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GPOL#1853183, VOL 0, ISS 0

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https://doi.org/10.1080/10406638.2020.1853183

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Color Characteristics and Content of Polycyclic Aromatic Hydrocarbons of Traditional Dry Fermented Sausages Throughout Processing in Controlled Conditions

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ABSTRACT

Petrovská klobása is traditional dry fermented sausage with intense red color, what differentiate it from other products of the same type. Very important factor in traditional sausage production is smoking, providing unique surface color of *Petrovská klobása*. On the other hand, smoking produces certain types of potentially carcinogenic chemicals, such as polycyclic aromatic hydrocarbons (PAH). Having in mind that assignment of the science is to help in transition of production from small household conditions to industrial ones, primarily with shortening fermentation and ripening time, and keeping the standard quality of the product the main aims of this work were to investigate the effects of autochthonous starter culture (*Staphylococcus xylosus*) addition on color formation of *Petrovská klobása*; and to determine the content of PAH in *Petrovská klobása* smoked in industrial conditions. Instrumental color characteristics (CIE $L^*a^*b^*$ system), contents of 13 US-EPA PAH (from Environmental Protection Agency list), pH values and moisture contents were determined during the processes of smoking, drying and ripening (throughout 60 days of production). Sensory evaluation of color was performed additionally, at the end of drying period (day 60 of production). Dry fermented sausages produced with addition of autochthonous starter culture had significantly ($P < 0.05$) higher value of redness-a^* (27.94) and better sensory score ($P < 0.05$) for color (4.3) comparing to control group of sausages (25.23; 3.9, respectively) at the end of drying period. Results of this study demonstrated that addition of *S. xylosus* had positive effect on color of the *Petrovská klobása*. Contents of benzo[a]pyrene and PAH4 were below the limits of detection in all analyzed samples of *Petrovská klobása* smoked in industrial conditions (2 days per 5 hour). These results indicated the safety of sausages produced in this manner, from the PAH point of view.

RESEARCH HIGHLIGHTS

- Effect of autochthonous starter culture on color of dry fermented sausages was analyzed
- *Staphylococcus xylosus* had positive effect to color of the *Petrovská klobása*
- BaP and PAH4 values were below the limit of detection in samples of *Petrovská klobása*
- Sausages smoked in industrial conditions (2 day per 5 hour) were safety from the PAH point of view

ARTICLE HISTORY
Received 13 January 2020
Accepted 15 November 2020

KEYWORDS
Petrovská klobása; *Staphylococcus xylosus*; CIE $L^*a^*b^*$ color parameters; PAH; industrial smoking conditions

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Introduction

Petrovská klobása is traditional dry fermented sausage, with protected designation of origin at national level, from the north of Serbia. It is produced according to original recipe of the ancestors, in traditional manner, without added starter culture or additives. The required microflora originates from the meat itself or from the environment, and constitute a part of the so-called "house-flora." Sausages are produced in small household enterprises only during winter, when the atmospheric temperatures are low (around 0 °C or lower), undergoing prolonged processes of drying and ripening.[1–3] Assignment of the science is to help in transition from small household production conditions of traditional products (such as *Petrovská klobása*) to industrial ones. The main tasks are shortening fermentation and ripening time, and keeping the standard quality of the product.[1]

The quality of meat and meat products consumers often evaluate based on color, that is why both the color formation and stability are important indicators of dry fermented sausage quality.[4–6] One of the essential features of *Petrovská klobása* is its intense red color, which differentiate it from other products of the same type.[7]

Some Staphylococcus species have nitrate-reductases, proteolytic and lipolytic activities, all of which could contribute to the red color of meat products.[6,8–11] However, the activity of starter cultures in dry fermented sausages may be influenced by the used ingredients (origin of the meat and fat, salt, fermentable sugars, spices, etc.) and/or processing (pre-treatment of the meat, mincing, mixing, stuffing and drying) conditions.[12] Thus, starter cultures cannot be equally effective for during/ripening of different types of fermented sausages. For this reason, it is good to confirm justification for the use of specified starter culture in manufacturing of particular dry fermented sausage.

