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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.

Key words: *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 (*Marcinčak*, 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 (*Janjić 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}±0.19	6.5^{B}±0.29	6.93^{A}±0.19	6.5^{B}±0.29	6.93^{A}±0.19	6.57^{AB}±0.34
Odor and flavor	5.71^{B}±0.27	5.14^{B}±0.24	6.93^{A}±0.19	5.64^{B}±0.56	5.5^{B}±0.41	6.36^{A}±0.38

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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References

Anon. (2000). Interim guidelines for the control of verotoxinogenic *Escherichia coli* including *E. coli* O157:H7 in ready to eat fermented sausages containing beef or a beef product as an ingredient. Guideline no. 12. Issued by Food Directorate. Health Protection Branch, Health Canada.

Anon. (2001). Performance standards for production of processed meat and poultry products; proposed rule. U.S. Department of Agriculture, Food Safety and Inspection Service.

Bösinger, S., Luf, W. & Brandl, E. (1993). Oxysterols: their occurrence and biological effects. *International Dairy Journal 3*, 1–33.

Broncano, J. M., Petrón, M. J., Parra, V., & Timón, M. L. (2009). Effect of different cooking methods on lipid oxidation and formation of free cholesterol oxidation products (COPs) in Latissimus dorsi muscle of Iberian pigs. *Meat Science, 83,* 431–437.

Cassens, R. G. (1997). Composition and safety of cured meats in the USA. *Food Chemistry 59*, *(4)*, 561–566.

Chien, J. T., Wang, H. C. & Chen, B. H. (1998). Kinetic model of the cholesterol oxidation during heating. *Journal of Agricultural and Food Chemistry, 46(7)*, 2572–2577.

Demeyer, D. (2007). Composition and Nutrition. In F. Toldra (Ed.), *Handbook of fermented meat and poultry* (pp. 243–256). New York: Springer.

Derewiaka, D. & Obiedzinski, M. (2010). Cholesterol oxides content in selected animal products determined by GC-MS. *European Journal of Lipid Science and Technology 112*, 1130–1137.

Ducic, M., Klisara, N., Markov, S., Blagojevic, B., Vidakovic, A. & Buncic, S. (2016). The fate and pasteurization-based inactivation of *Escherichia coli* O157, *Salmonella* Typhimurium and *Listeria monocytogenes* in dry, fermented sausages. *Food Control, 59*, 400–406.

European Union. (2011). Commission regulation (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives. *Official Journal of the European Union*, L 295, 1–2.

Georgiou, C. & Kapnissi-Christodoulou, C. (2012). Qualitative and quantitative determination of COPs in Cypriot meat samples using HPLC determination of the most effective sample preparation procedure. *Journal of Chromatographic Science*, *51*, 286–291.

Hur, S. J., Park, G.B. & Joo, S.T. (2007). Formation of cholesterol oxidation products (COPs) in animal products. *Food Control* 18, 939–47.

ISO 2918 (1975). Meat and meat products – Determination of nitrite content (Reference method), International Organization for Standardization.

ISO 4121:2003 (2003). Sensory analysis – Guidelines for the use of quantitative response scales. International Organization for Standardization.

Janjić, J., Katić, V., Ivanović, J., Bošković, M., Starčević, M., Glamočlija, N. & Baltić, M. (2016). Temperatures, cleanliness and food storage practices in domestic refrigerators in Serbia, Belgrade. *International Journal of Consumer Studies*, *40*, 276–282.

Kerry, J. P., Gilroy, D. A. & O'Brien, N. M. (2002). Formation and content of cholesterol oxidation products in meat and meat products. In Guardiola, F., Dutta, P.C., Codony, R. & Savage, G.P. (Eds.) *Cholesterol and phytosterol oxidation products: analysis, occurrence, and biological effects* (pp. 162–185) Champaign, IL, AOCS Press.

Kim, S. K., & Nawar, W. W. (1993). Parameters influencing cholesterol oxidation. *Lipids, 28(10)*, 917–922.

