prevent allergic reactions continues to be avoidance of the triggering foods (*FAO and WHO*, 2022). In 1995, the Food and Agriculture Organization of the United Nations (FAO) held a Technical Consultation (*FAO*, 1995) that led to the recognition of eight specific foods or food groups that are responsible for causing food allergies. Those are: cereals containing gluten (wheat, rye, barley, oats, spelt or their hybridized varieties and products of these; crustacea and their products; eggs and egg products; fish and fish products; peanuts, soybeans and their products; milk and milk products (lactose included); tree nuts and nut products; and sulphite in concentrations of 10 mg/kg or more (*FAO and WHO*, 2018). The list covers the most prevalent allergens that account for the majority of allergic reactions, even though approximately 170 different foods have been associated with such reactions (*Boyce et al*., 2011). Today, numerous domestic and foreign legal regulations define strict and clear labelling of all food ingredients that exhibits allergenic properties (*Official Gazette RS*, 41/009 and 17/2019; *Official Gazette RS*, 19/2017, 16/2018, 17/2020, 118/2020, 17/2022, 23/2022, 30/2022 and 61/2024; *Official Gazette RS*, 73/2010; *EU Regulation* 1169/2011, *FALCPA* 2004). In a threeyear study conducted in Belgrade, Serbia (2021–2024) on 212 commercial food items, peanut DNA was found in nine products across the sampled categories. Detection indicated undeclared peanut material regardless of label claims: peanut DNA appeared in frozen desserts (3), biscuits (4) and snacks (2); six of the positive items carried a precautionary allergen label, while three had no indication of peanut presence (*Janković et al*., 2025). In a second study by the same author, 114 out of 179 (63.69%) food products listed allergens on their ingredient labels. The most commonly declared allergen was soy, present in 27.4% of food products, followed by milk in 13.4%, egg in 11.7%, celery in 6.1%, mustard in 5.6%, and sesame in 4.5% (*Janković et al*., 2024).

This paper outlines the requirements, methodologies, and practical considerations for performing allergen risk assessments in compliance with current IFS standards.

2. Materials and Methods

A risk assessment regarding the presence of allergens in an FBO's production facility was conducted in accordance with IFS Food Version 8 requirements. The pre-existing checklist, compiled for control of the production premises in the facility, was expanded to contain additional considerations for evaluating potential allergen contamination (*Codex Alimentarius*, 2020). The FBO's food safety team appointed an allergen management team consisting of three technologists, while an external expert team was also appointed. Both these teams carried out the risk analysis in order to balance individual evaluations that can be subjective and differ between observers (*Petrović et al*., 2024). The final checklist was then used under field conditions to assess and determine the potential risk of allergen contamination in the FBO's production facility (Table 1). An explanation of the marking system used, which was in accordance with IFS audit requirements, is presented in Table 2.

The obtained control results were validated by taking swabs from sanitary equipment, tools, and workers' hands and clothing after exiting the kitchen. This approach aimed at providing an objective assessment of the effectiveness of all the changed work practices, as well as evaluating the possibility of allergen contamination by the workers themselves in the production facility, upon returning to their workstations after a break from the kitchen.

Table 1. Checklist for evaluation of potential allergen presence in the FBO production premises

Demand	KO A B C D	Remark
It is essential to ensure raw material specifications that list allergens requiring declaration. The organization must always have a current and valid list of all raw materials with allergens used on its premises, which also includes all mixtures and formulas to which such allergenic raw materials are added.		
The production of products containing allergens that require declaration must be conducted in a manner that minimizes cross-contamination to the greatest extent possible.		
Finished products with allergens must have declarations in accordance with current legal provisions. In cases of accidental presence, the labeling of legally declared allergens and traces must be based on a risk analysis.		
Production Areas must be segregated, clearly marked. Designate specific areas for the production of allergen-containing products to prevent cross-contact with non-allergenic foods.		
During production, special attention must be oriented to handling spices that contain allergens and are prepared for addition during product formulation. Until the moment of incorporation into the filling, spices must be kept in a closed container that is opened just before use. Care must be taken to prevent airborne contamination of the area.		
When adding spices to the cutter bowl, the lid must remain closed, and the spices should be introduced through an opening in the lid, ensuring that the used packaging is disposed of properly without creating dust.		
All used packaging from allergenic components or spice blends containing allergens must be removed from the production area as soon as possible, placed in a closed bag, and disposed of in accordance with the established procedure for handling packaging waste from the production facility.		
Daily production plan must be scheduled in order primarily produce allergen free foods prior to products with allergens, in order to reduce contamination risk.		
Rigorous and thorough Cleaning and Sanitation procedures must be staged between production runs to remove allergenic residues from equipment and surfaces. Validate cleaning efficacy through swab tests or other verification methods in accordance with self-control plan.		
Where possible, separate, use dedicated, equipment and utensils for allergenic and non-allergenic product in order to avoid shared surfaces.		