Another very important factor in color formation (surface) is smoking process. It is known that smoking provides unique sensory characteristics (color and aroma) of *Petrovská klobása* and reduces growth of microorganisms.[7,13–15] On the other hand, in the sausages production smoking produces certain types of potentially carcinogenic chemicals, such as polycyclic aromatic hydrocarbons (PAH).[15–19]

Having in mind the previously mentioned assignment of the science to help in transition from traditional production conditions to industrial ones, to shorten the fermentation and ripening time, and to keeping the standard quality and safety of the final product the main aims of this work were to:

- investigate the effect of autochthonous starter culture (*S. xylosus*) addition on color formation of *Petrovská klobása*;
- determine the content of 13 US-EPA PAH (from European Protection Agency list) in *Petrovská klobása* smoked in industrial conditions.

Materials and methods

Sausage preparation

Raw sausage mixture (lean pork meat, pork fat, home-made red-hot paprika powder, salt, sugar, crushed garlic, and caraway) was made according to recipe described by Škaljac et al.[2] Half of the obtained raw sausage mixture was inoculated with 0,015% of autochthonous starter culture which contained *S. xylosus* (S group). *S. xylosus* was previously isolated from *Petrovská klobása* produced in a traditional manner[1] and identified by 16S rRNA sequencing. Other half of obtained raw sausage mixture was assigned as control (C group). Both groups of sausages were stuffed in collagen casings (55 mm in diameter) and after one day of resting they were subjected to the smoking process in industrial conditional. Smoke was produced by smoke generator using sawdust from

beech wood. The smoke was transported to the smoking chamber through the pipes (indirect smoking). The full smoking process lasted for 2 days. Each day the sausages were smoked for 5 h. Relative humidity during smoking process was from RH_{max}=91.6% to RH_{min}=85.1%, while temperature was in the range from t_{min}=13.6 °C to t_{max}=19.9 °C. After the smoking process, sausages underwent drying/ripening process in industrial ripening room (relative humidity was from RH_{min}=70.3% to RH_{max}=86.5% while temperature was from t_{min}=12.1 °C to t_{max}=15.7 °C) for 2 months.

Instrumentally obtained color characteristic, pH value and moisture content were determined during the processes of smoking, drying and ripening, on day 0 and on days 6, 12, 30, and 60 of production. Additionally, at the end of drying period (day 60 of production) sensory evaluation of color was performed. All determinations were made in three samples from each batch (C and S group) in duplicate ($n = 3 \times 2$).

The contents of 13 US-EPA PAH were determined at the beginning of production (day 0), at the end of smoking period (day 6 of production) and at the end of drying period (day 60 of production) in control group of sausages (C group).

Instrumental measurement of color characteristics

Color measurements were performed by the method described by Škaljac et al.[7] using the MINOLTA Chroma Meter (Model CR-400, Konica Minolta Inc., Osaka, Japan), with aperture of 8 mm in the measuring head and standard observer angle of 2°. Analysis of color was performed on the fresh cut of sausages by adequative instrumental method using CIE $L^*a^*b^*$ system detection (lightness-L^*; redness-a^*; yellowness-b^*; hue angle-h and chroma-C^*). Hue angle (h) and chroma (C^*) were calculated by Equations (1) and (2). Presented data were obtained from six different cross-section areas (thickness 2 cm), where only color of lean meat was measured, avoiding fat parts.

$$h = \tan^{-1}\left(\frac{b^*}{a^*}\right) \tag{1}$$

$$C^* = \sqrt{a^{*2} + b^{*2}} \tag{2}$$

pH value and moisture content determination

pH value was determined using the portable pH meter (Testo 205, Testo AG, Lenzkirsch, Germany) equipped with combination electrode with temperature probe.[20] Moisture content (g/100g) was obtained using the method recommended by the International Organization for Standardization.[21]

PAH determination

The content of 13 US-EPA PAH (Phe—phenanthrene, Fln—fluorine, Acy—acenaphthylene, Ant - anthracene, Pyr—pyrene, CHR—chrysene, BaA—benz[a]anthracene, BbF—benzo[b]fluoranthene, BaP—benzo[a]pyrene, BkF—benzo[k]flouranthene, BgP—benzo [ghi]perylene, IcP—indeno[1,2,3-cd]pyrene and DhA—dibenz[a,h]anthracene) was determined by the method described by Škaljac et al.[2] on a gas chromatograph with mass spectrometer detector (GC-MS, Agilent, USA). Detection limits (LOD) and recovery method meet the criteria set by the Regulation European Commission.[22]