Lercker, G., & Rodriguez-Estrada, M. T. (2000) Cholesterol oxidation: presence of 7-ketocholesterol in different food products. *Journal of Food Composition and Analysis*, *13*, 625–631.

Marco, A., Navarro, J. L., & Flores, M. (2006). The influence of nitrite and nitrate on microbial, chemical and sensory parameters of slow dry fermented sausage. *Meat Science*, *73*, 660–673.

Marcinčak, S. (2016) Lipid oxidation of fermented meat products. In Zdolec, N. (Ed.) *Fermented Meat Products: Health Aspects* (pp. 488–511). Boca Raton, USA: CRC Press.

Morrissey, P.A. & Kerry, J.P. (2004). Lipid oxidation and the shelf-life of muscle foods. In Steele, R. (Ed.), *Understanding and measuring the shelf-life of food* (pp. 357–395). Cambridge, UK: CRC Press.

Muguerza, E., Ansorena, D., & Astiasaran, I. (2004). Functional dry fermented sausages manufactured with high levels of n-3 fatty acids: Nutritional benefits and evaluation of oxidation. *Journal of the Science of Food and Agriculture*, *84*, 1061–1068.

Nam, K. C., Du, M., Jo, C., & Ahn, D. U. (2001). Cholesterol oxidation products in irradiated raw meat with different packaging and storage time. *Meat Science*, 58, 431–435.

Reig, M. & Toldra, F. (2010). Detection of Chemical Hazards. In Toldra, F. (Ed.) *Handbook of Meat Processing* (pp. 469–480). Ames, USA: Blackwell Publishing.

Rodriguez-Estrada, M., Garcia-Llatas, G., & Lagarda, M. (2014). 7-Ketocholesterol as marker of cholesterol oxidation in model and food systems: When and how. *Biochemical and Biophysical Research Communications*, *446*, 792–797.

Ruiz, J. (2007). Ingredients. In Toldra, F. (Ed.) *Handbook of Fermented Meat and Poultry* (pp. 59–76). Ames, USA: Blackwell Publishing.

Serbia. (2013). Regulation on food additives. *Official Gazette of the Republic of Serbia,* 63.

Sindelar, J. J, & Milkowski, A. L. (2011). Sodium nitrite in processed meat and poultry meats: A review of curing and examining the risk/benefit of its use. *American Meat Science Association*. AMSA white paper series, No. 3.

Talon, R., Leroy, S., Lebert, I., Giammarinaro, P., Chacornac, J.P., Latorre-Moratalla, M., Vidal-Carou, C., Zanardi, E., Conter, M. & Lebecque, A. (2008). Safety improvement and preservation of typical sensory qualities of traditional dry fermented sausages using autochthonous starter cultures. *International Journal of Food Microbiology 126*, 227–234.

Ubhayasekera, S. J. K. A., Verleyen, T. & Dutta, P. C. (2004). Evaluation of GC and GC-MS methods for the analysis of cholesterol oxidation products. *Food Chemistry 84*, 149–157.

Ventanas, S., Estévez, M., Tejeda, J. F. & Ruiz, J. (2006). Protein and lipid oxidation in Longissimus dorsi and dry cured loin from Iberian pigs as affected by crossbreeding and diet. *Meat Science*, *72*, 647–655.

Vicente, S. & Torres, E. (2007). Formation of four cholesterol oxidation products and loss of free lipids, cholesterol and water in beef hamburgers as a function of thermal processing. *Food Control 18*, 63–68.

Wasowicz, E., Gramza, A., Hes, M., Jelen, H., Korczak, J., Malecka, M., Mildner-Skzudlar, S., Rudzinska, M., Samotyja, U & Zawirska-Wojtasiak, R. (2004). Oxidation of lipids in food. *Polish Journal of Food and Nutrition Sciences*, *13(54)*, 87–100.

Zanardi, E., Ghidini, S., Battaglia, A. & Chizzolini, R. (2004). Lipolysis and lipid oxidation in fermented sausages depending on different processing conditions and different antioxidants. *Meat Science*, *66*, 415–423.

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