Demand	KO A B C D	Remark
Staff on allergen management must be regulary trained with doccumented outcome of the training (importance of hand washing, changing protective clothing, and understanding cross-contamination risks).		
All ingredients and end products must be clearly and properly Labelled and stored. All allergenic ingredients are stored closed, separately in marked area, below non-allergenic ones to prevent accidental spillage or contact. Where a commodity is bagged, bags should be clean and those used for allergenic commodities should be identified (e.g. with different colours). Bags that have been used for an allergenic commodity should not be reused for a different commodity		
FBOs should ensure that the area where commodities are dried is clean and physical barriers are in place to prevent spillage and allergen cross-contact. Materials or containers used to lay, hang or bag commodities should be cleaned to remove allergenic residue.		
Transportation of foodstuff should be carried out using a clean transport vehicle that is dry and free of the previous load to prevent or minimise the potential for allergen cross-contact. As necessary, transport containers should be cleaned before use. At unloading, transport containers containing allergenic commodities should be emptied of all cargo and cleaned as appropriate to prevent or minimise the potential for allergen cross-contact of the next load.		
All suppliers are listed in the "List of Suppliers", and cooperation is rated through previous trend analysis. Suppliers must ensure they also follow strict allergen management practices and provide detailed allergen specifications for raw materials.		
Management with risk is regularly monitored and documented through Allergen Risk Assessment. Conduct regular risk assessments to identify potential cross-contamination points and develop strategies to mitigate them.		
Physical barriers (walls, partitions) are used to separate allergenic ingredients during storage and processing.		
Allergen Control Program is implemented, developed and maintained as part of broader food safety management system with all necessary data (procedures and corrective actions)		
On the floor of the production area, a yellow line clearly marks the path of allergens in the production facility, including the path of raw materials containing allergens.		
There is a clearly designated and defined production line for the manufacturing of products with allergens, and if this is not the case, the temporal separation and scheduling of production times for products with allergens are clearly documented.		

Table 2. Marking system for allergen risk assessment for the on-site evaluation (source IFS, Ver 8)

Mark	Description
KO (Knock Out)	These are fundamental requirements. Receiving a KO can result from a critical non-conformity and usually means that the certification cannot be granted until corrective actions are implemented. KOs reflect the most critical points of compliance.
A	Indicates full compliance with the requirement. The practice or process meets the standard completely without any issues.
B	Represents almost full compliance but with some minor deviation or non-conformity that needs addressing. Recieving a B does not represent a significant risk, but shows improvement is required.
C	Shows a clear non-conformity. Recieving a C means the practice does not meet the standard's requirement and poses a certain level of risk, demanding prompt corrective action.
D	Reflects major issues or serious non-conformance. Like KO, receiving a D for any requirement likely means failing the audit and requires urgent action to implement corrective measures.

2.1 Study Setting and Sampling Design

A total of 100 environmental swabs were collected from the meat processing facility in accordance with the FBO's established, HACCP-based self-control plan, with ten samples taken at the start of the workday over a period of ten weeks. Sampling was conducted during the 2025–2026 production cycle, specifically targeting periods after wet cleaning and sanitation, but prior to the commencement of allergen-free production runs. The monitoring schedule for determining allergen presence is presented in Table 3.

Table 3. Monitoring procedure for allergen presence in the production premises

Sampling Location	Allergens to Test	Test Method	Frequency	Responsible Personnel	Number of samples
Food Contact Surfaces (n=60)					
Meat Grinders	Soy, Gluten	LFT Swabs*, Swab tests**	Weekly	Allergen Management Team	15
Bowl cutters	Soy, Gluten	LFT Swabs, Swab tests	Weekly	Allergen Management Team	15
Stuffers	Soy, Gluten	LFT Swabs, Swab tests	Weekly	Allergen Management Team	15
Stainless steel preparation tables	Soy, Gluten	LFT Swabs, Swab tests	Weekly	Allergen Management Team	15
Non-Food Contact Surfaces (n=40)					
Employee hands	Egg, Milk, Mustard	LFT Swabs, Swab tests	Weekly	Allergen Management Team	10
Employee clothing	Egg, Milk, Mustard	LFT Swabs, Swab tests	Weekly	Allergen Management Team	10
Allergen path floor	Soy, Gluten	LFT Swabs, Swab tests	Weekly	Allergen Management Team	10
Wall panels	Soy, Gluten	LFT Swabs, Swab tests	Weekly	Allergen Management Team	10