Sensory analysis of color characteristics

Sensory analysis of color was performed by 8 trained panelists with previous experience in evaluation of dry fermented sausages. A training session for the panelists was performed prior to evaluation with the aim to ensure consistency and accuracy of sausage color characteristics evaluation between the panelists. Color scoring was according to quantitative descriptive analysis, using a scale from 0 (atypical color) to 5 (optimal color), with a sensitivity of 0.25 points. Score distribution was thoroughly discussed until a consensus among panel members was reached. Sausage samples were sliced into 4 mm thick slices and served on white paper plates; each sample was identified by a three-digit number. Also, surface color of sausages in casings was sensory evaluated. The sensory evaluation of color was performed in individual booths under white fluorescent lighting at room temperature.

Statistical analysis

Statistical analysis was carried out using analytic software package (STATISTICA 12.0). All determined parameters data were presented as mean value ± standard deviation (SD). The variance analysis (ANOVA) and Duncan's multiple range test were used to test the differences. A criterion of $P < 0.05$ was considered to indicate statistical significance of 95%.

Results and discussion

Color analysis

The color development of *Petrovská klobása* was determined by color characteristics: lightness-L^*; redness-a^*; yellowness-b^*; hue angle-h and chroma-C^* (Table 1). During processes of drying and ripening values of color characteristic were decreasing. Both groups of examined sausages (C and S) had significantly lower ($P < 0.05$) values of color characteristics at the end of drying (C group L^*=39.32; a^*=25.23; b^*=25.82; h = 45.55; C^*=36.12; S group L^*=45.62; a^*=39.28; b^*=27.94; h = 27.04; C^*=44.05) compared to sausages at day 0 of production (C group L^*=44.67; a^*=32.15; b^*=37.00; h = 48.85; C^*=49.10; S group L^*=45.62; a^*=33.57; b^*=38.05; h = 48.58; C^*=50.76). The average pH values of the raw sausages were 5.41 (C group) and 5.51 (S group) and they decreased slightly during drying/ripening, up to 5.16 (C group) and 5.15 (S group). The moisture content decreased from 58.87% (C group) and 59.05% (S group) up to 34.30% (C group) and 34.44% (S group) (Table 2). Decrease of water content and pH value during ripening period influences on formation of darker color in fermented sausages. Results of our study were in agreement with literature data.[23,24] Color formed on the surface of *Petrovská klobása* at the end of drying period was darker red compared with day 0 of production. The L^* values of *Petrovská klobása* were at the same level as for some other dry fermented traditional sausages from Argentina,[25] Croatia,[26] Italia,[27] and Spain,[28] but lower than for traditional dry fermented sausages from the China[8,24] and Portugal.[29] Furthermore, the values of a^* and b^* color parameters were higher than for other traditional dry fermented sausages from Italia, Portugal, Croatia, Spain, China, and Argetina.[8,24–29] According to the literature data, domestic red-hot pepper powder with intensive red color had essential influence on the formation of color characteristics of sausages.[4,30–32] In addition, nitrates from paprika have an important role in the development of color in sausages produced according traditional recipe without added additives. Colavita et al.[33] examined content of nitrates in samples of commercial and traditional chili paprika powder and they determined contents in the range from 325 mg/kg to 531 mg/kg. Nitrates in sausage samples (added by paprika) are reduced to nitrites by nitrate reductase activity of microflora (e.g., Staphylococcus strains; Micrococcus …). Then nitrites are converted by chemical reactions to NO, which is able to bind with Fe^{2+} in hem stable typical red nitrosomyoglobin (MbFeNO)

Table 1. Instrumental (CIE $L^*a^*b^*$ system) and sensory color characteristics of dry fermented sausages (*Petrovská klobása*) produced in controlled conditions.

