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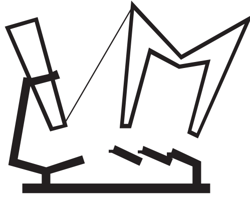

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Original scientific paper

Pigs and cattle slaughter process hygiene in a large-scale and a small-scale abattoir: A report from one county in Serbia

Boris Mrdovic[1], Mladen Raseta[1], Ivana Brankovic-Lazic[1], Milan Milijasevic[1], Branislav Baltic[1], Ivan Nastasijevic[1]

A b s t r a c t: Microbiological data on hygiene indicators are important for assessment of hygiene levels in abattoirs and control of carcass contamination. Risk categorization of slaughterhouses should be based on a process hygiene output through the use of indicator organisms – Total Viable Counts (TVC), Enterobacteriaceae counts (EC) and Salmonella spp. – monitored on carcasses. The level of TVC indicates the overall hygiene in abattoirs (equipment, tools, workers), while the presence of EC on carcasses can indicate fecal contamination which can occur during slaughter/dressing. Detection of Salmonella spp. indicates the presence of pathogens, with potential origin from the farm. The aim of this study was to assess the slaughter process hygiene in abattoirs of different sizes and throughputs, a large- and a small-scale abattoir, and to analyze differences in process hygiene levels based on implementation of their self-control plans, throughout a period of five years for cattle and six years for pigs. In the large-scale abattoir, TVC levels were slightly higher on cattle carcasses than on pig carcasses, but were always within the regulatory satisfactory or acceptable ranges. In the small-scale abattoir, low counts of EC were observed on cattle and pig carcasses, with slightly higher levels on cattle carcasses, but the counts were always within the regulatory satisfactory range. Higher TVC levels on both cattle and pig carcasses were observed in the large-scale abattoir versus the small-scale abattoir, but both abattoirs still showed process hygiene levels within the regulatory satisfactory or acceptable range. Salmonella was recovered only from two pig carcasses in the large-scale abattoir. The study revealed that TVC levels differed more on pig carcasses, while EC levels differed more on cattle carcasses. Process hygiene levels in both abattoirs were always in the allowed regulatory range and also were similar to the hygiene levels in other, developed European countries.

Keywords: *abattoir, process hygiene, Total Viable Count, Enterobacteriaceae, Salmonella.*

Introduction

An abattoir is a place where animals are humanely killed, under the supervision of authorized/official veterinarian, in order to provide meat for human consumption. During slaughter, many processing steps can contribute to cross-contamination of carcasses with the microorganisms originating from animal hide/skin, utensils and equipment, food-contact surfaces, workers and, most importantly, from the gastro-intestinal tract (*Veterinary Directorate*, 2007). Evisceration is the phase that contributes most to the finding of bacteria on the surface of the carcasses, especially because after skinning, at the slaughter line there is no longer any primary treatment phase that could reduce the number of bacteria (*Raseta et al*., 2015).

The implementation of Good Manufacturing Practice (GMP), Hazard Analysis and Critical Control Points (HACCP) and various interventions such as physical interventions (hygienic de-hiding, scalding/singeing/polishing, evisceration) and chemical interventions (carcass decontamination) (*Antic*, 2010) by slaughter and meat processing facilities play a large role in enhancing the safety of meat products (*Bohaychuk et al*., 2011). Such interventions can lead to significant decrease of microbial numbers on carcasses. However, the physical methods cannot be substituted and/or replaced simply by chemical interventions, but rather must be supplemented with multiple decontamination procedures which have additional biocidal effect and therefore increase the level of microbial reductions. Additionally, contamination from the environment can also be significant during primary processing at abattoirs (*Australian Meat Processor Corporation*, 2013).

From the standpoint of food spoilage and foodborne disease, enteric bacteria are of great concern because they are frequently encountered in red meat production. Although various foods can

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serve as vehicles of foodborne illness, meat and meat products remain important sources of human infections with a variety of foodborne pathogens, i.e. *Salmonella spp*., *Campylobacter jejuni/coli*, *Yersinia enterocolitica*, human pathogenic Verotoxigenic *Escherichia coli*, and *Listeria monocytogenes* (*Nørrung et al*., 2009). Foodborne disease provoked due to the presence of pathogenic bacteria in the food usually manifests itself in episodes of gastro-intestinal disease (diarrhea, vomiting, etc.) (*Nørrung et al*., 2009).

The intestines of animals contain large numbers of microorganisms, e.g. *E. coli* levels are usually greater than 10^5 cfu g^{-1}, and amongst these microorganisms can be found foodborne pathogens such as *E. coli* O157, *Salmonella* and *Campylobacter* (*Australian Meat Processor Corporation*, 2013). The important meat hygiene indicators are *Enterobacteriaceae* (EC) and Total Viable Counts (TVC). The deep muscle tissues of healthy, slaughtered livestock contain few, if any, microorganisms (*Veterinary Directorate*, 2007). However, their exterior surfaces (hide, hair, skin) are naturally contaminated with a variety of microorganisms, as well as their gastro-intestinal tracts. The most common order of event anticipating foodborne diseases involves the existence of a primary source, e.g. healthy animals intended for meat production, which can intermittently fecally shed the pathogens that can be further spread in the process of primary production (on-farm), processing (in abattoir and meat processing), as well as handling by consumers (*Nørrung and Buncic*, 2008).

Regulation (EC) No. 2073/2005 and the Regulation on the general and specific food hygiene requirements at any stage of production, processing and trade (*Serbia*, 2010) require the obligatory control of TVC and EC, which are defined as hygiene indicators in slaughter processes. *Martelli et al*. (2017) stated that TVC are defined as indicators of the overall slaughter hygiene (equipment, tools, workers, environment), while EC are recognized as indicators of fecal contamination on carcasses and in abattoirs. *Salmonella* is the second most commonly reported zoonotic gastro-intestinal pathogen in the European Union (*EFSA*, 2016), and a significant proportion of the cases is linked to the consumption of contaminated pork (*Martelli et al*., 2017). *Salmonella* is regularly found in the intestines of humans and animals, and by fecal shedding it can also reach the environment. Many *Salmonella* serovars which are closely related to foodborne outbreaks, e.g. *S.* Typhimurium, *S.* Enteritidis, *S.* Newport, and *S.* Heidelberg, have reservoirs in farm animals (*Andino*, 2015). The most common primary reservoirs for *S.* Typhimurium are pigs, cattle and poultry (*Nørrung et al*., 2009). Pork is especially important because pigs are one of the main meat-producing species that can asymptomatically carry *Salmonella*, periodically shedding the bacteria through feces (*Martelli et al*., 2017).

It has been estimated that approximately 1% of *Salmonella* infections in humans are caused by the consumption of contaminated pork or processed foods derived from pork in the United States (*Guo*, 2011), and 0.02 % in the EU (*EFSA*, 2016). On the other hand, the average percentage of all positive cattle carcasses contaminated with *Salmonella* in the EU along the meat chain (pre-harvest/on-farm, harvest/slaughter and post-harvest/meat-processing), during 2008 and 2009 was 0.2% (EFSA, 2013). The most commonly isolated *Salmonella* serovars originating from cattle carcasses were *S.* Typhimurium and *S.* Dublin. According to EFSA report from 2015, the overall herd prevalence was 12.4% and 2.1%, for pigs and cattle, respectively, while *Salmonella* was detected in 1.7% and 0.2% of pork and cattle meat samples, respectively (*EFSA*, 2016).

In addition, the main reservoir of *E. coli* O157 is cattle, which can shed the pathogen fecally, and therefore, this alimentary pathogen can subsequently contaminate the foods originating from cattle, most commonly, meat. Direct fecal contamination of carcass with pathogens originating from penetrated intestines during evisceration (leakage from guts onto the meat) is relatively rare in modern slaughterhouses, while microbiological contamination from hides (direct contact, knife, equipment, air) is a crucial and relatively common event (*Antic et al*., 2010; *Koohmaraie et al*., 2005). In Serbia, *Nastasijevic et al*. (2008) found that the prevalence of *E. coli* on the skin of slaughtered cattle was 28.2%, while *Blagojevic et al*. (2011) found that the prevalence was 52 to 64%. Under conditions of simulated direct skin-meat contact, this transfer amounts to 0.5% of TVC and 2% of EC from the numbers on cattle skins (*Antic et al*., 2010). However, despite this low transfer rate, the high level of bacterial contamination of the skin, as well as the regularity of bacterial transfer to the body during skin removal, indicate that the risk of bacterial contamination of the carcass from the skin is very significant (*Nastasijevic et al*., 2016).

According to the 1989 regulation on the conditions that must be fulfilled by abattoirs and meat processing establishments (*SFRJ*, 1989) and until 2011, abattoirs in Serbia were divided by structure, technical equipment, capacity, work and veterinary inspection organization into: (i) large-scale abattoirs (industrial abattoirs), (ii) small-scale abattoirs, and (iii) community abattoirs. However, meat

produced in small abattoirs can have a significant influence on consumers taking into consideration the level of their exposure to the meat delivered by these abattoirs, based on the market share. One of the best examples is in the UK, where 51% of abattoirs are small-scale, accounting for 22.7% of the meat market share (*UK*, 2008).

Changes to the Serbian Rulebook on Veterinary-Sanitary Requirements, and general and special conditions of hygiene of food of animal origin (*Serbia*, 2011) and its harmonization with EU law (*EC*, 2004) on the hygiene of foodstuffs of animal origin, did not recognize differences between large- and small-scale abattoirs. In fact, the terms *large-scale abattoirs* and *small-scale abattoirs* has been used to describe production volume, the number of employees, the craft of financial assets and the annual profit. For example, small-scale UK abattoirs have a maximum of 50 employees, while large establishments have a minimum of 250 employees (*UK*, 2008). No matter whether the abattoir is small-scale or large-scale, it must satisfy specific conditions related to the construction methods, technical/technological equipment, veterinary/sanitary conditions, working methods, hygiene level and workers' training. The aim of this research was: a) to determine if there are any differences regarding process hygiene level at slaughter between selected large- and small-scale abattoirs in one Serbian county, and b) to assess to which extent those abattoirs achieved satisfactory level of the slaughter process hygiene.

Materials and methods

The study encompassed one small-scale and one large-scale (industrial) abattoir in one county in Serbia, regularly inspected by the competent authority (veterinary inspection). Abattoirs that were the object of this research had different production capacities. The industrial abattoir had a daily production capacity of 700 pig carcasses and 70 cattle carcasses. Daily capacity of the small-scale abattoir was 90 pig carcasses and 30 cattle carcasses. Altogether, 1180 wet-dry swabs were collected in the large- and small-scale abattoirs during a five year period (2012–2016) for cattle carcasses and during a six year period (2011–2016) for pig carcasses. Therefore, swab (sample) collection took place throughout 2011 and until 2016. Samples were taken in accordance with the local regulation on the general and specific food hygiene requirements at any stage of production, processing and trade (*Serbia*, 2010). A random sampling strategy was followed, which means that swabs were regularly collected once per month. The sampling was based on standard Serbian-ISO harmonized methods (*ISO*, 2009). The sampling was also in line with the self-control plans developed by HACCP teams in both abattoirs and was regularly approved by the veterinary inspector in charge. The number of samples by year was not the same for large- and small-scale abattoirs, due to observed differences in their self-control plans. Therefore, the sampling frequency varied for large-and small-scale abattoirs depending on the year during the period 2011–2016. The sampling of cattle and pig carcasses was conducted by an authorized person in both abattoirs and testing was performed by an external laboratory accredited in accordance with ISO 17025 (*ISO*, 2005) for TVC, EC and *Salmonella* detection and enumeration. TVC and EC detection was performed according to ISO 4833 (*ISO*, 2003) and ISO 21528–2 (*ISO*, 2004), respectively. The interpretation of results was carried out according to EU Regulation 2073/2005 (*EC*, 2005). Detection of *Salmonella* spp. was carried out according to ISO 6579 (*ISO*, 2002), and results were recorded as *Salmonella* presence or absence (*EC*, 2005).

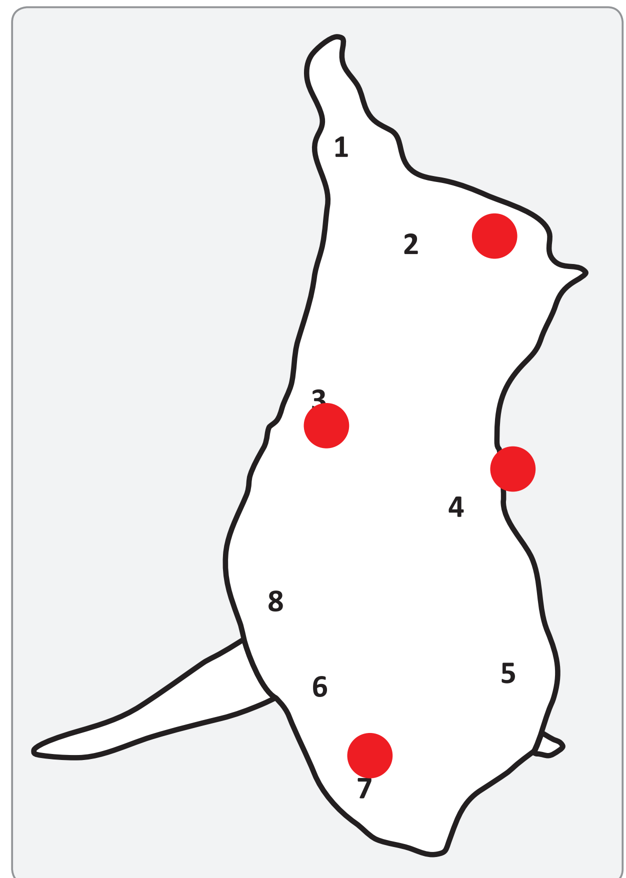

Figure 1. Sampling sites on pig carcass

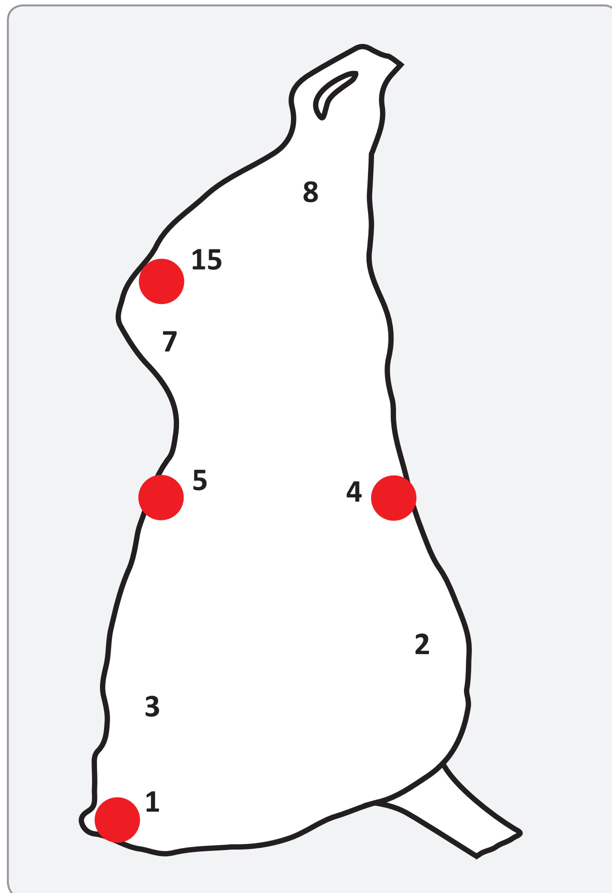

Figure 2. Sampling sites on cattle carcass

Swabs were collected in each abattoir at regular time intervals during this study. During each sampling session, swabs were taken from five cattle and/or five pig carcasses randomly selected at the end of slaughter line, after the final wash but before chilling. The swabs were taken at four sites on each carcass, e.g. pig carcasses (rump, belly, back and jaw; Figure 1) and cattle carcass (rump, belly, thorax and neck; Figure 2) following the recommendation of the harmonized national standard (*ISO*, 2009; it has to be taken into consideration that this version of the standard was used during the time when this study was conducted, from 2011–2016).

Sterile, pre-moistened sponges were used to swab four adjacent areas (Figure 1) covering a total area of 400 cm² (100 cm² per each area) on one half of each chosen carcass. *Salmonella* was recovered from sponges used to swab the corresponding half of each carcass, from a 400 cm² area. In total, 1180 carcasses were examined from 2011–2016. Altogether, 640 carcasses (cattle n=270, pig n=370) were swabbed in the industrial, large-scale abattoir, while 540 carcasses (n=cattle 340, pig n=200) were swabbed in the small-scale abattoir.

The obtained microbial results/data were analyzed using Microsoft Office Excel 2007. Firstly, the average logarithm value of TVC and EC for each carcass was calculated (based on previously estimated log values for each of four corresponding sites on each carcass), and then the average daily logarithm was defined. The average daily logarithm of *Salmonella spp.* was not calculated, taking into account the regulatory requirement defining only the absence or presence of *Salmonella spp*.

Results

Overall, in the period from 2012 to 2016 for cattle carcasses and 2011 to 2016 for pig carcasses, TVC counts ranged from 1.17–2.33 log cfu cm^{-1} and from 1.34–3.10 log cfu cm^{-1}, for cattle and pig carcasses, respectively (Tables 1 and 2). The EC levels varied from 0.01–0.37 log cfu cm^{-1} and 0.11–0.82 log cfu cm^{-1}, for cattle and pig carcasses, respectively (Tables 1 and 2).

Table 1. The level of cattle carcass contamination in a large-scale and a small-scale abattoir, 2012–2016 (n=610)

		2012		2013		2014		2015		2016	
		A	B	A	B	A	B	A	B	A	B
n		85	55	50	75	35	85	55	50	75	35
%		31.48	16.18	18.52	22.06	12.96	31.48	16.18	18.52	22.06	12.96
TVC – log cfu cm^{-1} ($\bar{x}$±SD)		0.60±0.26	0.24±0.15	1.12±0.10	0.82±0.19	1.63±0.11	1.43±0.17	2.16±0.21	1.99±0.14	2.85±0.25	2.57±0.23
EC – log cfu cm^{-1} ($\bar{x}$±SD)		0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.04	0.00±0.00	0.17±0.04	0.11±0.12	0.97±0.66	0.84±0.38
Salmonella spp.	*n*	0	0	0	0	0	0	0	0	0	0
	%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Legend: A – large-scale abattoir; B – small-scale abattoir, n – number of carcasses

Table 2. The level of pig carcass contamination in a large-scale and a small-scale abattoir, 2011–2016 (n=570)

		2011		2012		2013		2014		2015		2016	
		A	B	A	B	A	B	A	B	A	B	A	B
n		35	5	55	20	50	35	90	50	80	35	60	55
%		9.46	2.50	14.86	10.00	13.51	17.50	24.32	25.00	21.62	17.50	16.22	27.50
TVC – log cfu cm^{-1} ($\bar{x}$±SD)		0.39±0.18	0.00±0.00	1.15±0.12	0.47±0.33	1.44±0.07	1.01±0.18	1.94±0.27	1.60±0.25	2.63±0.17	2.26±0.14	3.40±0.28	3.06±0.39
EC – log cfu cm^{-1} ($\bar{x}$±SD)		0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.37±0.14	0.89±0.71	1.30±0.55	0.06±0.10
Salmonella spp.	n	0	0	0	0	0	0	0	0	2	0	0	0
	%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00

Legend: A – large-scale abattoir; B – small-scale abattoir, n – number of carcasses

Total Viable Counts

In the large-scale abattoir, TVC were isolated from all cattle carcasses (n=300; 100%) and from all pig carcasses (n=370, 100%). Mean TVC values for cattle carcasses were within the satisfactory range (<2.8 log cfu cm^{-1}) in 75% (n=225) of tested cattle carcasses, while in 25% (n=75) of tested carcasses, TVC levels were within the acceptable range, between 2.8–4.0 log cfu cm^{-1}. The established TVC levels on pig carcasses were within the satisfactory range (<3.3 log cfu cm^{-1}) in 74.33% (n=275) of tested carcasses, while in 25.67% (n=95) of tested pig carcasses, the TVC levels were within the acceptable range, between 3.3 and 4.3 log cfu cm^{-1}. In the small abattoir, TVC were isolated from all cattle carcasses (n=300; 100%), with levels within the satisfactory range. Further, TVC were isolated from all tested pig carcasses (n=200, 100%), but again, all of them were within the satisfactory range. Apparently higher TVC levels were observed in the large-scale abattoir versus the small-scale abattoir, but both abattoirs still showed process hygiene levels within the satisfactory range (Table 3).

Enterobacteriaceae Counts

In the large-scale abattoir, EC were isolated from 165 (55%) cattle carcasses, while on 135 carcasses, the EC levels were below the detection limit; EC values were always within the satisfactory range, below 1.2 log cfu cm^{-1}. On 230 (62.16%) of pig carcasses, the EC levels were below the detection limit, while on 140 (37.84%) of pig carcasses, the detected EC levels were within the satisfactory range (<1.3 log cfu cm^{-1}). In the small abattoir, low counts of EC were also determined. EC were confirmed on 85 (28.33%) of the cattle carcasses and 90 (45%) of the pig carcasses. In both cases, cattle and pig carcasses, the observed EC levels were always within the satisfactory range as defined by the legislation (*EC*, 2005; *Serbia*, 2010).

Salmonella species

The presence of *Salmonella* spp. was detected only on two pig carcasses originating from the large-scale, industrial abattoir.

Table 3. Summary view of cattle and pig carcass contamination in a large-scale and a small-scale abattoir, 2011–2016 (n=1180).

	TVC – log cfu cm^{-1} ($\bar{x}$±SD)		**EC – log cfu cm^{-1} ($\bar{x}$±SD)**		***Salmonella spp. detected***	
	Cattle	Pig	Cattle	Pig	Cattle	Pig
A	(n=270)	(n=370)	(n=270)	(n=370)	(n=270)	(n=370)
	1.53±0.98	1.99±1.01	0.20±0.49	0.30±0.62	0	2*
B	(n=340)	(n=200)	(n=340)	(n=200)	(n=340)	(n=200)
	1.40±0.97	1.86±1.10	0.17±0.47	0.26±0.61	0	0

Legend: A – large scale abattoir; B – small scale abattoir; n – number of carcasses *during 2015, *Salmonella* spp. was detected on two pig carcasses.

Discussion

TVC is the general indicator for hygienic operations indicating the overall hygiene in abattoirs (equipment, tools, workers), while EC counts indicate fecal contamination of carcasses. The TVC/EC levels detected on the carcasses do not serve *per se* for decision-making on carcass/meat acceptance or rejection, but rather serve as general indicators of slaughter hygiene (*Delhalle et al*., 2008). Low levels of TVC were detected at all times, either within the satisfactory or acceptable range. In both abattoirs, TVC on cattle carcasses were always within the regulatory requirements. Some other studies reflecting TVC levels on cattle carcasses showed different variations, such as a study carried out in Ethiopia where TVC levels were 5.21 log cfu cm^{-1} (*Gebeyehu et al*., 2013), in Algeria, 4.48 log cfu cm^{-1} (*Nouichi and Hamdi*, 2009), in Switzerland, between 2.1–3.1 log cfu cm^{-1} (*Zweifel et al*., 2005) and in Australia, 2.42 log cfu cm^{-1} (*Phillips et al*., 2001).

In both abattoirs, the EC levels were at all times within the satisfactory range for cattle and pig carcasses, respectively. A similar result was reported in another study conducted in Serbia, where the slaughter process hygiene was assessed in two large- and two small-scale abattoirs, accounting for 58.5% of Serbia`s national production of beef/pork meat (*Nastasijevic et al*., 2016). Our low *Salmonella* prevalence was also in accordance with an earlier study carried out in Serbian abattoirs (*Blagojevic et al*., 2011). In the current study, *Salmonella* was absent from cattle carcasses, while it was detected on two pig carcasses in the large-scale abattoir. It is worth noting the current legislation (*EC*, 2005; *Serbia*, 2010), by which the presence of *Salmonella* on 2 of 50 cattle carcasses and on 5 of 50 pig carcasses is acceptable.

Also, based on available results from developed countries such as the USA and Ireland, as well as other countries such as Algeria and Turkey, where average contamination of bovine carcasses was 1.5% (Sofos, 2005), 7.6% (*Keogh et al*., 2001), 10% (*Nouichi and Hamdi*, 2009) and 10% (*Akkaya et al*., 2008), respectively, it can be concluded that slaughter hygiene in the chosen Serbian slaughterhouses was similar or better than that in some other developed countries.