*LFT Swabs — Lateral Flow Test (Limit of detection — LOD ≤ 5 ppm)

**Swab test — ELISA (Limit of quantification — LOQ mg/kg)

Selection of Sampling Locations

Sampling locations were selected based on the risk assessment of the production line. The 100 swabs were distributed across:

Food contact surfaces (n=60) comprised surface swabs from meat grinders (n=15), bowl cutters (n=15), stuffers (n=15), and stainlesssteel preparation tables (n=15). Swabs were taken to determine the presence of those allergens identified by the risk assessment, specifically gluten and soy.

Non-food contact surfaces (n=40) comprised surface swabs from employee hands (n=10), employee clothing (n=10), yellow-taped "allergen path" floor zones (n=10), and wall panels in the production premises (n=10). Swabs were taken from the hands and work clothes of staff immediately following their break and upon exiting the kitchen to return to work, to determine the presence of allergens identified by the risk assessment, i.e., eggs, milk (casein and β-lactoglobulin), and mustard. Simultaneously, the floors and walls of the facility were tested for soy and gluten, allergens for which there was a reasonable suspicion of cross-contamination risk.

Swab Collection Technique

Sterile, pre-moistened allergen collection swabs (Hygiena AlerCheck and 3M™ Allergen Swabs) were used. A standardized area of 10 cm × 10 cm (100 cm^2) was swabbed for flat surfaces, while irregular surfaces (e.g., equipment joints) were swabbed thoroughly to capture potential residues. The two test types were conducted at the same locations as a form of additional verification and to evaluate the reliability of the rapid testing method.

Target Allergens

The study focused on the major allergens declared in the FBO's raw material specifications. The primary targets were; soy and gluten – commonly used as binding ingredients in meat processing and egg, milk and mustard (*Sinapis alba*) were used as ingredients in the kitchen for the preparation of employee meals.

Analytical Methods

Two complementary methods were employed for detection to ensure accuracy:

Rapid Lateral Flow Tests (LFT): Used for on-site screening to provide immediate qualitative results (Presence/Absence). The limit of detection (LOD) was set at 5 ppm, in alignment with 2026 industry safety thresholds (*Lupo*, 2025; *Eurofins*, 2026)

Enzyme-Linked Immunosorbent Assay (ELISA): Any swabs yielding weak positive or ambiguous results on LFT were subjected to quantitative ELISA analysis in an ISO 17025 accredited laboratory to confirm the concentration of the allergen protein. Swabs that produced weak positive or ambiguous LFT results were stored at 4 °C and shipped to the ISO 17025 accredited laboratory on the same day under chain-of-custody documentation. ELISA assays were run using a validated commercial kit specific to the target allergen, following the manufacturer's instructions and laboratory SOPs. Each swab was analyzed in duplicate, with reagent blanks, positive controls, and a calibration curve prepared from certified reference standards included on every plate. The laboratory reported concentrations in mg/kg (or ng/mL as appropriate), and results below the assay's limit of quantification (LOQ) were reported as <LOQ while those between the limit of detection (LOD) and LOQ were reported as detected but not quantifiable. Where ELISA results contradicted the LFT, a third replicate or an orthogonal method (e.g., mass spectrometry) was performed to resolve discrepancies. All analytical data, including raw absorbance values, calibration curves, QC performance, and chain-of-custody records, were retained according to ISO 17025 record-keeping requirements (*ISO*, 2017). ISO 17025:2017 specifies record-keeping requirements in clauses 8.4 (management system records) and 7.5 (technical records), including retention, legibility, retrievability, protection, and audit trail provisions.

Quality Control and Validation

Negative controls (swabs of unused clean surfaces) and positive controls (surfaces spiked with a known allergen concentration) were processed alongside every batch of 10 samples. Data were recorded within the FBO's food safety management system (FSMS) for traceability.

Statistical Analysis

Statistical data analysis was performed using Microsoft Excel (Windows).

3. Results

Alongside the evaluation of allergen contamination in the production department, sampling for allergen presence was conducted. The results obtained are presented in Table 4.