Characteristics of color	Drying/ripening period									
	Day 0		Day 6		Day 12		Day 30		Day 60	
	C	S	C	S	C	S	C	S	C	S
Lightness (L^*)	$44.67^{a,b}$ ±3.30	45.62^{a} ± 2.25	41.47^{cd} ±1.77	$44.71^{a,b}$ ±3.87	$44.33^{a,b,d}$ ±3.23	45.36^{a} ±2.02	$42.79^{a,b,d}$ ±3.32	$42.13^{b,c,d}$ ±1.70	39.32^{c} ±2.20	39.28^{c} ±2.86
Redness (a^*)	$32.15^{c,d}$ ±1.03	33.57^{d} ±2.62	$28.78^{a,b}$ ±1.36	30.82^{c} ±1.09	$30.26^{b,c}$ ±1.79	$32.13^{c,d}$ ±0.71	$28.62^{a,b}$ ±2.58	$28.40^{a,b}$ ±2.29	25.23^{e} ±2.34	27.94^{a} ±1.22
Yellowness (b^*)	$37.00^{c,d}$ ±4.27	38.05^{d} ±2.86	$27.32^{a,b}$ ±2.52	$34.43^{c,d}$ ±3.69	$33.42^{c,e}$ ±5.29	$34.55^{c,d}$ ±2.83	$30.20^{b,e}$ ± 3.68	$28.78^{a,b}$ ±4.70	25.82^{a} ± 3.32	$27.04^{a,b}$ ± 1.52
Hue angle (h)	48.85^{b} ± 3.66	48.58^{b} ±1.49	43.44^{d} ± 2.74	$48.03^{a,b}$ ± 2.95	$47.54^{a,b}$ ±3.70	$47.00^{a,b,c}$ ±2.28	$46.43^{a,b,c}$ ±2.91	$45.13^{a,c,d}$ ±2.81	$45.55^{a,c,d}$ ±2.22	$44.05^{c,d}$±1.34
Chroma (C^*)	$49.10^{d,e}$ ±2.97	50.76^{e} ± 3.65	39.72^{b} ± 2.15	$46.27^{c,d}$ ±2.98	45.16^{c} ± 4.76	$47.22^{c,d,e}$ ±2.03	41.66^{b} ± 3.99	40.48^{b} ± 4.83	36.12^{a} ± 3.81	$38.89^{a,b}$ ± 1.76
Sensory evaluation of color									3.94^{a} ± 0.18	4.31^{b} ± 0.26
Sensory evaluation of color surface									4.97^{ns} ±0.09	4.97^{ns} ±0.09

[a–e]The values in the same row with different letters in the superscript are significantly different (95%).
Results are expressed as mean ± standard deviation (n = 6).
C—sausages produced without autochthonous starter culture.
S—sausages produced with autochthonous starter cultur.

Table 2. pH and moisture content during the drying/ripening process of *Petrovská klobása* produced in controlled conditions.

	Drying/ripening period									
	Day 0		Day 6		Day 12		Day 30		Day 60	
Parameters	C	S	C	S	C	S	C	S	C	S
pH value	$5.41^{f} \pm 0.01$	$5.51^{g} \pm 0.01$	$5.38^{e} \pm 0.01$	$5.41^{f} \pm 0.01$	$5.22^{d} \pm 0.03$	$5.23^{d} \pm 0.04$	$5.06^{a} \pm 0.01$	$5.12^{b} \pm 0.01$	$5.16^{c} \pm 0.02$	$5.15^{c} \pm 0.01$
Moisture content (%)	$58.87^{g} \pm 0.26$	$59.05^{g} \pm 0.25$	$56.18^{f} \pm 0.03$	$53.39^{e} \pm 0.42$	$52.31^{d} \pm 0.34$	$52.71^{d} \pm 0.25$	$42.50^{b} \pm 0.05$	$44.91^{c} \pm 0.77$	$34.30^{a} \pm 0.35$	$34.44^{a} \pm 0.09$

$^{a-g}$The values in the same row with different letters in the superscript are significantly different (95%).
Results are expressed as mean ± standard deviation (n = 6).
C—sausages produced without autochthonous starter culture.
S—sausages produced with autochthonous starter culture.

