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Microbial ecosystem of processing units during production process of *Petrovská klobása*

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A b s t r a c t: Production of traditional dry-fermented sausages is associated with natural contamination by environmental flora. This microbiota is usually referred to as "house flora". This contamination occurs during slaughtering and increases during manufacturing. The diversity of microbiota in small-scale processing units, during production process of the traditional fermented dry sausages – Petrovská klobása, is reviewed in the present paper. Test samples were collected in two village households in Bački Petrovac, where the preparation of Petrovská klobása samples was performed in a traditional manner. The total number of 43 samples was subjected to laboratory examination. Generally, before stuffing, Listeria monocytogenes and Staphylococcus aureus were detected in 6.97 and 9.30%, respectively. Escherichia coli was found in 18.60%. The tested samples of end product at the end of the storage period (270th day) were safe with presence of bacteria populations from the working environment, such as: aerobic bacteria, Micrococcaceae, Lactic acid bacteria and Enterococcus spp. Examination of the hygienic status of the food processing environment, equipment, raw materials and final product provides an overview of growth trends and the disappearance of bacterial populations.

Key words: *Petrovská klobása, house flora, processing environment, growth trends.*

Introduction

In many European countries, the demand for traditional food products has increased. Moreover, food and gastronomy form an inherent link with tourism in Europe, with a renewed interest of consumers in typical and regional food. *Petrovská klobása*, traditional and autochthonous dry – cured sausage, presents a part of gastronomic heritage of Slovaks in Vojvodina. Nowadays, they are manufacturing the product in a traditional way according to the original recipe of their ancestors, without the use of nitrate/nitrite, glucono delta-lactone (GDL) and microbial starters. In rural households, in the Municipality of Bački Petrovac, this sausage is made by the end of November and during December. *Petrovská klobása* is made by mixing partly cooled (cca 4 h p.m) or cold (cca 24 h p.m) medium chopped lean pork and fat (up to10 mm) with addition of powdered red hot spicy paprika, salt, crushed garlic, caraway and sugar. A well-mixed filling, which is prepared within 15–30 minutes by using a unique technique of manual mixing with kneading and overturning, is stuffed into natural casings consisting of the rear part of pig intestines (colon), forming units 35–45 cm long and 4.5–5.0 cm in diameter. After stuffing, the sausages are left to drain for a while and then subjected to cold smoking for about 10-15 days, using specific kinds of wood (cherry wood in particular). When the smoking process is finished, the sausage is kept in a dry and well ventilated place to dry and ripen, until it achieves an optimum quality, which takes about four months (*Tasić*, 2012; *Janković, et al.*, 2013; Šojić et al., 2014). *Petrovská klobása* is a product of protected geographical origin, under number 44, based on the order issued by the Republic Bureau for Intellectual Property, number 9652/06 G-03/06, on 21/05/2007. In order to achieve a recognizable product of standardized supreme quality which will be continually produced in the controlled conditions, the aim of this study is to determine the parameters of typical house flora during the production

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process of *Petrovská klobása*, which is crucial because of the safety of the final product.

Materials and methods

1. Samples

Test samples were collected from two village households (A and B) in Bački Petrovac, where the preparation of *Petrovská klobása* samples was performed in a traditional manner. Testing included examination of 43 samples, such as: swabs – workers' hands (n = 11), working surfaces (n = 1), equipment before beginning the operation (n = 9), equipment after the operation (n = 9), and other swabs from the working area – wall, drains etc. (n = 7) and samples of fillings (n = 2) and sausages after the drying process (n = 4). The sample area was swabbed using a maximum recovery diluent (MRD, Oxoid) moistened cotton swab. The samples were stored at 0-4°C (coolbox) and then transported to the laboratory under chilled conditions 0−4°C. The samples were examined within 24 hours.