All examined allergens (soy, gluten, egg, milk, mustard) were below detection limits (LOD, ≤ 5 ppm) or quantification limits (LOQ, mg/kg) across all examined surfaces. This indicated, based on the sampling methods and detection limits, that there were no detectable levels of the specific allergens in the monitored areas.

Following the inspection and the collection of results, Table 1 was further adapted by the Allergen Management Team and harmonized to more adequately meet the regular production needs of the FBO. The results of this are presented in Table 5.

Table 5 served as a basis, alongside monitoring via swab tests, for establishing a regular sampling plan for the control of allergen presence in the production premises (Table 6), as part of the FBO's self-monitoring plan.

Table 5 assessed allergen handling in the FBO's production setup, focusing on key areas as specified by the IFS framework. Allergen Management System confirms a documented Allergen Control Program exists within the FBO's food safety system. Raw Material Management validates allergen specifications in raw materials, global compliance and supplier details. Production Process and Control covers cross-contamination reduction, including segregation, production planning, spice handling, packaging disposal, physical barriers, and clear allergen pathways. Cleaning and Sanitation verifies effective,

Table 4. Results of the allergen presence monitoring in the FBO's production facility

Sampling Location	Allergens to Test	Sampling Method	Number of samples	Number of positive samples	Results
Food Contact Surfaces (n=60 samples)					
Meat Grinders	Soy, Gluten	LFT Swabs, Swab tests	15	0	< LOD* < LOQ**
Bowl cutters	Soy, Gluten	LFT Swabs, Swab tests	15	0	< LOD < LOQ
Stuffers	Soy, Gluten	LFT Swabs, Swab tests	15	0	< LOD < LOQ
Stainles steel preparation tables	Soy, Gluten	LFT Swabs, Swab tests	15	0	< LOD < LOQ
Non-Food Contact Surfaces (n=40 samples)					
Employee hands	Egg, Milk, Mustard	LFT Swabs, Swab tests	10	0	< LOD < LOQ
Employee clothing	Egg, Milk, Mustard	LFT Swabs, Swab tests	10	0	< LOD < LOQ
Allergen path floor	Soy, Gluten	LFT Swabs, Swab tests	10	0	< LOD < LOQ
Wall panels	Soy, Gluten	LFT Swabs, Swab tests	10	0	< LOD < LOQ

LFT Swabs – Lateral Flow Test (Limit of detection – LOD* ≤ 5 ppm)
Swab test — ELISA (Limit of quantification – LOQ** mg/kg)

Table 5. Results of the on-site inspection of allergen handling procedures within the FBO production premises

Demand	Mark	Remark
Remark		
Is there a documented Allergen Control Program implemented as part of the food safety management system?	A	Allergen Control Program is implemented, developed and maintained as part of broader food safety management system.
Raw material management		
Are raw material specifications in place that list allergens requiring declaration?	A	Raw material specifications are in place with all listed allergens requiring declaration to comply with 2026 global food safety standards.
Is there a current and valid list of all raw materials with allergens, including mixtures and formulas?	A	FBO have a current and valid list of all raw materials with allergens used on its premises, which also includes all mixtures and formulas.
Do suppliers provide detailed allergen specifications for raw materials?	A	Suppliers follow strict allergen management practices and provide detailed allergen specifications for raw materials
Production Process And Control		
Is the production of allergen-containing products conducted to minimize cross-contamination?	A	The production of allergen-containing products is organized on separate production days and on dedicated production lines; if this is not possible, it is scheduled after the production of allergen-free products, in the second half of the workday
Are production areas segregated and clearly marked, with designated areas for allergen-containing products?	A	Production areas are segregated and clearly marked. Specific areas for the production of allergen-containing products are marked, while the "allergen path" during production is marked with yellow floor tape within the production facility
Are temporal separation and scheduling of production times for products with allergens clearly documented if dedicated lines are not used?	A	There is a clearly designated and defined production line for the manufacturing of products with allergens, and if this is not the case, the temporal separation and scheduling of production times for products with allergens are clearly documented
Is there a daily production plan that prioritizes allergen-free foods before products with allergens?	A	Daily production plan is scheduled in order primarily produce allergen free foods prior to products with allergens
Are special precautions taken when handling spices containing allergens (e.g., closed containers, controlled introduction)?	A	During production, special attention is oriented to properly handling spices that contain allergens. Spices are kept in a closed container. When weighing the necessary spices for each production batch, labelled and sealed plastic containers are used in order to prevent airborne contamination.
Is used packaging from allergenic components promptly removed and disposed of properly?	A	All used packaging from allergenic components or spice blends containing allergens are removed from the production area during breaks, while they are kept until that time in closed, dedicated, and labelled plastic containers