pigment of sausages.[6,9] Sánchez and Leroy[34] in their study reported that nitrate reductase activity and efficiency nitrosation of myoglobin in dry fermented sausages were dependent on many parameters: temperature during fermented process, pH values, type of salt, redox potential of meat, pigment concentration and water content. Because of that, conditions during production of *Petrovská klobása* affected on nitrate reductase activity and that was important factor for ensuring red color formation. Bosse et. al.,[6] Chen et al.[24] and Gøtterup et al.[5] founded that nitrate reductase activity of microflora was key point in the process of redness and color formation of meat products. On the contrary, Bedia et al.[12] in semi ripening Salami did not determine positive effect of nitrate reductase from Staphylococci species on redness of sausages. This result can be attributed to the low pH of sausages (pH $<$ 5.00) which affected to the nitrate reductase activity. Results of our study showed that, sausages inoculated with *S. xylosus* had slightly higher values of color characteristics (S group L^*=44.71; a^*=30.82; b^*=34.43; h = 48.03; C^*=46.27) compared with control group of sausages (C group L^*=41.47; a^*=28.78; b^*=27.32; h = 43.44; C^*=39.72) on the day 6 of production. Also, at the end of drying period sausages inoculated with *S. xylosus* had slightly higher a^* values and sensory score for color (S-27.94; 4.31 respectively) than control group of sausages (C-25.23; 3.94 respectively). According to the literature data,[35] the use of starter cultures (*S. carnosus and S. xylosus*) may reduce the need for chemical additives, such as nitrites and nitrates. Furthermore, the lower residual levels of nitrates and nitrites detected in fermented meat products inoculated with starter cultures are due to the ability of starters to metabolize those compounds. It is already known that alternative strategies for color formation in meat product without using nitrate/nitrite salts are NO formation across nitric oxide synthase (NOS). NOS is active enzyme in *S. xylosus* which catalysis the hydroxylation of L-arginine to NO and L-citrulline. Further, NO interacts with myoglobin and builds form of nitrosomyoglobin.[5,10,11] To the best of our knowledge, there are not studies about contribution of NOS from *S. xylosus* to the redness of dry fermented sausages. Results of our examination confirmed that addition of *S. xylosus* had positive effect on redness and formation of optimal color of *Petrovská klobása.* Therefore, selection of starter cultures based on NOS and nitrate reductase activity is an important factor which ensures good color development in fermented dry sausages.

PAH analysis

In the second part of this research the content of 13 US-EPA PAH in dry fermented sausages (*Petrovská klobása*) smoked in industrial conditions (Table 3) was determined. PAHs are very important compounds for safety of smoked meat products. Total content of 13 US-EPA PAH in raw sausages was 22.01 μg/kg. PAH compounds determined in samples on day 0 of production were Phe (13.3 μg/kg), Fln (2.1 μg/kg), Ant (4.9 μg/kg) and Pyr 1.7 (2.1 μg/kg), and they origin from spices and meat used for production of sausages. At the end of smoking period contents of PAH compounds (Phe-16.0; Fln-5.3 μg/kg; Acy-3.6 μg/kg; μg/kg; Ant-12.9 μg/kg and Pyr-3.0 μg/kg) in *Petrovská klobása* were significantly ($P < 0.05$) higher compared with contents of PAHs at the begin of production process (day 0). Smoking in industrial conditions influenced on increase of total content of 13 US-EPA PAH and in this study the total content of 13 US-EPA PAH was 40.8 μg/kg. Many parameters including smoking temperature and time, type of wood, method of smoking, etc. affect the content of PAH compounds during smoking.[15,17,19,36] Contents of PAH in smoked fermented sausages decrease during drying period because of decomposition influenced by light, whereas increase in PAH contents is caused by migration of soot particles from the surface to the center of sausage and because of the reduction of moisture content.[14,37] Consequently, in this study the PAH content in sausage samples at the end of drying period—day 60 of production (moisture content $<$ 35%) was determined. At the end of drying period of *Petrovská klobása* the determined contents of PAH compounds Acy (3.6 μg/kg), Fln (5.3 μg/kg) Ant (16.0 μg/kg), Phe (12.9 μg/kg) and Pyr (3.0 μg/kg) were significantly ($P < 0.05$) higher

Table 3. Content of polycyclic aromatic hydrocarbons in dry fermented sausage (*Petrovská klobása*) smoked in industrial conditions.

