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Genetic polymorphism of lactic acid bacteria isolated from "Pirot 'ironed' sausage" from Serbia

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Abstract: The "Pirot 'ironed' sausage" (Pis) is a traditional, dry, fermented product originating from the town of Pirot, situated in southeastern Serbia. This product is made from different types of meat (goat, sheep, beef and donkey) and spices, without additives or starter cultures. Pis is an organic, unprocessed product, without heat or smoke treatment. The aim of this study was to characterize 120 isolates of lactic acid bacteria (LAB) from Pis produced by six different brands during a two-year period using phenotypic and genetic identification. Preliminary characterization of the LAB was based on general morphology and biochemical tests. Repetitive elements such as REP, BOX and GTG_5, found in the genome of these bacteria, and randomly amplified polymorphic DNA-polymerase chain reaction (RAPD PCR) sequences were used for determination of genetic polymorphism. Identification by 16S rRNA gene sequencing showed the presence of only two LAB species, *Lactobacillus sakei* (76%) and *Leuconostoc mesenteroides* (24%). However, genetic polymorphism was detected using fingerprinting methods. In comparison with other primers, the profiles obtained with GTG_5 showed the highest heterogeneity for most of the tested isolates, with sequencing results additionally confirming its discriminatory power. In addition, M13 RAPD primer also produced satisfactory separation of the tested isolates.

Keywords: Pirot 'ironed' sausage; GTG_5 PCR; *Lactobacillus sakei*; *Leuconostoc mesenteroides*

INTRODUCTION

The Pirot 'ironed' sausage is a traditional, dry, fermented product made from top quality meat, mixed with spices, such as paprika (sweet or hot), salt, pepper and garlic. This traditional product originates from the town of Pirot which is situated in southeastern Serbia. For production of the so-called flat, 'ironed' sausage, several different types of meat are used, principally selected pieces of non-fat goat meat, donkey or horse meat, sheep and beef. The best taste is achieved by combining different types of meat. While the ingredients are not secret, the recipe is always kept in the family circle. There is no universal recipe, and each producer has their own process of production. Pis is an organic, unprocessed product without preservatives, starter cultures, artificial additives, heat treatment or exposure to smoke. The sausage is ironed with a glass bottle to increase its surface. In this form, the sausage becomes thinner and making it easier to release excess water. The ironing procedure is repeated twice every day during ripening. Drying of the sausage is carried out once a year, during wintertime, and the ripening process starts with the first cold days when the temperature is between -5°C and +5°C. All these factors and the specific microbial load lead to a great variety of flavor, taste and recognizable texture of the product. The specific process of production, the unusual combination of meat and the absence of any type of technological processing make the sausage very interesting for research, as fluctuations of bacterial communities depend on the meat type and drying methods. Generally, the sausages produced using traditional methods with long ripening times have a better flavor and overall quality when compared to

industrially produced sausages with defined starter cultures that depend on the composition and metabolic activity of domestic microflora [1]. The microbial profile of fermented sausages consists mainly of LAB, which are specially adapted to the ecology of meat fermentation. LAB have a great influence on the entire process of fermentation and preservation by preventing the growth of other opportunistic bacteria [1-3]. The ability of LAB to decrease the pH by producing acid from sugars prevents the growth of pathogens and ensures the stability and safety of the final product [4]. In addition, LAB also show some other characteristics that can contribute to a better quality of meat products, such as production of aromatogenic substances and bacteriocins. Thus, they can be used for food preservation, as starter cultures in the production of various products, but also as probiotics [5]. Among LAB, the most commonly determined species in meat products are *Lactobacillus sakei*, *L. curvatus* and *L. plantarum,* with *L. sakei* the most frequently isolated species [4,6]. Classical tests used for LAB identification can be problematic, difficult to interpret, time-consuming, and sometimes, in cases of uncultivable organisms, impossible [7,8]. Therefore, the correct classification and identification of LAB are difficult without the support of genotypic techniques which are a faster alternative [9,10]. These methods are reliable, straightforward and their application avoids subjective interpretation [11]. Thus, PCR amplification of repetitive bacterial DNA elements (rep-PCR) is recognized as a simple PCR-based technique with a high discriminatory power and low cost and it is used for classifying and typing a wide range of Gram-negative and several Gram-positive bacteria [8]. Rep-PCR fingerprinting methods can give comparative results, even when using only one primer, such as GTG_5, which was proven to be one of the best elements for *Lactobacillus* determination [2,8,10,12]. On the other hand, RAPD requires the use of more than one primer for strain characterization in most cases [13].