Each sample was tested on the presence of the set of the following bacteria: (1) Total Viable Count/ TVC (SRPS ISO 4833-1; Plate Count Agar -PCA, Oxoid); incubated at 30°C for 72h; (2) Total bacterial count of the *Micrococaceae* family (Manitol salt phenol – red agar, Oxoid), incubated at 30°C for 72 h; (3) Total *Enterobacteriaceae* count (SRPS ISO 21528-2; Violet Red Bile agar with glucose − VRBG, Oxoid), incubated at 30°C for 72 h; (4) Total count of β – glucuronidase positive *E. coli* (SRPS ISO 16649-2; Tripton Bile x Glucuronide agar/TBX, Oxoid), incubated at 44°C for 24 h; (5) *Enterococcus* spp. counts (Bile esculin azide agar, Biokar diagnostics), incubated at 37°C for 48 h; (6) Total count of coagulase positive staphylococci (SRPS ISO 6888 – 1, Baird Parker, Oxoid), incubated at 37°C for 24 h; (7) *Pseudomonas* spp. counts, (Pseudomonas Selective Agar – Cetrimide Agar, Merck), incubated at 35°C for 48 h; (8) Total count of sulphate-reducing bacteria, which grow in anaerobic conditions (SRPS ISO 15213; Iron Sulfite Agar, Oxoid), incubated at 37°C for 48 h; (9) Total count of *Clostridium perfringens* (SRPS ISO 7937; Sulfite cycloserine Agar, Oxoid), incubated at 37°C for 20 h; (10) *Salmonella* spp. (SRPS ISO 6579; modified Rappaport Vasilidis Soft Agar), incubated at 42°C for 24 h; (Rambach, Merck), incubated at 37°C for 24 h; (11) Lactic acid bacteria presence in samples of chunk meat and filling (ISO 15241; Man-Ragosa Sharpe/MRS, Merck, Darmstad, Germany), incubated at 30°C for 48−72; (12) *Listeria monocytogenes* presence and total count (SRPS ISO 11290−1, 2; ALOA, Merck).

2. Immunoenzymatic assay

For detection of *Listeria monocytogenes,* a Vidas − *L. monocytogenes* Xpress (LMX, BioMérieux) was used. In case of food samples, 25 g of sample (analytical unit) was aseptically added to 225 mL of LMX broth in a stomacher bag (Seward). In case of environmental samples, for each swab, 10 mL of LMX broth was aseptically added for each swab. Incubation period was 30 ± 1°C for 22 − 24 h for food samples or 24 − 26 h for environmental samples. After a specific period of incubation, 1− 2 ml broth was removed into a sterile test-tube (Sigma Aldrich), which was heated at 95 to 100°C for 5 ± 1 min. The tube was cooled down and 250 µl of the enriched sample was taken to test. All positive results obtained were confirmed by the reference SRPS ISO 11290-1 method or by using the ALOA chromogenic agar.

3. Statistical analysis

Statistical analysis was carried out using STATISTICA 9.1 (StatSoft, Inc., Tulsa, OK, USA). All data were presented as a mean value with the standard deviation indicated (mean ± SD).

Results

Results of testing are presented in Tables 1, 2, 3, 4 and 5. The environment of processing units was colonized at variable levels by resident spoilage and technological microbiota, with sporadic contamination by pathogenic microbiota. In the households A and B (Tables 1, 2, 3 and 4), the presence of aerobic bacteria, *E. coli*, enterococci, *Staphylococcus aureus*, *Enterobacteriaceae* and *Listeria* spp. was detected. In household A (Table 1), the aerobic bacteria counts ranged from 1.26 ± 0.17 $\log_{10}$cfu/cm^2 (knife) up to 8.04 ± 0.91 $\log_{10}$cfu/cm^2 (saw after cutting). *E. coli* was present in two samples (saw after cutting and table), while enterococci were found in all experimental samples, with a range between 2±0 $\log_{10}$cfu/cm^2 (workers hands) and 5.67 ± 0.06 $\log_{10}$cfu/cm^2. (workers' hands after slaughtering). *Staphylococcus aureus* was found in only one sample (workers' hands). *Enterobacteriaceae* had total counts that ranged between 2.67 ± 0.31 $\log_{10}$cfu/cm^2 (table) and saw after cutting (5.04 ± 0.4 $\log_{10}$cfu/cm^2). Other groups of bacteria were not detected. Household

Table 1. Microbiological contamination of processing environment, food contact surfaces, equipment and workers' hands in the household A during the meat production process (X ± SD, log_{10}cfu/cm^2).