It is worth noting that industrial-type, large-scale production meat establishments mostly have more developed risk-based meat safety management systems (HACCP-based) and more intensive cooperation with professional/scientific institutions and laboratories compared with small-scale establishments. This fact can be sometimes related to the higher level of slaughter process hygiene in large-scale meat establishments versus small-scale establishments. On the other hand, it should be kept in mind that small abattoirs with a smaller slaughter throughput have more opportunities to thoroughly conduct cleaning and disinfection protocols and to devote more attention to monitoring critical control points, which subsequently contributes to the higher level of hygiene.

Conclusion

Microbiological data on hygiene indicators are important for assessment of hygiene levels in abattoirs and control of carcass contamination. Risk categorization of abattoirs should be based on a process hygiene output through the use of indicator organisms, TVC and EC, monitored on carcasses. The indicators monitored on carcasses (TVC, EC) indicate whether the process hygiene functions acceptably, but they do not indicate control of the hazards *per se*. The presence of TVC indicate the overall hygiene in abattoirs (equipment, tools, workers), while the presence of EC on carcasses can indicate fecal contamination which can occur during slaughter/dressing. In addition, *Salmonella* spp. can serve as an indicator of the presence of pathogens on carcasses/meat. In this study, we compared the process hygiene levels between two selected abattoirs, a large-scale and a small-scale abattoir, over a period of five years for cattle carcasses and six years for pig carcasses. In total, 1180 cattle or pig carcasses were examined. The results revealed that in the large-scale abattoir, TVC were isolated from all cattle and pig carcasses. Mean TVC values for cattle carcasses were within the satisfactory range in 75% (n=225) of tested carcasses, while for 25% (n=75) of carcasses, TVC levels were within the acceptable range. TVC levels on pig carcasses were within the satisfactory range in 74.33% (n=275) of tested carcasses, while for 25.67% (n=95) of pig carcasses, TVC levels were within the acceptable range. In the small abattoir, TVC were also isolated from all cattle/pig carcasses, with levels always within the satisfactory range. Apparently higher TVC levels were observed in the large-scale abattoir versus the small abattoir, but both abattoirs still showed process hygiene levels were within the regulatory satisfactory range. In the large-scale abattoir, EC were isolated from 55% (n=165) of cattle carcasses and were always within the satisfactory range. On 62.16% (n=230) of tested pig carcasses, the EC levels were below the detection limit, while on 37.84% (n=140) of pig carcasses,

the detected EC levels were within the satisfactory range. In the small abattoir, low EC levels were observed on cattle/pig carcasses and were always within the regulatory satisfactory range.

Salmonella spp. prevalence was low, as this pathogen was not detected on cattle carcasses over the five year period, while only two positive findings were observed among the pig carcasses over six years.

Overall, the observed process hygiene levels in both abattoirs did not differ significantly, and were rather similar to process hygiene levels in other, developed EU countries. Development and vigorous implementation of self-control plans intended for monitoring hygiene indicators (TVC, EC) on cattle or pig carcasses can lead to achievement of satisfactory levels of slaughter hygiene, no matter the size and throughput of the abattoir. Further research should be carried out to establish an evidence-based interface between slaughter process hygiene, risk categorization of abattoirs and the frequency of inspection visits.

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Original scientific paper

The impact of triticale diet on production characteristics and meat quality in pigs

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A b s t r a c t: Triticale (Triticosecale) is a hybrid crop which inherited the excellent grain quality and high yield potential of wheat (Triticum spp.) and the good tolerance to biotic and abiotic stresses factors of rye (Secale cereale). As an energy crop, triticale has now been used for human food and livestock feed for many years. The main agronomic goal has been to improve the properties of triticale grains over those of wheat, corn, rye, barley, etc., making triticale an attractive option for increasing global food production. This paper discussesthe advantages of using triticale as a pig feed.

Keywords: *triticale characteristic, pig feed, meat quality, carcass quality.*

Introduction

Triticale (*Triticosecale*) is the only cereal crop successfully developed by man within the last 140 years. This crop species developed from crosses between wheat (*Triticum spp.*) and rye (*Secale cereale*). Triticale has high grain yield potential and quality that a rise from its wheat ancestry, plus broard adaptability, and ability to tolerate low soil fertility, drought and extremes of soil pH, all derived from rye (*Glamoclija,* 2012). Because of these characteristics, triticale is a well-established livestock feed ingredient that is widely available for use and readily accessible by most feed mills (*Glamoclija et al.,* 2017a). Triticale can be used in livestock diets like other cereals as a whole grain, or for fodder production like hay, silage, straw or chaff, and it compares favorably with respect to quantity and quality attributes. The grain is primarily used for feeding pigs, but it can be also used for poultry, and for ruminant animals such as cattle and sheep. As forage, the crop is grazed by cattle and sheep, or harvested for silage or hay for those animals. Triticale also produces an abundant amount of straw, which can be used for many animal species (*Van Barneveld,* 2002). Early interest in triticale as a feed grain was generated because of its higher protein concentration and better amino acid balance as compared to other feed grains.

The production characteristics of pigs and quality of the pork produced from the animals are influenced by numerous genetic and non-genetic factors, including dietary factors. The strains of pigs referred to as PIC (*Pig Improvement Company,* www.pic.com) swine have been developed for optimal, lean production, excellent growth rate and feed efficiency, and low backfat levels (*Turyk et al.,* 2011). Because of its agronomic attributes and dietary quality, triticale has the potential for broad application in animal feeds and has attracted international interest for its application as a major ingredient of swine feed. Triticale can be used as a substitute for corn or barley in the diets of growing-finishing pigs, without compromising muscle quality or palatability (*Jaikaran et al.,* 1998).

Nutrient composition of triticale

Triticale is primarily included in livestock diets as an energy source (*Glamoclija et al*., 2017b). It is characterized by moderate protein and high gross energy content because of its high contents of starch and other carbohydrates (*Widodo et al.*, 2015). Early on, interest in triticale as a feed grain was generated because of its higher protein concentration and better amino acid balance as compared to other feed grains such as maize and wheat (Table 1, Table 2) (*Boros,* 2002, *Glamoclija et al.,* 2017a).

The proximate chemical composition of triticale grains is, in general, intermediate between the two parent species of wheat and rye (Table 3) (*Myer and Lozano del Rio,*2004).

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Table 1. Comparative composition of triticale, maize and wheat grain (on an as-fed basis) (adapted from *Boros,* 2002; *Glamoclija et al.,* 2017a)

Item	Triticale	Maize	Wheat
Crude protein (%)	12.0	8.5	11.5
Lysine (%)	0.40	0.24	0.34
Crude fiber (%)	2.8	2.2	2.4
Acid detergent fiber (%)	3.8	2.8	3.5
Neutral detergent fiber (%)	12.7	9.6	11.0
Crude fat (%)	1.8	3.8	1.8
Calcium (%)	0.05	0.02	0.05
Phosphorus (%)	0.33	0.25	0.33
Metabolizable energy in pigs (kcal/kg)	3200	3350	3350

Table 2. Comparative chemical composition and energy value of triticale and other common grains for feeding animals in Europe (adapted from *Boros,* 2002; *Glamoclija et al.,* 2017a)

	Chemical composition (g kg^{-1}dry matter)						
Item	Maize	Wheat	Triticale	Rye	Oats	Sorghum	Millet
Crude protein	106	130	140	116	120	120	128
Crude fat	47	23	22	22	55	35	38
Cellulose	24	27	27	27	112	29	95
NFE[a]							
Starch	700	680	620	640	440	700	590
Sugar	20	31	55	50	18	15	10
Mineral mater	15	18	20	22	33	20	43
Calcium	0.4	0.8	0.9	0.9	1.2	0.4	0.5
Phosphorus	3.1	4.0	3.6	3.2	3.8	3.3	3.4
Digestible energy in pigs ($MJkg^{-1}$ dry matter)	16.4	16	15.8	15.7	13.2	15.8	13.6

[a] NFE-Nitrogen free extract

Table 3. Proximate chemical composition of triticale, wheat and rye (% on dry basis) (*Myer and Lozano del Rio,*2004)

Cereal	Protein	Starch	Crude fibre	Ether extract	Free sugars	Ash
Spring triticale	10.3–15.6	57–65	3.1–4.5	1.5–2.4	3.7–5.2	1.4–2.0
Winter triticale	10.2–13.5	53–63	2.3–3.0	1.1–1.9	4.3–7.6	1.8–2.9
Spring wheat	9.3–16.8	61–66	2.8–3.9	1.9–2.2	2.6–3.0	1.3–2.0
Winter wheat	11.0–12.8	58–62	3.0–3.1	1.6–1.7	2.6–3.3	1.7–1.8
Spring rye	13.0–14.3	54.5	2.6	1.8	5.0	2.1

Pig production characteristics

Triticale is often the preferred feed grain for pigs in many countries worldwide. Triticale can be included without restriction as a high value, consistent quality cereal grain in least-cost formulations for growing pigs. It canbe used in either ground or pelleted form (*Myer and Barnett,*2000; *Van Barneveld,* 2002; *Salmon et al.,* 2004; *Sullivan et al.,* 2007; *Woyengo et al.,* 2014).

Even though triticale grain contains more proteinthan maize or grain sorghum, diets should be formulated to meet the essential amino acid (especially lysine) requirements of the pig rather than the crude protein requirements. If diets containing triticale were formulated on the basis of crude protein alone, lysine levels could be in adequate and pig performance would suffer (*Van Barneveld and Cooper,*2002). The crude protein concentration of triticale-based diets is usually higher than that of comparable maize-based diets when both diets contain equal levels of lysine (*Myer,*2002).

Typical pig diets formulated with triticale are given in Table 4 (*Myer and Lozano del Río,* 2004).

There are several advantages in feeding pigs triticale. Triticale is superior to barley in digestible energy levels for pigs and protein composition. Triticale digestible energy in pig diets is equivalent to wheat, and in young pig diets it is equivalent to corn, but triticale is cheaper than wheat or corn. Digestibility of dry matter and amino acids in the ileum of pigs fed triticale was generally higher than for barley (*Van Barneveld,* 2002). The superior protein quality and high yield potential of triticale grain has maintained international interest in using the crop as a pig feed (*GRAIN,* 2004). Generally, reports show that using triticale as a pig feed has been very successful. Producers have been able to replace other cereals, (e.g. wheat, corn, barley or millet) with triticale without losing productivity

Table 4. Examples of typical pig diets using triticale grain (*Myer and Lozano del Río,* 2004)

	Grower (20–50 kg)	**Finisher I (50–80 kg)**	**Finisher II (80–110 kg)**
Base feed constituents			
Ground triticale (%)	74.25	82.75	90.00
44% soybean meal (%)[a]	22.5	15.0	8.0
Base feed mix[b]			
Dicalcium phosphate (%)[c]	1.25	0.75	0.625
Limestone ground (%)	1.000	1.000	0.875
Salt (%)	0.50	0.25	0.25
Vitamin-trace mineral premix (%)[d]	0.50	0.25	0.25
Total	*100*	*100*	*100*
Calculated composition (as-fed basis)			
Crude protein (%)	18.8	16.5	14.4
Lysine (%)	0.96	0.77	0.60
Calcium (%)	0.75	0.62	0.55
Phosphorus (%)	0.64	0.53	0.48
Metabolizable energy (kcalkg^{-1})	3150	3170	3200

Legend: [a]Can replace ten parts of 44 percent soybean meal with nine parts of 48 percent soybean meal and one part of triticale.

[b]A complete mineral-vitamin premix or a complete mineral premix and separate vitamin premix canbe used instead of the suggested base mix. Follow manufacturer guidelines.

[c]Defluorinated phosphate or mono-dicalcium phosphate, if available, can be substituted for dicalcium phosphate. However, if a substitution is made, the diets need to be reformulated since these products contain different calcium and phosphorus levels than does dicalcium phosphate.

[d]Amounts shown are typical for many commercial products. Follow manufacturer guidelines.

Table 5. Feeding and carcass production results for market hogs in the 27–110 kg class, comparing corn, barley and triticale (*Jaikaran et al.,* 1998)

	Corn	Barley	Triticale	Barley/Triticale
Feeding results				
Daily feed intake (kg) (F)	2.50	2.53	2.50	2.66
Daily gain (kg) (G)	88.5	91.5	89.9	93.5
Feed efficiency (kg) (F/G)	2.85	2.87	2.81	2.86
Carcass production results				
Shipping weight (kg)	109.7^{ab}	109.1^{b}	110.7^{ab}	112.4^{a}
Shrink (%)	4.86	5.45	5.22	4.66
Dressing (%)	79.5^{a}	78.2^{b}	78.6^{ab}	79.0^{ab}
Backfat (mm)	19.7^{ab}	17.5^{b}	17.9^{b}	20.7^{a}
Estimated lean yield (%)	59.4^{ab}	60.2	60.2^{a}	58.7^{b}
Carcass cutout lean yield (%)	55.6^{ab}	56.6^{ab}	56.9^{a}	55.0^{b}

*Values with different superscript letters in a row are significantly different (p<0.05)

Table 6. Meat and carcass quality of market hogs fed on corn, barley or triticale (*Robertson et al.,* 1998)

	Corn	Barley	Triticale	Barley/ triticale
Final live weight and carcass data				
Shipping weight (kg)	109.7 ^{ab}	109.1 ^{a}	110.7^{ab}	112.4^{a}
Final live weight at abattoir (kg)	105.1	104.3	104.7	105.5
Warm carcass weight (kg)	87.8 ^{b}	86.2 ^{a}	86.8^{ab}	87.5^{ab}
Rib eye area (12th rib) (cm^2)	35.70	36.46	37.90	35.35
Total cut out yield (g kg^{-1})	556.3	565.6	568.9	550.2
Meat quality (longissimus thoracis)				
pH 45 min	6.27	6.25	6.31	6.31
pH 48 h	5.55	5.53	5.59	5.57
Lightness (L*)	48.6^{a}	50.6^{b}	50.8^{b}	50.1^{ab}
Chroma (Cab*)	9.3	8.7	8.2	8.6
Drip loss (mgkg^{-1})	29.1^{a}	38.7^{b}	28.3^{a}	30.7^{a}
Maximum shear value (kg)	4.85	4.77	5.00	4.88
Moisture (mgkg^{-1})	747.9^{ab}	748.4^{ab}	749.0^{b}	744.9^{a}
Intra-muscular fat (mgkg^{-1})	17.9	19.1	18.2	21.1
Total protein (mgg^{-1})	220.6	218.8	219.6	221.1
Boiled chop overall tenderness	5.39^{a}	6.18^{b}	5.55^{ab}	5.45^{a}

Values with different superscript letters in a row are significantly different (p<0.05)

Table 7. Comparison of carcass characteristics of pigs fed barley or triticale based diets (*Turyk et al.,* 2011)

Specification	Barley	Triticale	SEM*	Significance§
Body weight at slaughter(kg)	105.17	110.83	1.41	p<0.01
Cold carcass weight(kg)	83.70	87.00	1.32	p<0.05
Cold dressing(%)	79.62	78.45	1.87	NS
Meatiness(%)	57.47	58.40	0.47	p<0.05
Carcass length(cm)	80.17	81.00	0.39	NS
Backfat thickness(cm)				
Over the shoulder	3.12	3.30	0.20	NS
Mid back	2.00	1.90	0.24	NS
Over loin I	1.72	1.65	0.15	NS
Over loin II	1.10	0.93	0.11	p<0.05
Over loin III	2.00	1.92	0.17	NS
Average offive measurements	1.97	1.86	0.19	NS
Loin eye area (cm^2)	42.93	45.85	0.88	p<0.01
Weight of suet(kg)	1.20	0.94	0.09	p<0.01

*SEM – standard error of the mean; § – Student's t-test; NS – non significant

Table 8. Comparison of physical and chemical properties of meat from pigs fed barley or triticale based diets (*Turyk et al.,* 2011)

Specification	Barley	Triticale	SEM*	Significance§
Muscle *longissimus lumborum*				
Dry matter (%)	27.33	27.46	0.41	NS
Crude ash (%)	1.16	1.12	0.02	NS
Crude protein (%)	22.82	23.29	0.18	NS
Crude fat (%	3.45	3.31	0.72	NS
Muscle *semimembranosus*				
Dry matter (%)	25.13	25.12	0.19	NS
Crude ash (%)	1.18	1.17	0.01	NS
Crude protein (%)	22.94	23.08	0.12	NS
Crude fat (%)	1.33	1.06	0.01	NS
Muscle water holding capacity (%)				
longissimus lumborum	20.00	23.93	1.55	p<0.01
semimembranosus	17.60	19.07	1.35	NS
Muscle meat colour (L*)				
longissimus lumborum	48.22	47.30	0.76	NS
semimembranosus	44.02	41.78	0.89	p<0.05
pH45 min	6.34	6.32	0.07	NS
pH24 h	5.82	5.79	0.08	NS

*SEM – standard error of the mean; § – Student's t-test; NS – non significant

Table 9. Growth performance of pigs fed balanced diets containing triticale compared with wheat, barley or sorghum *(Van Berneveld and Cooper,* 2002)

	Gain ($gday^{-1}$)	**Feed conversionratio (kg)**	**Gain ($gday^{-1}$);EBW* basic**	**Feed conversionratio (kg); EBW basic**	**Backfat depth at P2 position (mm)**
Triticale	681	2.4	415	4.0	10.1
Wheat	677	2.5	400	4.2	10.3
Barley	662	2.7	377	4.7	9.3
Sorghum	653	2.6	369	4.6	10.2

*EBW – Empty body weight

or product quality. Triticale is also more cost-effective than its competitors, as its high lysine content means less protein supplement is required (*Mergoumet al.,* 2009).

Two Canadian studies (*Robertson et al.,* 1998; *Jaikaran et al.,* 1998) compared the grain source of 100% triticale with 100% corn, 100% barley and a 50:50 mix of barley and triticale. The studies compared 25 pig production, carcass and meat quality characteristics. Triticale performed similarly to the corn (control) diet for 24 characteristics, and similarly to the 50:50 barley and triticale mixture in all cases. The conclusion was that triticale could be successfully substituted for corn or barley in the diets of growing-finishing (25–110 kg) pigs. Pig production results during/after feeding with corn, barley, triticale or the mix of barley plus triticale are shown in Tables 5 and 6 (*Robertson et al.,* 1998; *Jaikaran et al.,* 1998; *Myer and Lozano del Rio,* 2004).

Turyk et al. (2011) concluded that the greater body weight of pigs fed triticale diets resulted in significantly ($p<0.05$) greater cold carcass weight at comparable dressing percentages. Pigs fed triticale-based diets had larger loin eye area and smaller suet weight (both $p<0.01$), and tended to have slightly thinner backfat over loin compared with animals on barley-based diets (Table 7). The same authors conducted physical and chemical analyses of meat from pigs fed barley-or triticale-based diets (Table 8).

Van Berneveld and Cooper (2002) (Table 9) concluded that use of triticale in pig diet results in better daily weight gain than diets based on wheat, barley or sorghum.

Conclusion

The general scientific belief is that triticale is an excellent feed choice for pigs. Its use results in few to no feeding problems, and it is a suitable substitutefor other grains. When triticale substitutes other grains, ration costs are lower because less soymeal or other protein meal supplements are needed. Triticale varieties are used in pig diets to supply levels of digestible amino acids and digestible energy equal or better to those in wheat-, barley-, or corn-based diets. Finally, the performance of growing pigs, production characteristics and meat quality parameters are equal or better than when pigs are fed wheat-, barley-, or corn-based diets. Overall, the available scientific knowledge indicates that triticale can be used in pig diets without restriction.

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Original scientific paper

Effect of different sodium butyrate levels in weaned pig diet on the antioxidant capacity of selected organs

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A b s t r a c t: The aim of this study was to evaluate the influence of different sodium butyrate levels on the antioxidant capacity of selected organs (liver and kidney) in pigs. The study was conducted on 48 weaned piglets (28 to 54 days old) fed one of three diets (group C had no added sodium butyrate, group E-I had 3 g and group E-II 5 g of sodium butyrate added per kg of diet). The guaiacol peroxidase and pyrogallol peroxidase activities in liver tissues of E-II pigs were significantly lower than those in E-I pigs, while the activities of these enzymes in kidney tissues were significantly lower than in control pigs for both butyrate levels. Glutathione peroxidase activity in liver tissues was not upregulated by either level of dietary sodium butyrate. Also, the contents of malondialdehyde, indicative of lipid peroxidation, were not significantly different among the pig dietary groups. No significant differences in the enzyme activities (catalase, superoxide dismutase, glutathione S-transferase or the oxidation product, reduced glutathione) of control pigs and of animals consuming 3 g or 5 g sodium butyrate per kg of diet was found. The results showed that oral administration of sodium butyrate had an impact only on guaiacol peroxidase and pyrogallol peroxidase enzyme activities.

Keywords: *piglet diet, antioxidative enzymes, liver, kidney, lipid peroxidation***.***

Introduction

For young animals, weaning is a critical stage because of alterations in the gastrointestinal tract morphology and function, often challenged by post-weaning stresses including diarrhea, low feed intake, and body weight loss, and these stresses can adversely affect intestinal health and function (*Song et al*., 2011). The organic acids have positive effects on growth performance of all pig categories, including weaned piglets (*Galfi and Bokori*, 1990; *Witte et al*., 2000; *Mazzoni et al*., 2008; *Piva et al*., 2002). Short chain fatty acids (SCFA), which are produced in the large intestine of mammals during microbial fermentation, are an important source of energy for animals (*Cortyl*, 2014). Large intestinal cells can use the produced SCFA, especially butyric acid, as a metabolism substrate (*Jozefiak et al*., 2004). Butyric acid is produced by bacterial fermentation of undigested carbohydrates in the intestine of human and animals and recent studies have shown effects on antoxidative activity (*Mentschel and Claus*, 2003; *Biagi et al*., 2007; *Guilloteau et al*., 2010).

Butyrate supplementation could improve antioxidative stress ability and piglet performance (*Lu et al.* 2008; *Ma et al.* 2012). *Song et al*. (2011) demonstrated that sodium butyrate can reduce diarrhea through a reduction in intestinal permeability and increasing the expression of mucosal tight junction proteins on the intestinal mucosa. Because of its functional properties and accessibility, sodium butyrate is widely used as a feed additive. Numerous studies have demonstrated that dietary inclusion of sodium butyrate did not disturb normal biochemical and physiological processes in animals (*Inan et al.*, 2000; *McCracken and Lorenz*, 2001; *Kotunia et al*., 2004; *Claus et al.*, 2007; *Lu et al*., 2008; *Guilloteau et al*., 2010; *Sunkara et al.*, 2011).

The present study, therefore, investigated the effect of sodium butyrate addition to weaned piglet diet on antioxidative capacity of the feed mixture, and on antioxidant enzyme activities in liver and kidney tissues.

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Materials and Methods

Animal ethics

The experimental protocol was approved by the Veterinary Directorate of the Serbian Ministry of Agriculture, Forestry and Water Management and the Ethics Committee of the Faculty of Veterinary Medicine, University of Belgrade.

Animal, housing and trial

The study was conducted on 48 weaned piglets (50% male and 50% female), of the same origin, Yorkshire x Landrace crossbreed. Sodium butyrate (commercial preparation of chemically protected sodium butyrate with 54% activity, Butirex C4, Novation, Spain) was included as a feed additive in sows' diet seven days before farrowing, and this continued during lactation until the day of insemination, and 30 days after insemination. The sodium butyrate preparation was used at the levels recommended by the manufacturer. The daily feed intake of lactating sows was 5 to 6 kg per day. Piglets were farrowed within a day, fed on sows' milk and from days 7 to 10 of life, started to feed on pre-starter with 2 g added sodium butyrate (Butirex C4, Novation, Spain) per kg of feed. Before weaning, piglets were housed with sows in the same facility, with the same preconditions including microclimate, before entering the trial. Weaned, 28-day-old piglets were randomly allocated and housed in one of three weaning pens (dimensions 2x2.3 m) within the same weaning facility, on concrete slatted floors, in groups of 16 animals per pen (stocking density was 4 animals m^{-2}). Weaned piglets were provided *ad libitum* with feed and water. The trial was conducted over a 26-day period (when piglets were from 28 days old to 54 days old), during which animals consumed their respective experimental diets.