The aim of this study was to determine the phenotypic and genetic identification of LAB isolates from Pis produced by six different brands over a two-year period, and to establish their potential differences due to their different meat content. In addition, we investigated which fingerprinting method best reflected the inter- and intraspecies genetic heterogeneity among *Lactobacillus* and *Leuconostoc* isolates.

MATERIALS AND METHODS

Bacterial strains

A total of 120 bacterial isolates from Pis manufactured by six different producers in their personal households located around the city of Pirot in Serbia (43.1557° N, 22.5857° E) were used for this research. The Pis were made exclusively in the home environment, i.e. they did not require the existence of any production unit, and the production capacity was in smaller quantities. The analysis was comprised of isolates sampled during a two-year period (2013 and 2014) from producers 1 (27 isolates), 3 (32 isolates) and 4 (29 isolates), whereas in the case of producers 2 (10 isolates), 5 (12 isolates) and 6 (10 isolates), only samples isolated in 2014 were used. The isolates obtained in 2013 are marked with the prefix pK, whereas isolates from 2014 have no prefix, and the first number is the serial number of the producer. Different producers were chosen based on the meat types used in the sausage-making process, on variations in their percentage distribution, as well as on different drying methods, as shown in Supplementary Table S1.

The isolates were sampled from randomly chosen sausages produced by a certain producer (two sausages per producer were used and pooled into one sample), by making cuts in 5-6 places with a sterile scalpel. Ten grams of the inner part of the sausages was sampled and immersed into 90 mL of saline solution with the addition of 0.9 g of peptone [4]. Serial dilutions were made after 20 min of shaking, and these were plated on de Man, Rogosa and Sharpe (MRS) agar and incubated at 30°C for 24 h under aerobic conditions. A total of 120 isolates were chosen according to colony morphology as LAB.

Bacterial isolation and biochemical characterization were performed in the Laboratory of the Agrifood School of Professional Studies during 2013 and 2014, while the molecular analysis carried out at the Department of Microbiology at the Faculty of Biology University of Belgrade during 2015 and 2016.

Biochemical characterization of bacterial strains

One hundred and twenty isolates were subjected to morphological and biochemical analyses. After Gram staining, all isolates were checked for gas production

from glucose, the ability to grow at different temperatures (+15 and +45°C), tolerance to different salt concentrations (4 and 6.5% of NaCl), as well as for arginine and aesculin hydrolysis [14].

DNA fingerprint analysis

Genomic DNA was isolated according to the method described in Dimkić et al. [15]. Rep-PCR and RAPD PCR fingerprinting analysis were performed according to the optimized programs for each of the primers (REP1R-I (IIIICGICGICATCIGGC) and REP2-I (IIICGNCGNCATCNGGC) [10]; BOXA1R (CTACGGCAAGGCGACGCTGACG) [6]; GTG_5 (GTGGTGGTGGTGGTG) [10]; and M13 (GAGGGTGGCGGTTCT) [16]). The PCR amplification was performed in a 25-μL reaction mixture containing: 0.1-1 μg of template DNA, 10×KAPA Taq Buffer, $MgCl_2$ at a final concentration of 25 mM, a 200-μmol/L concentration of each dNTP, each primer at a final concentration of 10μM and 5 U/μL of KAPA Taq polymerase (KAPA Biosystems, USA). The PCR reactions were performed under the following conditions: initial denaturation step at 95°C for 7 min (for REP and BOX PCR), and for 5 min (for GTG_5 and RAPD PCR), followed by 35 cycles at 94°C for 1 min (for REP and RAPD PCR) and 30 s (for GTG_5 PCR), and 30 cycles at 94°C for 1 min (for BOX PCR); primer annealing at 40°C for 1 min (for REP PCR) or for 20 s (for RAPD PCR), or primer annealing for 1 min (for GTG_5 PCR) at 45°C, and for 1 min (for BOX PCR) at 52°C, and a 65°C polymerization step for 8 min (for REP and BOX), or at 72°C for 4 min (for GTG_5 PCR) and for 2 min (for RAPD PCR). The final extension step was performed at 65°C for 16 min (for REP and BOX), or at 72°C for 7 min for (for GTG_5 PCR), and at 75°C for 5 min in the case of RAPD PCR.