Demand	Mark	Remark
Are physical barriers used to separate allergenic ingredients during storage and processing?	A	Physical barriers (walls, partitions) are used to separate allergenic ingredients during storage and processing. All allergenic ingredients are closed and properly marked in separated dedicated storage areas
Is a yellow line clearly marking the "allergen path" on the production floor?	A	On the floor of the production area, a yellow line clearly marks the "allergen path", ensuring that it is not erased or worn off due to mechanical transport or sanitation
Cleaning and sanitation		
Are rigorous and thorough cleaning and sanitation procedures in place between production runs to remove allergenic residues?	A	Rigorous and thorough documented sanitation (mechanical cleaning, degreasing, and disinfection) procedures are staged between production runs in order to remove all allergenic residues
Is cleaning efficacy validated through methods like swab tests?	A	Swab tests for the presence of allergens are performed regularly in accordance with the FBO's self-control plan
Equipment and Utensils		
Is dedicated equipment and utensils used for allergenic and non-allergenic products where possible?	A	All equipment and utensils for allergenic and non-allergenic product are separated where is possible. In cases where this is not possible, the equipment is labelled and sent for sanitation immediately after use.
Staff Training		
Is staff regularly trained on allergen management, including hand washing, changing clothing, and cross-contamination risks?	A	Employees undergo regular training on proper allergen handling in production, with a special emphasis on handwashing, replacing soiled equipment, and understanding procedures that can lead to cross-contamination. Training is conducted quarterly by the technologist (internal training), while external training is held annually by external experts, including a test to evaluate employee knowledge, after which certificates are issued.
Is the outcome of training documented?	A	Records of each quarterly training session are maintained within the FBO's food safety management system, while certificates are issued annually for external training.
Labelling and Storage		
Are all ingredients and end products clearly and properly labelled and stored?	A	All ingredients and end products are clearly and properly labelled and stored. All ingredients and end products containing allergens are clearly labelled and kept in sealed, undamaged packaging, ensuring they are clearly visible to all employees who have access to them.
Are allergenic ingredients stored closed, separately, and marked, preferably below non-allergenic ones?	A	All allergenic ingredients are stored closed, separately in marked area, below non-allergenic ones

Demand	Mark	Remark
Are bags used for allergenic commodities identified (e.g., different colors) and not reused for other commodities?	A	Plastic containers used for spices with allergenic mixture are red coloured, with 5–10 cm wide yellow track. All other Bags, or differently marked plastic containers, are not reused for keeping or transportation of allergens
Drying and handling commodities		
Is the drying area clean, with physical barriers to prevent spillage and cross-contact?	A	Dedicated marked sanitation zone where plastic containers for allergen handling are kept during drying is clean and physically separated to prevent cross-contamination
Are materials/containers used for commodities cleaned to remove allergenic residue?	A	Swab testing is performed on regular basis to validate that materials or plastic containers are cleaned of allergenic residues
Transportation		
Is transportation carried out using clean, dry vehicles free of previous loads?	A	Transportation of end products are carried out with a clean and sanitized transport vehicle that is dry and free of the previous load
Are transport containers cleaned before use if necessary and after unloading allergenic commodities?	A	Transport containers are sanitized and dried before use. During unloading, all transport containers containing allergenic commodities are primarily emptied of all cargo and appropriately cleaned
Supplier management		
Is there a "List of Suppliers" maintained?	A	All suppliers are listed in the *List of Suppliers*
Are supplier evaluations conducted on a regular basis?	A	The supplier evaluation system is conducted regularly in accordance with the rating scale specified in Table 3 (A → KO)
Risk assessment		
Is Allergen Risk Assessment regularly monitored and documented?	A	Management with risk is regularly monitored and documented through Allergen Risk Assessment. Regular risk assessments are conducted yearly, or more frequently as needed, by the FBO Food Safety Team.
Finished Product Labelling		
Do finished products with allergens have declarations in accordance with current legal provisions?	A	Finished products with allergens have declarations in accordance with current legal provisions.
Who is responsible for applying labels to individual and bulk packaging, and who is responsible for the accuracy of the information on the label?	A	The employee is responsible for applying the labels, the Packing Department supervisor is responsible for checking the presence of the labels, while the Food Safety Team Leader and the technologist are responsible for the label content.
Is accidental presence of allergens handled based on risk analysis for labeling?	A	In cases of accidental presence, the labelling of legally declared allergens and traces must be based on a risk analysis.