Experimental diets

From the start (28-day-old piglets) until the end (54-day-old piglets) of the trial, each of the three groups of animals (16 animals per group) was fed one of three experimental diets. These comprised the same standard mixture for weaned piglets

Table 1. Ingredients of the pig diets (per kg of diet)

Ingredient (%)	**Diet**		
	C	**E-I**	**E-II**
Corn	45.56	45.45	45.37
Barley	18.0	18.0	18.0
Soybean meal	11.31	11.33	11.34
Soybean grits	4.5	4.5	4.05
AK 530 soy isolate	9.0	9.0	9.0
Potato protein	2.5	2.5	2.5
Whey 72%	2.5	2.5	2.5
Monocalcium phosphate	1.43	1.38	1.37
Cattle chalk	0.91	0.92	0.93
Cattle salt	0.52	0.33	0.2
Premix 1.5%*	1.5	1.5	1.5
Lysolecithin	0.05	0.05	0.05
Soybean oil	1.74	1.74	1.74
Mycotoxin adsorbent	0.2	0.2	0.2
Zinc oxide	0.3	0.3	0.3
Sodium butyrate	0.0	0.3	0.5
Σ	**100**	**100**	**100**

Legend: *Premix composition (per kg): Lysine 202.94g; Methionine 72.65g; Threonine 65.44g; Tryptophan 20.00 g; St. Dig. Lysine 202.90g; St. Dig. Methionine 72.65g; St. Dig. Meth&Cyst 72.65g; St. Dig. Threonine 65.44g; St. Dig. Tryptophan 20.00 g; Calcium137.16g; Vitamin (Vit). A 800100i.e; Vit. D 380030i.e; Vit. E 10952.56mg; Alpha tocopherol 9966.80mg; Vit. K3 306.83mg; Vit. B1 153.53mg; Vit.B2 306.83mg; Vit. B6 233.33mg; Vit. B12 1.54mg; D-pantotenic acid 780.03mg; Niacin 1533.47mg; Cholinchloride 16666.77mg; Biotin 15.47mg; Mn 3133.43mg; Fe 15066.80mg; Cu 11000.03mg; Zn 8000.07mg; I 15.47mg; Cobalt-II-carbonate 33.37mg; Se 26.83mg; Phytase 33333.40FYT; Fungal xylanase (3.2.1.8) 13333.40FXU

(starter diet), formulated to meet the maintenance and growth requirements of animals used in this study, but which differed in the addition of sodium butyrate. The diet for the experimental group C had no added sodium butyrate, the diet for experimental group E-I contained added 3 g of sodium butyrate per kg of mixture, while the diet for experimental group E-II contained added 5 g of sodium butyrate per kg of mixture (Table 1).

Chemical composition of the animal diets

Chemical analyses to determine protein, moisture, cellulose, fat, and ash of the feed were conducted according to AOAC methods (*AOAC*, 1990). Antioxidant capacity in diet is based on formation of the $ABTS^{\bullet+}$ cation [2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)], and its scavenging by antioxidant sample constituents measured by spectrophotometry (decay of green/blue chromophore absorbance is inversely associated with antioxidant sample content, while the control antioxidant is Trolox, a hydrophilic vitamin E analog) (*Miller et al*., 1993).

Biochemical analyses

At the end of the study, animals were transported to the slaughterhouse, individually weighed, electrically stunned and immediately slaughtered. Subsequently, animals were processed using standard industrial techniques and hot carcass, liver and kidney weights were recorded and samples of the organs were taken. Homogenates of liver and kidney were used with phosphate buffers (pH=7.0) for further biochemical analysis. Activities of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD-1), glutathione peroxidase (GSHPx), guaiacol peroxidase (GPx), pyrogallol peroxidase (PPx), glutathione S-transferase (GST), reduced glutathione and lipid peroxidation were measured in selected tissues (liver and kidney). The CAT activity was assayed by the method of *Aebi* (1984). The SOD-1 activity was determined according to *Kakkar et al*. (1984). The GSH-Px activity was determined using the method of *Paglia and Valentine* (1967). The GPx activity was measured by following the H_2O_2 dependent oxidation of guaiacol at 470 nm (*Agrawal and Laloraya*, 1977). The activity of PPx was measured using pyrogallol as the substrate according to *Chance and Maehly* (1955). The formation of purpurogallin was followed at 430 nm. GST activity in samples was evaluated using 1-chloro-2, 4-dinitrobenzene (CDNB) as the substrate, as previously described by *Habig et al*. (1974). Reduced glutathione (GSH) was performed according to the method described by *Sedlak and Lindsay* (1968). Malondialdehyde (MDA) level was analyzed with 2-thiobarbituric acid using the method of *Ohkawa et al*. (1979).

Statistical analyses

Statistical analyses of the results were conducted using software GraphPad Prism version 7.0 for Windows (GraphPad Software, San Diego CA, USA, www.graphpad.com). All parameters for each group of weaned piglets are shown in tables as mean±standard deviation. One-way ANOVA with Tukey's post-hoc test was performed to assess the significance among experimental groups.

Results and Discussion

The chemical composition of the feed, i.e. protein, moisture, fat, ash, fiber, calcium, phosphorus and NFE content is shown in Table 2. Diets for all groups of weaned piglets differed only in the amount of added sodium butyrate (0 g kg^{-1}, 3 g kg^{-1} or 5 g kg^{-1}).

Table 2. Chemical composition of the animal diet

Parameter	
Moisture	9.85
Ash	5.83
Crude Protein	18.68
Crude Fat	4.5
Crude Fiber	3.64
Calcium	0.97
Phosphorus	0.64
NFE*	57.50

*Nitrogen Free Extract

The results of chemical analyses showed that the diets for all piglets were in accordance with technological and legislative norms (*Official Gazette RS*, 2010), and the nutrient content fully satisfied the needs of weaned piglets (*NRC*, 1998). The sodium butyrate preparation was used at the levels recommended by the manufacturer.

Among the available analytical techniques, the Trolox equivalent antioxidant capacity assay is the most frequently used for assessing antioxidant

Table 3. Antioxidant activity of different feed extracts, measured by applying an improved ABTS test (mixture)

Feed	Extract			
	70% acetone	70% methanol	70% ethanol	Puffer pH=4.5
C	0.566±0.012^{AB}	0.446±0.012^{AB}	0.421±0.093^{a}	0.247±0.008^{aA}
E-I	0.630±0.012^{AC}	0.506±0.037^{AC}	0.499±0.037	0.263±0.025^{aB}
E-II	0.653±0.003^{BC}	0.569±0.023^{BC}	0.542±0.034^{a}	0.296±0.010^{AB}

Legend: Values expressed as mean± SD;
ANOVA test with *post hoc* Tukey's test.
^{A,B} Means within column with same superscript significantly differ at p<0.01.
^{a} Means within column with same superscript significantly differ at p<0.05.
C – diet without added sodium butyrate; E-I – diet with 3 g sodium butyrate added per kg; E-II – diet with 5 g sodium butyrate added per kg.

properties of feed extract components. The antioxidant capacity of different feed mixture extracts (acetone, methanol, ethanol and puffer) are shown in Table 3. The total antioxidant capacity of acetone and methanol extracts from diet with 5 g of added sodium butyrate was significantly higher (p<0.01) compared to the other diets (control and E-I diets). Significant differences were observed in antioxidant capacity of ethanol and puffer extracts between the experimental diets. The acetone, methanol, ethanol and puffer extracts of the diet with 5 g sodium butyrate added were fast and effective scavengers of the ABTS radical, so butyrate supplementation significantly improved the antioxidant properties of the

Table 4. Effect of sodium butyrate on antioxidant enzyme activities and lipid peroxidation in pigs' liver tissues

Parameter	Pig group		
	C	E-I	E-II
CAT (IU mg protein^{-1})	18.56±2.08	17.99±1.87	18.21±1.09
SOD-1 (IU mg protein^{-1})	10.77±2.48	10.42±1.67	8.47±2.03
GSHPx (IU mg protein^{-1})	30.96±4.22	32.51±3.32	32.08±1.86
GPx (IU mg protein^{-1})	6.41±0.17^{A}	6.83±0.24^{AB}	6.38±0.19^{B}
PPx (IU mg protein^{-1})	24.40±1.02	25.00±0.98^{a}	23.80±0.88^{a}
GST (IU mg protein^{-1})	190.87±16.88	181.89±12.00	191.11±14.25
Reduced glutathione (µmol GSH per mg protein)	20.11±2.87	19.44±2.07	21.36±2.00
Lipid peroxidation (nmol MDA per mg protein)	1.38±0.09	1.45±0.11	1.29±0.14

Legend: Values expressed as mean± SD.
ANOVA test with *post hoc* Tukey's test.
^{A,B} Means within row with same superscript significantly differ at p<0.01.
^{a} Means within row with same superscript significantly differ at p<0.05.
C – pigs consumed diet without added sodium butyrate; E-I – pigs consumed diet with 3 g sodium butyrate added per kg; E-II – pigs consumed diet with 5 g sodium butyrate added per kg.
CAT – catalase; SOD-1 – superoxide dismutase; GSHPx – glutathione peroxidase; GPx – guaiacol peroxidase; PPx – pyrogallol peroxidase; GST – glutathione S-transferase; GSH – reduced glutathione; MDA – malondialdehyde.

feed. Animal feeds contain a range of different compounds that possess antioxidant activities, including vitamin E (consisting of eight compounds tocopherols and four tocotrienols), carotenoids (more than 600 compounds), flavonoids (more than 8000 compounds), ascorbic acid and some other compounds that contribute to anti-oxidant/pro-oxidant balance in animals and that have positive effects on major physiological functions (*Surai*, 2007).

Effects of sodium butyrate on the activities of endogenous antioxidant enzymes and lipid peroxidation in the liver tissue of pigs are shown in Table 4. The inclusion of sodium butyrate induced significant change in the activity of the measured enzymes, GPx and PPx. Significant decreases in GPx and PPx activities were observed in E-II pigs' livers (Table 4; $p<0.05$; $p<0.01$). There were no significant differences in the other measured enzyme activities (CAT, SOD-1, GSHPx, GST and reduced glutathione) between the control pigs and animals treated with 3 g or 5 g sodium butyrate per kg of feed mixture ($p>0.05$). A decrease in SOD after weaning in our study indicated that oxidative stress was present in weaning pigs, which caused increased free-radical generation. The MDA level increased somewhat in livers of pigs consuming 3 g sodium butyrate per kg of the feed compared with in livers of control and E-II pigs, but not significantly.

The mechanism of butyrate effects on inflammatory and oxidative status were presented by *Canani et al.* (2011). Butyrate has anti-inflammatory effects, primarily via inhibition of nuclear factor κB (NF-κB) activation, which can result from the inhibition of histone deacetylase. NF-κB regulates many cellular genes involved in early immune inflammatory responses, including IL-1b, TNF-α, IL-2, IL-6, IL-8, and IL-12. Butyrate can act on immune cells through specific G-protein-coupled receptors (GPRs) for SCFAs, i.e., GPR41 (or FFA3) and GPR43 (or FFA2), which are both expressed on immune cells, including polymorphonuclear cells, suggesting that butyrate might be involved in the activation of leucocytes. The possible immune-modulatory functions of SCFAs are highlighted by a recent study on GPR43 -/- mice in which they exhibit aggravated inflammation, related to increased production of inflammatory mediators and increased immune cell recruitment.

Data related to factors influencing the activity of antioxidant enzymes in pig tissues are limited. Antioxidant enzyme activities differ between different tissue types (*Pradhan et al*., 2000; *Hernández et al*., 2002). Variations in the activity of these enzymes between animals of a single species and different genetic types could lead to differences in oxidative stability of the tissues (*Hernández et al*., 2004). Antioxidant enzymes are indispensable key factors against oxidative stress induced by xenobiotic factors in animals' defense systems (*Hwang et al*., 1993). The antioxidant enzyme defense system consists of CAT, SOD and GSH-Px. SOD converts radicals ($HO._2$-/$O._2$-) to the less toxic H_2O_2,while CAT and GSH-Px detoxify H_2O_2 into O_2 and H_2O (*Ahmad et al*., 2012). After treatment with 5 g kg^{-1} sodium butyrate, GSH, one of the non-enzymatic antioxidant components, increased in our pig liver tissue, whereas MDA, a source of free radical mediated lipid peroxidation injury, decreased compared with the control (Table 4). Butyrate had no effect on other enzymes (including CAT and SOD-1). These results, therefore, suggest that the mechanism by which butyrate exhibits its effects may not be fully due to antioxidant stress (*Song et al.,* 2011). The alteration in antioxidant indices by sodium butyrate, including in the amounts of MDA and GSH detected, suggest an improvement in the level of oxidative stress in the liver cells, which could result in improved healing. Previously, studies suggested that sodium butyrate improves the intestinal tight junction and depresses permeability by improving antioxidant ability. According to *Sunkara et al*. (2011), butyrate strongly induces synthesis of endogenous HDPs (Host Defense Peptides) and their expressions in different cell and tissue types including HD11 macrophages, primary monocytes, bone marrow cells, jejunum and cecal explants as well as in crop, cecum, and cecal tonsils of chickens, thus inhibiting the harmful proinflammatory response. The present study demonstrated that treatment with sodium butyrate did not enhance overall antioxidative ability in pig livers.

Table 5 presents the activity of the measured antioxidant enzymes and MDA levels in pig kidney tissue. Compared to the control group, treatment with sodium butyrate significantly decreased PPx activity and GPx activity in the kidney tissues of pigs ($p<0.01$). No significant alterations in other antioxidant enzymes (CAT, SOD-1, GSHPx, GST and reduced glutathione) between the control pigs' kidneys and those of animals with sodium butyrate added to their diets were observed.

Some in vitro studies indicated that butyrate could increase the activity of antioxidant enzymes. The activity of antioxidant enzymes in non-malignant human colon cells significantly increased after exposure to a butyrate environment (*Jahns et al*., 2015). Namely, butyrate could contribute to chemoprotection in colon cells by reducing the growth of tumor cells, committing them to more rapidly go into

Table 5. Effect of sodium butyrate on antioxidant enzyme activities and lipid peroxidation in pigs' kidney tissues

Parameter	Pig group		
	C	E-I	E-II
CAT (IU mg protein^{-1})	22.46±2.85	23.69±4.01	21.87±2.11
SOD 1 (IU mg protein^{-1})	21.20±1.46	22.18±3.50	23.59±4.40
GSHPx (IU mg protein^{-1})	29.33±3.05	30.22±2.54	30.10±2.00
GPx (IU mg protein^{-1})	2.18±0.08^{AB}	1.96±0.09^{A}	1.95±0.12^{B}
PPx (IU mg protein^{-1})	64.32±1.75^{A}	62.02±2.20^{B}	57.18±1.89^{AB}
GST (IU mg protein^{-1})	97.31±7.87	94.14±9.25	95.46±6.07
Reduced glutathione (μmol GSH per mg protein)	15.75±1.52	14.89±1.30	15.07±1.02
Lipid peroxidation (nmol MDA per mg protein)	1.92±0.09	2.05±0.14	1.95±0.15

Legend: Values expressed as mean± SD.

ANOVA test with the *post hoc* Tukey's test.

^{A,B} Means within row with the same superscript significantly differ at p< 0.01.

C – pigs consumed diet without added sodium butyrate; E-I – pigs consumed diet with 3 g sodium butyrate added per kg; E-II – pigs consumed diet with 5 g sodium butyrate added per kg.

CAT – catalase; SOD-1 – superoxide dismutase; GSHPx – glutathione peroxidase; GPx – guaiacol peroxidase; PPx – pyrogallol peroxidase; GST – glutathione S-transferase; GSH – reduced glutathione; MDA – malondialdehyde.

apoptosis, serving as a survival factor for normal, non-transformed colon cells, enhancing mucin synthesis and via the mechanism of favorably altering patterns of drug metabolism (*Scharlau et al.*, 2009). The antioxidant properties of feed additives which contain a complex mixture of antioxidants (including ascorbate, carotenoids, vitamin E and other phenolics such as the flavonoids) can also act within the digestive tract and improve overall gut functions (*Halliwell et al.*, 2000). Apart from the positive effect on antioxidant feed capacity, the changes in liver and kidney antioxidant enzyme activity, observed in this study, indicated that the sodium butyrate generally did not improve the antioxidant properties in the animal tissues, which is not in agreement with above-mentioned studies.

Conclusion

Results from the present study showed that oral intake of sodium butyrate had no effect on lipid peroxidation or antioxidative enzymes activity of pigs' kidney and liver tissues, with the exception of GPx and PPx. For these, addition of 3 g sodium butyrate per kg of pig diet generally had better influence than the addition of 5 g per kg. The lower-level sodium butyrate supplementation increased GPx enzyme activity in liver, but reduced it in kidney tissues. The addition of 5 g sodium butyrate had negative effects on PPx activities in both tissues. Sodium butyrate did have a positive effect on the antioxidant capacity of the feed.

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Determination of weight loss and temperature of broiler carcasses during air cooling with intermittent water spraying – case study

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A b s t r a c t: The aim of this study was to determine the broiler carcasses weight loss and temperature during air cooling with intermittent water spraying (3.1 m sec^{-1}, −1.5°C, 120 min) in order to optimize the poultry chilling process. Weight and temperature of broiler carcasses were measured before and after cooling. Measurements were made once a week (during six weeks) on randomly selected broiler carcasses (eight carcasses per week) on the slaughterline. The most common broiler hybrids on the domestic market were used in the study (Ross-308, Cobb 500), of different ages (34 to 41 days old), which had been transported 70 to 260 km to the slaughterhouse. In broilers originating from flocks with higher average bird live body weight (2.2 to 2.4 kg), which were held longer (41 days), slight weight losses of carcasses occurred (0.32 to 0.76%) after cooling. On the other hand, broilers which were held 34–38 days with smaller average bird live weight (1.58 to 2.1 kg) produced slight increases in carcass weights after cooling (0 to 2.18%). Temperatures of the broiler carcasses before cooling ranged from 33.00 to 41.37°C and after cooling were from 1.58 to 5.46°C. The achieved temperature of broiler carcasses depended on carcass size, and was adequate (0 to 4°C) for broilers weighing less than 2.13 kg, but carcasses of larger birds did not meet temperature regulations. The transport length influenced weight loss of live broilers, but did not affect the weight loss of broiler carcasses after cooling. Also no differences were observed in accordance to the broiler hybrid type used (Ross-308, Cobb 500).

Keywords: *broiler carcasses, air cooling, water spraying, weight loss, meat temperature.*

Introduction

In poultry processing, chilling is a crucial step that can prevent microbial growth to a level that will maximize both product safety and shelf life (*Wang and Sun,* 2003; *Carroll and Alvarado*, 2008). Also, it improves carcass appearance, but the prime purpose of chilling is to limit the growth of both pathogenic and food spoilage microorganisms (*James et al.,* 2006). In order to control pathogens in broiler processing, enhanced insight into contamination dynamics can be provided with mathematical calculation of cooling processes (*McCarthy et al*., 2017). The most significant broiler meat pathogen is *Salmonella* spp., which is one of the main causes of zoonotic diseases triggered by ingestion of contaminated meat or eggs (*Pajic et al*., 2015), followed by *Campylobacter* spp. (especially *Campylobacter jejuni*) on broiler carcasses, a worldwide problem (*Ivanovic et al*., 2008).

The most common methods for chilling poultry include water immersion or cold-air blast chilling with or without an intermittent water spray (*Mead et al.,* 2000). Air chilling is also used to chill raw poultry carcasses and parts (*Anonymous*, 2014). In Europe, the usage of air chilling to cool poultry carcasses is very common, and there are restrictions on the usage of immersion chilling (*Young and Smith*, 2004). For air chilling as opposed to immersion chilling, cross-contamination can be lower because carcasses are hung individually on the line, depending on the presence of water spray incorporated in the air chill system (*Fluckey et al*., 2003). Spraying carcasses during air chilling, which is not allowed in the European Union, can cause aerosols with bacteria to spread from carcass to carcass due to the fans blowing in air-chilled systems (*Mead et al*., 2000).

In immersion chilling, carcasses are moved through tanks containing chilled water or a mixture of ice and water (*James et al*. 2006). In the case of broilers undergoing immersion chilling, the immersion chilling process should meet hygiene criteria as specified by the competent authority and

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the reduction in carcass temperature should be as rapid as possible (*Anonymous*, 2005). Immersion chilling is a relatively low cost and fast chilling technique, largely employed in the South and North American poultry industries (*Rodrigues et al*., 2014). This system is used a lot in the United States of America and Brazil, two of the biggest poultry producers in the world (*Cariofi and Laurindo*, 2007). During immersion, carcasses uptake water into the intercellular spaces created during the rigor mortis (*Dufour and Renou*, 2002). If broiler carcasses are immersion cooled, the maximum amount of water absorbed into the carcasses and the base parts of the carcass must not exceed 5%, according to domestic legislation (*Anonymous*, 1988). The cooling rate is influenced by the size, shape, and fat of the carcass, as well as by the temperature and flow pattern (and stirring level) of water inside the tanks. In air chillers, the cooling rate depends on the air relative humidity and flow conditions (*Savell et al.,* 2005).

The temperature of chilled broiler meat should be between 0°C to 4°C, as stated in domestic legislation (*Anonymous*, 2014a), and only then it can be released to market (*Anonymous*, 1988). The carcass of slaughtered poultry, the edible parts and internal organs should be cooled so that the deep muscle tissue of the carcass is up to 4°C, while edible parts and organs must be up to 3°C (*Anonymous*, 2010). The FSIS Compliance Guide recommended that all poultry slaughtered and eviscerated in official establishments are chilled immediately after processing so that the internal temperature of broiler carcasses and major portions weighing under 1.8 kg are reduced to 4.4°C or below within 4 hours of processing, and carcasses weighing 1.8 to 3.6 kg within 6 hours of processing. Once chilled, poultry needs to be packaged and shipped at 4.4°C or less (*Anonymous*, 2014).

During transportation from the farm to the slaughterhouse, broilers are exposed to various stressful factors that influence post-mortem muscle metabolism and consequently meat quality (*Babic et al.*, 2014). Transport is an entirely new environment to which the animal has not been adapted, and which can which have adverse effects manifesting from mild agitation to death (*Karabasil et al*., 2013).

In the modern broiler meat industry, the hybrids of broilers that are being grown are genetically engineered for great productivity. The selection of hybrids is based on production characteristics, while increasingly neglecting the health of livestock (*Maslic-Strizak et al*., 2012). Despite all the changes suffered by broilers, with controlled production conditions and adequate biosecurity measures, the selectors have managed to produce broilers with breeding length reduced to 34 to 40 days, achieving a final weight of 2 to 2.8 kg, with a food conversion rate of 1.47 (*Mitrovic et al.*, 2010). The previous standard broiler breeding period lasted for 42 days, or an extended duration of 56 days (*Ramzija et al.,* 2010). No other species of domestic animals have been exposed to the extreme changes in body weight that poultry have been subjected to (*Ljubojevic et al*., 2011), whereby new ways of improving the yield and quality of broiler meat are always sought through nutrition (*Markovic et al*., 2009).

The aim of this study was to determine weight loss and temperature changes of broiler carcasses during air cooling with intermittent water spraying, as well as to determine whether there are differences among two common broiler hybrid types, and to estimate the effect of transport on weight loss.

Materials and Methods

A total of 48 birds during 6 weeks period (8 birds week^{-1}) were randomly collected from an in-line poultry processing plant that has air chilling with intermittent water spraying.

Data were collected from the slaughterhouse documentation regarding hybrid types, the ages of flocks, the length of transport and the average live bird weights.

Broiler carcasses were removed from the processing line before cooling, weighed, labeled, and returned to the cooling line. After cooling, but before packaging, the carcasses were again weighed in an identical manner. The weights of the carcasses before and after cooling were determined on calibrated electronic scales (Waagen K-PZ2-03-010).

The microclimate cooling conditions (chamber temperature, air circulation rate) were measured manually with a combined thermometer-aerometer 405-V1 Air Velocity Stick Meter (Testo, Germany).

Temperatures of the broiler carcasses were measured manually with a thermometer BT20 (Trotec, Germany). The probe was manually stabbed deep into the chest musculature, near the chest bone and directly read and recorded before and after cooling. During chilling of broiler carcasses, the highest temperature is located in the breast geometric center, where chilling effectiveness was evaluated.

Data in this case study were collected directly during the cooling process in defined conditions and then obtained results were statistically evaluated using Microsoft Office 2010, Excel 2010, and presented in Tables 1 and 2 as mean±SD.