Phylogenetic analysis

The total DNA from the selected isolates was used as a template for PCR amplification of the 16S rRNA gene, with universal primers $UN1_{16s}F$ (GAGAGTTTGATCCTGGC) and $UN1_{16s}R$ (AGGAGGTGATCCAGCCG). The PCR reaction mixture was prepared as described above. The PCR reactions were performed with primer annealing at 50°C for 1 min. The PCR products were purified using a column of QIAquick PCR Purification and Gel Extraction KIT/250 (QIAGEN GmbH, Hilden, Germany) and sequenced commercially by the Macrogen sequencing service (Netherlands). The obtained sequences were searched for homology with deposited genes in the GenBank database using the National Center for Biotechnology Information Blast search program for nucleotides. All sequences were aligned using ClustalW multiple sequence alignment implemented in BioEdit 7.1.3 program, and phylogenetic trees were constructed in MEGA 6 using the neighbor-joining method based on a pair-wise distance matrix with the Kimura two-parameter nucleotide substitution model. The obtained DNA fingerprint profiles were used for dendrogram construction by free download software PyElph 1.4 [17].

RESULTS

Bacterial strains and biochemical characterization

Preliminary phenotypic identification of 120 LAB isolates showed two different types of colony morphologies. The first morphology was characterized as small, white, smooth and opaque (type I), and the second as slimy and transparent (type II). Gram staining showed that all isolates belong to Gram-positive rods or coccoid rods, indicating that most of them probably belong to *Lactobacillus* and *Leuconostoc* genera, respectively. Biochemical tests showed that all isolates had the ability to grow at 15°C in the presence of 4% and 6.5% of NaCl, while production of CO_2 from glucose, growth at 45°C, arginine and aesculin hydrolysis were not detected.

DNA fingerprint profiling

With the aim of determining the best discriminatory fingerprinting technique for LAB and for evaluating heterogeneity of bacterial isolates from six different producers of Pis, 77 isolates, all sampled in 2014, were tested using REP, BOX and GTG_5 rep-PCR primers, as well as the M13 RAPD-PCR primer. Dendrograms were constructed using the UPGMA algorithm for interpretation of rep- and RAPD PCR fingerprinting patterns. The summarized results regarding the

number of different fingerprinting profiles obtained by dendrogram analysis are presented in Table 1.

Table 1. The number of different fingerprinting profiles obtained using different primers for all analyzed producers.

Producers	Number of tested isolates	REP	BOX	GTG_5	M13
1	15	7	10	6	6
2	10	5	4	7	3
3	17	6	11	11	10
4	14	6	3	8	7
5	12	5	8	7	7
6	9	4	2	2	4
Σ	77	33	38	41	37

Compared to the other primers used in this study, GTG_5 PCR produced the greatest number of different patterns – 41 out of 77 tested isolates (Fig. 1). Slightly less discriminatory than GTG_5 PCR were BOX and M13 primers (Supplementary Figs. 1 and 2), which produced 38 and 37 different patterns, respectively. Using REP-PCR, 33 different patterns were obtained (Supplementary Fig. 3). After choosing GTG_5 PCR as the best profiling method, we screened the entire collection (all 120 isolates) using this primer and selected 51 isolates with different patterns to be subjected to 16S rRNA gene sequencing. The isolates were clearly grouped in two clusters on the phylogenetic tree (Fig. 2). The majority of the isolates (76%) were grouped together with the referent strain *L. sakei* NBRC 15893, and the rest of the isolates (24%) were grouped with the referent strain *Leuconostoc mesenteroides* ATCC 8293. In comparison with other primers, GTG_5 revealed the highest heterogeneity between the tested sausage producers (Fig. 1). In this study, all isolated strains belonging to producers 1 and 6 are listed as *L. sakei*, even though they were placed into six different clusters based on their GTG_5 pattern similarities (sausage from producer 1). However, the LAB content of sausages made by producer 2 showed a higher presence of *L. mesenteroides* (isolates 2-1; 2-6; 2-7; 2-8; 2-17, and 2-18). In the Pis from producers 3, 4 and 5, *L. sakei* was the dominant species and *L. mesenteroides* was presented