Tabela 1. Mikrobiološka kontaminacija radne sredine, radnih površina, pribora i ruku radnika u okviru domaćinstva A tokom proizvodnje mesa (X ± SD, log_{10}cfu/cm^2)

Microbiological contamination/ Mikrobiološka kontaminacija	Workers' hands/ Ruke radnika	Workers' hands after slaughtering/ Ruke radnika posle klanja	Saw/ Testera	Saw after cutting/ Testera posle klanja	Knife/Nož	Knife after cutting/ Nož posle klanja	Table/Radna površina	Wall/ Zid
Total viable count/ Ukupan broj bakterija	4.13±0.16	6.09±0.52	7±0	8.04±0.91	1.26±0.17	1.33±0.17	6.83±0.02	7±0
Micrococcaceae	ND	ND	ND	ND	ND	ND	ND	ND
E. coli	ND	ND	ND	3.11±0.16	ND	ND	1.98±003	ND
Enterococcus spp.	2±0	5.67±0.06	3.04±0.07	3.35±0.31	2.13±0.08	4.74±0.04	5.04±0.04	3.94±0.03
S. aureus	2.24±0.21	ND	ND	ND	ND	ND	ND	ND
Pseudomonas spp.	ND	ND	ND	ND	ND	ND	ND	ND
Sulphite-reducing bacteria	ND	ND	ND	ND	ND	ND	ND	ND
Clostridium perfringens	ND	ND	ND	ND	ND	ND	ND	ND
Enterobacteriace	ND	ND	ND	5.04±0.4	ND	3.8±0.29	2.67±0.31	ND
Salmonella spp.	ND	ND	ND	ND	ND	ND	ND	ND
L. monocytogenes	ND	ND	ND	ND	ND	ND	ND	ND

Legend/Legenda: ND – not detected/nije otkriven

Table 2. Microbiological contamination of processing environment, food contact surfaces, equipment and workers' hands in the household B during the meat production process (MS ± Sd, log_{10}CFU/cm^2).

Tabela 2. Mikrobiološka kontaminacija radne sredine, radnih površina, opreme i ruku radnika u okviru domaćinstva B tokom proizvodnje mesa (MS ± Sd, log_{10}cfu/cm^2)

Microbiological contamination/ Mikrobiološka kontaminacija	Workers' hands/ Ruke radnika	Workers' hands/ Ruke radnika	Workers' hands/ Ruke radnika	Saw/ Testera	Saw after cutting/ Testera posle klanja	Knife/ Nož	Knife after cutting/ Nož posle klanja	Chopper/ Mašina za sečenje	Chopper after Cutting/ Mašina za sečenje posle sečenja	Apron/ Kecelja	Drain/ Odvod
Total viable count/ Ukupno bakterija	3.6±0.53	4.05±1.69	3.72±0.09	5.83±0.56	6.29±0.03	5.58±0.17	6.1±0.35	2.57±0.24	6.44±0.17	6.46±0.19	7.19±0.15
Micrococcaceae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
E. coli	ND	ND	ND	ND	3.41±0.36	ND	1.87±0	ND	3.41±0.23	ND	3±0
Enterococcus spp.	ND	ND	ND	4.28±0.25	4.45±0.08	ND	3.66±0.16	ND	4.39±0.05	ND	3.33±0.28
S. aureus	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pseudomonas spp.	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sulphite-reducing bacteria	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Clostridium perfringens	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Enterobacteriaceae	ND	ND	ND	3.52±0.17	5.07±0.5	ND	5.73±0.2	ND	4.64±0.06	ND	2.37±0
Salmonella spp.	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
L.monocytogenes.	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.28±0.02

