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CHARACTERIZATION OF LACTIC ACID BACTERIA ISOLATED FROM BOSNIAN ARTISANAL DRY FERMENTED SAUSAGE (SUDŽUK) DURING FERMENTATION

Josip ČOLO[1], Sanja MIHAJLOVIĆ[2], Maja TOLINAČKI[2], Mersiha ALKIĆ[1], Dušanka POPOVIĆ[2], Milan KOJIĆ[2], Amarela TERZIĆ-VIDOJEVIĆ[2*]

[1]Faculty of Agriculture and Food Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
[2]Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia

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Bosnian sudžuk is a dry fermented sausage produced in a rural household near the town of Visoko in central Bosnia and Herzegovina. This kind of sausage was manufactured only from beef and spices in a traditional way without the addition of a starter cultures. To identify lactic acid bacteria (LAB), a total number of 160 LAB strains were isolated from five samples of Bosnian sudžuk collected over 28 days of fermentation. Preliminary identification by phenotypic tests and 16S rDNA sequencing were performed for all 160 of the LAB isolates. Identification of LAB strains from traditionally produced Bosnian sausage at the species level revealed the presence of six genera: *Lactococcus* sp., *Enterococcus* sp., *Leuconostoc* sp., *Lactobacillus* sp., *Pediococcus* sp. and *Weissella* sp.. Among the 15 distinct species identified, the species *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Enterococcus faecalis* and *Enterococcus durans* were present throughout the entire process of fermentation. *Leuconostoc mesenteroides*, *Lactobacillus plantarum* and *Lactococcus lactis* prevailed, with 21.8%, 19.3% and 13.1%, respectively, of total LAB strains during the entire fermentation process. Significant negative correlations (r = 0.892 and r = 0.829,

Corresponding author: Amarela Terzić-Vidojević,Institute of Molecular Genetics and Genetic Engineering, University of Belgrade,Vojvode Stepe 444a, P.O.Box 23, 11010 Belgrade, Serbia. Telephone: +381 11 397 59 60; Fax: +381 11 397 58 08; E-mail: amarela@imgge.bg.ac.rs; lab6@imgge.bg.ac.rs,http://www.imgge.bg.ac.rs

respectively) between the presence of *Weissella* sp. and *Lactobacillus* sp., and between the presence of *Weissella* sp. and *Lactococcus* sp. were recorded. *Lactobacillus plantarum*, *Enterococcus durans* and *Leuconostoc mesenteroides* were the best producers of aromogenic compounds while 32.3% of *Lactobacillus plantarum* and 28.6% of *Leuconostoc mesenteroides* were produced exopolysaccharides.

Key words: Bosnian sudžuk, fermented sausages, lactic acid bacteria, 16S rDNA sequencing

INTRODUCTION

Spontaneously fermented meat products have a long tradition of production in certain regions of the world. Distinct production processes and artisanal microflora lead to a great diversity and variety of fermented sausage in the market. Lactic acid bacteria (LAB) are one of the main group of bacteria that is considered technologically important in meat preservation and the fermentation process of the sausages (COPPOLA *et al*., 1998). LAB convert the sugars to lactic and acetic acids, decreasing pH and contributing to the inhibition of spoilage and growth of pathogenic microorganisms (LEBERT *et al*., 2007). Moreover, the modification of raw material by LAB improves the flavor, color and texture of fermented sausages (BONOMO *et al*., 2008). Although industrial sausage production is controlled in terms of the microbial community and manufacturing process, artisanal products are widely appreciated for their sensory characteristics and authenticity (TALON *et al*., 2007).

Sudžuk is a dry fermented sausage produced for centuries over a wide area of the Middle East, Central Asia and the Balkans. In the Balkans sudžuk is mainly produced in Bosnia and Southwest Serbia. Despite the industrial production of sudžuk, there is a long tradition of producing Bosnian sudžuk without any starter cultures, additives or preservatives. The most promising bacteria for use as a starter culture are those that are isolated from the indigenous microflora of traditional products, since environmental conditions in each geographic region affect the specific properties of predominant native microflora. These microorganisms are well-adapted to the meat environment and have important technological properties and bacteriocin production capabilities that could be used successfully in the meat industry (HAMMES, 1990).