Results and discussion

In Table 1, hybrid type, age, transport length, number of broilers examined, as well as the average weight of live broilers are presented.

Table 2 presents the weights and temperatures of broiler carcasses before and after air cooling with intermittent water spraying.

In air chilling, weight loss between 1 to 1.5% is common and can be as high as 3% depending upon the capacity and system requirements (*James et al*., 2006). In our study, weight loss was lower: 0.32% (in broilers of 2.2 kg live weight) up to 0.76% (in broilers of 2.4 kg live weight), or no weight change was recorded (in broilers 2.1 kg and lower live weight), or up to 2.18% increase of carcass weight was detected (in broilers 1.58 kg live weight). The results obtained in our study were similar to other published findings (*James et al*., 2006) on air cooling of broiler carcasses with water spray, in which post-cooling weight changes varied from −2% to +1.7%, in accordance with carcass weight.

In the United States, an increase of broiler carcass weight during cooling is not recommended. All poultry that is slaughtered and eviscerated in official establishments is chilled immediately after processing so that the internal temperature of poultry carcasses and major portions weighing less than 1.8 kg is reduced to 4.4°C or below within 4 hours of processing (*Anonymous*, 2014). Conventional chilling methods such as forced air and water immersion are used in the industry to chill poultry carcasses from approximately 40°C to 4°C, which is essential to improve safety (*Rodrigues et al*., 2014). Comparing these statements with the results obtained in our study, there was a match with temperature requirements, while there were differences in carcass weight increases, especially for the lighter weight carcasses (1.58 kg carcasses increased weight by 2.18% during air cooling with intermittent water spray).

Spraying carcasses during air chilling is not allowed in the European Union because of cross-contamination with aerosols (*Mead et al*., 2000; *Demirok et al*., 2013). Some authors claimed that air-chilled products have better microbial quality than immersion-chilled products because air chilling without water spray can injure or kill bacteria as a result of skin surface dehydration during chilling (*Berrang et al*., 2008; *Carroll and Alvarado*, 2008). In accordance with the high level of compliance of

Table 1. Hybrid type, age, transport length, number of broilers examined, and average live bird weight

Week	Hybrid type	Broiler age (days)	Transport length (km)	No of broilers examined	Average live bird weight (kg)
I	Cobb 500	41	70	8	2.40
II	Cobb 500	41	107	8	2.20
III	Cobb 500	34	79	8	1.58
IV	Cobb 500	38	79	8	2.13
V	Ross 308	38	260	8	2.11
VI	Ross 308	38	260	8	2.12

Table 2. Mean broiler carcass weights and temperatures before and after cooling, and mean percentage change of carcass weights

Week	Mean weights of carcasses before cooling (kg±SD)	Mean weights of carcasses after cooling (kg±SD)	Mean temperatures of carcasses before cooling (°C±SD)	Mean temperatures of carcasses after cooling (°C±SD)	Carcass weight change (%)
I	1.720±0.17	1.707±0.17	40.92±0.43	5.46±0.62	– 0.76
II	1.586±0.09	1.581±0.09	41.30±0.46	4.91±0.68	– 0.32
III	1.055±0.11	1.078±0.10	33.00±1.06	1.67±0.56	+ 2.18
IV	1.436±0.16	1.437±0.16	41.37±0.52	4.01±0.44	+ 0.06
V	1.202±0.19	1.204±0.19	38.93±1.27	2.41±1.15	+ 0.16
VI	1.254±0.15	1.254±0.14	39.21±1.22	2.58±0.56	/

Serbia with regard to European Union integration processes, with the ultimate goal of becoming a full member in the near or further future, urgent reforms in almost all areas have become inevitable. Certainly, the areas of food production, processing and trade cannot be bypassed (*Buncic and Rudan*, 2006). The experiences of European Union countries with well-developed and advanced standards and obligatory legislation in force, in the production of safe food, have shown that manufacturing safe food is only possible by control of the complete food chain. This control is based on the preventive approach (*Nastasijevic et al.*, 2005).

In our study, adequate cooling temperatures (1.67±0.56°C to 4.01±0.44°C) were achieved for broiler carcasses with average pre-cooling weights of 1.055±0.11 kg to 1.436±0.16 kg, respectively (Table 2). We note the latter group of broilers reached only the borderline temperature limit in accordance with domestic regulations (*Anonymous*, 1988; *Anonymous*, 2010) and foreign recommendations (*Anonymous*, 2014). Therefore, the defined conditions measured during air cooling (air flow 3.1 m sec^{-1}, air temperature −1.5°C, 120 min) with intermittent water spraying adequately cooled those broiler carcasses that weighed up to 1.436 kg before cooling, where the average live broiler weight was 2.13 kg or less.

The larger broiler carcasses, 1.586±0.09 kg to 1.720±0.17 kg reached temperatures of only 4.91±0.68°C and 5.46±0.62°C, respectively (Table 2). These temperatures after air cooling with water spraying were inadequate and did not accord with the previously mentioned regulations and recommendations.

The transport length influenced the weight loss of live broilers, but did not affect the weight loss of broiler carcasses during cooling. Also no differences were observed in accordance to the broiler hybrid type used in study (Ross-308, Cobb 500).

Conclusion

Weight loss and temperature of broiler carcasses during air cooling with intermittent water spraying depended (apart from the microclimate cooling conditions in the chamber, which were a constant temperature, air flow rate, and cooling length), on live bird weight and carcass weight. The broiler carcasses originating from birds with average live weight of 2.2 kg underwent 0.32% weight loss, and those originating from birds with average live weight of 2.4 kg lost 0.76% of weight due to cooling. Carcass weight did not change when broilers originated from birds with average live weight of 2.1 kg. The carcass weight of lighter broilers (1.58 kg average live weight) increased by 2.18% after cooling.

Adequate cooling temperatures (1.67 and 4.01°C) were achieved for broiler carcasses with pre-cooling weights of 1.055 kg (average live broiler weight of 1.58 kg) and 1.436 kg (average live broiler weight of 2.13 kg), respectively. However, since these heavier carcasses reached only 4.0°C on average, they were at the borderline temperature limit. The average temperatures of even heavier broiler carcasses (1.586 kg, average live broiler weight of 2.20 kg and 1.720 kg, average live broiler weight of 2.40 kg) were 4.91 and 5.46°C, respectively after the chilling regime examined. Therefore, these larger carcasses did not achieve adequate chill temperatures after the air cooling with water spraying.

The transport length influenced the weight loss of live birds, but did not affect the weight loss of broiler carcasses after cooling. The two broiler hybrids behaved similarly with regard to carcass weight changes after chilling.

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Quality assessment of Srpska sausage from nine different manufacturers in Serbia

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A b s t r a c t: The aim of this study was to assess the quality of 11 different samples of Srpska sausage from 9 manufacturers in Serbia who responded to a public call of the Chamber of Commerce and Industry of Serbia in order to improve the brand of Srpska sausage. For characterising properties of the Srpska sausages chemical composition analysis and sensory descriptive analysis were used. The chemical analysis showed that all the samples had a meat protein content higher than the stipulated minimum (12%), that the total phosphorus content (expressed as P_2O_5, g/kg), the content of $NaNO_2$ (mg/kg), and the content of collagen in the meat protein (%) is lower than the maximum permitted level (8.00 g/kg, 150.00 mg/kg, and 20.00%, respectively). In the sensory analysis following attributes were evaluated: external appearance, cross-section appearance (including colour), texture, odour, flavour and overall sensory quality. The best-evaluated was a sample with a mean cumulative score of 87.23±8:33, and the worse-evaluated was a sample with a mean cumulative score of 51.65±10.05. There were not significantly differences between four samples with highest cumulative scores. The sausage with the highest score was evaluated as best by 35% of panellists, followed by third (25% of panellists), second and fourth (15% of panellists). The quality parameters of the highest ranked "Srpska sausage" will be submitted to the competent state authorities as amendments to the current Regulation.

Keywords: *Srpska sausage, Regulation, cooked sausage quality, chemical composition, sensory evaluation.*

Introduction

Unlike fermented and precooked-cooked sausages, which have a very long history, being mentioned in the Greek literary works about 500 BC and in the scriptures of Leo VI the Wise from the early 10th century, raw-cooked sausages are products of more recent times (*Vukovic*, 2006; *Trojan and Piotrowski*, 2007). According to some data, the first raw-cooked sausage from a mixture of pork and beef, so called frankfurter sausage, was made by the Viennese butcher Johann Georg Lahner in 1805 (*Vukovic*, 2006). Taking into account the production quantities of cooked sausages in many countries, including central Europe, they are the most common meat products. There are hundreds of these types of sausages worldwide that are often classified under a common concept – cooked, and the only differences between themselves are some local or regional characteristics (*Radetic*, 2000). By definition, raw-cooked sausages contain meat (muscle, fatty and connective tissue) and non-meat ingredients which are processed raw, i.e. uncooked by comminuting and mixing. The resulting viscous mix/batter is portioned in sausages or otherwise and thereafter submitted to heat treatment, i.e. "cooked"–"pasteurised", with or without smoking (*FAO*, 2007). Production technology of cooked sausages includes a complex of biochemical, chemical and physical changes, particularly the process that relates to a water binding capacity and adipose tissue (fat) emulsifying, which is often in a very close relationship with the process of comminution (*Radetic*, 2000).

In the group of cooked sausages, there are a large number of products that differ amongst themselves by its composition, the comminution grade of the stuffing, type and diameter of the casings. Based on the comminution grade of the stuffing, raw-cooked sausages are divided into finely chopped cooked sausages, coarsely chopped cooked sausages, cooked sausages with meat pieces and meat loaves (*Vukovic*, 2006). According to the current Regulation on the quality of ground meat, meat preparations and meat products (*Official Gazette of the Republic of Serbia*, 2015,2017), coarsely chopped

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cooked sausages are produced and placed on the market under the name of Srpska sausage, Moravska sausage, Tirolska sausage and Mortadella, or under a different name. The same Regulation defines Srpska sausage as a meat product derived from meat, firm fatty tissue and blood preparations with the addition of salt, the curing salt, water, spices, spice extracts, sugars, additives, smoke flavour and natural flavouring agents. Srpska sausage is stuffed itno the small intestine of pigs or adequate artificial casings, followed by pasteurisation and smoking as a heat treatment. Also, the quality of Srpska sausage is specified by its chemical quality parameters such as meat protein content, a content of collagen in meat proteins, total phosphorus content and nitrite content (*Official Gazette of the Republic of Serbia*, 2015, 2017; *Official Gazette of the Republic of Serbia*, 2013). In previous regulations on the quality of meat products, Srpska sausage was in a group of semi-durable sausages, it had to contain at least 25% of meat of all categories, 20−50% of meat batter and water up to 55%, and its production was defined by the manufacturer's specification, which was brought by manufacturer and which had to be approved by the competent Ministry (*Official Gazette of the Socialist Federal Republic of Yugoslavia,* 1968; *Official Gazette of the Socialist Federal Republic of Yugoslavia,* 1974, 1978, 1980). This indicates that the Srpska sausage belonged to the group of so-called "related products" and the non-meat proteins were allowed by this product which affected its quality, and therefore in the following regulations, it became necessary that the quality of Srpska sausage should be precisely defined.

Suitable formulations for production of meat products and the provision of uniform quality, within the unchanged use of this product, are one of the tasks with which the meat processing industry faces every day (*Sveinsdóttir et al.,* 2009). Quality and composition of meat product in all organised countries is regulated by regulations adjusted to consumer habits, technological capability and development of the country, control possibilities, religious demands (*Arihara*, 2006). Besides of regulations of the Republic of Serbia, that determine the chemical composition of the Srpska sausage, cooked sausages are extremely variable group of meat products, and therefore on the markets we can find sausages that do not meet the specified conditions, or Srpska sausages, that despite adequate chemical composition, do not satisfy certain sensory characteristics that are also an important indicator of quality (*Kurcubic et al.*, 2012).

The aim of this study was to assess the quality of 11 different samples of Srpska sausage, by determination of chemical composition and sensory evaluation, from 9 manufacturers in Serbia who responded to a public call of the Chamber of Commerce and Industry of Serbia in order to improve brand of Srpska sausage and competitiveness of domestic producers.

Materials and Methods

Nine different manufacturers, who responded to a public call of the Chamber of Commerce and Industry of Serbia, have made 11 different Srpska sausages in accordance with Article 75. and 77. of the current Regulation (*Official Gazette of the Republic of Serbia*, 2015, 2017). The filling is stuffed into anatural casing (pig small intestine – salted, calibrated, diameter 32−34 mm). Sausages were vacuum-packed in a packaging of 1.5 kg, stored at 2−4ºC until they were delivered for sensory evaluation and chemical analysis.

Twenty trained panellists participated in the sensory evaluation and were selected according to Serbia/ISO standard (*SRPS*, 2015; *SRPS*, 2012a). The evaluation was conducted in a sensory laboratory designed according to Serbian/ISO standard (*SRPS*, 2012b). The quantitative descriptive sensory analysis was used to evaluate the sensory profile of the samples (*SRPS*, 2001). Six attributes were used for sensory evaluation, including external appearance, cross-section appearance (including colour), texture (hardness, juiciness, chewiness and fattiness), odour, flavour and overall sensory quality. From this parameters, cumulative score for each sample was calculated by multiplying all scores by coefficients of importance and addition of adjusted ratings. Scores were in the range from 1 (unacceptable) to 5 (excellent), and the coefficients of importance were 2, 3, 5 and 6. The level of acceptability was half of the value obtained by multiplying the highest score by a coefficient of importance for each attribute. The example of the sensory ballot is shown in Table 1 (*Baltic*, 1994). Panellists marked with a number the intensity for each attribute.

Before sensory evaluation, a half of the sausages were boiled at 80°C for 5 min. The sausages were served with casings, cold and boiled. Then, the tips of the sausages were cut off and discarded. Slices (1 cm thick) were cut and used for evaluation. Each panellist tasted 2−3 slices from the same sausage. Samples were labelled with random three digit numbers and served in random orders.

Table 1. The example of sensory ballot for evaluation of Srpska sausage (*Baltic*, 1994)

Panellist code:		Date:	
Sample code:		Meat product:	
Attributes	Score 1–5 (A)	Coefficients of importance (B)	Adjusted rating (AxB)
External appearance		2	
Cross section appearance		6	
Texture		2	
Odour		3	
Flavour		5	
Overall sensory quality		2	
		Cumulative score:	

Chemical composition

After sensory evaluation, 3 samples from each Srpska sausage were taken for chemical composition analysis. Total protein content, total fat content, a content of $NaNO_2$, P_2O_5 and NaCl was determined using standard reference methods (*SRPS*, 1992; *SRPS*, 1998; *SRPS*,1999a; *SRPS*,1999b; *SRPS*, 1999c). Collagen content was calculated by multiplying hydroxyproline content (%) by factor 8 (hydroxyproline content was determined by method *SRPS*, 2002) and the proportion of collagen in meat proteins is further calculated as follows: content (quantity) of the collagen in proteins (%) = collagen content (%) × 100 / total protein content (%). Additionally, nutritional (water/protein and fat/protein ratio) and energetic (total energy content and the percentage of protein and fat energy) parameters of examined sausages were calculated.

Statistical analysis

Statistical analysis of the results was conducted using the software GraphPad Prism Version 5.00 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). The results of sensory evaluation were expressed as mean±SD and reported in the table, while results of chemical analyses were shown in figures. One-way ANOVA and post hoc Tukey's test were performed to assess the significance of differences among samples of sausages. Values of $p<0.05$ and $p<0.01$ were considered significant.

Results and Discussion

Chemical analyses

The quality of cooked sausages is defined by the meat protein content, regarding total protein content and the relative content of the connective tissue protein in total proteins. Coarsely chopped cooked sausages should contain at least 12% of protein and maximum 20% of collagen in the meat protein (*Vukovic*, 2006). Average protein content in the examined Srpska sausages was 16.84±1.37%, while average content of collagen in the meat protein was 9.20±2.32%. All of the samples, by these parameters, were in accordance with the requirements set by the Regulation (2015, 2017) (Figure 1; Figure 2). Kurcubic et al. (2012) found that of the 94 examined coarsely chopped cooked sausages from different producers in Serbia, 11.11% of the samples had total protein content lower than allowed, and 25% of the samples had a content of collagen in the meat protein higher than permissible. Of these, 12 were Srpska sausages, from which two samples had a higher content of collagen in meat protein than allowed. Also, *Saicic et al.* (2006) found that of 67 coarsely chopped cooked sausages, 13.92% did not meet the quality requirements prescribed by current Regulation. *Krausse* and *Kotter* (1971) consider that the main factor that determines the quality of the meat products is the quantity and quality of protein. In this way, the quality of the sausages would be assessed, at the same time excluding the possibility of the presence of proteins derived from other foods (soy, milk), by determining the content of high value protein.

Additional indicators of sausages quality are water/protein ratio and fat/protein ratio. For Kranjska sausage, it is determined that the water/protein ratio should be 3.3, and the fat/protein ratio 2.4 (*Anon.*, 2016). These ratios for 11 Srpska sausages were ranged from 2.98 to 4.55 and from 0.98 to 1.61, respectively (Table 2). Thus, according to the parameter water/protein ratio, even 54.55% of the examined sausages did not meet the conditions, while in terms of fat/protein ratio, all sausages met the requirement. According to *Krausse* and *Kotter* (1971), if the percentage of the protein content,

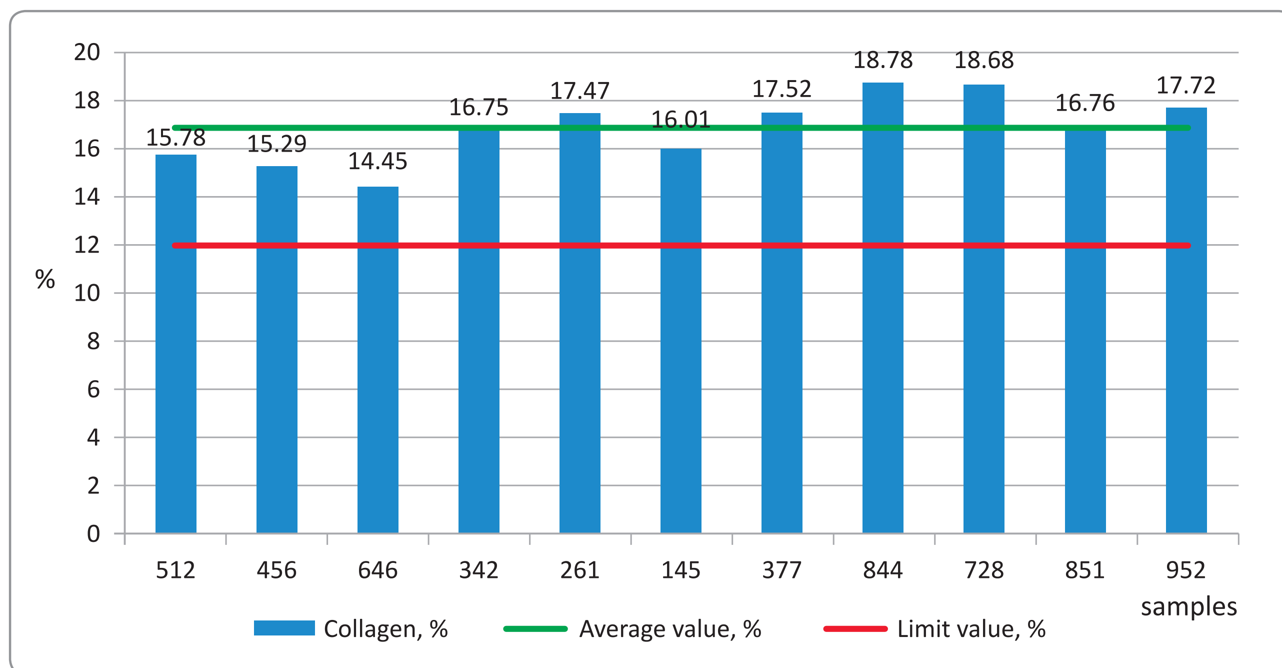

Figure 1. Protein content of examined Srpska sausages

which does not include the connective tissue protein, is satisfactory, the ratio of fat/protein and water/protein of the meat product can be disregarded.

Sausages are considered to be products with a highfat content. The fat content in the examined samples of Srpska sausages ranged from 16.28% (sample 646) to 26.69% (sample 851) (average fat content of 11 samples was 22.68±3.54%), where 81.82% of sausages had a fat content higher than 20% (Figure 3). *Dojcinovic et al.* (2015) found that most of the finely chopped cooked sausages on the market in Banja Luka (frankfurters and Parisian sausages) had fat content in the range of 15–20% (44.74% and 46.15%, respectively). National Food Survey figures for 1995 estimate that meat and meat products currently contribute 18.1 g of fat or 23% to the total daily intake of which 4.9 g is derived from carcass meat, 2.2 g from uncooked poultry meat, 26 g from bacon and ham, and 8.4 g from other meat

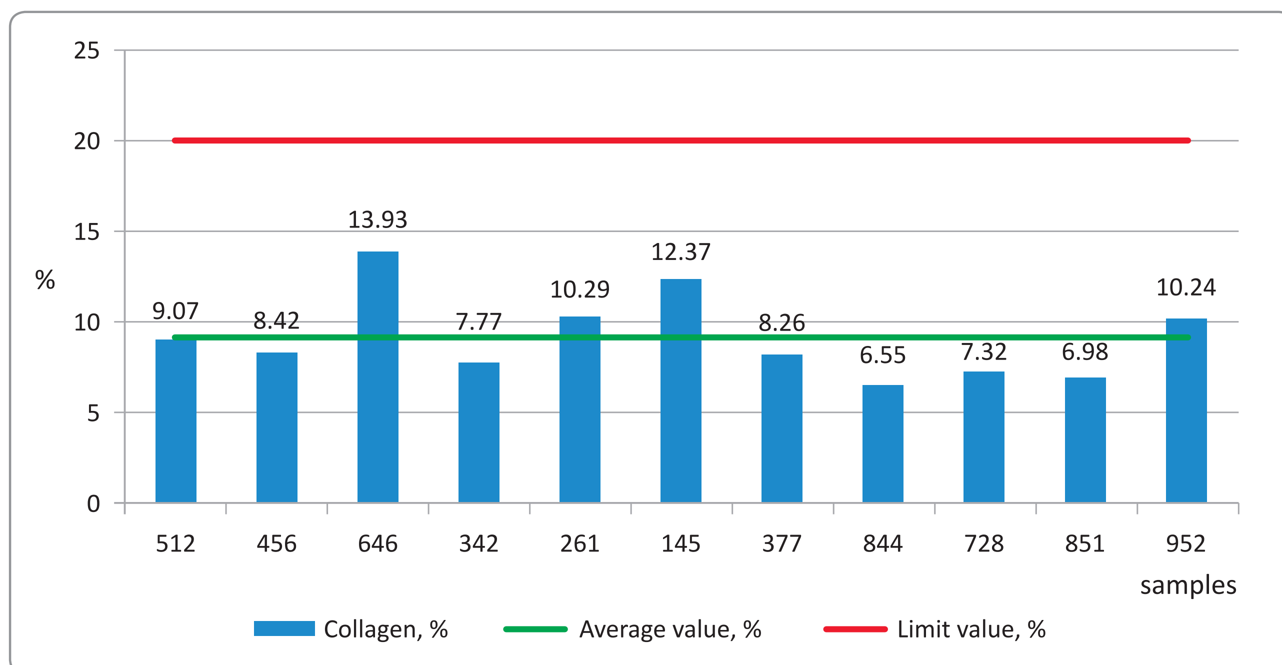

Figure 2. Collagen content in meat proteins of examined Srpska sausages

Table 2. Selected nutritional and energetic parameters of examined sausages

Sample	water: protein ratio	fat: protein ratio	total energy (kJ)	protein energy (%)	fat energy (%)
512	3.77	1.39	1089.87	24.25	75.75
456	3.75	1.60	1176.62	21.76	78.24
646	4.55	1.13	855.45	28.29	71.71
342	3.53	1.23	1054.49	26.60	73.40
261	3.56	0.98	936.92	31.23	68.77
145	3.42	1.61	1241.80	21.59	78.41
377	3.02	1.52	1298.75	22.59	77.41
844	2.92	1.27	1210.19	25.99	74.01
728	2.89	1.28	1210.40	25.85	74.15
851	3.19	1.59	1286.39	21.82	78.18
952	3.20	1.26	1139.69	26.04	73.96

products (*Ministry of Agriculture, Fisheries and Food,* 1996; *Sheard et al*., 1998).