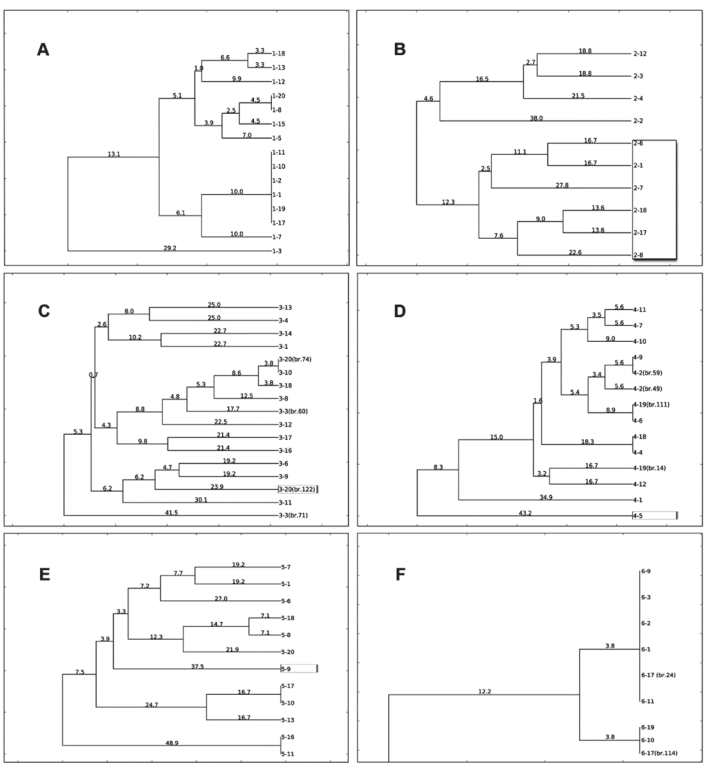

Fig. 1. Dendrograms obtained by UPGMA analysis of the tested isolates from producers 1 (**A**), 2 (**B**), 3 (**C**), 4 (**D**), 5 (**E**) and 6 (**F**) using GTG_5 PCR. Genetic distances are presented with numbers placed on the branches. Isolates in rectangles were identified as *L. mesenteroides*.

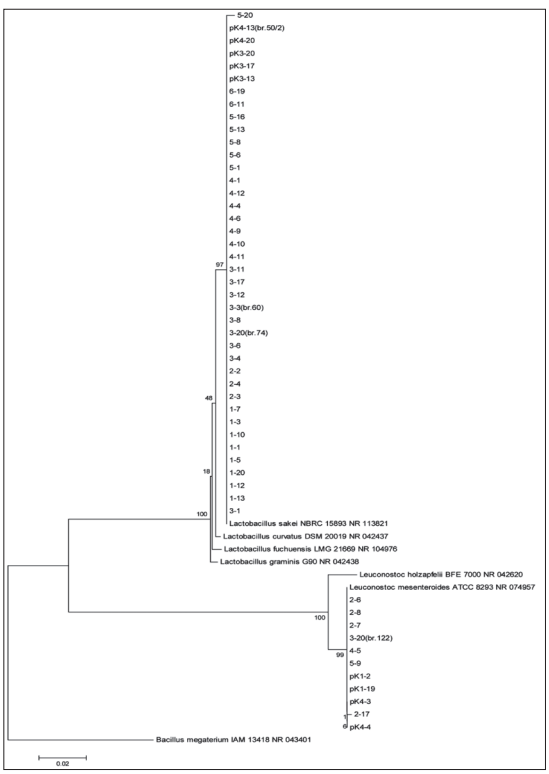

Fig. 2. Neighbor-joining phylogenetic tree according to 16S rRNA gene sequencing of the chosen isolates from six different producers of Pis.

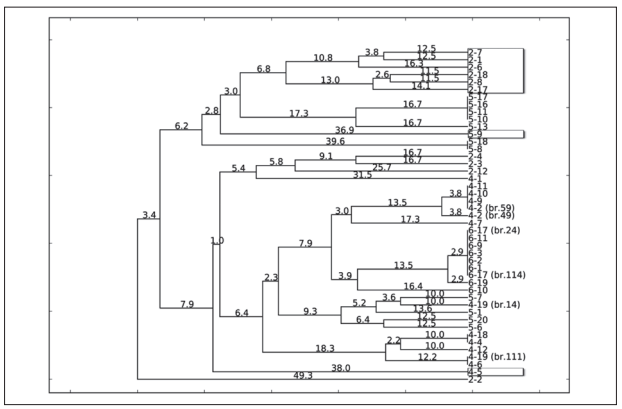

Fig. 3. Dendrograms obtained by UPGMA analysis of the tested isolates from producers 2, 4, 5 and 6 using GTG_5 PCR. Genetic distances are presented with numbers placed on the branches. Isolates in rectangles are identified as *L. mesenteroides.*

only as one monophyletic branch with isolates 3-20, 4-5 and 5-9, respectively. All of them exhibited high similarity levels with other strains of *L. sakei*.

