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The influence of starter cultures on the lactic acid bacteria microbiota of Petrovac sausage

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PAPER

Abstract

Petrovac sausage (*Petrovská klobása*) is a high-quality fermented dry sausage produced traditionally in the municipality of Bački Petrovac (Vojvodina, Serbia). The product is characterised by specific and recognised texture, aroma and colour, produced without additives or preservatives. Lactic acid bacteria (LAB) microbiota plays an important role in production of the sausage. The aim of the paper is to monitor the changes in LAB during the production of Petrovac sausage. Samples of sausages were prepared without and with the addition of starter culture *Staphylococcus xylosus* as well as combined starter culture *Lactiplantibacillus plantarum* and *S. xylosus*, and produced at two different temperature ranges. A total number of 495 strains were isolated from 33 samples of Petrovac sausage during 120 days of production process. Characterisation of the isolates was performed by phenotypic tests, while molecular identification of the representative strains was done by 16S ribosomal DNA sequencing. The total number of LAB was about 8 log (Colony Forming Unit (CFU))/g in all samples, while the number of staphylococci was about 4 log CFU/g. Molecular identification confirmed that all isolates belonged to the following species: *Levilactobacillus brevis*, *Leuconostoc mesenteroides*, *Lactiplantibacillus plantarum* and *Pediococcus pentosaceus. Lactobacilli* and *Leuconostoc* spp. dominate the total LAB strains, while *P. pentosaceus* was isolated at the lowest frequency.

Keywords: fermented sausages, lactic acid bacteria, Petrovac sausage, 16S rDNA sequencing

Introduction

The production of fermented sausages correlate with the diversity of microbiota present in meat batter as well as those added in the form of starter culture (Cocconcelli and Fontana, 2008; Toldra, 2002). Starter cultures contribute to the functional properties of fermented products and play a major role in the improvement of organoleptic, technological, nutritional and health characteristics of fermented sausages (Laranjo *et al.,* 2017). Different types of microorganisms (Lactic Acid Bacteria [LAB], staphylococci and micrococci, mould, and yeasts) can be used for autochthonous and commercial starter cultures in the production of fermented sausages (Casaburi *et al*., 2008; Kovacevic *et al.,* 2010). Combination of *Lactobacillus* spp. and *Staphylococcus* spp. used as starter cultures in the sausages production can contribute to the pleasant aroma of sausages and they possess antimicrobial properties against unwanted microorganisms and pathogen microbiota (Hosseini and Pilevar, 2017). Owing to acidification, lipolysis, and proteolysis, and production and development of volatile aroma compounds, LAB microbiota (*Lactobacillus sakei*, *Lactiplantibacillus pentosus*, *Lactobacillus curvatus*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, *Levilactobacillus brevis* etc.) play an essential role of a starter culture in meat fermentation (Ammor and Mayo, 2007; Kumar *et al.,* 2017). Production of lactic and acetic acid reduces pH of the meat batter resulting in the formation of characteristic sausage aroma and consistency. The acidification process plays an important role in the inhibition and inactivation of pathogenic microorganisms contributing to the prolonged shelf life and safety of fermented sausages (Leistner, 1995; Martinovic and Veskovic-Moracanin.,

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2006). LAB species can produce bacteriocins as antimicrobial products of fermentation. *L. sakei*, *L. curvatus, L. plantarum* and *L. paracasei,* which are often used as starter cultures, contribute to the safety and stability of fermented sausages because of strong antibacterial activity against *Escherichia coli* and *Listeria monocytogenes* (Pidcock *et al.,* 2002; Veskovic-Moracanin, 2010). In addition to the safety of product, some strains of lactobacilli used as starter cultures (*L. sakei*, *L. curvatus* and *L. plantarum*) promote the degradation of peroxide (Martinovic and Veskovic-Moracanin., 2006).

Gram-positive cocci used as starter cultures (*Staphylococcus carnosus*, *Staphylococcus xylosus* and *Micrococcus varians*) play an important role in the reduction of nitrates and nitrites, decomposition of peroxides, lipolysis stabilisation and development of texture (Skocińska *et al.,* 2016). *S. carnosus* and *S. xylosus* as starter cultures contribute to the development of desirable colour and aroma in fermented sausages. Owing to antioxidant properties, growth on optimal salt concentrations and growth on optimal pH, *S. carnosus* and *S. simulans* are often used as starters in fermented sausages (Casaburi *et al*., 2005).