Table 6. Sampling plan for allergen presence in the production premises

Sampling Location	Allergens to Test	Sampling Method	Frequency	Responsible Personnel	Remarks
Raw Material Storage Area	Soy, Gluten	Swab tests on surfaces	Monthly, more frequently if needed	Quality Assurance Team	Ensure proper labeling and storage separation.
Ingredient Mixing Area	Milk, Egg, Soy	Swabs, Composite sampling	Monthly	Production Manager	Focus on cross-contamination risks.
Processing Equipment	All identified allergens	Swab tests	Monthly	Hygiene Supervisor	After maintenance and deep cleaning activities.
Finished Product Storage	All declared allergens	End product testing	Monthly	Quality Control Analyst	Verify label accuracy and product segregation.
Employee Handrails & Gloves	All common allergens	Swab tests	Monthly	Health and Safety Officer	Ensure consistent hygiene practices.
Packaging Area	Gluten, Soy	Air sampling	Monthly	Packaging Supervisor	Monitor airborne allergens during packaging.

documented cleaning, necessary so the swab tests can remove any allergenic residues from surfaces. Equipment and Utensils ensures dedicated/labelled equipment for allergenic products and immediate sanitation for shared items. Staff Training regulates training courses on allergen management, documented with assessments and certificates. Labelling and Storage defines labels and stores ingredients/products clearly, with proper allergen separation and identification. Drying and Handling Commodities defines practices of cleaning and drying of equipment subjected to swab testing for allergenic residue removal. Transportation covers the use of clean, allergen-free transport vehicles and containers. Supplier Management maintains a supplier list with regular evaluations. Risk Assessment regularly monitors and documents allergen risk assessment. Finished Product Labelling ensures legal allergenic declarations and handles accidental presence through risk analysis.

The sampling plan is an integral part of the FBO's self-control plan, developed in accordance with domestic (*Official Gazette RS*, 19/2017, 16/2018, 17/2020, 118/2020, 17/2022, 23/2022, 30/2022 i 61/2024; *Official Gazette RS*, 30/2024) and international (*EU Regulation* 1169/2011; *EU Regulation* 828/2014) legal regulations. This plan needs regular updates and validation to ensure ongoing compliance and effectiveness. Each production segment, each production unit requires a different approach to risk assessment for the presence of allergens, so the approach must be tailored to the current and real hazards specifically associated with the risk in that production step.

4. Discussion

The need for a documented allergen control system, which was the starting point of our research and the first item on our checklist, is also stated by *López-Calvo* et *al*. (2025) as the fundamental problem-solving approach in their study conducted in Costa Rica. The issue of allergies arising from human exposure to specific food components has been recognized as immunologically mediated hypersensitivity since the 1990s (*Muthukumar* et *al*., 2020). On a global scale, the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) have emphasized the significance of addressing this public health challenge. This has led to the establishment of a systemic approach to the control and identification of food allergens, which currently constitutes a fundamental requirement for food business operations. In line with a systemic approach to ensuring