Comparative analysis of isolates from sausages made by different producers

In order to detect potential differences in dominant microflora among sausages that differ only in the percentage of types of meat and in the duration of ventilation, from producers 2 (beef 30%, sheep 15%, goat 15%, donkey 40%; 23 days of ripening), 4 (beef 40%, sheep 10%, goat 25%, donkey 25%; 30 days of ripening), 5 (beef 50%, sheep 20%, goat 20%, donkey 10%; 28 days of ripening) and 6 (beef 60%, sheep 25%, goat 10%, donkey 5%; 20 days of ripening), the GTG_5 PCR patterns were compared (Fig. 3). Two clusters and one separate monophyletic branch with the isolate 2-2 were obtained on the dendrogram. Isolates from producers 2 and 5 were mainly grouped within the first cluster, whereas most of the LAB isolated from the Pis of producers 4 and 6 were associated in the second cluster. The dominant meat type in sausages produced by producers 4, 5 and 6 was beef and for producer 2 donkey meat was the main component (Supplementary Table S1). Also, sausages from producers 2 and 5 had almost equal percentages of goat and sheep meat. Regarding species distribution, *L. mesenteroides* isolates were grouped together and clearly separated from the *L. sakei* strains, especially for producer 2. For producers 4 and 5, two *L. mesenteroides* strains (4-5 and 5-9) were also observed as distant single branches.

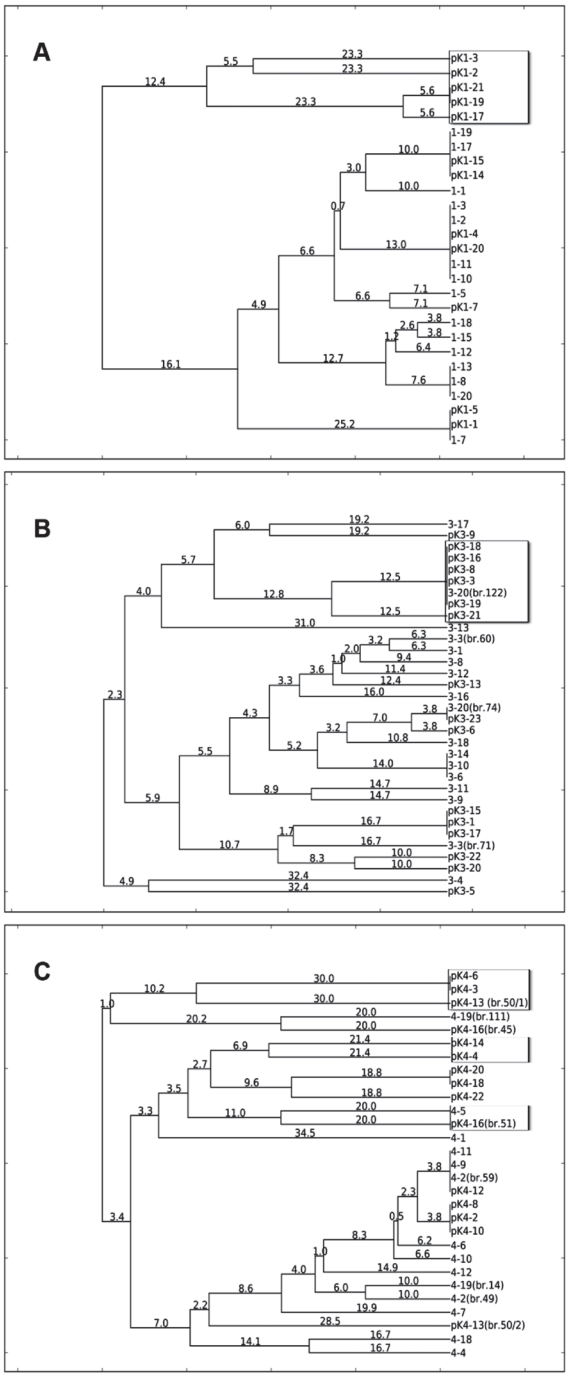

Fig. 4. Dendrograms obtained by UPGMA analysis of the tested isolates from producers 1 (**A**), 3 (**B**) and 4 (**C**) after using GTG_5 PCR. Genetic distances were presented with numbers placed on the branches. Isolates in rectangles are identified as *L. mesenteroides.* Isolates obtained in 2013 are marked with the prefix pK; isolates from 2014 have no prefix and the first number represents the serial number of the producer.

To ascertain whether there was a difference in the distribution of LAB isolates during the two-year period, dendrogram analysis of isolates from producers 1, 3 and 4 was performed (Fig. 4). Almost all isolates of producer 1 (Fig. 4A) from 2013 were clustered together with the isolates from 2014, and according to sequence analysis, they belong to *L. sakei* species. However, five isolates (pK1-2, pK1-3, pK1-17, pK1-19, pK1-21), identified as *L. mesenteroides*, were detected only in the samples from 2013. Three distinct clusters were obtained on the dendrogram for producer 3 (Fig. 4B). A number of the isolates from 2013 were clustered together with one from 2014 (3-20(br.122)) and identified as *L. mesenteroides* (pK3-3; pK3-8; pK-3-16; pK-3-18; pK3-19, and pK3-21), while the other strains were identified as *L. sakei*. The isolate distribution for producer 4 is presented in Fig. 4C. Three major clusters were formed. The first cluster was dominated by *L. mesenteroides* isolates from both years. In the second cluster, isolates from 2013 prevailed (both *L. mesenteroides* and *L. sakei*). The largest, third group was composed of isolates from 2014, with *L. sakei* as the exclusive species.