The calculated total energy content of examined samples of sausages was between 855.45 kJ and 1298.75 kJ (Table 2), and in all sausages, more than 20% of energy come from protein. The nutritive value of the meat products originates mainly from proteins and fats, they do not contain carbohydrates or their quantity in the meat products is negligible. Calories intake by eating sausages come from degradation of proteins and fats, and these sources of energies are physiologically favourable for the human body (*Oluski*, 1974).

Salt is involved in water holding, texture, colour, taste and aroma development and enhancement of the microbiological safety of cooked sausages. The salt content is usually between 1.5% and 2.5%, but lower and higher contents are frequently seen (*Toldrá*, 2010; *Albarracín*, 2011). Consumption of 100 g of examined Srpska sausages implies average intake of 2.14±0.31 g of salt (Figure 4). Estimated average salt intake in the adult population of Novi Sad is relatively high at 12.12±4.79 g compared to the recommended amount of 5 g per day (*Popovic*, 2013). While, the average total daily sodium intake per individual in developed countries is 4–5 g of Na (10–12 g of NaCl), which is up to 25 times greater than the minimum adult requirement (0.5 g of NaCl) (*Albarracín*, 2011).

The content of nitrite in 11 Srpska sausages ranged from 0.03 mg/kg to 99.45 mg/kg (average nitrite content of 11 samples was 46.91±30.99), thus all the samples had nitrate content lower than the maximum stipulated limit (Figure 5). Nitrite is added to most cooked sausages at levels of 120–150 mg/kg, but there is a constant trend to reduce these levels (*Toldrá*, 2010). The average total phosphates content (which includes the natural phosphorus and added phosphorus content) of examined Srpska sausages was 4.60±0.53 g/kg and all 11 samples were in accordance with the requirements set by the Regulation (8 g/kg) (Figure 6). Additionaly, the Serbian regulation on additives defines the amount of added phosphates (P_2O_5) as 5 g/kg (0,5%) (*Official Gazette of the Republic of Serbia*, 2013). Also, *Kurcubic et al.* (2012) showed that total P_2O_5 content (g/kg) in all of 272 examined samples was compatible with the values permitted by the Regulation, indicating a strict adherence to regulations when using phosphate as one of the technologically most important additives in the production of cooked sausages. In most countries, the use of phosphates is allowed and the levels used vary from 0.15% to 0.3%, given as P_2O_5 (in the EU, the maximum is 0.5%) (*Toldrá*, 2010).

Sensory evaluation

Appearance determines how consumers perceive quality and significantly influences purchasing behaviour (*Resurreccion*, 2004). Sensory analysis is a useful and irreplaceable method for precise determination of the quality of food products (*Radovanovic and Popovic-Raljic*, 2001). By using descriptive method it is possible to define the quality characteristics of the selected product and to identify potential product defects (*Grujic et al*., 2010). Mean values for each evaluated sensory attribute of the 11 studied sausages are shown in Table 3. Assessing of the external appearance of Srpska sausages depend on the adjustment of the casings and whether there

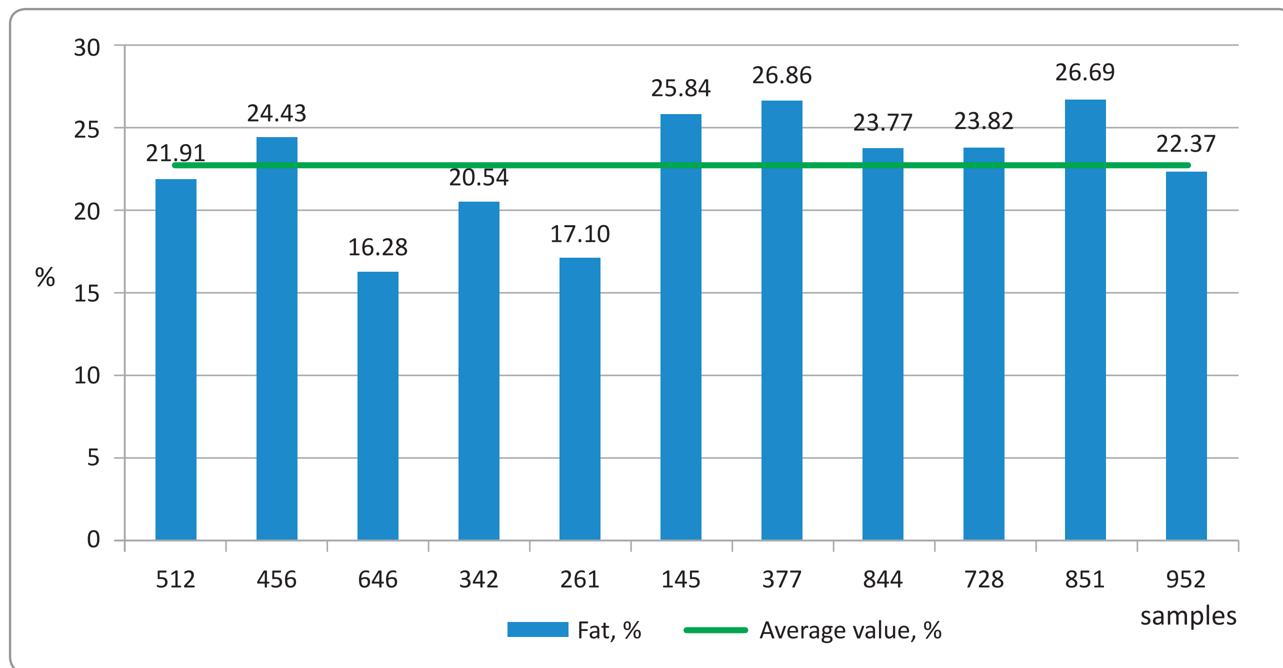

Figure 3. Fat content of examined Srpska sausages

is a separation of jelly and grease, while evaluation of cross sectional appearance takes into consideration that the meat is uniform with stable colour and that the components of the filling are evenly distributed and connected (*Official Gazette of the Republic of Serbia*, 2015, 2017). With the lowest scores for external and cross sectional appearance (including colour) were evaluated samples 145 and 377 (fat content >25%), while the highest scores obtained samples 844 and 952. According to these attributes, all samples were acceptable. The appearance of cooked sausages can be influenced by pH, meat source, packaging conditions, freezing history, the rate of thawing, fat content, added ingredients (salt, nitrite, phosphate) and preservation treatments. Additionally, these factors change the ratio of different forms of myoglobin; the main pigments responsible for the ultimate colour of meat (*Whyte*, 2006).

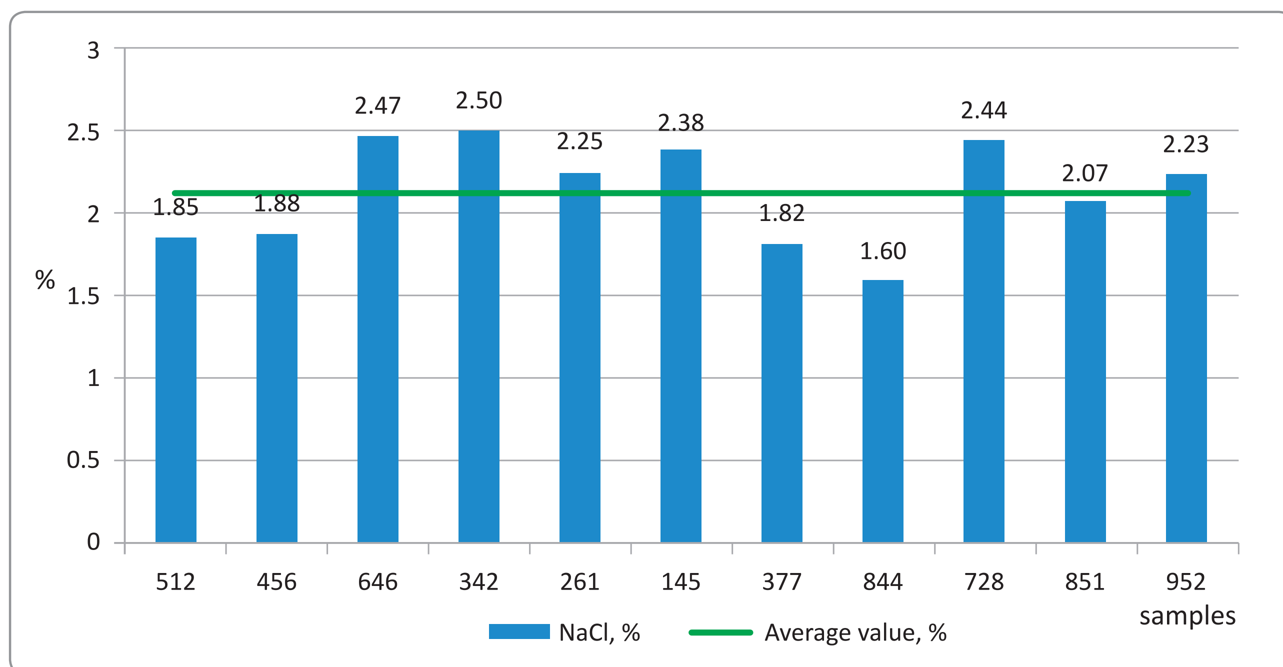

Figure 4. Sodium chloride content of examined Srpska sausages

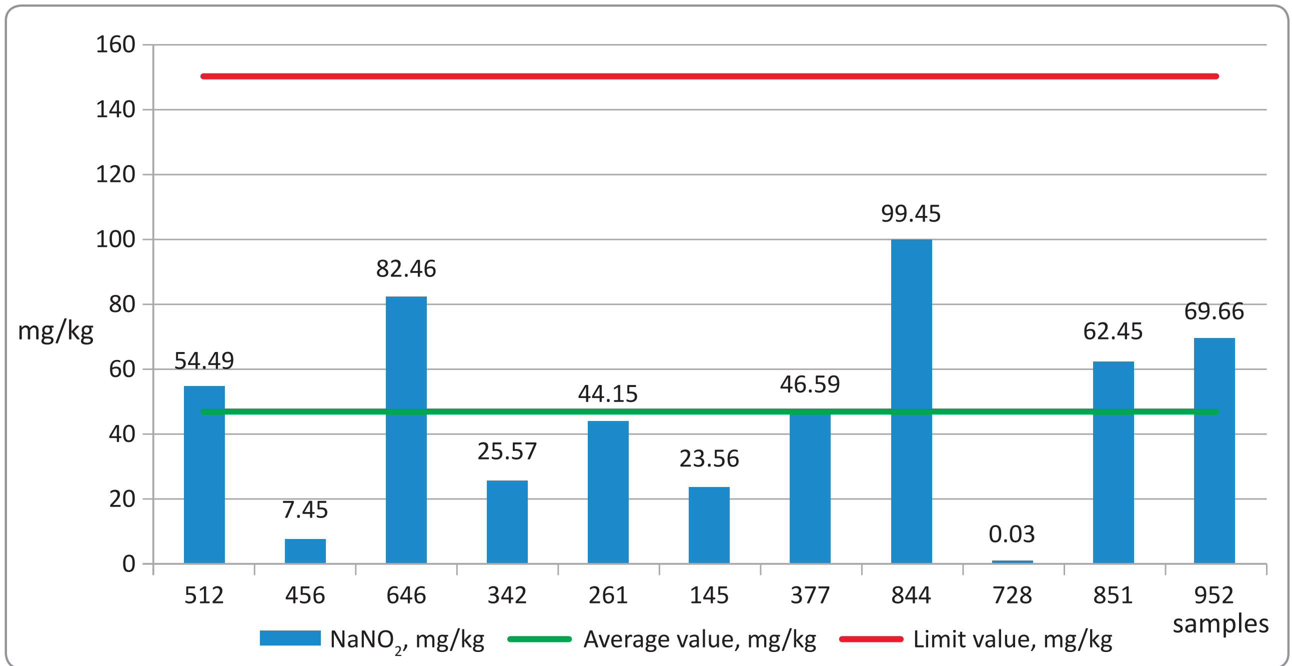

Figure 5. Sodium nitrite content of examined Srpska sausages

Texture includes a variety of characteristics, such as hardness, juiciness, chewiness and fattiness and among them, hardness is the most important to the consumer (*Grujic et al*., 2014). Samples 145 and 377 were evaluated with the lowest scores for texture (6.00±1.56 and 6.05±1.19, respectively), while the highest score was assigned to a sample of 952 (8.60±0.82) and by this attribute, sample 952 significantly differed from samples 145 and 377 ($p<0.01$). Factors that contribute to the texture of cooked sausages are the content of connective tissue, fat content, phosphates and especially salt. Many authors showed that salt influence on hardness and juiciness of sausages and that a reduction in salt (NaCl) content of less than 2.0% resulted in sausages of less firm texture (*Matulis et al*., 1995; *Ruusunen et al*., 2003). Namely, in meat products, salt contributes to water and fat binding by expanding the filament

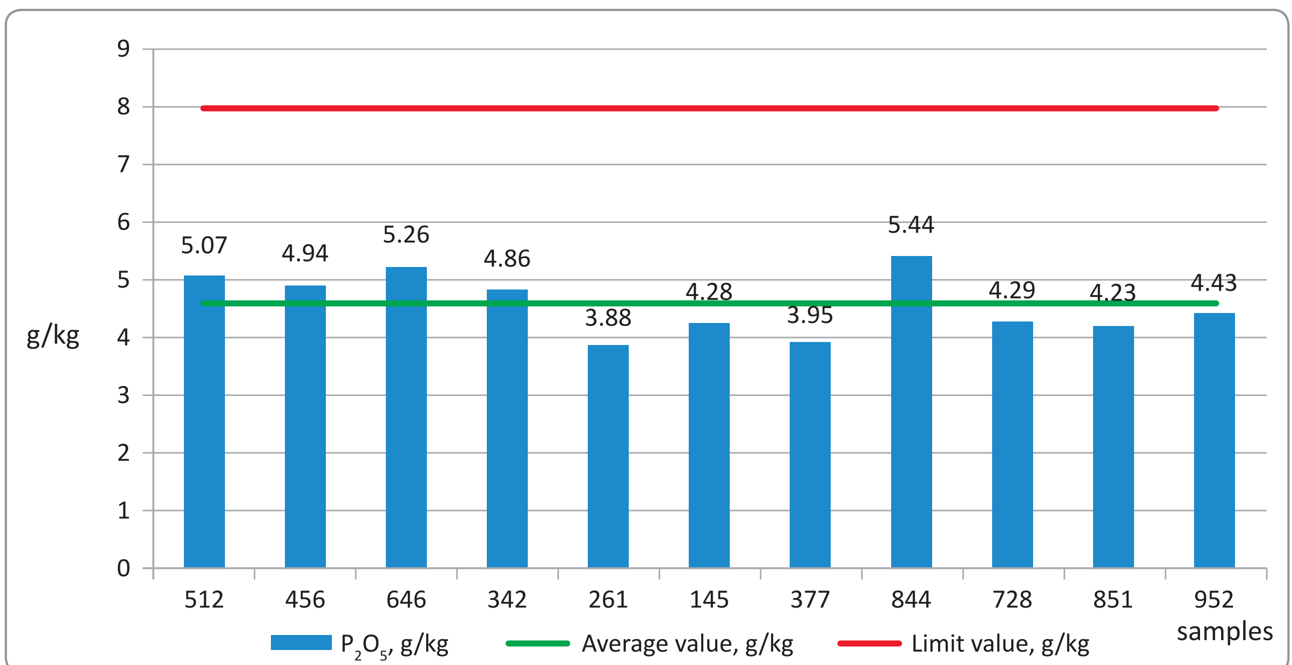

Figure 6. Total P_2O_5 content of examined Srpska sausages

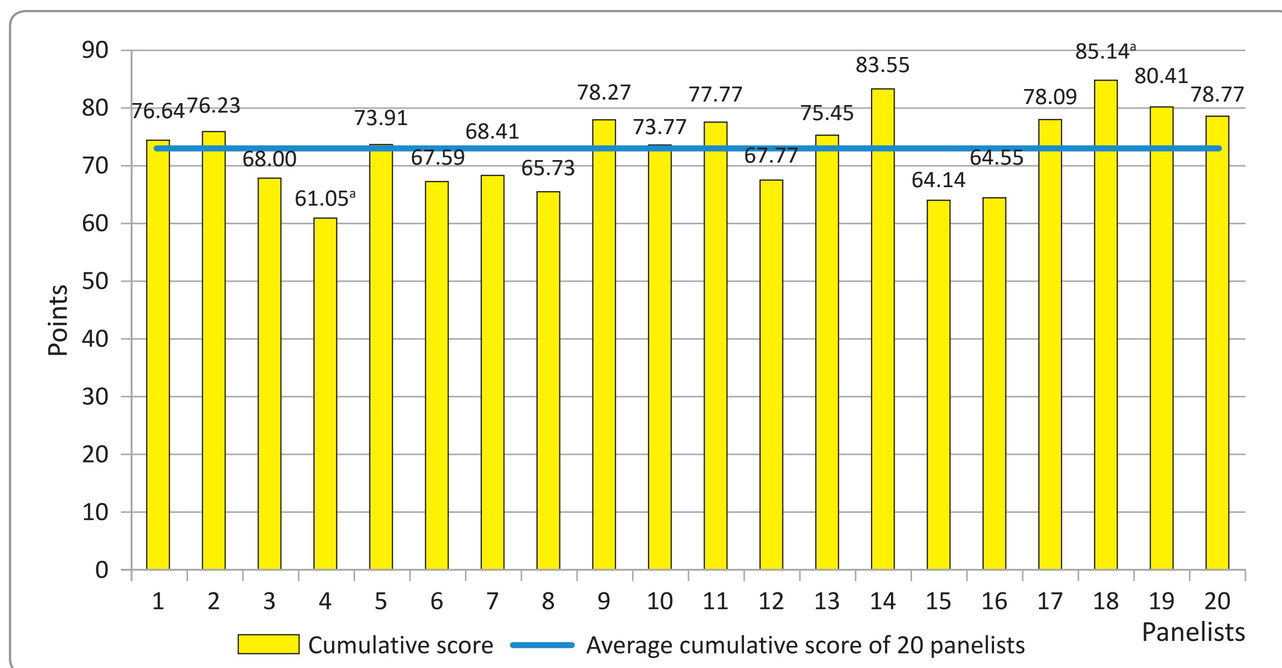

Figure 7. Cumulative scores of 20 panelists for 11 samples of Srpska sausage ([a] $p<0.05$)

lattice of myofibrils and (*Ruusunen et al*., 2003). by partially solubilizing the myofibrillar proteins

Sample 377 is assessed with lowest scores for the odour, flavour and overall sensory quality, while for the same parameters highest scores obtained sample 952. Also, the sample 952, by these parameters, was not significantly different from the samples 844, 728 and 851 ($p>0.05$). In addition, for flavour and overall sensory quality, sample 377 was below the limit of acceptability and by these two properties was significantly different from all other samples ($p<0.01$), except for samples of 261 and 145. Sausage odorants come from raw materials (e.g. spices and flavourings) or are generated through chemical reactions during cooking. It is shown that fat level influenced the release of volatile compounds

Table 3. Sensory evaluation of 11 Srpska sausages

Sample	Attributes						
	External appearance	Cross sectional appearance	Texture	Odour	Flavour	Overall sensory quality	Cumulative score
512	7.35 ± 1.35^{abcd}	21.45 ± 4.38^{ABa}	7.95 ± 1.47^{AB}	10.20 ± 1.86^{abcA}	16.63 ± 3.37^{ABaCD}	7.05 ± 1.28^{aAbcB}	70.63 ± 11.92^{ABaCD}
456	8.65 ± 1.04^{ABC}	23.40 ± 3.97^{bCD}	7.55 ± 1.57^{Ca}	12.30 ± 1.92^{BCD}	18.38 ± 4.31^{bEFc}	7.65 ± 1.69^{CDE}	77.93 ± 12.30^{EFG}
646	8.40 ± 1.19^{eDf}	22.05 ± 3.12^{EF}	8.25 ± 1.21^{DE}	11.68 ± 2.36^{dEF}	19.38 ± 3.33^{GHI}	7.75 ± 1.02^{FGH}	77.45 ± 10.58^{HIJ}
342	7.85 ± 1.27^{E}	22.35 ± 3.83^{GH}	7.20 ± 1.32^{bc}	10.73 ± 1.90^{Ge}	17.50 ± 2.92^{dJK}	7.05 ± 1.10^{dIefJ}	72.68 ± 10.02^{KLbM}
261	7.00 ± 1.62^{AeFGHg}	19.20 ± 4.71^{bIcJK}	7.10 ± 1.21^{de}	9.15 ± 2.62^{BdHIJK}	14.38 ± 4.58^{bGLMNO}	6.05 ± 1.70^{CFKLMN}	62.88 ± 14.77^{EHNOPQ}
145	5.95 ± 1.85^{aBDEIJKL}	15.60 ± 4.63^{ACEGLMNO}	6.00 ± 1.56^{ACDFGHI}	8.93 ± 2.60^{CELMNO}	13.75 ± 4.33^{EHdPQRS}	5.65 ± 1.76^{aDGdOPQR}	55.88 ± 14.82^{AFIKRSTU}
377	6.95 ± 1.47^{CfMNOh}	15.30 ± 4.34^{BDFHPQRS}	6.05 ± 1.19^{BaEJKLM}	7.80 ± 2.16^{aDFGPQRS}	10.75 ± 2.94^{AFIJTUVW}	4.80 ± 1.32^{AEHISTUV}	51.65 ± 10.05^{BGJLVWXY}
844	8.90 ± 1.02^{bFIM}	24.00 ± 3.08^{ILP}	8.40 ± 0.88^{FJ}	12.45 ± 1.76^{bHLP}	21.13 ± 2.98^{BLPT}	8.50 ± 0.89^{beKOS}	83.38 ± 8.62^{aNRV}
728	8.85 ± 1.23^{cGJN}	$23.25\pm4,.23^{cMQ}$	7.80 ± 1.58^{GK}	12.08 ± 2.20^{IMQ}	20.50 ± 4.02^{aMQU}	8.05 ± 1.47^{LPT}	80.53 ± 13.32^{OSW}
851	8.80 ± 1.15^{dHKO}	24.75 ± 3.21^{JNR}	8.55 ± 1.05^{bdHL}	12.75 ± 1.92^{cJNR}	21.13 ± 2.36^{CNRV}	8.60 ± 0.75^{cfMQU}	84.58 ± 8.67^{CbPTX}
952	8.55 ± 1.32^{gLh}	25.95 ± 2.44^{aKOS}	8.60 ± 0.82^{ceIM}	13.13 ± 1.88^{AeKOS}	22.25 ± 2.80^{DcKOSW}	8.70 ± 1.08^{BJNRV}	87.23 ± 8.33^{DMQUY}

Legend:$^{A\text{-}Y}$ Within a column, values with a common superscript letter are significantly different, $p<0.01$
$^{a\text{-}h}$ Within a column, values with a common superscript letter are significantly different, $p<0.05$

during mastication, but only if mastication lasts more than 1 min. In addition, fat content could influence sausage flavour by changing the water/fat ratio and modifying the concentration in each phase of compounds such as salt and emulsifying proteins but also the concentrations of odour and taste active compounds (*Carrapiso*, 2007). Also, for sausage flavour, an important step of processing is cooking (*Carrapiso*, 2007). Besides, salt is usually reported to enhance the total flavour intensity of meat products (*Matulis et al*., 1995; *Ruusunen et al*., 2003).

The sample 377 was evaluated by the lowest cumulative score, at the level of acceptability and differed significantly from all samples ($p<0.01$), except samples 261 and 145. The best-evaluated sample, a sample of the best quality was 952, with a mean cumulative score of 87.23±8.33. However, samples 844, 728 and 851 also had a score higher than 80 and did not significantly differ from sample 952 ($p> 0.05$). The mean cumulative score for all 20 palenellists was 73.16±6.85. Most of the panellists, 35% of them, assessed that the best sausage was sample labelled with 952, 25% of panellists chose sample 728, while 15% chose samples 844 and 851. Panellist no. 4 was the strictest in the evaluation, with a mean cumulative score of 61.05±12.36, while the most lenient evaluator was panellist no. 18, with a mean cumulative score of 85.14±13.89. ($p<0.05$) (Figure 7).