allergen control in production, we emphasized the importance of raw material intake control, a finding corroborated by other studies (*Decker et al.*, 2008; *Röder* and *Weber*, 2016). Control measures encompass aspects such as identifying food allergens, avoiding cross-contamination with allergens during transportation, managing the storage of raw materials and food additives (RW&FA), overseeing the production process, and ensuring proper storage of finished products. The subsequent section of the checklist comprised inquiries aimed at enabling food business operators to clearly define and evaluate all associated risks. This ensures appropriate handling of allergen-containing products, thereby preventing the intermingling of raw materials during routine production and mitigating the risk of cross-contamination. This approach is consistent with methodologies employed in other studies sharing the same objective (*Blom et al*., 2021, *Elrahi et al*., 2023, *Smječanin*, 2023). The necessity of delivering precise and transparent information regarding allergens present in products by individuals in the food industry responsible for market distribution is undeniable. For those with allergies, proper allergen labeling serves as a crucial tool to mitigate exposure risks and avert anaphylaxis. Examining the conditions for the physical segregation of allergens, as specified in the production process control section of Table 5, prevalence of food allergies is reduced or eliminated. Similar approach is used in researches of *Zhou et al* (2024) and *Gunal-Koroglu et al* (2025). We emphasized the necessity for clear and visible marking of allergen transport routes within the production facility, a measure that aligns with the recommendations of *Boye and Godefroy* (2011). Their work highlights the requirement for 'Dedicated Traffic Pathways' as a fundamental control measure. The scientific rationale for this approach lies in preventing the mechanical transfer of allergens via forklift tires or employee footwear. Although specific color is not prescribed, industrial best practices typically utilize yellow lines — the international symbol for caution and boundary — to designate zones where allergens are handled. Furthermore, production shifts or cycles must invariably commence with allergen-free products, ensuring that manufacturing begins when equipment cleanliness is at its peak following the comprehensive sanitation protocols conducted at the end of the previous operational period. This practice is consistent with the findings of *Crevel* (2007). In accordance with IFS standards, food business operators are encouraged to schedule the processing of allergen-containing products at the conclusion of the production day, immediately preceding the primary sanitation and equipment cleaning cycle. We emphasized the necessity of conducting regular employee training on allergen control within the framework of the implemented operational food safety management system. A similar requirement is documented in the research by *Gonzalez-Mancebo* et *al*. (2019). Sanitation protocols within production facilities handling food allergens must be rigorous, comprehensive, and meticulously documented. This documentation should encompass every stage, including preparatory mechanical cleaning and effective sanitation phases such as disinfection. A consistent approach is advocated in the study by *Nantob-Bikatui* (2020). Canadian Allergen management guide (2022) particular attention points toward equipment maintenance, specifically regarding components that are difficult to access for cleaning and maintenance. These areas often contain 'dead zones' where allergens can accumulate, posing a risk of persistent contamination. This approach aligns with the allergen control checklist developed based on the IFS. This framework stipulates the mandatory segregation and dedicated storage of tools and equipment utilized in the manufacturing of allergen-containing products. Furthermore, the storage protocols for allergen-containing products outlined in the aforementioned guide — specifically their placement in clearly designated areas, segregated from non-allergenic goods, and positioned on lower shelving units to prevent cross-contact — align with the criteria established in our checklist derived from IFS standards. These requirements were fully satisfied, as evidenced by the 'A' rating documented in the audit record (Table 5).

Scientific evidence demonstrates that surface swabbing is the most reliable method for identifying 'hotspots' on equipment where meat residues and spice traces may persist, such as joints, blades, and filler corners (*Jackson et al*, 2008). The allergen management team, operating within the FBO's HACCP team and supported by an external expert group, evaluated the risk of allergen contamination in meat products during regular production processes. The risk of allergen presence was assessed based on the IFS Food Version 8 guidelines, which served as the foundation for developing a checklist (Table 1); this was used to evaluate the current state, marked according to Table 2. The results are presented in Table 5. Simultaneously with the evaluation, planned swabbing of potentially contaminated sites was initiated. The sampling procedure was systematically designed and is presented in Table 3. Swabs were collected in ten series of ten samples each; simultaneously, rapid swabs for

immediate allergen detection were performed alongside swabs for ELISA laboratory diagnostics. The obtained test results are presented in Table 4. The results of the allergen risk evaluation (Table 5) and the results of the swab monitoring (Table 4) served as the basis for establishing a regular allergen control plan within the production facility (Table 6) as an integral part of the FBO's self-control plan.

Allergen risk assessment represents a fundamental pillar of modern food safety management, and the International Food Standard (IFS) provides a comprehensive framework for implementing these assessments systematically across food production operations. The IFS standard directly addresses allergen risk mitigation through Requirement 4.19, which comprises three interconnected sub-requirements that form a holistic approach to allergen management. Requirement 4.19.1 mandates a thorough risk assessment for all raw materials and allergenic ingredients, requiring documented allergen risks and up-to-date records of allergenic ingredients. This foundational component ensures that organizations have complete visibility into their allergen landscape from ingredient sourcing through finished product declaration. Requirement 4.19.2 obligates companies to implement risk-based measures to minimize cross-contamination, moving beyond the precautionary principle to evidence-based control strategies. These measures include cleaning validation protocols, personnel hygiene training, process zoning, and monitoring across transport, storage, and production environments. The shift toward process-oriented (HACCP-based) risk assessment rather than precautionary labeling represents a significant evolution in allergen management thinking, allowing organizations to implement proportionate controls based on actual contamination risks (*Allergenen Consultancy*, 2018). Requirement 4.19.3 emphasizes evidence-based allergen labeling, leveraging scientific thresholds such as eliciting dose (ED): ED01 and ED05 reference doses to determine when accidental or unavoidable cross-contamination must be declared. ED is a critical metric used for quantitative allergen risk assessment and determining the necessity of precautionary allergen labelling (*IFS*, 2023a) This approach aligns with FAO/WHO guidance on risk-based labeling frameworks, ensuring labels are both accurate and legally compliant. The development of IFS Food Version 8 reflects the industry's recognition that traditional hazard-based approaches to allergen warning statements lack precision and limit consumer choice (*Kiwa*, 2023; *Henderson*, 2025). The FAO/WHO established a scientific framework based on three assessment criteria: prevalence, potency, and severity, in order to determine which foods warrant inclusion on a global priority allergen list (*FAO and WHO*, 2022; *Anaphylaxis Campaign*, 2024). This framework moves away from precautionary labeling toward an assessment of actual risk, considering both the likelihood and severity of allergic reactions (*Henderson*, 2023). Reference doses set by FAO/WHO consultations establish quantitative limits for handling unintended allergen presence (UAP), assisting companies in deciding when warning labels are scientifically supported. Integrating these thresholds into IFS-compliant risk assessments demonstrates that allergen management is based on international scientific agreement, not assumptions.