DISCUSSION

The preliminary phenotypic and biochemical identification of 120 LAB isolates was in accordance with previous studies of these isolates [2,18], indicating that they belong to *Lactobacillus* and *Leuconostoc* genera.

Phenotypic identification and characterization of LAB is insufficient, primarily because many bacterial strains have similar cell morphology, physiology and growth under the same conditions. Consequently, there is a need to complement the phenotypic characterization results with genetic characterization [13]. Highly conserved repetitive DNA such as REP, BOX, GTG_5 and M13 RAPD profiling elements are widely distributed in the genomes of many bacterial groups [19]. Because of such a distribution in the small genomes of prokaryotic organisms, they can be used as rapid and reliable methods for typing a wide range of Gram-negative and several Gram-positive bacteria [20-22]. Rep-PCR techniques have been used previously and compared for their ability to type *Lactobacillus* strains where oligonucleotide primes such as ERIC [23] or BOX [8,10] were used together with GTG_5. Hyytiä-Trees et al. [24] suggested that by using a combination of rep-PCR (BOX and REP) and RAPD primers an adequate level of discrimination among *L. sakei* strains can be achieved. Contrary to that, Tran et al. [8] indicated that molecular patterns generated by REP and BOX primers were not discriminatory enough for isolates from Vietnamese fermented sausages, unlike GTG_5, which created more complex and diverse patterns. Gevers et al. [10] also found that the discriminatory significance was not improved when combining BOX, REP and GTG_5 banding patterns, and that typing of LAB strains can be performed only using the GTG_5 primer instead of multiple primers. This fingerprinting primer can be used for identification and possibly for intraspecies differentiation and is especially useful for screening a large number of strains [10]. Based on sequencing data and comparison between fingerprinting profiles, it was clear that GTG_5 PCR could be a method of choice for further research into polymorphism within species of LAB. The sequencing results of isolates from the Pis pointed to the existence of only two species, *L. sakei* and *L. mesenteroides*. Among lactobacilli, *L. sakei* is the species that is best adapted to the fermentation processes, so it is not surprising that it was found in this type of meat product [1,4]. The domination of *L. sakei* in fermented sausages was also reported by several authors [16,25,26]. *Leuconostoc mesenteroides* appeared in a small percentage in the tested sausages. Its low presence was also confirmed in earlier studies [4,26,27]. In Petrovska sausage, which is also a traditionally manufactured product, *L. mesenteroides* (37.1%) comprises the dominant microflora with lactobacilli [2]. Generally, *Leuconostoc* strains are heterofermentative and undesirable in meat products because of their ability to produce CO_2 and form holes in the products [28]. On the other hand, they are able to produce acetic acid, acetaldehyde, diacetyl and ethanol, which form the characteristic taste and aroma of the product [29].

According to our analysis, the meat content and quantities of different kinds of meat, as well as the duration of drying, did not significantly affect the LAB microbial composition of the sausages (Fig. 3). There was no correlation between a particular mixture of meat in the sausages and distinct LAB isolate profiles. Rantsiou et al. [27] tested three types of sausages with different amounts and ratios of different meats, showing that in spite of these distinctions and differences in fermentation and drying length, the same

Lactobacillus species were always present (*L. curvatus* in Greek sausages; *L. sakei* in Hungarian; *L. curvatus* and *L. sakei* in Italian sausages). *L. plantarum*, *L. sakei* and *L. curvatus* are species found in all three types of sausages. Papamanoli et al. [1] also showed the absence of significant differences in microbiomes among two types of Greek sausages, both made of beef and pork meat, but with different meat contents, fermentation and duration of drying. Cluster analysis presented in an earlier study of *Lactobacillus* profiles [26] revealed that some strains were grouped in fermentation-specific clusters, which arose from the ingredients used in production. In the same study, *L. sakei* showed a higher degree of heterogeneity, which was confirmed by strains that did not group evenly. Results of this study revealed the presence of *L. mesenteroides* in 2013, which could be a consequence of factors that we did not follow. One of the reasons could be due to the year's batch of sheep small intestines used for sausage filling and/or the batch of spices used for curing the sausage (salt, garlic, pepper, and sweet or chili cayenne pepper). Apart from the marked presence of *L. mesenteroides* in 2013, our result suggests that the isolate distribution of *L. sakei* was not affected in any conceivable way by the year of production. It is an established fact that traditionally-made fermented sausages rely wholly on environmental microflora [30]. Thus, differences can appear as a consequence of different environmental factors, such as temperature variations or humidity, and even the human factor. Meat can come into contact with environmental microflora in many different ways. Some bacteria linger on animal skin or inside the natural casings used in the process of making traditional sausages. Also, every segment of preparing can lead to new contacts with environmental bacteria – knives, the working surface, skin or clothes of the manufacturer.