Legend/Legenda: ND – not detected/nije otkriven

Table 3. Microbiological contamination of processing environment, food contact surfaces, equipment and workers' hands in the household A during preparation of the filling (MS ± Sd, $log_{10}CFU/cm^2$)

Tabela 3. Mikrobiološka kontaminacija radne sredine, radnih površina, opreme i ruku radnika u okviru domaćinstva A tokom pripreme nadeva (MS ± Sd, $log_{10}cfu/cm^2$)

Microbiological contamination/ Mikrobiološka kontaminacija	Mincing machine (beginning)/ Mašina za mlevenje (početak)	Mincing machine (operation)/ Mašina za mlevenje (rad)	Stuffing machine (beginning)/ Mašina za punjenje (početak)	Stuffing machine (operation)/ Mašina za punjenje (rad)	Casing/ Crevo	Casing with the filling/ Crevo sa punjenjem	Workers' hands during grinding/ Ruke radnika tokom mlevenja	Workers' hands with spices/Ruke radnika sa začinima	Drain/ Odvod
Total viable count/ Ukupno bakterija	4.83±0.26	6.64±0.05	3.23±0.06	6.77±0.07	6.75±0.13	6.75±0.13	6.69±0.05	6.94±0.1	6.84±0.06
Micrococcaceae	ND	ND	ND	ND	ND	ND	ND	ND	ND
E. coli	ND	ND	ND	1.97±0.03	ND	ND	ND	2±0	ND
Enterococcus spp.	ND	5.27±0.25	ND	4.52±0.06	4.03±0.05	4.89±0.06	4.72±0.05	5±0	3.28±0.12
Staphylococcus aureus	ND	2±0	ND	ND	ND	ND	3.31±0.21	3±0	ND
Pseudomonas spp.	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sulphite-reducing bacteria	ND	ND	ND	ND	ND	ND	ND	ND	ND
Clostridium perfringens	ND	ND	ND	ND	ND	ND	ND	ND	ND
Enterobacteriaceae	ND	ND	ND	1.32±0.04	ND	ND	ND	ND	2.33±0.05
Salmonella spp.	ND	ND	ND	ND	ND	ND	ND	ND	ND
Listeria monocytogenes	ND	ND	ND	2. 03±0	ND	ND	ND	ND	ND

Legend/Legenda: ND – not detected/nije otkriven

Table 4. Microbiological contamination of processing environment, food contact surfaces, equipment and workers' hands in the household B during preparation of the filling (MS ± Sd, $log_{10}CFU/cm^2$)

Tabela 4. Mikrobiološka kontaminacija radne sredine, radnih površina, opreme i ruku radnika u okviru domaćinstva B tokom pripreme nadeva (MS ± Sd, $log_{10}cfu/cm^2$)

Microbiological contamination/ Mikrobiološka kontaminacija	Mincing machine (beginning)/ Mašina za mlevenje (početak)	Mincing machine (operation)/ Mašina za mlevenje (rad)	Stuffing machine (beginning)/ Mašina za punjenje (početak)	Stuffing machine (operation)/ Mašina za punjenje (rad)	Workers' hands after cutting the meat/ Ruke radnika posle sečenja mesa	Workers' hands after cutting the meat/ Ruke radnika posle sečenja mesa	Workers' hands after cutting the meat/ Ruke radnika posle sečenja mesa	Workers' hands after mixing the filling/ Ruke radnika posle punjenja	Drain/ Odvod
Total viable count/ Ukupno bakterija	3.18±0.14	6.56±0.08	2.21±0.02	4.21±0.26	6.21±0.62	6.1±0.19	5.2±0.17	6.75±0.12	7.19±0.15
Micrococcaceae	ND	ND	ND	ND	ND	ND	ND	ND	ND
E. coli	ND	ND	ND	ND	ND	ND	ND	ND	3±0
Enterococcus spp.	ND	3.33±0.24	ND	3.57±0.27	ND	4.3±0.11	4.14±0.36	4.3±0.3	3.33±0.28
Staphylococcus aureus	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pseudomonas spp.	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sulphite-reducing bacteria	ND	ND	ND	ND	ND	ND	ND	ND	ND
Clostridium perfringens	ND	ND	ND	ND	ND	ND	ND	ND	ND
Enterobacteriaceae	ND	2.33±0.58	ND	ND	2.33±0	2.33±0	ND	2.11±0.58	2.37±0
Salmonella spp.	ND	ND	ND	ND	ND	ND	ND	ND	ND
Listeria monocytogenes	ND	2.02±0.46	ND	ND	ND	ND	ND	ND	ND