5. Conclusion

A thorough review of the IFS standard requirements enabled the FBO's allergen management team, with support from an external expert team, to assess the potential for allergan contamination of raw materials and finished products. Swab samples taken from food contact surfaces and the environment demonstrated the effectiveness of consistent implementation of IFS Food Version 8 and permitted the development of a tailored allergen control plan as an integral part of the self-monitoring plan. By following the requirements and methodologies outlined in this paper, it is anticipated the FBO will ensure compliance, help protect consumer health, and maintain the highest levels of food safety throughout the supply chain.

The current IFS Food Version 8 framework offers a comprehensive, standardized approach to risk assessment, process control, and compliance in food manufacturing. Performing risk assessment in accordance with IFS Food Version 8 demands a systematic, risk-based approach that aligns with global food safety standards. To manage allergens, FBOs must develop and maintain comprehensive written policies and procedures that are tailored to their own, specific operations. Proper allergen management strategies yield several benefits. They enable a FBO to show it is making all required efforts to eliminate or minimize the chance of an allergen being inadvertently included in food products. They enhance the precision of declarations regarding allergenic ingredients. They offer a chance for FBOs to showcase their competence and understanding of effective allergen management practices. They help mitigate the risks posed to consumers with food allergies by limiting the presence of unintended allergens.

Uspostavljanje plana kontrole prisustva alergena u pogonu na osnovu analize rizika pomoću odrednica Međunarodnog standarda za hranu (IFS)

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INFORMACIJE O RADU

Ključne reči:
IFS standard
Analiza rizika
Alergeni
Unakrsna kontaminacija
Uzimanje briseva na prisustvo alergena

APSTRAKT

U radu su predstavljeni zahtevi, metodologija i praktična razmatranja u vezi sasprovođenjem procene rizika od alergena kod subjekta u poslovanju hranom u skladu sa IFS standardima. IFS verzija 8, koja je stupila na snagu u januaru 2024. godine, pruža sveobuhvatan i standardizovan okvir za procenu rizika, kontrolu procesa i usklađenost u proizvodnji hrane. Usklađenost sa IFS verzijom 8 zahteva sistematičan pristup zasnovan na riziku, koji je integralni deo globalnih standarda bezbednosti hrane. Postupanjem po stavkama zahteva standard i korišćenjem definisane metodologije, proizvođači hrane mogu da unaprede nivo bezbednosti proizvodnje i da doprinesu u zaštititi zdravlja potrošača, uz istovremeno održanje najvišeg nivoa bezbednosti hrane kroz ceo lanac snabdevanja. Validacija postavljenog sistema kontrole alergena izvršena je uzimanjem 100 briseva sa površina na kojima postoji mogućnost kontaminacije alergenima; tom prilikom nisu dobijeni pozitivni rezultati, što dokazuje funkcionalnost primenjenog sistema kontrole. Dobijeni rezultati monitoringa briseva i evaluacije prisustva alergena i mogućnosti kontaminacije su poslužili kao osnov za razvoj sopstvenog plana kontrole alergena kao integralnog dela plana samokontrole subjekta u poslovanju hranom.

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STATISTICA (Data Analysis Software System) (2006). v.7.1., StatSoft, Inc., USA (www.statsoft.com).

- Websites:

Technical report on the Food Standards Agency project G010008 (2002). Evaluating the risks associated with using GMOs in human foods, University of Newcastle, UK (http://www.foodsafetynetwork.ca/gmo/gmnewcastlereport.pdf).

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