Dry fermented sausages are produced in three phases: formulation, fermentation and ripening/drying (SANZ *et al*., 1998). The ripening technique has an effect on the LAB composition, which develops during the fermentation process. Among LAB, *Lactobacillus* (especially *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum*), *Leuconostoc*, *Weissella*, *Enterococcus*, *Pediococcus* and *Lactococcus* are the genera most widely described in fermented sausages (DI CAGNO *et al*., 2008; RANTSIOU *et al*., 2006). Lactobacilli are often present in the early stages of sausage fermentation, whereas there is no pattern of distribution of the other LAB groups during fermentation (URSO *et al*., 2006).

Phenotypic identification of LAB is frequently time consuming and problematic, due to ambiguous biochemical or physiological features (AMMOR *et al*., 2005). The application of molecular-based techniques offers a rapid and specific alternative. To better qualify LAB strains, a combination of classic physiological and biochemical tests with modern molecular genetic techniques should be applied. The development of PCR-based molecular techniques enables faster and more reliable identification and characterization of bacterial isolates. As a result, the knowledge of the biodiversity of microbial ecosystems has significantly increased (DANILOVIĆ *et al*., 2011).

Little is known about a microflora of Bosnian sudžuk although this type of sausage is common in the diet of Bosnia and Herzegovina population. Therefore, the aim of this study was to examine the natural LAB population of an artisanal Bosnian sausage (sudžuk) produced in a rural household near the town of Visoko in central Bosnia and Herzegovina. The sudžuk was produced only from beef meat and spices in a traditional way, without the addition of starter culture. To identify and characterize the LAB from the sausage during the fermentation process, conventional microbiological, biochemical and molecular genetic tests were applied for all of the 160 selected isolates. The identified LAB from Bosnian sudžuk could facilitate the selection of certain strains with good technological properties as a starter culture for the industrial production of sudžuk.

MATERIALS AND METHODS

Manufacturing process and sampling of the Bosnian sudžuk

Sudžuk is a dry fermented sausage which has traditionally been produced without the addition of starter culture in a rural household of Gračanica village in central Bosnia and Herzegovina (altitude 420 m above sea level). The highest quality parts of the fresh beef and 3% beef tallow were first mixed and 2.5% salt was added. After 8 to12 hours the following ingredients were added and mixed well: garlic (1%), black pepper (0.05%) and sugar (0.02%). The mixture was packed into natural casings 40 mm in diameter and linked in a horseshoe shape. The sausages were ripened in a traditional way that includes smoking by cool procedure for 28 days at 15-20ºC with low circulation of air. Smoking and drying were done with smoke from beech wood with no open flame. The samples of the sausages for microbiological analysis were taken from the same batch after 0 (sausage mixture), 3, 7, 14, and 28 days of fermentation. All samples were taken aseptically and transported to the laboratory under refrigeration. The designation of samples is given in Table 1.

Table 1. Sample nomenclature of Bosnian sudžuk sausage (BS) during fermentation

Sample of Bosnian sudžuk	Fermentation days (d)
BS0	0 d (sausage mixture)
BS3	3 d
BS7	7 d
BS14	14 d
BS28	28 d

pH measurements

Ten grams of each sample were diluted and homogenized in 90 ml of distilled water. The pH of the sudžuk samples was measured using a pH meter (InoLab model ph 720; WTW GmbH, Hamburg, Germany). Three independent measurements were obtained from each sample. Means and standard deviations were calculated.

Isolation and phenotypic characterization of LAB

Samples were analyzed by conventional microbiological methods. Twenty five grams of each sample was mixed with 225 ml of saline solution (8.5 g/l NaCl) and homogenized for 1.5 min in a Stomacher Lab Blender 80 (PBI, Milan, Italy). Serial dilutions were prepared (10^{-1} to 10^{-7}) and inoculated onto plate count agar (PCA, Oxoid LTD, Basingstoke, Hampshire, England), MRS agar (Oxoid) and M17 agar (Oxoid) supplemented with 0.5% (w/v) glucose (GM17). The PCA was used for enumeration of aerobic mesophilic bacteria, and MRS and GM17 agar for enumeration of LAB. An agar overlay plate method was used to establish microaerophilic conditions for growth of LAB on MRS and GM17 agar plates. The aforementioned process was carried out in triplicate. The plates were incubated at 30ºC and 45ºC for 48 to 72 h. Five to ten different LAB colonies from MRS and GM17 plates, for each sampling point and for each fermentation day, were randomly selected according to HARRIGAN and MC CANCE (1976) and streaked on new agar plates for purification. Single colonies from each plate were examined by Gram staining, for catalase production and microscopic morphology. The pure cultures were stored at -80ºC in GM17 or MRS broth supplemented with 20% (w/v) glycerol (Posh, Gliwice, Poland).