Legend/Legenda: ND – not detected/nije otkriven

Table 5. Microbiological contamination of sausage batter and final product after a drying process (X ± SD, $log_{10}CFU/cm^2$)

Tabela 5. Mikrobiološka kontaminacija nadeva i kobasica nakon procesa sušenja (X ± SD, $log_{10}CFU/cm^2$)

Sample/ Uzorak	Total viable count/ Ukupno bakterija	Micrococcaceae	Enterococcus spp.	Lactic Acid Bacteria	*Enterobacteriaceae*	*L. monocytogenes*
Batter A/Masa A	7.03±0.05	4.23±0.42	3.24±0.24	ND	3.89±0.02	ND
Batter B/Masa B	7.05±0.07	4.51±0.45	3.19±0.17	ND	4.19±0.12	ND
Sausage A1/ Kobasica A1	4.19±0.22	2.75±0.35	< 2	5.3±0.15	ND	ND
Sausage A2/ Kobasica A2	4.28±0.23	3.4±0.22	< 2	6.4±0.22	ND	ND
Sausage B1/ Kobasica B1	4.5±0.05	2.64±0.1	< 2	5.7±0.15	ND	ND
Sausage B2/ Kobasica B2	4.31±0.02	2.62±019	< 2	6.2±0.30	ND	ND

Legend/Legenda: ND – not detected/nije otkriven

B (Table 2) showed similar situation with regard to the presence of microorganisms (aerobic bacteria, *E. coli*, enterococci and *Enterobacteriaceae*). The working surfaces, machines, tools and worker's hands had total aerobic counts between (chopper) and 7.19 ± 0.15 $log_{10}cfu/cm^2$. For *E. coli*, contamination level was 1.87 ± 00 $log_{10}cfu/cm^2$, 3.41 ± 0.23 $log_{10}cfu/cm^2$, 3 ± 0 $log_{10}cfu/cm^2$, respectively. *Enterobacteriaceae* were found in six samples, with maximum of 5.73 ± 0.2 $log_{10}cfu/cm^2$ (knife after cutting). The presence of *L. monocytogenes* was detected in swabs from the drain 2.28 ± 0.2 $log_{10}cfu/cm^2$ (Table 2). In households A and B, during preparation of the filling (Tables 3 and 4), the presence of *L. monocytogenes* was detected in swabs from the stuffing (2.03 ± 0 $log_{10}cfu/cm^2$) and mincing machine (2.02 ± 0.46 $log_{10}cfu/cm^2$). Also, *L. monocytogenes* was detected in a sausage batter A (2.07 ± 1270.07 $log_{10}cfu/cm^2$) and sausage batter B (2.08 ± 0.08 $log_{10}cfu/cm^2$) (Table 5).

In regard to the final product – sausage after drying process (Table 5), the presence of aerobic bacteria, micrococci, enterococci and Lactic Acid Bacteria (LAB) was detected while other groups of bacteria were not detected.