Polycyclic aromatic hydrocarbons (μg/kg)		day 0 of production	End of smoking process	End of drying process
Phenanthrene	Phe	13.3[a]± 0.20	16.0[b]± 1.12	21.2[c] ± 1.98
Fluorene	Fln	2.1[a] ± 0.14	5.3[b]± 0.42	6.8[c]± 0.85
Acenaphthylen	Acy	< 0.3	3.6[a]± 0.29	13.6[b] ± 1.34
Anthracene	Ant	4.9[a]± 0.42	12.9[b]± 1.27	12.8[b]± 1.06
Pyrene	Pyr	1.7[a]± 0.07	3.0[b]± 0.21	4.0[c] ± 0.49
Chrysene	CHR	< 0.4	< 0.4	< 0.4
Benz[a]anthracene	BaA	< 0.3	< 0.3	< 0.3
Benzo[*b*]fluoranthene	BbF	< 0.3	< 0.3	< 0.3
Benzo[a]pyrene	BaP	< 0.3	< 0.3	< 0.3
Benzo[k]fluoranthene	BkF	< 0.3	< 0.3	< 0.3
Benzo[ghi]perylene	BgP	< 0.6	< 0.6	< 0.6
Indeno[1,2,3-cd]pyrene	IcP	< 0.4	< 0.4	< 0.4
Dibenz[a,h]anthracene	DhA	< 0.5	< 0.5	< 0.5
Σ 13 US-EPA PAHs		22.01[a]± 0.83	40.8[b] ± 1.07	58.3[c]± 1.77

[a–c]The values in the same row with different letters in the superscript are significantly different (95%).
Results are expressed as mean ± standard deviation (n = 6).

compared to the PAH compounds at the end of smoking period. Results of this study shown that at the end of drying process sausages smoked 2 days per 5 h (totally 10 h) had 58.3 μg/kg total content of 13 US-EPA. Škaljac et al.[2] determined that *Petrovská klobása* with collagen casing indirectly smoked 1 day per 6 h had 31.3 μg/kg total content of 13 US-EPA. Mastanjević et al.[19] reported higher values for the 13 EPA PAH (95.69 μg/kg) for Croatian dry fermented sausages with collagen casing which were indirectly smoked 4 days per 3 h (12 h). Croatian and Serbian sausages had the same type of casing, the same diameters, same type of wood was used and smoking methods were the same, only the shorter smoking time could influence lower PAH content in Serbian sausages. Codex Alimentarius Commission[38] recommended the investigation and the definition of smoking conditions for the reduction of PAH content in meat products. Work should be directed on defining smoking time that ensures optimal smoke flavor to the *Petrovská klobása* and the lowest PAH content in sausages. The PAH contents determined in *Petrovská klobása* smoked in industrial (indirect) conditions were lower than those reported for Portuguese and Spanish dry fermented sausages produced in controlled conditions.[39–42] This can be explained by the fact that the traditional method of smoking has been held in Portugal and Spain, although these sausages are produced under controlled conditions. Consequence of the prolonged and more intense traditional smoking method is higher content of PAH compounds.[2,17,19,42]

In the present study, we demonstrated that a reduced smoking step allows the control of PAHs levels in dry-fermented meat sausages, while maintaining the products' sensory characteristics.

According to EU Regulation 835/2011[43] the maximum content of BaP and PAH 4 compounds (CHR, BaA, BbF and BaP) in smoked sausages is 2 μg/kg and 12 μg/kg, respectively. Examined samples of *Petrovská klobása* at the end of smoking period (day 6 of production) and at the end of drying period (day 60 of production) had content of BaP and PAH4 lower than limit of detection. These results proved safety of sausages which were smoked (2 days per 5 h, indirect smoking procedure) and dried (60 day) in controlled conditions, from PAH point of view. The objectives of this research have applied interest for the meat industry, because of importance to define smoking conditions which improve safety of meat products from PAH point of view.

Conclusion

Petrovská klobása with addition of autochthonous starter culture had significantly ($P < 0.05$) higher values of redness-a^* (C-25.23; S-27.94) and better sensory scores ($P < 0.05$) for color (C-

3.9; S-4.3) compared to sausages from control group at the end of drying period. Our results confirmed that addition of *S. xylosus* had positive effect to redness and formation of optimal color of *Petrovská klobása*. Therefore, selection of starter cultures based on NOS and nitrate reductase activity is an important factor which ensures good color development in fermented dry sausages.

Additionally, BaP and PAH4 values were below the limit of detection in analyzed samples of *Petrovská klobása* smoked in industrial conditions (2 days per 5 hour) and these results were indicators that the sausages produced in this way were safe from the PAH point of view.

Funding

The present study was in the frame of the Projects No. 451-03-68/2020-14/200134 and 451-03-68/2020-14/200222 financed by the Ministry of Education, Science and Technological Development, Republic of Serbia.

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