One hundred sixty isolates from different samples (0, 3, 7, 14, and 28 days fermentation) were subjected to a set of tests as follows: growth at 15ºC, 30ºC and 45ºC; growth in 2%, 4% and 6.5% NaCl broth; gas production from glucose in reconstituted MRS broth tubes containing inverted Durham bells; production of acetoin by Voges-Proskauer test (IJUTOV, 1963); and L-arginine hydrolysis. The presumptive identification of enterococci was tested by hydrolysis of esculin on bile esculin agar (Himedia). Utilization of citrate was tested as described by KEMPLER and MCKAY (1981). Diacetyl production was tested only qualitatively. After incubation for 16 h at 30ºC or 37ºC, depending on the strain, 1 ml of coagulated milk was mixed with 0.1 g of creatinine (Molar Chemicals, KFT, Budapest, Hungary) and 1 ml of 30% NaOH (w/v). Diacetyl generation was indicated by the formation of a red halo at the top of the tubes after 2 h of incubation at room temperature. Exopolysaccharides (EPS) production was monitored on MRS medium supplemented with sucrose as described by VAN DER MEULEN *et al*. (2007).

DNA extraction and 16S rDNA sequencing

The total DNA from all the 160 LAB isolates was extracted as described by (MARTIN-PLATERO *et al*., 2007). The PCR amplification of 16S rDNA, was performed with 0.4 µM WO1 [5`-AGAGTTTGATC(AC)TGGCTC-3`] and 0.4 µM WO12 [5`-TACGCATTTCACC(GT)CTACA-3`] primers. The PCR reaction mixture (50 µl) contained 1 µl of template DNA, 5µl of 10xTaq reaction buffer, 1.5 mM of $MgCl_2$, 400 µM of dNTPs and 1 U of Taq DNA Polymerase (MBL, Còrdoba, Spain). PCR conditions were 94ºC for 4 min; 30 cycles at 94ºC for 30 sec, 50ºC for 30 sec, and 72ºC for 60 sec; and 72ºC for 2 min. The obtained PCR amplicons were purified with a PerfectprepGel Cleanup kit (Eppendorf, Hamburg, Germany) and sequenced (Macrogen, Amsterdam, Netherlands). The BLAST algorithm was used to determine the most closely related sequences in the NCBI (National Center for Biotechnology Information) nucleotide sequence database (http://www.ncbi.nlm.nih.gov/BLAST). The 16S rDNA sequences were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/HG798366-HG798525).

Statistical analysis

Two-way analysis of variance (ANOVA) was used to evaluate the significance of the differences between the bacterial count means during the fermentation process. Duncan's test was applied for post-hoc analysis. The data analysis was performed using SPSS software, version 20.0 (SPSS Inc., Chicago, IL, USA). The Pearson correlations between the identified LAB genera were performed by Statistica 7.0 software (Statsoft Ltd, Chicago, IL, USA). Significance for all the tests was determined at the $P < 0.05$ level.

RESULTS

pH measurements and enumeration of bacteria

The determination of pH values and the viable counts of bacteria throughout the fermentation process of Bosnian sudžuk are summarized in Fig. 1. During fermentation, the pH value rapidly decreased from an initial pH value of 6.26±0.04 to 4.95±0.01 on day 7. A slow but statistically significant decrease continued until the end of the fermentation process when the final pH value was 4.84±0.01 (Fig. 1). The two-factor analysis of variance showed the interaction between the fermentation process and bacterial growth. The mean count of aerobic mesophilic bacteria significantly increased from the beginning of the fermentation period to day 7, when it reached a maximum value of 7.951 log CFU/g (Table 2 and Fig. 1). A 1.1-fold decrease of aerobic mesophilic count occurred by the end of the fermentation process. A similar growth pattern was observed for the LAB that were grown in both MRS and GM17 broth. There was no significant difference between the mean LAB counts observed on the first day and third day of fermentation (Fig. 1). However, a significant 1.2-fold increase of the mean LAB count that were grown in MRS broth was obtained from day 3 to day 28. The mean count of LAB grown on GM17 reached a maximum of 10.856 log CFU/g on day 7 and significantly decreased to 9.875 log CFU/g at the end of the fermentation process.