Dry-fermented sausages represent the result of physical, chemical, biochemical, microbiological and sensory changes that occur during the ripening of meat batter (Hammes *et al.,* 2008). Petrovac sausage (*Petrovská klobása*) is a traditional dry-fermented product made in Bački Petrovac (Vojvodina, Serbia). As a high-quality fermented product with appropriate texture, aroma and colour, it is produced without additives or preservatives, and protected by Protected Denomination of Origin (PDO) at the national level (Ikonic *et al*., 2015; Petrovic *et al.,* 2007). Petrovac sausage can be produced without adding starter cultures (Danilovic *et al*., 2018). The traditional production excludes the addition of starter cultures (Ikonic *et al*., 2016; Jokanovic *et al*., 2017, 2010).

The aim of this work was to monitor the changes in LAB microbiota in the samples of Petrovac sausage (*Petrovská klobása*) prepared without and with the addition of starter culture *S. xylosus* and combined starter cultures *L. plantarum* and *S. xylosus* and produced under controlled conditions in two different temperature ranges. For this purpose, isolation, characterisation and identification of LAB microbiota were performed.

Materials and methods

Fermented sausage technology and sampling procedure

Fermented sausages were produced according to the traditional recipe in the Agro-Industrial Complex (AIC) 'Bačka Topola' (Vojvodina, Serbia). Meat batter was made of minced pork (85%) and solid back fat tissue (15%) with addition of the following ingredients (w/w): red hot pepper (2.5%), salt (1.8%), garlic (0.2%), caraway seeds (0.2%) and sucrose (0.1%). The meat batter was divided into three equal parts: control sausages (H) (without addition of starter), sausages (I) (with the addition of combined starter cultures of *S. xylosus* and *L. plantarum*) and sausages (J) (with the addition of starter culture of *S. xylosus*). The initial number of starter cultures in meat batter was the same for LAB and coagulase-negative cocci (CNC) (4.5–5.0 log (CFU)/g). Autochthonous starter cultures were previously isolated from traditionally produced Petrovac sausage (Danilovic, 2012). The mixture was stuffed into artificial collagen casings. Smoking, drying and ripening of the sausages were carried out under controlled conditions in the ripening chamber at the temperature range of 14–16°C (tag 1) and ~10°C (tag 2). All experiments were performed in triplicate. Samples were collected after 0 (meat batter), 6, 15, 60, 90 and 120 days of production.

Isolation and enumeration of bacteria

For microbiological analysis, 10 g of each sausage sample was aseptically homogenised in 90 mL of sterile saline peptone water (8 g/L NaCl + 1 g/L peptone) (Urso *et al*., 2006). The enumeration of microorganisms was performed in triplicate by the successive serial dilution method and represented as the mean value. Dilutions were prepared and plated on nutrition agar (NA, Torlak, Belgrade, Serbia), de Man, Rogosa and Sharpe (MRS) agar (Torlak, Belgrade, Serbia) and Mannitol Salt Agar (MSA) plates for determining the total number of mesophilic bacteria, LAB and staphylococci, respectively. After the incubation of plates (48 h, 30°C) and enumeration, randomly selected colonies from MRS agar plates were streaked to new MRS agar plates for purification.

Phenotypic identification and characterisation of LAB isolates

Basic characterisation of the isolates was performed through Gram reaction, cell morphology and catalase test with H_2O_2 (30% v/v). Gram-positive and catalase-negative isolates were subjected to the following physiological tests: CO_2 production, arginine and esculin hydrolysis, bacterial growth on MRS agar plates at different temperatures (15°C and 45°C) for 72 h, bacterial growth on MRS agar plates supplemented with NaCl (4%, 6.5% and 8%) for 72 h, bacterial growth on bile esculin agar, synthesis of exopolysaccharides and the synthesis of bacteriocines.

Arginine hydrolysis was performed in arginine broth (g/L: tryptophan 5, L-arginine 3, glucose 0.5 and K_2HPO_4 2), while esculin hydrolysis was performed in esculin broth (Torlak, Belgrade, Serbia). After incubation, a few drops of phenyl-red were added to the arginine broth (red colour indicates a positive reaction, and yellow colour a negative one), and a few drops of 2% $FeCl_3$ solution to the esculin broth (a positive reaction is the appearance of a black precipitate). For preliminary identification of enterococci, isolates were grown on bile esculin agar (Rocheux's Medium, Himedia, Mubai, India). The appearance of black colonies indicate the presence of enterococci. Exopolysaccharide production was detected visually (appearance of mucous colonies) after incubation of isolates on a modified MRS medium supplemented with maltose, sucrose, galactose, fructose, lactose and glucose (Merck GmbH, Darmstadt, Germany) at a temperature of 30°C for 48 h.