In conclusion, the microbial heterogeneity of the Pis was not significantly different from other naturally-fermented sausages previously described. However, this study recommends GTG_5 fingerprinting as the best rep-PCR technique for LAB typing, with the strongest discriminatory power. The high genetic heterogeneity of *L. sakei* isolates could be important for the specific nature of naturally-fermented Pis. Also, the higher number of *L. sakei* isolates in all 6 producers could be an advantage because of their potential characteristics (starter culture, production of potent bacteriocins), which can repress unwanted bacterial growth that might cause sliminess, malodor and other product defects. The differences in drying time, usage of unusual meat combinations, as well as the large number of producers, provide a good starting point for a diverse microflora and for the characterization of novel potential starter cultures, particularly because this product is made according to traditional procedures. All of these characteristics revealing the high quality of this authentic Serbian specialty that has great potential for expansion on world markets.

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Author contributions: ID, SS and TB conceived and designed the experiments; ID, SB and AJ wrote the paper; ID and TB checked the results and the language; SB, AJ, IN and BD performed the experiments; SS contributed the reagents, materials and analysis tools.

Conflict of interest disclosure: The authors declare that there is no conflict of interest.

REFERENCES

1. Papamanoli E, Tzanetakis N, Litopoulou-Tzanetaki E, Kotzekidou P. Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. Meat Sci. 2003;65(2):859-67.
2. Danilović B, Joković N, Petrović L, Veljović K, Tolinački M, Savić D. The characterisation of lactic acid bacteria during the fermentation of an artisan Serbian sausage (Petrovská Klobása). Meat Sci. 2011;88(4):668-74.
3. Hugas M, Monfort JM. Bacterial starter cultures for meat fermentation. Food Chem. 1997;59(4):547-54.
4. Urso R, Comi G, Cocolin L. Ecology of lactic acid bacteria in Italian fermented sausages: isolation, identification and molecular characterization. Syst Appl Microbiol. 2006;29(8):671-80.
5. Riboulet-Bisson E, Sturme MH, Jeffery IB, O'Donnell MM, Neville BA, Forde BM, Lawlor PG. Effect of *Lactobacillus salivarius* bacteriocin Abp118 on the mouse and pig intestinal microbiota. PLoS One. 2012;7(2):e31113.
6. Adiguzel CG, Atasever M. Phenotypic and genotypic characterization of lactic acid bacteria isolated from Turkish dry fermented sausage. Rom Biotechnol Lett. 2009;14(1):4130-8.
7. Rantsiou K, Cocolin L. New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: a review. Int J Food Microbiol. 2006;108(2):255-67.