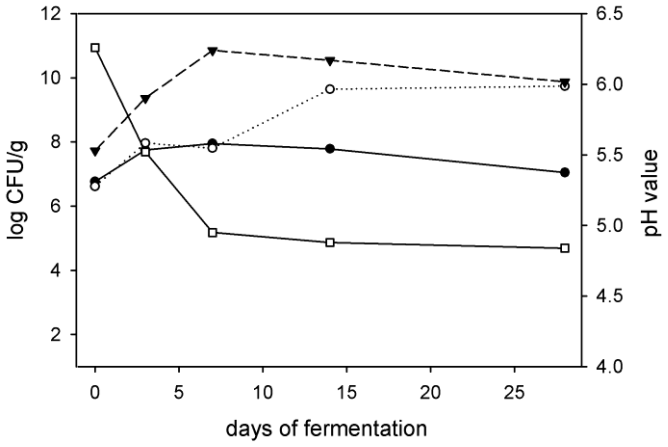

Figure 1. Changes in pH value (○) and population dynamics of aerobic mesophilic bacteria (▲) and LAB counted on MRS agar plates (♦) and GM17 agar plates (■) during spontaneous fermentation of artisanal Bosnian sudžuk sausage.

Table 2. Two-factor analysis of variance of the mean bacterial count of the Bosnian sudžuk during fermentation

Fermentation (days)	Growth medium		
	PCA	MRS	GM17
0	6.765[e]	6.618[d]	7.728[d]
3	7.747 [c]	7.966[d]	9.365[d]
7	7.951[a]	7.806[c]	10.856[a]
14	7.787[b]	9.643[b]	10.543[b]
28	7.050[d]	9.739[a]	9.875[c]

[a-d] Different superscripts within the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

Identification and characterization of LAB during fermentation of Bosnian sudžuk

A total of 160 Gram positive and catalase negative strains were isolated from 5 samples collected from the same batch during fermentation of the Bosnian sudžuk. The phenotypic and genotypic identification of the total isolates showed the presence of six LAB genera in the Bosnian sudžuk as follows: *Lactobacillus* sp. (36.9%), *Enterococcus* sp. (23.1%), *Leuconostoc* sp. (22.5%), *Lactococcus* sp. (13.1%), *Pediococcus* sp. (3.1%) and *Weissella* sp. (1.3%). The isolates were preliminarily characterized and grouped by biochemical tests. The general phenotypic properties of the 160 LAB isolates, identified by 16S rDNA sequencing are shown in Table 3.

Rod shaped cells were observed in 59 isolates and classified as lactobacilli. Forty-nine lactobacilli were arginine negative and belonged to the homofermentative group of lactobacilli (Table 3). Thirty-seven isolates formed black colonies on bile esculin agar plates and are determined to belong to *Enterococcus* sp. Our results have shown that 40.5% of enterococci isolated from the Bosnian sudžuk utilized citrate and produced acetoin and diacetyl (Table 3). Heterofermentative group of cocoid LAB forms, which were observed in 36 isolates, produced CO_2 from glucose and belonged to *Leuconostoc* sp. Overall, in this study, 62.9% of total isolated *Leuconostoc* sp. utilized citrate, 28.6 % produced EPS and 22.2% isolates produced acetoine (Table 3). Twenty-one LAB cocci, which were esculin negative belonged to lactococci. Although lactococci are a group of mesophilic bacteria, three lactococcal isolates had the ability to grow at 45°C (Table 3). Regardless of the common growth of lactococci in the presence of 4% NaCl, 27.3% of the lactococci showed growth in the presence of 6.5% NaCl. Fifty four percent of total lactococci isolated from Bosnian sudžuk had the ability to utilize citrate, 18.2% to produce acetoine and 13.6% to produce diacetyl. Five esculin-negative cocci formed tetrads and grew in broth with 6.5% NaCl and on GM17 agar plates at both 45°C and 15°C and were characterized to be pediococci. Two of them utilized citrate (Table 3).

All 160 isolated LAB strains have been molecularly identified by 16S rDNA sequencing. The accession numbers of all 160 LAB isolates are listed in a separate table (Table 5) and are shown in the supplementary data.

Table 3. Phenotypic properties of lactic acid bacteria isolated from the Bosnian sudžuk

[a] Number of isolates out total 160 isolates.