The bacteriocinogenic activity was performed using the agar well diffusion assay. Soft nutrition agar (0.7% w/v), containing indicator strain, was poured into plates with thin layer of MRS agar. After hardening of the medium, small diameter wells (10 mm) were made into plates. Into each well, aliquot (50 µL) of the supernatant of overnight culture (16 h) was poured. Also, a crystal of pronase E was added close to the edge of the bacteriocin-containing well. The plates were incubated at 30°C for 24 h. Appearance of a clear inhibition zone around the well was recorded as a positive signal for production of bacteriocin. For detecting bactericiongenic activity, *Bacillus subtilis, Listeria monocytogenes* and *E. coli* were used as pathogenic microorganisms. Production of bacteriocin against any of the analysed strains was stated as positive.

Molecular identification of LAB isolates

Isolation of the total genomic DNA as well as (GTG)5-PCR fingerprinting was performed as described previously (Nikolic *et al.*, 2008). For 16S ribosomal DNA (rDNA) sequencing method, PCR amplifications with primers UNI 16SF (5'-GAG AGT TTG ATC CTG GC-3) and UNI 16SR (5'-AGG AGG TGA TCC AGC CG-3') were performed with a Taq DNA polymerase kit (Fermentas UAB, Vilnius, Lithuania). The amplification of the samples was performed through GeneAmp® PCR system 2700 (Applied Biosystems) operated with the following parameters: the initial duration of DNA for 7 min at 95°C, 32 cycles of denaturation of 1 min at 94°C, polymerisation with a duration of 8 min at 65°C, and the final extension of incomplete product with a duration of 16 min at 65°C. Plasmide profiles were monitored on 1.5% (w/v) agarose gel with ethidiumbromide at a constant voltage of 60 V (at 4°C for 20 h) (Versalovic *et al.*, 1994). Visualisation of PCR products was performed by applying CCD camera Biometra BDR2/5/6 (Bio Doc Analyze). Specific PCR products were analysed by electrophoresis on 1% agarose gel and purified using QIAquick PCR Purification KIT/250 (Qiagen, Hilden, Germany). Purified PCR amplicons were sequenced using Macrogen sequencing service in Seoul, South Korea. The results were compared with the data stored in the National Centre for Biotechnology Information (NCBI) gene databank using BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST).

Results and discussion

Petrovac sausage is an indigenous fermented sausage produced of minced meat and spices without preservatives with specific and recognisable characteristics. The sausage fermentation process is greatly affected by the changes in the development and composition of LAB and staphylococci microbiota. In order to determine the changes in LAB microbiota during the production of Petrovac sausage with the addition of starter cultures, sausage samples were prepared without starter culture (sausages H), with combined starter culture *L. plantarum* and *S. xylosus* (sausages I) and with starter culture *S. xylosus* (sausages J). Production of the sausages was performed under controlled conditions at a temperature range of 14–16°C (tag 1) and ~10°C (tag 2).

During the production of Petrovac sausage, the change in the number of mesophilic bacteria was almost identical to the change in LAB regardless of using starters. In the sausages prepared without starter cultures (sausages H), the number of initial LAB and aerobic mesophilic bacteria was about 5 log CFU/g, while the number of staphylococci ranged about 4 log CFU/g. The maximum value of LAB and aerobic mesophilic bacteria (8–9 log CFU/g) was reached after 15 days and it remained stable till the end of the production process. The number of staphylococci at the end of the process was lower and was about 3 log CFU/g (Figure 1H). Similarly, in the sausages prepared with combined starter culture of *S. xylosus* and *L. plantarum* (sausages I), the initial number of LAB was almost identical to the initial number of aerobic mesophilic bacteria (about 5 log CFU/g). During the production of sausages I, similar changes in the number of both LAB and aerobic mesophilic bacteria were observed as in sausages H. The number of staphylococci in sausages I was in the same range as in sausages prepared without starter cultures (3–4 log CFU/g) (Figure 1I). In sausages prepared with the addition of starter culture *S. xylosus* (sausages J), the number of staphylococci at the end of production was higher in all samples produced at 14–16°C (about 3 log CFU/g) than in the samples produced at ~10°C (about 2 log CFU/g). Changes in the number of LAB and aerobic mesophilic bacteria were almost identical as in sausages I (Figure 1J).