Conclusions

Manufacturers in Serbia should be encouraged to produce the best possible Srpska sausage, which would not satisfyonly the requirements set by Regulation but also meet the nutritional and health needs of the consumers and their perception of quality.

Improving the quality of Srpska sausages can be achieved in several ways.One of them is related to the Regulation on labelling of agricultural and food products with the national code of higher quality “Serbian quality” (no. 90/2016). In addition, Srpska sausage could be protected by trademark or indication of geographical origin by The Intellectual Property Office of the Republic of Serbia. Regardless of the way for improving the quality, for Srpska sausage, it should be made standard modelled on Codex Alimentarius standards or some other standards.

Quality parameters of the highest ranked “Srpska sausage” from this study will be, on behalf of the Council of Technologists producers of meat and meat products, submitted to the competent state authorities as amendments tothe current Regulation on the quality of ground meat, meat preparations and meat products.

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Proteolysis and texture profile of traditional dry-fermented sausage as affected by primary processing method

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A b s t r a c t: In this study, proteolysis and texture profile of Petrovská klobása sausages produced with different raw batter mixing procedures (traditional, manual vs. mechanical) were investigated. Physicochemical analyses (pH value, moisture, nitrogen fraction), Labon-a-Chip (LoaC) electrophoresis-based protein analyses and texture profile analyses were performed in order to follow the drying/ripening process. The results obtained showed that manual mixing caused better water holding capacity of meat proteins, and thus, at the end of the drying process and under identical thermo-hygrometric conditions, control sausages had higher moisture content and lower hardness and chewiness values compared to their mechanically mixed counterparts. The increase in non-protein nitrogen and free amino acid nitrogen content and the results of electrophoretic separation of myofibrillar proteins indicated somewhat more intense proteolytic changes in manually-mixed sausages. However, the different mixing procedures did not significantly affect (p>0.05) sensory texture scores of Petrovská klobása at the end of the drying process.

Keywords: *dry-fermented sausage, mixing procedure, proteolysis, texture.*

Introduction

Traditional dry fermented sausages have great significance and economic value in all European countries. Such products are highly appreciated by consumers and are considered of high sensory quality. The wide variety of this type of sausage in the European geographic zone is a consequence of variations in raw materials, formulation and manufacturing processes, which arise from different traditional habits in countries and/or regions. Geographical indication often defines the name of the sausage (*Casaburi et al*., 2007; *Vignolo et al*., 2010). This is the case for *Petrovská klobása*, a dry-fermented sausage traditionally manufactured in north-western Serbia (Municipality of Backi Petrovac, Vojvodina province). Due to its production characteristics (smoking, plus long drying and ripening phases) and sensory attributes (aromatic and spicy-hot taste, dark red colour and hard consistency), it has been protected with a designation of origin (PDO) under Serbian legislation. In general, processing implies five well-defined phases: meat mincing, mixing with seasonings, stuffing, smoking and drying/ripening. Presently, this sausage is produced in the traditional manner according to original recipes, without the use of chemical additives or microbial starters. It is produced in micro-processing plants within village households during winter, and undergoes slow drying and ripening processes (*Petrovic et al.,* 2006; *Ikonic et al*., 2013; 2016). An important part of the production process is mixing the raw material (minced meat and back fat) with seasonings, and in traditional practice, this is performed manually. This introduces a portion of the indigenous microbiota into the sausage batter. In order to meet market demands and produce greater quantities of standard quality *Petrovská klobása*, it is necessary to apply more mechanisation and efficient control and management of thermo-hygrometric conditions during the drying/ripening phase than occurs in traditional conditions. Implementation of mechanisation implies the discharge of manual and the introduction of mechanical mixing for the sausage batter (*Marcos et al.*, 2016).

Proteolysis is one of the main biochemical phenomena which occurs during the ripening of fermented sausages, and is influenced by both muscle and microbial enzymes. Proteolysis results in the formation of several low molecular weight components, i.e. peptides, amino acids, aldehydes, organic acids and amines, which influence and define the final texture characteristics and aroma of

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the product (*Dalmıs and Soyer*, 2008; *Roseiro et al.*, 2008; *Spaziani et al.*, 2009). Regarding meat products, quality is strongly affected by their texture characteristics. The textural profile of dry-fermented sausages throughout the manufacturing process is usually determined by consecutive instrumental measurements, since sensory analysis can be applied on the final product (*Szczesniak*, 2002; *Gonzalez-Fernandez et al.*, 2006; *Barbut*, 2007).

Having in mind that any changes in production processes can affect the final quality of dry-fermented sausages, the aim of this study was to evaluate the effect of mixing procedure (manual vs. mechanical) on physicochemical traits, proteolysis and texture characteristics of *Petrovská klobása*.

Materials and methods

Preparation of Petrovská klobása

Petrovská klobása sausages were manufactured from a mixture of lean minced pork (80%) and back fat (20%) obtained from carcasses of large white cross breed pigs. The minced meat and fat mixture (Ø≈10 mm) was divided in two parts and the same amounts of seasonings (red hot paprika powder, salt, raw garlic paste, caraway and sucrose) were added. One part was mixed manually (control – C) and the other was mixed mechanically (experimental – E) (Fig. 1). Both parts were mixed until homogenous batter was obtained. After mixing, batters were immediately stuffed into collagen casings (500 mm long and 55 mm in diameter) and sausages were entirely processed in a traditional drying/ripening room during 60 days, until the required moisture content (<35%) was achieved (Serbia, 2015). The environmental conditions in this traditional room were highly dependent on outdoor climate conditions and were measured regularly during the processing. Average temperature and relative humidity during 60 days of processing were 8.3°C and 79.3%, respectively.

Samples

Samples of sausage types C and E were taken during processing on days 0, 2, 6, 9, 15, 30 and 60. On each sampling occasion, three sausages of each type were taken for physicochemical and texture profile analyses while the rest of the samples were prepared and stored at –20°C for electrophoretic separation of proteins. Sensory analysis was performed after 60 days of processing, at the end of the drying process.

Physicochemical analysis

The pH of sausages was measured using the portable pH meter Testo 205 (Testo AG, USA) equipped with a combined penetration tip with temperature probe. Moisture content was quantified according to the recommended ISO standard (ISO, 1997). The non-protein nitrogen (NPN) and free amino acid nitrogen (FAAN) were determined according to the methods described by Ikonic et al. (2013). The nitrogen fractions content were expressed as g/100 g dry matter (dm) of sausage.

Electrophoretic separation of proteins – LoaC method

The extraction and separation of myofibrillar proteins was performed as described by Ikonic et al. (2013), using Agilent 2100 bioanalyzer (Agilent

Figure 1. Traditional, manual (C) and mechanical (E) mixing of *Petrovská klobása*

Technologies, Santa Clara, CA) in combination with the Protein 230 Plus LabChip Kit and the dedicated 2100 expert software.

Texture Profile Analysis (TPA)

Texture analysis was performed as described by Bourne (1978), at room temperature, using TA.XT2 Texture Analyzer (Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems, Godalming, UK) equipped with a standard cylindrical plate of 75 mm in diameter. The samples (cylinders) 2 cm thick and 2.54 cm in diameter, after discarding the external layer of the sausage, were compressed twice to 50% of their original thickness at a constant test speed of 1 mm s^{-1}. The following parameters were determined: hardness (g), springiness, cohesiveness and chewiness (g). Hardness was defined by peak force during the first compression cycle. Adhesiveness was obtained from the negative force area under the curve obtained between cycles. Springiness was defined as the rate at which a deformed sample goes back to its un-deformed condition after the deforming force is removed. Cohesiveness was calculated as the ratio of the area under the second curve to the area under the first curve. Finally, chewiness was obtained by multiplying hardness, cohesiveness and springiness. Measurements were carried out six times for each sausage sample.

Sensory analysis

The sensory analysis was performed by an 8-member panel with previous experience in testing dry-fermented sausages. The casing was removed and the sausages were cut into slices of approximately 3 mm thickness and served at room temperature on white plastic dishes. Panel tasters were asked to score samples by using a 0 (worst) to 5 (best) scale for texture attributes.

Statistical analysis

The effects of drying time and mixing procedure on the variables studied were analysed by Factorial ANOVA (Statistica 13.2, Dell Inc., 2016). Duncan's post hoc test was performed for comparison of mean values. Differences were considered significant at $p<0.05$.

Results and Discussion

Changes in pH and moisture content during the drying period (Table 1) showed the prevailing tendencies observed in this type of product. Intensive pH decline was registered in both C and E sausages, reaching the isoelectric point (pI) of actomyosin (≈5.0) after 15 days of smoking/drying phase. In the next 15 days, pH continued to fall, achieving minimal values (C – 4.94, E – 4.99). Further on, slight pH increases in both sausages occurred, due to proteolysis processes, i.e. liberation of peptides, amino acids, ammonia and amines (*Kaban and Kaya*, 2009; *Spaziani et al.*, 2009). Similarly, moisture content in sausages decreased significantly ($p<0.05$) during the drying period, reaching values lower than 35%, required by national legislation for dry-fermented sausages (*Serbia*, 2015), after 60 days. Somewhat lower moisture content was registered in E sausages, indicating the slightly faster drying process of sausages produced by mechanically mixing the batter. Based on the results of pH and moisture content, it can be concluded that at 60

Table 1. Changes in pH and moisture content (%) of *Petrovská klobása* sausages during the drying process

Parameter	Sausage type	Time (days)							DT	MP	DT × MP
		0	2	6	9	15	30	60			
pH	C	5.74^{fg} ±0.04	5.75^{fg} ±0.02	5.67^{f} ±0.10	5.26^{d} ±0.15	5.06^{bc} ±0.06	4.94^{a} ±0.02	5.00^{ab} ±0.03	*	ns	*
	E	5.81^{g} ±0.02	5.72^{f} ±0.02	5.53^{e} ±0.04	5.10^{c} ±0.06	5.01^{abc} ±0.03	4.99^{ab} ±0.02	5.06^{bc} ±0.02			
Moisture content	C	54.3^{j} ±0.04	54.1^{j} ±0.06	53.1^{i} ±0.08	50.2^{f} ±0.32	48.6^{e} ±0.18	41.1^{c} ±0.05	33.9^{b} ±0.23	*	*	*
	E	55.6^{l} ±0.01	54.8^{k} ±0.02	51.7^{h} ±0.26	50.6^{g} ±0.02	48.2^{d} ±0.47	41.2^{c} ±0.31	32.3^{a} ±0.10			

Legend: Results are given as mean value ± standard deviation; $^{a-l}$ Means with different superscript letters are different ($p<0.05$); C – control, prepared from manually mixed batter; E – experimental, prepared from mechanically mixed batter; DT – drying time effect; MP – mixing procedure effect; ns – not significant; * $p<0.05$

Table 2. Evolution of NPN and FAAN content (expressed as g/100 g dm) in *Petrovská klobása* sausages during the drying process

Parameter	Sausage type	Time (day)							DT	MP	DT × MP
		0	2	6	9	15	30	60			
NPN	C	0.58^{a} ±0.01	0.62^{c} ±0.01	0.65^{d} ±0.01	0.66^{d} ±0.01	0.73^{f} ±0.01	0.82^{h} ±0.01	0.97^{j} ±0.01	*	*	*
	E	0.61^{b} ±0.00	0.62^{c} ±0.00	0.69^{e} ±0.00	0.69^{e} ±0.01	0.78^{g} ±0.01	0.89^{i} ±0.00	0.99^{k} ±0.00			
FAAN	C	0.20^{b} ±0.01	0.22^{c} ±0.01	0.20^{ab} ±0.00	0.22^{c} ±0.00	0.29^{d} ±0.00	0.33^{f} ±0.00	0.38^{h} ±0.00	*	ns	*
	E	0.20^{b} ±0.01	0.20^{b} ±0.01	0.19^{a} ±0.00	0.23^{c} ±0.00	0.31^{e} ±0.01	0.35^{g} ±0.01	0.37^{h} ±0.00			

Legend: Results are given as mean value ± standard deviation; $^{a-k}$ Means with different superscript letters are different ($p<0.05$); C – control, prepared from manually mixed batter; E – experimental, prepared from mechanically mixed batter; NPN – non-protein nitrogen; FAAN – free amino acids nitrogen; DT – drying time effect; MP mixing procedure effect; ns – not significant; * $p<0.05$

days, the ripening process in both *Petrovská klobása* sausages was not completely finished due to low pH values (C – pH 5.00, E – pH 5.06), although the drying process was considered finished. According to the Code of Practice for this traditional sausage (*Petrovic et al.*, 2006), the pH value of product ready for consumption has to be higher than 5.4.

NPN and FAAN content increased significantly ($p<0.05$) during processing in both analysed sausage types (Table 2), being approximately 65% and 88% higher in dried products compared to raw sausage batter, respectively. Nevertheless, the growth of these nitrogen fractions was slightly higher in C sausages, indicating more intensive proteolysis in manually mixed sausages, even though the differences in FAAN were not significant ($p>0.05$). Hence, the results obtained showed that degradation of proteins during drying/ripening of fermented sausages, i.e. liberation of polypeptides, peptides, amino acids, aldehydes, amines etc., was followed by an increase in NPN and FAAN content, as was previously reported by a number of authors (*Casaburi et al.*, 2007; *Dalmış and Soyer*, 2008; *Kaban and Kaya*, 2009; *Casquete et al.*, 2011).

Fig. 1 shows the LoaC gel-like electrophoretograms of myofibrillar proteins for control (C) and experimental (E) sausages. Generally, important changes in qualitative and quantitative composition of

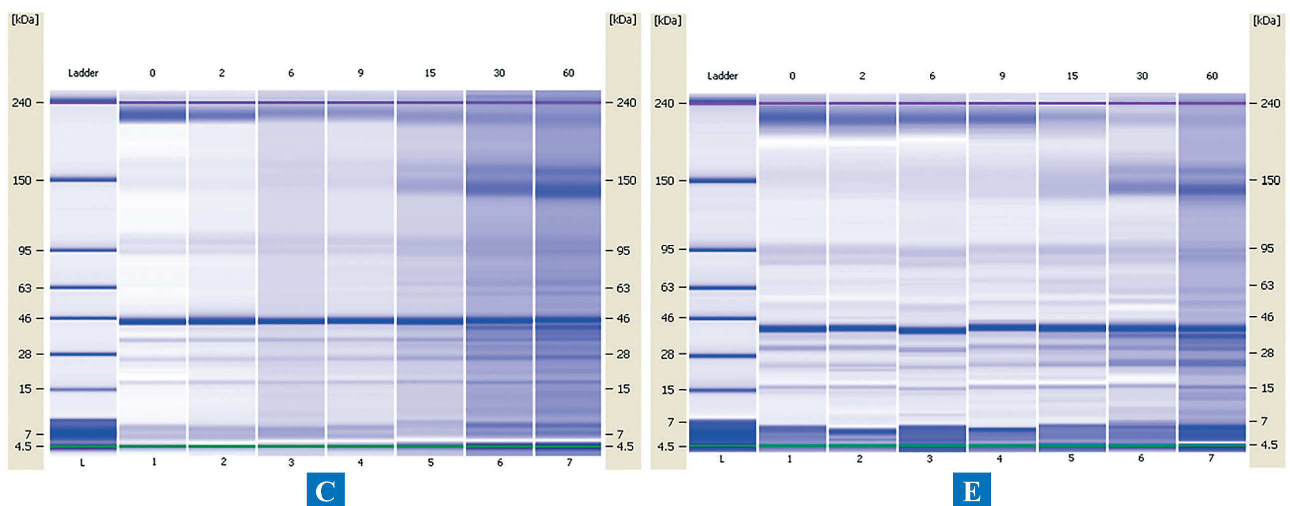

Figure 2. LoaC gel-like image of myofibrillar protein extracts during the drying of *Petrovská klobása*: C – control, prepared from manually mixed batter; E – experimental, prepared from mechanically mixed batter (lanes 1–7 correspond to days 0–60). Lane L – Ladder, molecular weight standards in the range of 4.5–240 kDa.

myofibrillar protein extracts were recorded during the drying period. Still, some differences in the intensity of certain protein fractions between the differently processed sausages were obvious. The concentrations of myosin (myosin heavy chain – MHC, ≈220 kDa) and actin (≈45 kDa), the most important myofibrillar proteins, were significantly reduced during processing compared to their initial levels. This phenomenon was particularly pronounced for MHC, since it was extensively hydrolysed in both analysed sausages (C ≈ 90%, E ≈ 94%). This result is in agreement with the findings of several authors (*Casaburi et al.*, 2007; *Roseiro et al*, 2008; *Spaziani et al*., 2009; *Ikonic et al*., 2013). However, actin showed greater stability. After 60 days of C and E sausage processing it lost about 55% and 40% of its initial concentration, respectively. Significant actin hydrolysis was previously reported in numerous publications dealing with dry-fermented sausages (*Dalmıs¸ & Soyer*, 2008; *Spaziani et al*., 2009). As a consequence of MHC degradation and co-migration of the resulting products, an important increase of protein bands at ≈145 and 160 kDa was observed in both sausage types in our study. This phenomenon was more pronounced for manually mixed sausages. Similar findings related to myosin degradation and formation of polypeptides with molecular weights in the range of 120–150 kDa were previously reported (*Martín-Sánchez et al*., 2011; *Spaziani et al*., 2009). Moreover, the intensity of protein bands at ≈74, 66, 41, 34, 31, 25 and 14 kDa increased during the ripening, while five (≈60, 40, 30, 22, 15 kDa) and three (≈105, 40, 22 kDa) newly-formed components were registered in sausage C and E, respectively. The appearance and accumulation of several polypeptides in the range of 14–100 kDa have also been observed by a number of authors (*Dalmıs & Soyer*, 2008; *Martín-Sánchez et al.*, 2011) during processing of dry-fermented sausages. Furthermore, a reduction in the protein band at ≈100 kDa (probably α-actinin) was observed throughout the drying period of both sausages, in concordance with findings of *Casaburi et al.* (2007) and *Dalmıs and Soyer* (2008).

Results of texture profile analysis are shown in Table 3. Hardness and chewiness of both types of sausages, as expected, increased progressively during processing. From day 9, hardness and chewiness values for E sausages were somewhat higher than those for C sausages. This phenomenon

Table 3. Evolution of texture profile of *Petrovská klobása* sausages throughout the drying process

Parameter	Sausage type	Time (day)						DT	MP	DT × MP
		2	6	9	15	30	60			
Hardness (g)	C	306^{a} ±44.9	499^{abc} ±68.8	624^{bc} ±164	923^{d} ±178	1562^{e} ±175	2611^{g} ±157	*	*	*
	E	298^{a} ±40.2	452^{ab} ±76.5	718^{cd} ±227	923^{d} ±127	1794^{f} ±208	3098^{h} ±432			
Springiness	C	0.39^{abcd} ±0.04	0.36^{afg} ±0.04	0.39^{abcd} ±0.02	0.42^{de} ±0.02	0.39^{abcd} ±0.03	0.34^{f} ±0.01	*	ns	ns
	E	0.38^{abc} ±0.05	0.38^{abg} ±0.03	0.44^{e} ±0.04	0.41^{bcde} ±0.02	0.42^{cde} ±0.03	0.34^{fg} ±0.02			
Cohesiveness	C	0.40^{a} ±0.03	0.35^{bc} ±0.03	0.38^{ac} ±0.03	0.40^{a} ±0.02	0.40^{a} ±0.03	0.33^{b} ±0.03	*	*	*
	E	0.40^{a} ±0.03	0.35^{bc} ±0.02	0.46^{d} ±0.06	0.46^{d} ±0.02	0.40^{a} ±0.02	0.33^{b} ±0.04			
Chewiness (g)	C	48.4^{a} ±11.1	63.2^{a} ±13.0	91.4^{ae} ±24.2	156^{bc} ±32.2	205^{c} ±100	292^{d} ±39.5	*	*	ns
	E	45.1^{a} ±9.33	59.4^{a} ±10.3	142^{be} ±38.3	171^{bc} ±32.3	304^{d} ±47.3	343^{d} ±75.3			

Legend: Results are given as mean value ± standard deviation; $^{a-h}$ Means with different superscript letters are different ($p < 0.05$); C – control, prepared from manually mixed batter; E – experimental, prepared from mechanically mixed batter; DT – drying time effect; MP – mixing procedure effect; ns – not significant; * $p<0.05$

could be due to the faster pH decrease of E sausages (5.10) toward the isoelectric point of protein (≈5.0). Decline of pH is followed by the solubilisation of myofibrillar proteins which aggregate to form a gel with an ordered protein network, and so contribute to sausage firmness (*Gonzalez-Fernandez et al.*, 2006; *Spaziani et al.,* 2009). At the end of drying process, hardness values were 2611g and 3098 g for C and E sausages, respectively ($p<0.05$). Chewiness values at the end of processing reached values of 292 g and 343 g for C and E sausages, respectively. Since the texture characteristics, are influenced by the drying process as well as the fermentation process (i.e. the pH decline), differences in hardness and chewiness values at the end of the drying process could be due to the difference in moisture content between sausages. Significant negative correlations ($p<0.05$) between pH and moisture content on one hand and hardness and chewiness on the other were registered by *Gonzalez-Fernandez et al*., (2006), *Bozkurt and Bayram* (2006) and *Casaburi et al.*, (2007). In our study on day 9, springiness, and on days 9 and 15, cohesiveness, was higher for E sausages than for C sausages. Lack of variability in cohesiveness was previously explained by *Spaziani et al*. (2009) as due to small difference in pH between samples when pH was close to the isoelectric point of proteins.

The results of sensory analysis did not reveal any significant difference ($p<0.05$) in texture between sausages at the end of the drying process. The observed differences in the instrumental analysis of texture were not detected in the sensorial analysis of *Petrovská klobása*. Hence, both C and E sausages showed relatively high scores for texture, being 3.92 and 3.95, respectively.

Conclusions

The influence of different mixing procedure (manual vs. mechanical) was analysed during the *Petrovská klobása* drying period. Most probably, manual mixing induced better water holding capacity of proteins, thus resulting in higher moisture content and lower hardness and chewiness values in C sausages at the end of the drying process. According to evolution of nitrogen fraction content and the results of LoaC electrophoretic separation of myofibrillar proteins, it can be concluded that manually mixed sausages underwent slightly more intense proteolytic changes. Nevertheless, different mixing procedures did not significantly affect the sensory attributes of texture. Further research will be related to transfer and adjustment of traditional technology to industrial conditions in order to achieve *Petrovská klobása* of optimal and standard quality.

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Effects of sodium nitrite and heat treatment on cholesterol oxidation products and sensorial characteristics of dry fermented sausages

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A b s t r a c t: Cholesterol oxidation products (COPs) and selected sensorial characteristics (color, odor and flavor) of Sremska sausages industrially produced without or with use of sodium nitrite as an additive and pasteurized at the end of drying were investigated. Both groups of sausages, nitrite free and with added nitrite, were divided into three subgroups and treated as follows: the first subgroup were not subjected to any heat treatment (unpasteurized); the second subgroup were heat-treated (pasteurized) at 47°C for 6.5 hours, while the third subgroup were pasteurized at 53°C for 22.1 minutes. Analysis of COPS were performed after maximum shelf life and eight cholesterol oxidation products common for this type of food were determined in all samples. Control sausages had the highest sum of COPs of all subgroups, while absence of sodium nitrite and application of pasteurization treatments resulted in significantly lower levels of COPs. Pasteurized sausages with added nitrite had lower levels of COPs compared to unpasteurized products, while the lowest levels of cholesterol oxidation were determined in pasteurized nitrite-free sausages. This study shown that the selected pasteurization regimes applied to dry fermented sausages produced without sodium nitrite as an additive did not increase oxidation of cholesterol, but even have potential to improve their lipid oxidation status. Additionally, the selected sensorial characteristics of all tested Sremska sausages were evaluated with high scores, regardless of the presence/absence of the additive, nitrite, or the application of pasteurization regimes.