8. Tran KT, May BK, Smooker PM, Van TT, Coloe PJ. Distribution and genetic diversity of lactic acid bacteria from traditional fermented sausage. Food Res Int. 2011;44(1):338-44.
9. Gancheva A, Pot B, Vanhonacker K, Hoste B, Kersters K. A polyphasic approach towards the identification of strains belonging to *Lactobacillus acidophilus* and related species. Syst Appl Microbiol. 1999; 22(4):573-85.
10. Gevers D, Huys G, Swings J. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiol Lett. 2001;205(1):31-6.
11. Rantsiou K, Drosinos EH, Gialitaki M, Metaxopoulos I, Comi G, Cocolin L. Use of molecular tools to characterize *Lactobacillus* spp. isolated from Greek traditional fermented sausages. Int J Food Microbiol. 2006;112(3):215-22.
12. Van Hoorde K, Vandamme P, Huys G. Molecular identification and typing of lactic acid bacteria associated with the production of two artisanal raw milk cheeses. Dairy Sci Technol. 2008;88(4-5):445-55.
13. Mohammed M, El-Aziz HA, Omran N, Anwar S, Awad S, El-Soda M. Rep-PCR characterization and biochemical selection of lactic acid bacteria isolated from the Delta area of Egypt. Int J Food Microbiol. 2009;128(3):417-23.
14. Greco M, Mazzette R, De Santis EPL, Corona A, Cosseddu AM. Evolution and identification of lactic acid bacteria isolated during the ripening of Sardinian sausages. Meat Sci. 2005;69(4):733-9.
15. Dimkić I, Stanković S, Nišavić M, Petković M, Ristivojević P, Fira D, Berić, T. The profile and antimicrobial activity of *Bacillus* lipopeptide extracts of five potential biocontrol strains. Front Microbiol. 2017;8:925.
16. Bonomo MG, Ricciardi A, Zotta T, Parente E, Salzano G. Molecular and technological characterization of lactic acid bacteria from traditional fermented sausages of Basilicata region (Southern Italy). Meat Sci. 2008;80(4):1238-48.
17. Pavel AB, Vasile CI. PyElph-a software tool for gel images analysis and phylogenetics. BMC bioinformatics. 2012;13(1):9.
18. Čolo J, Mihajlović S, Tolinački M, Alkić M, Popović D, Kojić M, Terzić-Vidojević A. Characterization of lactic acid bacteria isolated from Bosnian artisanal dry fermented sausage (sudžuk) during fermentation. Genetika. 2015;47(3):819-32.
19. De Vuyst L, Camu N, De Winter T, Vandemeulebroecke K, Van de Perre V, Vancanneyt M, Cleenwerck I. Validation of the $(GTG)_5$-rep-PCR fingerprinting technique for rapid classification and identification of acetic acid bacteria, with a focus on isolates from Ghanaian fermented cocoa beans. Int J Food Microbiol. 2008;125(1):79-90.
20. De Urazza PJ, Gomez-Zavaglia A, Lozano ME, Romanowski V, De Antoni G. DNA fingerprinting of thermophilic lactic acid bacteria using repetitive sequence-based polymerase chain reaction. J Dairy Res. 2000;67(63):381-92.
21. Lanoot B, Vancanneyt M, Dawyndt P, Cnockaert M, Zhang J, Huang Y, Swings J. BOX-PCR fingerprinting as a powerful tool to reveal synonymous names in the genus *Streptomyces*. Emended descriptions are proposed for the species *Streptomyces cinereorectus*, *S. fradiae*, *S. tricolor*, *S. colombiensis*, *S. filamentosus*, *S. vinaceus* and *S. phaeopurpureus*. Syst Appl Microbiol. 2004;27(1):84-92.
22. Wieser M, Busse HJ. Rapid identification of *Staphylococcus epidermidis*. Int J Syst Evol Microbiol. 2000;50(3):1087-93.
23. Stephenson DP, Moore RJ, Allison GE. Comparison and utilization of repetitive-element PCR techniques for typing *Lactobacillus* isolates from the chicken gastrointestinal tract. Appl Environ Microbiol. 2009;75(21):6764-76.
24. Hyytiä-Trees E, Lyhs U, Korkeala H, Björkroth J. Characterisation of ropy slime-producing *Lactobacillus sakei* using repetitive element sequence-based PCR. Int J Food Microbiol. 1999;50(3):215-9.
25. Cocolin L, Rantsiou K, Iacumin L, Urso R, Cantoni C, Comi G. Study of the ecology of fresh sausages and characterization of populations of lactic acid bacteria by molecular methods. Appl Environ Microbiol. 2004;70(4):1883-94.
26. Comi G, Urso R, Iacumin L, Rantsiou K, Cattaneo P, Cantoni C, Cocolin L. Characterization of naturally fermented sausages produced in the North East of Italy. Meat Sci. 2005;69(3):381-92.
27. Rantsiou K, Drosinos EH, Gialitaki M, Urso R, Krommer J, Gasparik-Reichardt J, Cocolin L. Molecular characterization of *Lactobacillus* species isolated from naturally fermented sausages produced in Greece, Hungary and Italy. Food Microbiol. 2005;22(1):19-28.
28. Ammor MS, Mayo B. Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. Meat Sci. 2007;76(1):138-46.
29. Lee JY, Kim CJ, Kunz B. Identification of lactic acid bacteria isolated from kimchi and studies on their suitability for application as starter culture in the production of fermented sausages. Meat Sci. 2006;72(3):437-45.
30. Santos EM, Gonzalez-Fernandez C, Jaime I, Rovira J. Comparative study of lactic acid bacteria house flora isolated in different varieties of 'chorizo'. Int J Food Microbiol. 1999;39(1):123-8.

Supplementary Data

Available at: http://serbiosoc.org.rs/NewUploads/Uploads/Bogdanovic%20et%20al_3425_Supplementary%20Data.pdf