The number of LAB in sausages produced without starter culture (H) during the first days of fermentation was in accordance with the results obtained for Tunisian dry-fermented sausage produced without starter culture (4.3 log CFU/g). However, the initial number of LAB in sausages I and J (about 5 log CFU/g) was lower than the number of LAB obtained for Tunisian sausages produced with combined starter culture of *S. xylosus* and *L. plantarum* (7.3 log CFU/g) (Essid and Hassouna, 2013). The rapid increase in LAB during the first days of fermentation is also in accordance with the rapid increase in dry-fermented poultry sausages prepared without starter cultures (8.3 CFU/g), with starter culture of *S. xylosus* or *L. plantarum* (8.9 CFU/g) and mixed starter culture of *S. xylosus* and *L. plantarum* (8.8 CFU/g) (El Adabi *et al.,* 2014). Also, the maximum level of number of LAB during the first 15 days (8–9 log CFU/g) was in accordance with the results obtained for Tunisian sausages produced with combined starter culture of *S. xylosus* and *L. plantarum* (8.1 log CFU/g) (Essid and Hassouna, 2013). Rapid increase in the total number of aerobic mesophilic bacteria in all samples during the first days of production process was in accordance with the results obtained for the samples of Petrovac sausages produced under traditional conditions (5–8.5 log CFU/g) and for the samples produced under controlled conditions (5–7 log CFU/g) (Danilovic *et al.,* 2018). The total number of aerobic mesophilic bacteria was in

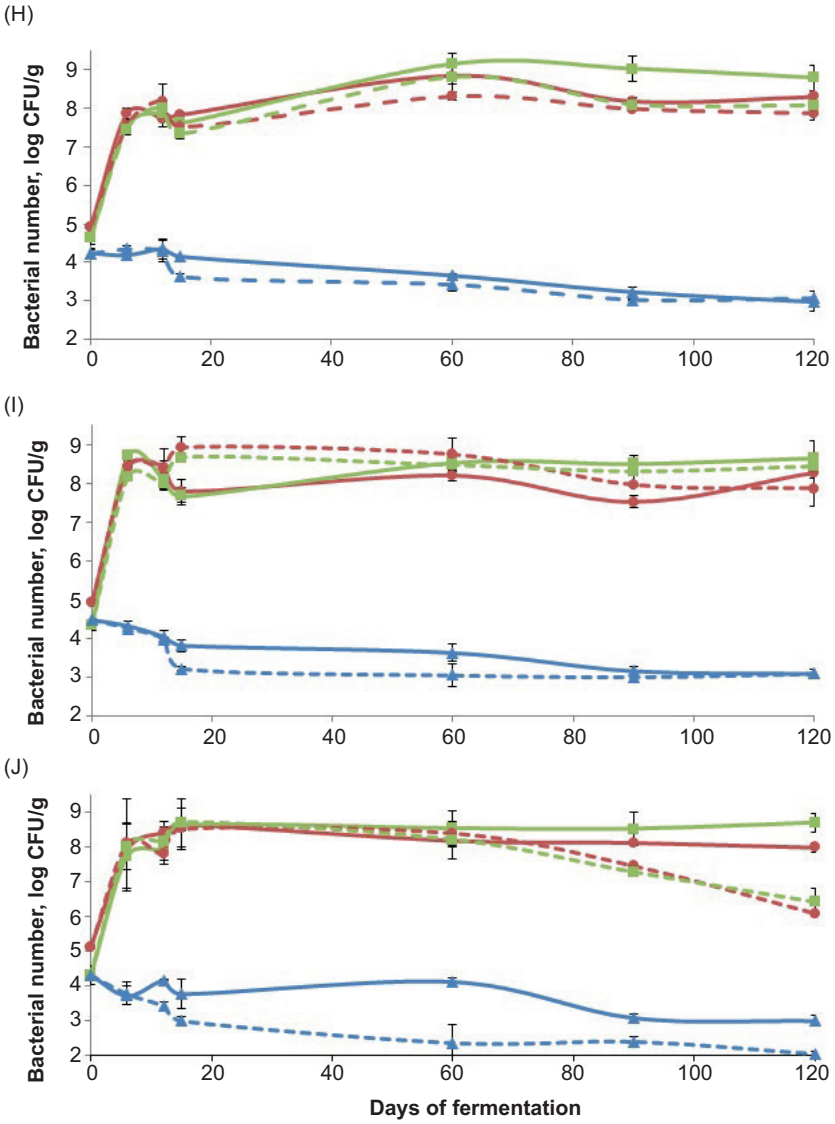

Figure 1. The number of aerobic mesophilic bacteria (●, red line), LAB (■, green line) and staphylococci (▲, blue line) during the production of Petrovac sausages prepared without starter cultures (H), with combined starter culture *S. xylosus* and *L. plantarum* (I), and with *S. xylosus* (J) and produced at 14–16°C (full line) and ~10°C (dashed line). Vertical error bars represent standard deviation.