Keywords: *dry fermented sausages, cholesterol oxidation products, sodium nitrite, pasteurization, sensorial characteristics.*

Introduction

Dry fermented sausages are highly valuable cured meat products with a long shelf life. However, high fat content makes these foods susceptible to lipid oxidation with possible further manifestation of rancidity as uncontrolled, excessive decomposition of fat. Rancidity is considered as one of the main causes for functional, sensory and nutritional quality deterioration in meat and meat products (*Marcincak*, 2016; *Ventanas et al*., 2006). Oxidation of lipids is related to free radical chain reactions in unsaturated fatty acids of phospholipids but, also, in cholesterol and other lipid compounds. The oxidation process can be induced by oxygen, light, metal ions, heating or enzymes like lipoxygenase (*Reig and Toldra*, 2010). There are more than 100 identified derivatives of cholesterol oxidation; to date, however, only six to eight of these substances are generally reported in animal-derived foods: 7α-hydroxycholesterol, 7β-hydroxycholesterol, α-epoxycholesterol, β-epoxycholesterol, cholestane-triol, 7keto-cholesterol, 20α- and 25-hydroxycholesterol (*Wasowicz et al*. 2004). Moreover, some cholesterol oxides as a part of lipid oxidation products are involved in the development of cardiovascular diseases and are also associated with cytotoxicity, mutagenesis and carcinogenesis (*Reig and Toldra*, 2010; *Muguerza et al*., 2004). On the other hand, it should be noted that controlled lipid oxidation has positive implications related to volatile compounds with pleasant flavor, which arise from oxidation of unsaturated fatty acids. This is especially important in production of fermented meat products (*Ruiz*, 2007).

Sodium nitrite ($NaNO_2$) is a well known food additive which contributes to enhancing microbial safety and to developing the typical color, aroma and taste of meat products. It is also considered as an antioxidant that delays rancidity of lipids and extends the shelf-life of cured meat products (*Sindelar and*

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Milkowski, 2011). On the other hand, a high intake of this additive can present a health risk with possible allergenic and vasodilator effects, metmyoglobin production *in vivo* and the production of carcinogenic nitrosamines (*Marco et al*., 2006). In that sense, consumers have become apprehensive about the use of this chemical preservative, and consequently, the European Commission will consider the possibility for further reductions of the current maximum limits of nitrites in all meat products in the near future (*European Union*, 2011). At the same time, due to a number of foodborne outbreaks involving fermented sausages, US Food Safety and Inspection Service (*Anon*., 2001) and Health Canada (*Anon*., 2000), required additional pathogen reduction measures and one of the suggested approaches is based on heat (pasteurization) treatments.

Therefore, the aim of this study was to analyze cholesterol oxidation products, as a measure of lipid oxidation status of Serbian dry fermented sausages (*Sremska*), which were industrially produced without or with the use of added sodium nitrite and pasteurized at the end of drying, then stored for maximum shelf life. In addition, the study also evaluated color, odor and flavor of the produced and additionally processed sausages, as a measure of their acceptance by consumers.

Materials and methods

Production of dry fermented sausages

A pork-based Serbian type of dry fermented sausage (Sremska) was used in the study.

Sausages without added sodium nitrite. Sausages were manufactured in a local meat industry according to traditional formulation: pork meat 70%, pork back fat 30%, NaCl 2.5%, spices 2.5% (paprika, chilies, coriander, garlic, pepper), dextrose 0.3%. Sausages were manufactured by natural fermentation, i.e. without starter cultures. Frozen meat trimmings and fatty tissue had been left to defrost in the chiller and then chopped in a cutter to particles of about 5 mm diameter and after that other ingredients were added and mixed. Batters for sausages were stuffed into natural pork casings (32–34 mm) and placed in a climate chamber for ripening.

Control sausages with added nitrite. Sausages were prepared from the chopped raw materials in the same proportions and in identical manner as described above, but with added NaCl-$NaNO_2$ mixture 2.5% (ratio 99.5%: 0.5%), instead of 2.5% of NaCl.

Ripening of sausages. The process of fermentation and drying of sausages lasted for 20 days under the following temperature/relative air humidity conditions: day 1: 20°C/95%; day 2: 19°C/90%; day 3: 18°C/85%; day 4: 17°C/ 80%; day 5: 16°C/75%; days 6 to 15: 15°C/75% and days 15 to 20 12°C/70%. Smoking was applied for 2 h daily, from days 3 to 5.

Post-processing pasteurization of sausages. After ripening, both batches (groups) of sausages (without added nitrite/with added nitrite) were divided into three subgroups. Sausages in the first subgroup were not subjected to any heat treatment (unpasteurized). Sausages in the second subgroup were heat-treated (pasteurized) at 47°C for 6.5 hours, while in the third subgroup, products were pasteurized at 53°C for 22.1 minutes. Temperature and duration of pasteurizations were pre-selected and calculated by taking into account previously experimentally determined D-values of *Salmonella* reduction in finished pork dry fermented sausages with intention of achieving 6.5 log reduction (*Ducic et al*., 2016).

Post-pasteurization storage of sausages. Products were wrapped into laminated paper sheets imitating packaging of sausages at the counter in the retail shops and were stored for three months (maximum extent of shelf life) at 9°C (the temperature at which majority of household refrigerators in Serbia operate (*Janjic et al*., 2016) and examined for cholesterol oxidation products (COPs). For this analysis, six samples from each of the six subgroups of sausages were used.

Physicochemical analysis

Water activity (a_w) in sausage samples was determined with LAB Swift-aw set Euro-plug&BAT equipment (Novasina, Switzerland), while pH was determined by a hand-held pH meter (Testo 205; Germany), both used according to the manufacturer's instructions. Content of nitrite in sausages was determined in accordance with ISO standard (*ISO 2918,*1975) on the start (day 0), at midprocess (day 10) and at the end of the process (day 20). For each analysis, six samples from both groups of sausages (nitrite-free/nitrite added) were used.

Sensory analysis

Color, odor and flavor of finished sausages were scored by a trained, seven-member sensory panel using a 1–7 point scale, according to ISO standard (*ISO 4121,* 2003). The evaluation was conducted in the sensorial analysis laboratory at the Faculty of Veterinary Medicine in Belgrade.

Cholesterol oxidation analysis

The cholesterol oxides were determined according to a method from *Ubhayasekera et al.*, (2004), with some modifications, followed by LC-MS/MS analysis. Homogenized samples were weighed (1 g) in 50 ml centrifuge tubes (Falcon), to which were added 100 µl of a hexane solution of 20 mg 10ml^{-1} of 5α-cholestan-3β-ol (Merck, 8.41513) as the internal standard. Three ml of dichloromethane (Merck, 1.06044), 7 ml of 2 M KOH (Merck, 1.05033) in 70% ethanol (Pharmachem, 09516) and 500 µl of butylated hydroxytoluene (BHT) (Sigma-Aldrich, B-1378) were added. The mixture was vortexed for 3 min and then transferred into ultrasonic bath (2 h, 60°C). After cooling the centrifuge tubes, 10 ml of water and 10 ml of diethyl ether (Merck, 1.00921) were added. The samples were mixed well, ultrasonicated for 15 min, and centrifuged for 10 min at 1700 × g (5810 centrifuge, Eppendorf). Then the greater part of polar phase was removed and 5 ml of 0.5 M KOH (Merck, 1.05033) in distilled water was added. The samples were ultrasonicated and centrifuged again. The greater part of polar phase was removed and 5 ml of 10% NaCl (Merck, 1.06404) was added. After ultrasonification and centrifugation, 5 ml of the organic layer was transferred to a glass flask and the solvent was evaporated (560 mbar, 40°C; Buchi Rotavapor R-250/215). The residue was diluted in 5 ml of acetonitrile and then transferred to a Supel™ QuE Z-Sep^{+} centrifuge tube (Supelco, 55296-U), mixed well and centrifuged at 3000 × g for 10 min. The supernatant was then transferred to a glass flask and the solvent was evaporated (94 mbar, 40°C; Buchi Rotavapor R-250/215). The residue was diluted in 1 ml of hexane. Thus, the samples were prepared for solid-phase extraction.

The solid-phase extraction was performed using a Strata Si-1 column (Phenomenex; 8B-S012-EAK). The pre-conditioning was carried out with 3 ml of hexane (Merck, 1.04371). One ml of prepared sample and 2 ml of hexane were loaded. The column was then washed with 3 ml of hexane (Merck, 1.04371): diethyl ether (Merck, 1.00921) (9:1, v/v). The elution was carried out with 3 ml of acetonitrile (Merck, 1.00030). The solvent was evaporated (94 mbar, 40°C; Büchi Rotavapor R-210/215) and the residue was diluted in 1 ml of acetonitrile (Merck, 1.00030):2-propanol (Merck, 1.00998) (55:45, v/v).

The samples were analyzed by LC–MS/MS. The LC system consisted of a model 1100 G1312A binary pump and a model G1330B autosampler (Agilent Technologies). This reversed-phase HPLC separation was carried out using a C18 column (100 mm × 2 mm internal diameter; 2.6 µm particle size; Kinetex, Phenomenex), which was protected by a C18 guard cartridge (Kinetex, Phenomenex). The mobile phase comprised water (A) and acetonitrile (B), and the following gradient was used: 0–3.0 min, 80–98% B; 3.0–7.5 min, 98% B; 7.5–7.6 min, 98–80% B; 7.6–12 min, 80% B. The column was maintained at 45°C, with an injection volume of 10 µl and a flow rate of 400 µl min−1. To identify and quantify the COPs, LC–MS/MS was used. A Micromass Quattro Micro Mass spectrometer equipped with an electrospray ionization source was operated in positive ion mode (Waters, Milford, MA, USA). The mass spectra were obtained with the following operating parameters: capillary voltage, 3.2 kV; cone voltage, 30 V; extractor, 2 V; source temperature, 120°C; desolvation temperature, 350°C; cone gas flow, 50 L h^{-1}; and desolvation gas flow, 400 L h^{-1}.

The data were acquired using multiple reaction monitoring, at the collision energy of 36 V. The following transitions were obtained: 5α-cholestan-3β-ol (371.66>109.00); 7α-hydroxycholesterol (368.18>>145.22); 7β-hydroxycholesterol (368.18>145.22); 20α-hydroxycholesterol (368.18>147.22); 22-hydroxycholesterol (368.18>147.22); 25-hydroxycholesterol (368.18>147.22); 7-ketocholesterol (401.64>175.40); cholesterol 5α,6α-epoxide (385.66>159.30); cholesterol 5β,6β-epoxide (385.66>159.30) and cholesterol (370.18>147.22). The data were processed using the Quantify function of the MassLynx V4.1 programme.

The COPs were identified according to their retention times, in comparison with the following standards: 7α-hydroxycholesterol (Steraloids Inc., C6420--000); 7β-hydroxycholesterol (H6891 SIGMA); 7-ketocholesterol; cholesterol 5α,6α-epoxide (C2773 SIGMA); cholesterol 5β,6β-epoxide (C2648 SIGMA); 20α-hydroxycholesterol (H6378 SIGMA-ALDRICH); 22-hydroxycholesterol (H9384 SIGMA); 25-hydroxycholesterol (H1015 SIGMA); (284122 ALDRICH); and cholesterol (C8667 SIGMA). The recovery of COPs was from 83% to 86%, with accuracies from 2.5% to 4.6%. The limits of detection were from 8 ng to 13 ng injected, and the limits of quantification from 30 ng to 47 ng injected. The experimental recoveries and quantification of the method for different COPs were determined by the standard addition method. The sample was spiked with all of the compounds analyzed at four spiking levels (0.5, 5, 25, 50 ng g^{-1}), by adding different volumes of a solution of the analytes.

Statistical analyses

Determination of mean values, standard deviation and analysis of variance (ANOVA) followed by Tukey's test for the significances of differences between grouped data was conducted by using

Statistica Version 12 software (StatSoft, Inc., Tulsa, USA). Values of $P<0.05$ were considered significant.

Results and discussion

Regarding a_w and pH values, both nitrite-free and nitrite-added unpasteurized/pasteurized sausages can be classified at the end of the ripening as low acid dry fermented products (*Aymerich et al*., 2003; *Lücke*, 2000). Residual nitrite in sausages produced with added sodium nitrite were analyzed throughout the production process and results are presented in Table 1.

The level of nitrite in Sremska sausages at first day of production was 139.7 mg kg^{-1} which is in accordance with standard value of maximum of 150 mg kg^{-1} in thermally non-treated products (*Serbia*, 2013). Regarding the balance between positive effects of nitrite on color, prolonging of shelf life and delaying the growth of microorganisms and the potentially negative effect on human health by forming the cancerogenic nitrosamines, *Cassens* (1997) recommended that concentration of residual nitrite in finished products should be 5 mg kg^{-1} to maximum 15 mg kg^{-1}.In our investigation, during the production process level of nitrite decreased to 10.5 mg kg^{-1} in finished sausages, which is in accordance with suggested values.

The analysis of cholesterol and cholesterol oxidation products (COPs) in dry fermented sausages after three months of storage are presented in Table 2. The total content of COPs were in the range of the values for this type of products reported in investigations of *Zanardi et al*., (2004), *Muguerza et al*., (2004) and *Derewiaka and Obiedzinski*, (2010), with the exception of higher levels in our control sausages that are similar to results reported by *Talon et al*., (2008). In that sense, it should be taken into account that ascorbic acid/ascorbate as an antioxidative additive was not included in recipes and that analyzed sausages were stored aerobically for three months, in common condition that influence lipid oxidation (*Kerry et al*., 2002). In addition, several authors highlight that oxidation of lipids in meat products depends to a large extent on the quality of the raw materials and their initial level of oxidation (*Demeyer*, 2007; *Hur et al*., 2007; *Lercker & Rodriguez-Estrada*, 2000).

The percentage of cholesterol oxidation products (% COPs) in the total content of cholesterol ranged from 0.14% in nitrite-free Sremska sausages pasteurized at 53°C/22.1 minutes, up to 0.73% in control unpasteurized sausages with added nitrite. In all subgroups of sausages, % COPs were higher than 0.1%, which is reported as minimal toxicity level for cultured cells (*Bösinger et al*., 1993). However, our findings are still far from the levels required to show toxic effects in *in vivo* trials with laboratory animals, which is, according to the previously mentioned authors, about 100 times higher than toxic levels determined in *in vitro* experiments. Unexpectedly, control sausages produced according to standard recipes and manner had markedly higher % COPs in compare with values of other subgroups of sausages. Regarding sausages without added nitrite, results showed that the % of COPs in the total content of cholesterol was lower than in sausages produced with added nitrite. In the case of sausages produced with added nitrite, sausages from both pasteurization regimes (47°C/ 6.5 h; 53°C/22.1 min) had significantly lower values of % COPs, compared with unpasteurized sausages. It should be noted that cholesterol content, probably because of high melting point, was not decreased in pasteurized sausages, although fat out as a consequence of melting of other lipids occurred to a mild extent during heat treatment. Pasteurization treatments in nitrite-free sausages also induced decrease of % COPs

Table 1. Water activity and pH values of Sremska sausages (without added/with added sodium nitrite) after ripening and concentration of nitrite during ripening of sausages produced with added sodium nitrite.

Parameters	Day	$+NaNO_2$	$-NaNO_2$
a_w	20	0.71 ± 0.01	0.7 ± 0.01
pH	20	5.64 ± 0.07	5.69 ± 0.04
Nitrite concentration (mg kg^{-1})	0	139.7 ± 1.2	N.P.
	10	71.9 ± 0.8	N.P.
	20	10.5 ± 1.1	N.P.

Legend: $+NaNO_2$ – sausages with added sodium nitrite; $-NaNO_2$ – sausages without added sodium nitrite; aw – water activity; N.P. – not performed.

Table 2. The effects of nitrites and/or selected post-processing pasteurization regimes on total cholesterol (mg $100g^{-1}$) and cholesterol oxidation products (COPs) (µg g^{-1}) in sausages stored at 9°C for 3 months (mean values±standard deviation)

Parameters	**Unpasteurized**		**Pasteurization regimes**			
			47 °C/6.5 h		**53 °C/22.1 min**	
Nitrite additive	$+NaNO_2$	$-NaNO_2$	$+NaNO_2$	$-NaNO_2$	$+NaNO_2$	$-NaNO_2$
mg g^{-1}±SD – Cholesterol oxides; mg $100g^{-1}$±SD – Cholesterol						
Chol	70.61±1.31	74.61±1.93	75.1±0.94	73.7±0.41	74.32±2.59	75.9±1.05
7α-OH	0.48^{BC}±0.23	0.59^{AB}±0.17	0.83^{A}±0.32	0.27^{BC}±0.07	0.82^{A}±0.03	0.24^{C}±0.07
7β-OH	0.26^{AB}±0.12	0.3^{A}±0.05	0.32^{A}±0.23	0.06^{B}±0.03	0.21^{AB}±0.11	0.19^{AB}±0.14
7-keto	3.19^{A}±0.22	0.29^{BC}±0.08	0.5^{B}±0.16	0.21^{C}±0.02	0.38^{BC}±0.1	0.32^{BC}±0.09
α-epoxide	0.21^{A}±0.17	0.06^{B}±0.02	0.04^{B}±0.03	0.02^{B}±0.01	0.04^{B}±0.04	0.03^{B}±0.02
β-epoxide	0.3^{A}±0.07	0.12^{B}±0.05	0.12^{B}±0.09	0.08^{B}±0.04	0.11^{B}±0.06	0.06^{B}±0.04
20α-OH	0.06±0.02	0.22±0.18	0.12±0.15	0.15±0.08	0.23±0.17	0.06±0.05
22-OH	0.34^{A}±0.03	0.14^{BC}±0.12	0.36^{A}±0.01	0.21^{B}±0.04	0.14^{BC}±0.07	0.08^{C}±0.08
25-OH	0.32^{A}±0.03	0.03^{C}±0.01	0.23^{A}±0.18	0.21^{AB}±0.04	0.07^{BC}±0.09	0.1^{C}±0.06
Total COPs	5.18±0.08	1.74±0.45	2.53±0.42	1.21±0.17	2.00±0.43	1.07±0.32
% COPs	0.73^{A}±0.01	0.24^{BC}±0.07	0.34^{B}±0.06	0.18^{C}±0.04	0.33^{B}±0.17	0.14^{C}±0.04

Legend: Chol (Cholesterol), 7α-OH (7α-hydroxycholesterol), 7β-OH (7β-hydroxycholesterol), 7-keto (7-ketocholesterol), α-epoxide (5α,6α- epoxycholesterol), β-epoxide (5β,6β-epoxycholesterol), 20α-OH (20α-hydroxycholesterol), 22-OH (22-hydroxycholesterol), 25-OH (25-hydroxycholesterol), % COPs (% of oxysterols among the total cholesterol content).
^{A,B,C} Mean values within a row with different letters are differs significantly, $p<0.05$.

compared to unpasteurized nitrite free subgroup, but not in the range of statistical significance. *Vicente and Torres* (2004) examined oxidation of cholesterol in beef minced meat – hamburgers cooked at moderately high temperature (80°C) and found that level of COPs decreased after application of heat. According to the same authors, this result might be explained by association of COPs with other molecules. They also suggested that reduction of cholesterol oxides could be due to release of cholesterol during processing and due to the lack of thermally induced conversion of cholesterol to his oxides. However, in our study, contents of cholesterol in sausages were not decreased after pasteurization, because of our application of mild heat. Regarding thermally induced formation of COPs, according to several studies (*Vicente and Torres*, 2004; *Chien et al.*, 1998; *Kim and Nawar*, 1993) no or very little conversion is expected at temperatures below 120°C.

In all Sremska sausages, eight common derivatives of cholesterol oxidation were detected (Table 2). The most abundant COPs were B-ring oxides as products of primary oxidation of cholesterol. The B ring oxides, 7-ketocholesterol, 7α-hydroxycholesterol and 7β hydroxycholesterol are common findings in meat according to *Georgiou and Kapnissi-Christodoulou* (2012). Since it is easily formed, 7-ketocholesterol is one of the most common oxysterols in meat, especially in stored products, and it is usually found in high levels (*Rodriguez-Estrada et al*. 2014). The concentrations of 22-hydroxycholesterol, 25- hydroxycholesterol and 20- hydroxycholesterol were lower than the B-ring oxides, probably due to fact that side-chain oxidation needs longer times with stronger prooxidant conditions and usually takes place in solid cholesterol matrices, according to *Morrissey and Kerry* (2004). Regarding such conditions, it can be assumed that side chain oxysterol production could be intensified only after a considerable part of the water has been evaporated from sausage during ripening. 5β,6β-epoxycholesterol and 5α,6α-epoxycholesterol, as secondary oxidation products had the lowest concentrations among the COPs in almost all Sremska sausages.

Regarding distribution of individual cholesterol oxidation products per subgroups, as expected,

Table 3. The effects of nitrites and/or selected post-processing pasteurization regimes on selected sensorial characteristics of dry fermented sausages after ripening.

Parameters	Unpasteurized		Pasteurization regimes			
			47 °C/6.5 h		53 °C/22.1 min	
Nitrite additive	$+NaNO_2$	$-NaNO_2$	$+NaNO_2$	$-NaNO_2$	$+NaNO_2$	$-NaNO_2$
Color	$6.93^{A}\pm0.19$	$6.5^{B}\pm0.29$	$6.93^{A}\pm0.19$	$6.5^{B}\pm0.29$	$6.93^{A}\pm0.19$	$6.57^{AB}\pm0.34$
Odor and flavor	$5.71^{B}\pm0.27$	$5.14^{B}\pm0.24$	$6.93^{A}\pm0.19$	$5.64^{B}\pm0.56$	$5.5^{B}\pm0.41$	$6.36^{A}\pm0.38$

Legend: ^{A,B} Mean values within a row with different letters are significantly different, $p<0.05$.

7-ketocholesterol was markedly higher in control than in all other sausages. The applied pasteurization, as well as absence of nitrite seems to have favored degradation of 7-ketocholesterol against its formation, as already observed by other authors in heat treated meat products (*Rodriguez-Estrada et al*., 2014; *Broncano et al*., 2009). As a major component, 7-ketocholesterol was also presented in the subgroup of nitrite free sausages treated by 53°C for 22.1 min, while in other subgroups of sausages, the most abundant derivate was 7α-hydroxycholesterol, and such results are in accordance with findings of other studies of heat treated meat products (*Broncano et al*., 2009; *Nam et al*., 2001). Statistical analysis showed that contents of 7 ketocholesterol, 5α,6α-epoxycholesterol, 5β,6β-epoxycholesterol, 25hydroxycholesterol, were significantly higher in control sausages produced with sodium nitrite compared to all other subgroups. It can be observed that all investigated derivatives had markedly lower concentrations in the group of sausages produced without added nitrite. Levels of 5α,6α-epoxycholesterol and 5β,6β-epoxycholesterol were similar in all subgroups, with exception of significantly higher levels in control sausages.

In general, qualitative and quantitative comparative analysis of COPs based on data available in the literature is very difficult because great discrepancies have been observed between different investigations. The high variations in results suggests that formation of oxysterols depends on many factors including composition of food, processing conditions and analytical techniques (*Derewiaka and Obiedzinski*, 2010; *Broncano et al*., 2009). Among other factors, it should be considered that the high variability of COP levels between subgroups of sausages in our study could be related to changes of growth of microbiota and their consumption of oxygen induced by pasteurization regimes (*Zanardi et al*., 2004).

In this investigation, an experienced sensory panel evaluated color, odor and flavor of both nitrite-free and nitrite-added unpasteurized/pasteurized sausages after ripening and results are presented in Table 3. Sausages produced with added nitrite (unpasteurized/pasteurized) had significantly higher scores for color than nitrite-free unpasteurized and nitrite-free pasteurized at 47°C/ 6.5 h subgroups of sausages. Nitrite free sausages pasteurized at 53°C for 22.1 min also rated lower compared to products with added nitrite, but difference was not in the range of statistical significance. These results are expected and they are in accordance with the well-known effect of sodium nitrite on improving of color of cured meat products. Nevertheless, sausages produced without additive were also evaluated with very high scores. Results of the sensorial panel showed that absence of nitrite as an additive did not induce any significant changes of odor or flavor in Sremska sausages. However, heat treated sausages (nitrite-added and pasteurized at 47°C/ 6.5 h, or nitrite-free and pasteurized at 53°C for 22.1 min) had significantly higher scores for odor and flavor ($P<0.05$) than control, which suggests that application of the selected pasteurization regimes could have potential to improve these sensorial attributes.

Conclusion

Both groups of Sremska sausages, nitrite-free and nitrite-added (unpasteurized/pasteurized), regarding physicochemical characteristics can be classified as low–acid, dry, fermented sausages. Sausages produced with added sodium nitrite had the regular content of this additive throughout the production process.