Discussion

Many authors support the belief that the microorganisms present in traditional sausages are derived from the raw materials or from the manufacturing (*Talon et al.,* 2007). This microbiota is usually referred to as "house flora". While the microbiota isolated from traditional sausages is well described, the resident microbiota in the environment of the processing unit is still poorly known. The presence of aerobic bacteria, enterobacteriaceae, enterococci and *L. monocytogenes* in A and B fillings, most probably resulted from the cross contamination of the sausage batter either with working surfaces or after the meat mincing and addition of spices; that is a consequence of the specific filling preparation technique by manual mixing on the wooden table for ca. 15-30 min (*Ikonić et al.,* 2010). Generally, *L. monocytogenes* and *S. aureus* were detected in 6.97 and 9.30%, respectively, while *E. coli* was found in 18.60%. Sausage samples at the end of the production cycle (270^{th} day) were safe in regard to the presence of bacteria populations from the working environment, such as: aerobic bacteria, *Micrococcaceae, Lactic acid bacteria* and *Enterococcus*. The results are in accordance with the results obtained by *Lebert et al.* (2007), *Janković et al.* (2013), *Lakićević et al.* (2014). Several critical points were identified such as the drain, saws, workers' hands, mincing and stuffing machines. The current study revealed that the majority of the sampling sites (control point) tested were (2 to 6 log cfu/cm^2) contaminated by spoilage flora (*Enterobacteriaceae*) with knives and saws, mincing machines (*Listeria monocytogenes*), workers' hands (*Staph. aureus, E. coli*), which surely indicates an inappropriate slaughtering process, and to a low level of personal hygiene. Detection of *Listeria monocytogenes* can be considered as a useful indicator of a deterioration in hygiene or process conditions during food production. Unclean, insufficiently or inadequately cleaned pieces of equipment

have often been identified as a source of pathogens. The results are unique and crucial for the improvement of hygiene control systems in traditional meat processing units (*Talon et al.,* 2007).

Conclusion

Traditional dry sausages rely on natural contamination by environmental flora. This contamination occurs during slaughtering and increases during manufacturing. The results, during the production of the *Petrovská klobása* in the traditional manner, showed that processing units were colonised at various levels by spoilage and technological microflora with excessive contamination levels. Sporadic contamination by pathogenic microflora was recorded. *L. monocytogenes* and *S. aureus* were detected in 6.97 and 9.30% of the samples, respectively, while *E. coli* was enumerated in 18.60% of the samples. The variability of the contamination emphasized the different cleaning, disinfecting and manufacturing practices routinely followed by these (A and B households) small-scale processing units. The technological flora (coagulase negative staphylococci and lactic acid bacteria) were both in the environment and in products. Enterococci were present all along the manufacturing period. Examination of the hygienic status of the processing environment, equipment, raw materials and food safety criteria of the final product provides an overview of growth trends and the disappearance of bacterial populations.

Further studies will be carried out to detail phenotypic, genotypic and physiological characterization of the isolated strains of staphylococci (CNS) and LAB from the *Petrovská klobása* with additional purpose of creating Serbian bank of autochthonous functional starter cultures specific for industrial production of the *Petrovská klobása* . In this way, the product will meet all regulations, as needed on a broad market, and it will remain autochthonous, safe and recognizable.

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Mikrobiološki ekosistem malih proizvodnih jedinica tokom proizvodnog ciklusa *petrovačke kobasice*

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R e z i m e: Proizvodnja tradicionalnih suvih-fermentisanih kobasica je u direktnoj vezi sa mikrobiološkim ekosistemom radne sredine (house flora), čije definisanje ima značajnu ulogu u mikrobiološkoj stabilnosti i bezbednosti gotovog proizvoda. U okviru rada, prezentovani su rezultati koji daju uvid u diverzitet microbiota u malim proizvodnim jedinicama, tokom proizvodnog ciklusa petrovačke kobasice. Test uzorci su sakupljeni u dva seoska domaćinstva u Bačkom Petrovcu, gde je izvršena priprema petrovačke kobasice na tradicionalan način. Ispitivanjem su obuhvaćena ukupno 43 uzorka. Generalno, Listeria monocytogenes i Staphylococcus aureus su detektovani u 6,97 i 9,30%, respektivno, dok je Escherichia coli detektovana u 18,60% uzoraka. Uzorci kobasica su na kraju perioda skladištenja (270. dan) bili bezbedni uz prisustvo sledećih grupa bakterija: aerobne bakterije, Micrococcaceae, bakterije mlečne kiseline i Enterococcus spp.

Ključne reči: *Petrovačka kobasica, trend rasta, radna sredina.*

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