accordance with the results obtained for Petrovac sausages produced under traditional and controlled conditions (7–8 log CFU/g; Danilovic *et al.*, 2018) and for the sausages produced from hot deboned meat (Danilovic *et al.*, 2011). Results obtained for Petrovac sausages indicated that LAB microbiota, being the dominant microbiota during the production process, were in accordance with the results of Casaburi *et al.* (2008), Casquete *et al.* (2012) and Zdolec *et al.* (2008). Domination of LAB microbiota in Petrovac sausages was in accordance with the results obtained for Alheira-fermented sausage produced in Portugal (Albano *et al.*, 2009), traditional Greek dry-fermented sausages (Ambrosiadis *et al.*, 2004; Papamanoli *et al.*, 2003), dry-fermented sausages produced with *L. sakei* (Bolumar *et al.*, 2006) and Tunisian dry-fermented beef sausage produced with combined starter culture of *S. xylosus* and *L. plantarum* (Essid and Hassouna, 2013). The initial number of staphylococci in sausages H, I and J (about 4 log CFU/g) was lower than the number of the same microbiota in Tunisian beef sausage produced without starter culture (5 log CFU/g) and with combined starter culture of *S. xylosus* and *L. plantarum* (7 log CFU/g) (Essid and Hassouna, 2013). The lower number of staphylococci at the end of production process was probably due to reduction in pH caused by lactobacilli (Johansson *et al.*, 1994; Lizaso *et al.*, 1999). Addition of starter culture had no effect on the total number of staphylococci. The higher number of staphylococci in the samples produced at higher temperature range (14–16°C) than the number presented in samples produced at lower temperature (~10°C) was in accordance with the results obtained for Italian fermented sausages, where the growth of *S. xylosus* was better at higher temperatures (Fiorentini *et al.*, 2010). Other results confirmed that increasing temperature from 10°C to 26°C increased growth of *S. xylosus*, *S. carnosus* and *S. equorum*, with strong synergy between temperature and pH (Søndergaard and Stahnke, 2002). The number of both aerobic mesophilic bacteria and LAB was identical regardless of the addition of starter cultures *S. xylosus* and *L. plantarum*. These results were in accordance with the results obtained for Tunisian dry-fermented sausages produced with the addition of starter cultures *S. xylosus* and *L sakei* (Najjari *et al.*, 2020).

A total of 495 Gram-positive and catalase-negative strains were isolated from 33 samples during the production of Petrovac sausage. Phenotypic grouping of strains by cell morphological characteristics divided all isolates into five groups (Table 1). The identity of the isolate was confirmed by (GTG)5-PCR and 16S rDNA sequencing. The 16S ribosomal RNA (rRNA) gene sequence analysis confirmed that all isolates belonged to *L. brevis*, *L. mesenteroides*, *L. plantarum* and *P. pentosaceus* species. DNA analyses of the PCR-amplified 16S rRNA gene fragments obtained from purified isolates during sausage production provided the fingerprints shown in Figure 2. The (GTG)5 fingerprints didn't show intraspecific biodiversity.

Gram-positive, catalase-negative and rod-shaped cells were classified as lactobacilli. Arginine-negative group of lactobacilli had the ability to grow well at 15°C and in the presence of 6%, 5% and 8% of NaCl. This group didn't

Table 1. Characterisation of LAB isolated during the production of Petrovac sausage.

Group	I	II	III	IV
No. of isolates	188	172	131	4
Cell morphology	rods	Rods	coccoid	cocci
CO_2 formation	–	–	+	–
Growth at				
45°C	–	–	–	–
15°C	+	+	+	+
Growth on NaCl				
4%	–	–		–
6.5%	+	+	+	+
8%	+	+	+	+
Hydrolysis of arginine	–	–	–	–
Hydrolysis of esculin	–	–	+	+
Black colonies on bile esculin agar	–	–	–	–
production of EPS from sucrose	–	+	+	–
Production of bacteriocines	v	V	–	v
Identified by 16S rDNA gene sequencing	*L. brevis*	*L. plantarum*	*L. mesenteroides*	*P. pentosaceus*

'+': positive; '–': negative; 'v': variable, 'EPS': exopolysaccharides.