After sausages underwent storage to the maximum shelf life, the content of cholesterol oxidation products of all subgroups of Sremska sausages was in the range of common values for this type of food. Absence of nitrite as an additive as well as pasteurization regimes did not induced increase of COPs, but instead levels of COPs in nitrite-free and pasteurized subgroups were lower than in control

sausages. Moreover, our investigation shown that sausages without sodium nitrite treated with pasteurization regimes had the lowest level of COPs. Such results suggest the investigated modifications of the sausage production process have the potential to improve lipid oxidation status of the final products.

Generally, color, odor and flavor of both groups of sausages – nitrite-free and with added nitrite (unpasteurized/pasteurized) were good. However, absence of nitrite resulted in somewhat less desirable color, while odor and flavor were not affected or were even slightly improved after application of the selected pasteurization regimes.

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Fatty acid composition of cow's milk: opportunities and challenges for Serbian dairy producers

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A b s t r a c t: The objective of this study was to investigate whether there is a difference in fatty acid (FA) profile, with emphasis on C18:1cis-9 and conjugated linoleic acids (CLA), of milk obtained from dairy cows under typical farming conditions practiced in Serbia, as well as to investigate variations in fatty acid composition among retail milks labeled differently in relation to fat content (3.2%, 2.8% or 1.5%). Accelerated solvent extraction of milk lipids followed by capillary gas chromatography with flame ionization detector was used for milk FA determination. The results obtained in this study showed that in raw milk, saturated fatty acids (SFAs) accounted for up to 73.73% of the total FAs, followed by monounsaturated fatty acids ($\bar{X}$23.44 to 26.92%) and polyunsaturated fatty acids (PUFA) ($\bar{X}$2.72 to 3.92%). Very similar results were obtained in retail milks. The FA composition of raw milk did not significantly differ between the geographical regions examined, except in the contents of C18:2n-6 and PUFA, which were higher in the milk produced in South Banat than in milks produced in Central Banat and in the area of West Backa and Syrmia. In relation to commercially processed milk, the FA contents varied significantly ($p<0.05$) between the milks with different declared fat levels. However, from the nutritive point of view, all milks examined should be considered as beneficial to human health regardless of fat content, although opportunities for improvement, as related to dietary guidelines, are present.

***Key words:** milk, fatty acid, conjugated linoleic acid (CLA), trans fatty acid, human health.*

Introduction

Milk is a very important source of nutrients in the human diet, providing energy, high quality protein, and essential minerals and vitamins. Besides milk protein, the level and composition of bovine milk fat is critical for milk quality with regard to its nutritive value (positive or negative effects on human health) as well as the processing and technological qualities of the milk (e.g., melting point and hardness of butter) (*Duchácek et al.,* 2014). Fat is the most variable component of milk (3.0% to 6.0%) and the fatty acid composition of milk fat is highly diverse (*MacGibbon and Taylor*, 2006; *Lock and Bauman*, 2011). This diversity is due to the presence of over 400 fatty acids (FAs) found in milk, which makes milk fat the most complex of all natural fats. FAs in bovine milk come from two sources: by *de novo* synthesis in the mammary gland or by being preformed in plasma lipids originating from absorption from the small intestine or from body stores of adipose tissue. *De novo* synthesized fatty acids include those 4 to 16 carbons in length, while preformed FAs from the circulatory system are 16 carbons in length or longer (Heck *et al.,* 2012). Furthermore, the FA composition of milk is also of interest because it might be useful as an indicator of the metabolic status of cows (*Stoop et al.,* 2009).

Although milk fat is largely composed of triacylglycerols (these comprise about 98% of the total fat; *MacGibbon and Taylor*, 2006), which have been claimed to contribute to heart disease, two other milk components considered to be beneficial for human health are the FAs conjugated linoleic acid (CLA) and butyric acid (C4:0). Biomedical studies with animal models have demonstrated a variety of effects attributed to CLA, including anticarcinogenic, antiatherogenic, antiobesity, immune system enhancement and antidiabetic properties (*Corl et al.,* 2003; *Larsson et al.,* 2005). Butyric acid, which is uniquely present in ruminant milk, has been claimed to have a role in preventing colon cancer (*Perrin et al.,* 1994). The cow's diet has a major influence on the content of CLA in milk fat, and these effects have been recently summarized (*Griinari et al.,* 2000; *Peterson et al.,* 2002; *Kelsey et al.*, 2003).

The composition of milk and its standardization are essential to guarantee the quality of dairy

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products/ingredients and so have great importance for the dairy industries (*Swensson and Lindmark-Mansson,* 2007); therefore, it is necessary to monitor milk quality (*Demment and Allen*, 2003). The dairy industry in Serbia is economically important for the country. According to official statistics, total milk production in Serbia is around 1500 million t year^{-1} (3477 t per milked cow) (*Statistical Office of the Republic of Serbia*, 2016).

The objective of this study was to investigate whether there is a difference in FA profile, with emphasis on C18:1cis-9 and CLA, of milk obtained from dairy farms under standard farming conditions as practiced in the north of Serbia, as well as to investigate variations in mean FA composition among differently labeled retail milks in relation to fat content (3.2%, 2.8% or 1.5%). In addition, this study provides information relevant for consumers on possible impacts on the risk of developing cardiovascular disease due to consumption of milks with different fat contents.

Materials and methods

Milks studied

In October and December 2016, using a stratified random sampling method, 24 raw cow's milks were collected from four different municipalities in Vojvodina Province, northern Serbia: South Backa District, South and Central Banat Districts, and West Backa and Syrmia. Two breeds of cattle form the basis of the cattle industry in Serbia: Simmental cattle or domestic spotted Simmental type cattle, which are most common in rural areas on family smallholdings, and Black and Red Holstein-Friesian cattle (dairy type cattle), which are mainly present in the organized manufacturing farm production that supplies raw milk to the dairy industry (*Petrovic et al.,* 2013). Feeding regimens were based on total mixed rations directly formulated on-farm by farmers or their technicians. The ingredient composition of the diet corresponded to the current level of individual daily milk yields, and feeding rations were completely balanced for energy, protein, and fat as well as mineral and vitamin content. In addition, a total of 15 commercially processed milks (sterilized) with different declared fat contents (3.2%, 2.8% or 1.5%) were purchased in a retail supermarket.

FA analysis by capillary gas chromatography

The total lipids were extracted from the milks by accelerated solvent extraction (ASE), (ASE 200, Dionex, Sunnyvale, CA, USA) with petroleum ether and isopropanol mixture (60:40, v/v) (as proposed by *Dionex Application Note No. 345*) at 100°C over three static cycles under nitrogen at 12 MPa. The solvent from the collected extracts was removed under a stream of nitrogen (Dionex Solvent evaporator 500) at 50°C until dry.

Fatty acid methyl esters (FAMEs) in the extracted lipids were prepared by esterification using 0.5 M sodium methoxide in anhydrous methanol as proposed by *Christie et al*. (2001). FAMEs were determined by gas-liquid chromatography (Shimadzu 2010, Kyoto, Japan) equipped with a flame ionization detector and capillary HP-88 column (length 100m, i.d. 0.25 mm, film thickness 0.20 µm). Injector and detector temperature were 250ºC and 280ºC, respectively. Nitrogen was used as the carrier gas at a flow rate of 1.87 mL min^{-1}. The injector split ratio was set at 1:50. A programmed column oven temperature starting at 50ºC and ending at 230ºC was applied. Total analysis time was 66.5 min. The chromatographic peaks in the samples were identified by comparing relative retention times of FAME peaks with peaks in Supelco 37 Component FAME mix standard (Supelco, Bellefonte, PA) and a standard mixture of methyl esters of *cis*-9,11 and *tran*s-10,12 isomers of conjugated linoleic acid (CLA) (O5632 ≥99%, Sigma-Aldrich, USA). Each sample was analyzed in duplicate. Results were expressed as mass of FA (g) in 100 g of FAs.

Statistical analysis

Statistical analysis of the data was performed using JMP software (SAS Institute Inc., version 10.0). Differences in individual FAs in milks from the different regions in Serbia or in retail milks with different declared fat contents were assessed using one-way ANOVA and Tukey's HSD post-hoc test. Differences were considered statistically significant at the levels of $p<0.05$, $p<0.01$ and $p<0.001$. The data structure and descriptive statistics relating to the milks studied are presented in Tables 1 and 2.

Results and discussion

The fatty acid profile of milk obtained from bovine dairy farms under the farming conditions practiced in northern Serbia is presented in Table 1, while fatty acid composition of milk with different declared fat contents from the Serbian retail market (3.2%, 2.8% or 1.5%) is presented in Table 2.

Short-chain saturated FA (SCSFA) were represented by butyric (C4:0), caproic (C6:0) caprylic (C8:0), capric (C10:0) and lauric (C12:0)

acid in the milks, and in general, their levels were lower than other saturated (SFA) and unsaturated (UFA) FAs. This SCSFA are de novo FA, synthesized in the mammary gland and are derived from acetic and butyric acids that are generated in the rumen by fermentation of feed components. The SCSFA (C6–10) do not appear to affect human health, whereas it has been claimed that butyric acid (C4:0) could perhaps prevent colon cancer (*Parodi*, 2001).

The levels of major medium and long-chain saturated FAs (LCSFA) (C14:0, C16:0 and C18:0) showed the highest concentrations among the different FAs in the milks studied (Table 1). These results agree with those reported by *Palmquist et al.* (1993), *Jensen* (2002) *Lock & Garnsworthy* (2011) for the FA composition of cow's milk. In the current study, 46% of the FAs were medium length (14–16), while long-chain FAs (≥18:0) and short-chain (<14:0) FAs corresponded to approximately 12% of total fat. Palmitic acid (C16:0) has a lower genetic effect and a higher herd effect compared with other even-chain *de novo* synthesized FA (*Garnsworthy et al*., 2006; *Craninx et al*., 2008; *Moate et al*., 2008). Therefore, C16:0 is considered to be partly synthesized *de novo* and partly derived from the blood (*Garnsworthy et al*., 2006; *Craninx et al.,* 2008; *Moate et al.,* 2008). The remaining SFAs and long-chain UFAs originate from dietary lipids and from lipolysis of adipose tissue triacylglycerols.

The medium-chain saturated FAs, lauric (C12:0) myristic (14:0) and palmitic (16:0) acids, and some *trans* FAs (TFAs), are nutritionally undesirable because they adversely affect plasma cholesterol levels. They decrease the level of blood low density lipids – LDL – which are thought to be associated with an increased risk of coronary heart disease. Approximately 2.7% of the FAs in milk are TFAs with one or more *trans*-double bonds (*Precht and Molkentin,* 1995). TFAs are not synthesized by the human body and are not required in the human diet.

TFAs are produced during biohydrogenation of polyunsaturated FA (PUFA) and isomerization of monounsaturated FA (MUFA) in the rumen. The major TFA is vaccenic acid (VA) (18:1, 11t), although small amounts of several other TFAs, including those with *trans* double bonds in positions 4-16 are also observed in milk fat *(MacGibbon and Taylor,* 2006). TFAs have a significant impact on dairy economics, causing important losses to dairy producers (*Lock et al.,* 2005**)**. According to *Hulshof et al.* (1999), in many European countries, approximately 50% of TFAs in the human diet come from dairy fat and they have adverse effects on blood lipids and lipoproteins similar to those from industrial sources when consumed in equal amounts. The American Heart Association recommended in 2006 that <20% of total energy content should be consumed in the form of MUFA. Overall, it is important for human health to reduce them in milk fat (*Cicero et al.,* 2012; *Imamura et al.,* 2012; *Lefevre et al.,* 2012).

In contrast to saturated fats, polyunsaturated fats, particularly those in the n-3 series have been shown to be very beneficial to human health. UFAs detected in the milks in the current study with the highest percentages were oleic (C18:1cis-9), linoleic (C18:2n-6) and VA (18:1, 11t) (25.40, 2.68 and 2.40%, respectively; Table 1). The level of linoleic acid (C18:2n-6) in the milks studied varied significantly ($p<0.05$) in the range 1.54 to 2.68%. The significant differences in PUFA levels observed ($p<0.05$) were a direct result of differences in linoleic acid content between the groups of milks. This was probably due to differences in the feeding during the year. The percentages of SFA and UFA in the milks ranged from 69.87 to 73.83 % and 25.16 to 30.11%, respectively (Table 1).

In regard to the FA profile of commercially processed sterilized retail milks with different declared fat contents (3.2%, 2.8% or 1.5%), processing raw milk into sterilized commercial milk did not seem to result in any changes in the FA profiles of the milk. In all retail products, the percentage of SFAs was higher than the percentage of UFAs (Table 2). However, the FA content was reduced according to the fat content (Table 2). When FAs in sterilized milk are expressed as mg of FAs per 100 g (*Greenfield & Southgate*, 2003) (Table 2), the obtained data are more important for consumers and demonstrate the nutritional value of the milk consumed per specific milk serving.

Several national and international organizations and authorities have formulated Dietary Reference Values or recommendations for the intakes of total fat, FAs, and cholesterol. The WHO/FAO (2003) set population nutrient intake goals for SFA at <10% of energy intake (E%), and for n-6 PUFA at 5 to 8 E% with a total intake of PUFA of 6 to 10 E%. In a report on health risks and benefits of TFAs in food, the European Food Safety Authority states that daily consumption of total TFAs higher than 2 E% gives rise to a significant increase in the risk of cardiovascular disease. Therefore, they recommended this value as a consumption level that should not be exceeded (*EFSA*, 2010).

The FA composition of cow's milk is influenced by many factors, both internal (cattle breed, age, stage of lactation, etc.) (*White et al.,* 2001; *Kelsey et al.*, 2003; *Auldist et al*., 2004; *Kay et al*., 2005; *Soyeurt et al.*, 2006) and external (feeding systems,

Table 1 Fatty acid composition ($\bar{X}$±SD for % of total fatty acids) in 24 raw cow milks from four regions in northern Serbia

Fatty acids	South Backa	South Banat	Central Banat	West Backa and Syrmia
C4:0	2.97±0.06	2.71±0.19	2.71±0.06	2.66±0.08
C6:0	2.15±0.18	2.01±0.22	1.97±0.02	1.98±0.10
C8:0	1.35±0.19	1.28±0.20	1.18±0.01	1.24±0.08
C10:0	2.98±0.49	3.04±0.61	2.65±0.06	2.93±0.28
C12:0	3.35±0.54	3.53±0.73	3.08±0.11	3.45±0.43
Total	**12.8±0.29**	**12.57±0.39**	**11.59±0.05**	**12.26±0.19**
C14:0	11.34±1.49	12.58±1.18	13.47±0.61	12.95±0.82
C15:0	1.04±0.06	1.08±0.19	1.12±0.04	1.18±0.17
C16:0	33.83±2.52	34.10±1.72	36.17±2.44	36.90±2.17
C17:0	0.51±0.06	0.54±0.07	0.59±0.03	0.57±0.14
C18:0	11.40±0.61	10.25±1.88	9.78±1.41	9.84±1.32
C22:0	0.08±0.04	0.07±0.02	0.09±0.02	0.06±0.02
C24:0	0.08±0.02	0.06±0.02	0.08±0.03	0.06±0.01
Total	**58.28±0.68**	**58.68±0.72**	**61.3±0.65**	**61.56±0.66**
C16:1	1.52±0.53	1.45±0.12	1.56±0.13	1.47±0.19
C18:1trans-9	2.09±0.39	2.40±0.65	2.10±0.50	1.60±0.25
C18:1cis-9	25.40±1.10	23.77±2.20	22.77±1.64	21.97±2.36
Total	29.01±0.67	27.62±1.0	26.43±0.75	25.04±0.93
C18:2n-6	2.10±0.69^{ab}	2.68±0.34^{a}	1.54±0.36^{b}	1.77±0.21^{b}
C20:0+C18:3n-6	0.24±0.12	0.23±0.04	0.25±0.05	0.24±0.03
C18:3n-3	0.26±0.15	0.34±0.06	0.28±0.09	0.22±0.04
c9. t11CLA	0.51±0.03	0.62±0.09	0.70±0.19	0.46±0.06
C20:3n-6	0.32±0.03	0.28±0.09	0.23±0.06	0.26±0.06
Total	3.43±0.20	4.15±0.12	3.0±0.15	2.95±0.08
SFA	69.87±1.46	70.88±2.05	72.91±1.79	73.83±2.43
MUFA	26.92±1.25	25.20±2.15	24.33±1.51	23.44±2.24
PUFA	3.19±1.22^{ab}	3.92±0.42^{a}	2.75±0.37^{b}	2.72±0.25^{b}
Lipids %	3.66±1.41	3.82±0.98	2.60±0.41	3.36±0.87

Legend: $^{a, b, c}$ Means with different letters within the same row are significantly different (p≤0.05)

seasonal changes, milking frequency and milking system, etc.) (*Jensen,* 2002; *Kalac* and *Samková*, 2010; *Morales-Almaráz et al.*, 2010). Another important factor is geographical, which determines the edible plant variety underlying the feeding of ruminants (*Frelich et al.*, 2009; *Rutkowska and Adamska,* 2011).

It is well established that the FA composition of milk can be modified by manipulating the cow's diet. However, the lipid profile in bovine milk does not exactly match the lipid profile of the cow's diet because of the biohydrogenation of most of the dietary PUFA (*Jenkins et al.,* 2007). Ruminants consume a large proportion of UFAs. These UFAs are,

Table 2 Fatty acid composition of 15 commercially processed sterilized milks from retail with different declared fat contents (3.2%, 2.8% or 1.5%) ($\overline{X}$±SD for % of total fatty acids and; mg 10^{-2} g)

Fatty acids	milk 3.2% fat		milk 2.8% fat		milk 1.5% fat	
	(%)	(mg 10^{-2} g)	(%)	(mg 10^{-2} g)	(%)	(mg 10^{-2} g)
C4:0	2.62±0.03	79.23^{x}	2.68±0.11	71.04^{y}	2.66±0.07	37.70^{z}
C6:0	1.85±0.01	56.09^{x}	1.94±0.07	51.46^{x}	1.97±0.06	27.93^{y}
C8:0	1.16±0.02	35.08^{x}	1.20±0.04	31.75^{y}	1.20±0.03	17.08^{z}
C10:0	2.80±0.02	84.82^{x}	2.87±0.06	76.07^{y}	2.85±0.06	40.40^{z}
C12:0	3.34±0.02	101.15^{x}	3.37±0.02	89.30^{y}	3.35±0.03	47.56^{z}
Total	**11.77±0.02**	**356.37**	**12.06±0.06**	**319.62**	**12.03±0.05**	**170.67**
C14:0	$13.78±0.05^{a}$	416.86^{x}	$13.36±0.16^{ab}$	353.64^{y}	$13.00±0.07^{b}$	184.27^{z}
C15:0	$1.10±0.01^{a}$	33.26^{x}	$1.04±0.01^{b}$	27.65^{y}	$1.06±0.01^{b}$	15.02^{z}
C16:0	36.78±0.82	1112.23^{x}	35.82±0.19	947.80^{y}	34.91±0.07	494.85^{z}
C16:1	1.44±0.01	43.55^{x}	1.38±0.02	36.65^{y}	1.40±0.01	19.91^{z}
C17:0	$0.57±0.01^{a}$	17.24^{x}	$0.53±0.01^{b}$	14.02^{y}	$0.53±0.01^{b}$	7.58^{z}
C18:0	10.14±0.19	306.78^{x}	10.27±0.16	271.88^{y}	10.57±0.01	149.83^{z}
C22:0	$0.07±0.01^{ab}$	2.27^{x}	$0.06±0.01^{b}$	1.45^{y}	$0.09±0.01^{a}$	1.35^{y}
C24:0	nd	1.06^{x}	nd	0.93^{x}	nd	nd
Total	**63.88±0.84**	**1933.25**	**192.46±0.08**	**1654.13**	**61.56±0.03**	**872.81**
C18:1trans-9	$2.07±0.02^{b}$	62.75^{x}	$2.09±0.03^{b}$	55.30^{y}	$2.44±0.03^{a}$	34.66^{z}
C18:1cis-9	22.61±0.53	683.88^{x}	22.41±0.15	593.10^{y}	23.15±0.38	328.15^{z}
Total	**24.68±0.27**	**746.63**	**24.5±0.09**	**648.40**	**25.60±0.20**	**362.81**
C18:2n-6	$2.04±0.05^{b}$	61.69^{x}	$2.09±0.04^{b}$	55.30^{y}	$2.30±0.01^{a}$	32.67^{z}
C20:0+C18:3n-6	0.22±0.02	6.65^{x}	0.22±0.01	5.95^{x}	0.20±0.01	2.83^{y}
C18:3n-3	0.30±0.02	9.22^{x}	0.31±0.01	8.20^{y}	0.33±0.01	4.75^{z}
c9. t11CLA	0.54±0.06	16.48^{x}	0.52±0.02	13.76^{x}	0.51±0.06	7.30^{b}
C20:3n-6	0.08±0.01	2.42^{x}	0.07±0.01	1.85^{x}	0.08±0.01	1.20^{x}
Total	**3.18±0.03**	**96.46**	**3.21±0.02**	**85.06**	**3.42±0.02**	**48.75**
SFA	72.98±0.71	2206.92^{x}	73.18±0.27	1936.34^{y}	72.20±0.44	1023.51^{z}
MUFA	24.05±0.54	727.42^{x}	23.79±0.16	629.62^{y}	24.55±0.36	348.07^{z}
PUFA	3.18±0.06	89.66^{x}	3.21±0.09	79.91^{x}	3.24±0.08	46.00^{y}

Legend: $^{a, b, c}$ Means with different letters within the same row are significantly different ($p≤0.05$); $^{x, y, z}$ Means with different let ters within the same row are significantly different ($p≤0.01$); nd Not detected

to a large part, biohydrogenated by the rumen bacteria to SFAs (long-chain), and during this process, several intermediates, mainly TFAs, are formed (*Jenkins et al.,* 2008). Therefore, milk contains mostly SFAs. A small proportion of the feed UFAs and a small proportion of the biohydrogenation intermediates are not completely biohydrogenated to SFA (C18:0) and are secreted into milk. Under the farming conditions practiced in Serbia, the nutrition of cows is based mainly on corn silage and hay supplemented with grains. Those fodders contain much more SFA than do green fodder materials, and so their consumption results in correspondingly high contents of long-chain SFAs in milk.

Increasing the overall total PUFAs in the diet is considered beneficial to human health with respect to reducing cardiovascular disease; additionally, cancer research has mainly focused on the benefits of increasing these FAs as well. However, the high level of unsaturation increases lipid oxidation that could affect the quality of dairy products due to the development of off-flavors (*Hu and Willett*, 2002).

Conclusion

Variability in milk composition is a result of genetic, physiological, nutritional, and environmental factors. This study showed that the major FAs in Serbian cow's milk were palmitic (C16:0), oleic (C18:1cis-9), myristic (C14:0), and stearic (C18:0) acids. These results will help to explain to farmers and dairy product manufacturers the importance of raw milk quality factors that fluctuate depending on dairy cow feeding practices and dairy plant management. In the future, this study needs to be repeated on a larger scale in order to achieve statistical analysis relevant to the bovine dairy herds over the entire country, and to enable the factors (feeding regime, breeding etc.) that could affect the FA composition of milk fat to be further studied. Furthermore, detailed work is essential to understand the regional and seasonal effects on milk fat composition.

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Common abbreviations:

- CFU colony forming units, capitalised, common and so is never spelled out
- kg kilogram, common and so is never spelled out
- L litre, common and so is never spelled out
- Longissimus dorsi (LD) is redundant and so is not used. For the whole muscle, use Longissimus thoracis et lumborum (LTL). The correct terms for the two parts of this muscle are Longissimus thoracis (LT) or Longissimus lumborum (LL).
- mL millilitre, common and so is never spelled out
- µm micrometre, common and so is never spelled out
- mol mole, common and so is never spelled out
- M molar, common and so is never spelled out
- PCR polymerase chain reaction, common and so is never spelled out
- SD standard deviation, capitalised, common and so is never spelled out
- SE standard error, capitalised, common and so is never spelled out
- sp. species (singular), common and so is never spelled out (not capitalised, full-stop)
- spp. species (plural), common and so is never spelled out (not capitalised, full-stop)
- UV ultraviolet, capitalised, common and so is never spelled out
- aw water activity
- h hour(s)
- min minute(s)
- 25°C (no gap after the numeral)
- 20±1°C (no gaps between numbers, sign and unit in-text and in tables/figures)
- p<0.05, p<0.01 (not italicised, not capitalised, no gaps)
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- found in 20.05% of cats…(no gap in-text)

Conclusion This section provides a review of the most important facts obtained during the research.

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