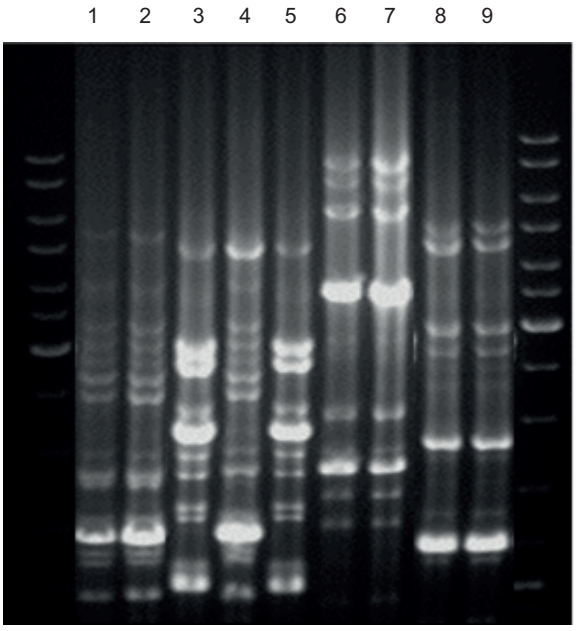

Figure 2. Reference PCR profiles of the amplified 16S rRNA gene of the isolates: *L. brevis* (1, 2, 4), *L. plantarum* (3, 5), *P. pentosaceus* (6, 7) and *L. mesenteroides* (8, 9).

produce CO_2 and was not able to grow at 45°C. On the basis of morphological characteristics, two groups of lactobacilli were observed. (GTG)5-PCR fingerprinting (Figure 2) confirmed that two groups belonged to *L. brevis* and *L. plantarum.* Some *L. brevis* and *L. plantarum* strains synthesised bacteriocines, which was in accordance to the data found in literature that these species could be active against *L. monocytogenes* (Tosukhowong *et al.*, 2011). Also, nitrite-reduction capability is one of the most important characteristics of *L. brevis* (Paik and Lee, 2014). Additionally, *L. plantarum* leads to rapid decrease of pH in fermented sausages and contributes to the organoleptic properties of the fermented product (Heinz and Hautzinger, 2007).

The arginine-negative and esculin-positive isolates that produced CO_2 from glucose and had the ability of forming slimy colonies on MRS agar plates with sucrose were identified by 16S rDNA sequencing as *L. mesenteroides. Leuconostoc* spp. produce lactic acid, acetic acid, dextran, acetaldehyde, diacetyl, ethanol and other metabolites that contributes to the development of aroma and flavour in production of fermented sausages (Lee *et al.,* 2006). As heterofermentative strains, *Leuconostoc* spp. produce CO_2, which is considered as one of the main causes in forming holes in meat products; this property classifies them as undesirable microbiota (Ammor and Mayo, 2007). *Leuconostoc* spp. may synthesise spectra of bacteriocines (mesentericin Y105, produced by *L. mesenteroides* spp. *mesenteroides*; leucocin A-UAL 187, produced by *L. gelidum*; carnosin 44A, produced by *L. carnosum*; and leuconocin S, produced by *L. paramesenteroides*) that exhibit strong microbial activity against *Listeria* spp (Stiles, 1994). The prevalence of *Leuconostoc* spp. in sausages is in correlation with the results obtained for Petrovac sausages produced from hot deboned meat (Danilovic *et al,* 2011) as well as for sausages ripened under the traditional and controlled conditions (Danilovic *et al.*, 2018).

Only four isolates (0.8%) were esculine-positive cocci. They all had the ability to grow at 15°C as well as on MRS agar plates with addition of NaCl (6%, 5% and 8%). Some of cocci produced bacteriocines (Table 1). Esculine-positive cocci, which formed tetrads, were identified by 16S rDNA sequencing as *P. pentosaceus* (Figure 2). As a result of low catabolism of amino acids, pediococci don't play a major role in the formation of organoleptic properties in fermented sausages (Leroy *et al*., 2006). Among pediococci, *P. acidilactici* and *P. pentosaceus* were often isolated from European sausages (Albano *et al.* 2007; Kozachinski *et al.* 2008). *P. acidilactici* produced pediocin that inhibits the growth of food-borne pathogens *L. monocytogenes* and *Clostridium perfringens* in Spanish dry-fermented sausages (Nieto-Lozano *et al*., 2010). In addition, *P. pentosaceus* showed strong inhibitory effect against *S. aureus* (Erdogrul *et al*., 2002). *P. pentosaceus* and *P*. acidilactici are commonly used as starters in the United States in producing dry sausages (Rantsiou and Cocolin, 2006). Besides bacteriocines, some strains of pediococci produce EPS (Semjonovs and Zikmanis, 2008). The low frequency of isolation of pediococci is correlated with the results obtained for Bosnian Sudzuk, Alheira sausage and Croatian sausage (Albano *et al.,* 2009; Kozachinski *et al.,* 2008). In Petrovac sausages produced from hot deboned meat, pediococci were isolated at the highest percentage after ninth day of production process (Danilovic *et al.,* 2011).

Total isolated LAB microbiota constituted *L. brevis* (37.9%), *L. plantarum* (34.7%), *L. mesenteroides* (26.4%) and *P. pentosaceus* (0.8%). Sausages prepared without starter cultures (H1 and H2) were characterised by the prevalence of *leuconostoc* spp. during the first 15 days of fermentation regardless of temperature. Complete replacement of *leuconostoc* spp. was observed after 15 days and lactobacilli were the dominant microbiota. On the 60th day of production process, *L. plantarum* rapidly increased up to 80% in sausages H1, while in sausages H2, almost equal distribution of *L. brevis* and *L. plantarum* was detected. Later stages of production process were characterised by the prevalence of *L. plantarum. P. pentosaceus* was isolated only from sausages H2 in a 90-day-old sample with a representation of 1.4% (Figure 3). On the other hand, in sausages prepared with the addition of combined starter cultures of *S. xylosus* and *L. plantarum* (sausages I1 and I2), the highest percentage of

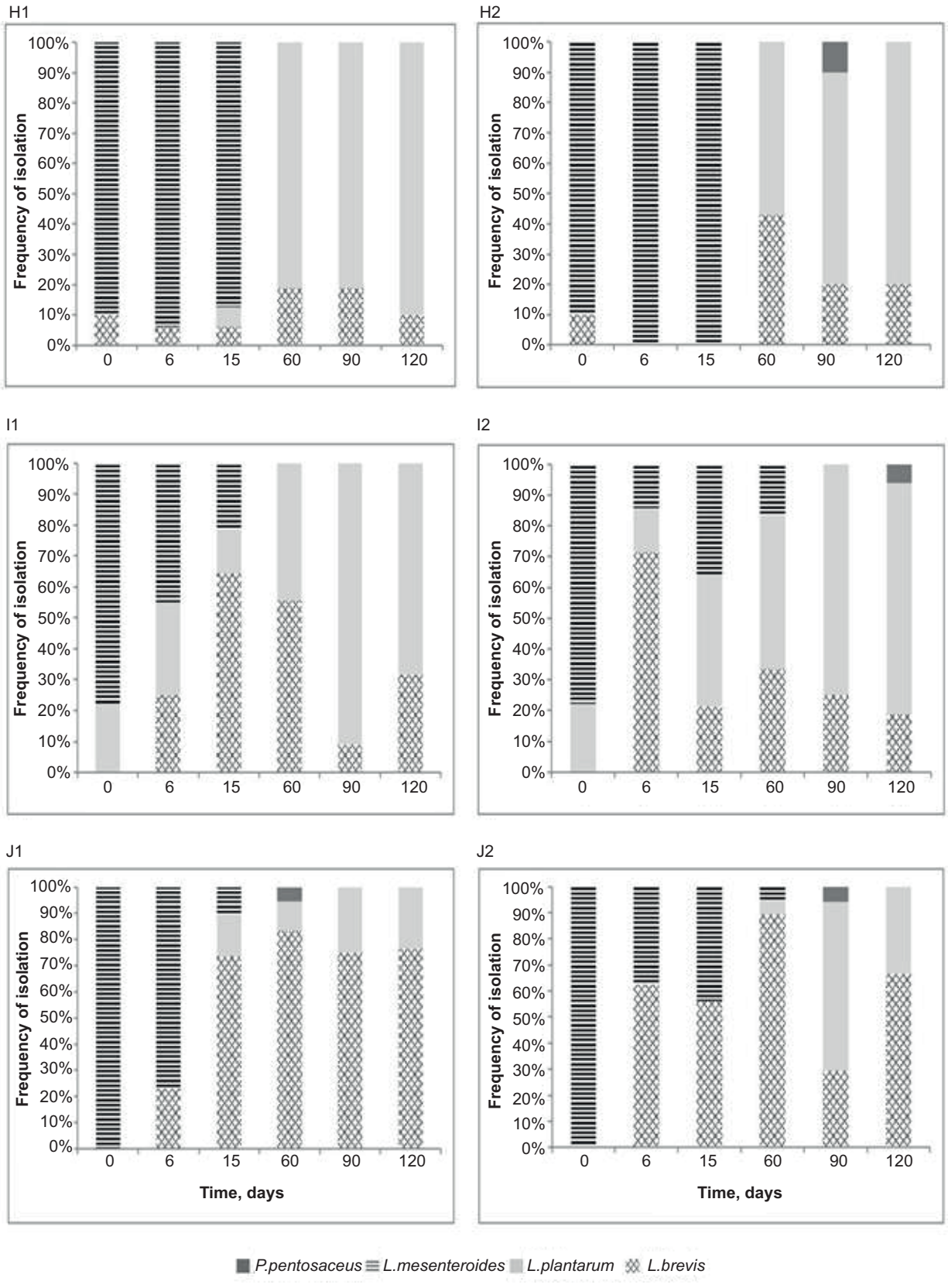

Figure 3. Changes in microbial population during the production of Petrovac sausages prepared without starter cultures (sausages H), with combined starter cultures of *S. xylosus* and *L. plantarum* (sausages I) and with starter culture of *S. xylosus* (sausages J) produced at 14–16°C (samples with tag 1) and ~10°C (samples with tag 2).

leuconostoc strains was detected only in the meat batter. *Leuconostoc* spp. decreased immediately after preparation of sausage mixture but remained still up to the 15th day of production in sausages I1 and up to the 60th day of production in sausages I2. After this period, depletion of leuconostoc strain was observed and lactobacilli were the dominant microbiota (Figure 3). Pediococci were isolated only at the end of production process in sausages I2 with a share of 1%. In sausages prepared with the addition of starter culture of *S. xylosus* (sausages J1 and J2), the domination of *L. brevis* was detected at all stages of production process except in the meat batter, where the full presence of *L. mesenteroides* (100%) was detected. *P. pentosaceus* was detected on the 60th and 90th day of production in sausages J1 and J2, respectively. This increase in the content of lactobacilli was observed during production, with the presence of 100% lactobacilli in the sample after 120 days of production.

During the production of Petrovac sausage, the prevalence of *L. mesenteroides* was observed in the meat batter prepared with and without adding starter cultures. Also, *L. mesenteroides* strains were present during the early stages of fermentation process regardless of temperature. The high frequency of *L. mesenteroides* at the beginning of production process was in accordance with the results obtained for Serbian traditional fermented sausages Sremski kulen, Lemeski kulen (Vasilev *et al*., 2015) and Užička sausage (Borovic *et al*., 2017). On the contrary, these results were not in accordance with the results obtained for Italian fermented sausage (Comi *et al*., 2005; Urso *et al*., 2006), where low frequency of *leuconostoc* spp. was detected at the beginning of the production process. Regardless of the production conditions, in all sausages, lactobacilli were the dominant microbiota from 15 days till the end of production process. This is in accordance with the results obtained for Užička sausage (Borovic *et al*., 2017). Also, the high frequency of lactobacilli was presented in Sremski and Lemeški kulen (77.1 and 54.3%, respectively). *L. brevis* was the most dominant lactobacilli species in these sausages (61.5% and 57.9%, respectively) (Vasilev *et al*., 2015). High frequency of *L. brevis* was in accordance with the results obtained for traditional fermented Užička sausage (Borovic *et al*., 2017); *P. pentosaceus* was isolated in the smallest percentage in the final stages of production, while in sausages ripened under traditional and controlled conditions, pediococci were present only in the meat batter (1.7% of the total microbiota) (Danilovic *et al.,* 2018). Pediococci were isolated in small percentage from Iberian dry-fermented sausages—Salcichon and Chorizo (Benito *et al.,* 2008) and Italian fermented sausages—Salami (Bonomo *et al.,* 2008). On the contrary, Pediococci were isolated at high frequency from the fermented sausages produced in the United States, where *P. acidilactici* and *P. pentosaceus* are commonly added as starter cultures (Anba-Mondoloni *et al*., 2015).

Conclusion

Petrovac sausage is an artisanal Serbian sausage appreciated for its sensory characteristics. In order to preserve the quality of the industrial production process, there is a need to understand the effect of starter cultures on the level of microbiota and composition. The results indicate that application of starter culture *S. xylosus* and combined starter culture *S. xylosus* and *L. plantarum* didn't influence the total number of LAB during process. On the other hand, temperature range of 14–16°C increased the number of staphylococci, compared with the application of ~10°C temperature. Comparison of the effect of different starter cultures with the composition of microbiota resulted in the achievement of similar microbiota composition as for traditional sausages when combined starter culture was used. According to the results, combined starter culture of *S. xylosus* and *L. plantarum* could be the most promising solution for the production of Petrovac sausage, although further sensory analysis is required to be conducted.

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