LAB species identified by 16S rDNA sequencing	Growth at		Growth in broth with			Hydrolysis of arginine	Utilization of citrate	Production of			
	15 °C	45 °C	2% NaCl	4% NaCl	6.5% NaCl			CO_2	Acetoin	Diacetyl	EPS
Lb. plantarum (31)[a]	+ (77.4)[b] – (22.6)	+ (6.5) – (93.5)	NT	+	+ (93.5) – (6.5)	–	+ (45.2) – (54.8)	–	+ (87.1) – (12.9)	–	+ (32.3) – (62.7)
Lb. casei (12)	+ (83.3) ± (16.7)	–	+	+ (75.8) – (25.0)	+ (75.8) – (25.0)	–	+ (58.3) – (42.7)	–	+ (66.7) – (33.3)	–	–
Lb. brevis (7)	+ (85.7) ± (14.3)	+ (14.3) ± (85.7)	+	+ (71.4) – (28.6)	+ (71.4) – (28.6)	+ (57.1) ± (42.9)	+ (14.3) – (85.7)	+	+ (14.3) – (85.7)	–	–
Lb. fermentum (3)	+	+ (33.3) – (66.7)	+	+ (66.7) – (33.3)	+ (33.3) – (66.7)	+ (66.7) ± (33.3)	–	+	–	–	–
Lb. curvatus (1)	+	–	NT	NT	+	–	–	–	–	–	–
Lb. sakei (5)	+	–	NT	NT	+	–	–	–	–	–	–
Ec. faecalis (13)	+	+	NT	NT	+	+	+ (23.1) – (76.9)	–	+ (76.9) – (23.1)	–	–
Ec. durans (12)	+	+	NT	NT	+	+	+ (50.0) – (50.0)	–	+ (25.0) – (75.0)	+ (50.0) – (50.0)	–
Ec. faecium (12)	+	+	NT	NT	+	+	–	–	+ (16.7) – (83.3)	+ (50.0) – (50.0)	–
Ln. mesenteroides (35)	+ (94.3) ± (5.7)	+ (5.7) ± (2.9) – (91.4)	+ (91.4) – (8.6)	+ (82.8) ± (8.6) – (8.6)	+ (17.1) ± (2.9) – (80.0)	– (74.3) ± (25.7)	+ (62.9) – (37.1)	+	+ (20.0) – (80.0)	–	+ (28.6) – (71.4)
Ln. lactis (1)	+	–	NT	+	–	±	–	+	+	–	–
Lc. lactis (21)	+	+ (13.6) – (86.4)	+	+ (22.7) – (77.3)	+ (27.3) – (72.7)	+	+ (54.5) – (45.5)	–	+ (18.2) – (81.8)	+ (13.6) – (86.4)	–
P. pentosaceus (5)	+	+	NT	NT	+	+ (60.0) ± (40.0)	+ (40.0) ± (40.0) – (20.0)	–	–	–	–
W. viridescens (1)	+	–	+	±	±	–	–	+	+	–	–
W. confusa (1)	+	–	NT	+	±	–	–	+	+	–	–

[b] Percent of isolates.

+ positive reaction; – negative reaction; ± weak reaction; NT- no tested.

Lb. - *Lactobacillus* sp.; *Lc.* - *Lactococcus* sp; *Ln.* - *Leuconostoc.* sp. *Ec.* - *Enterococcus* sp.; *P.* - *Pediococcus* sp.; *W.* - *Weissella* sp..

Distribution of LAB during fermentation of Bosnian sudžuk

The fluctuation of the LAB population during 28 days of fermentation was observed. During the entire fermentation process, the dominant LAB genera were *Lactobacillus*, *Enterococcus* and *Leuconostoc* (Fig. 2). The domination of lactobacilli compared to other genera was observed in the Bosnian sudžuk throughout fermentation (Fig. 2). After seven days of fermentation, they made up 47.1% of the total microflora. Among them, 31 isolates (52.4%) belonged to *Lb. plantarum* and 12 isolates (20.34%) belonged to *Lb. casei* (Table 4). A lower number of isolates was detected of the species *Lb. brevis* (7 isolates or 11.86%), *Lb. sakei* (5 isolate or 8.47%), *Lb. fermentum* (3 isolates or 5.03%)) and *Lb. curvatus* (1 isolate or 1.69%).

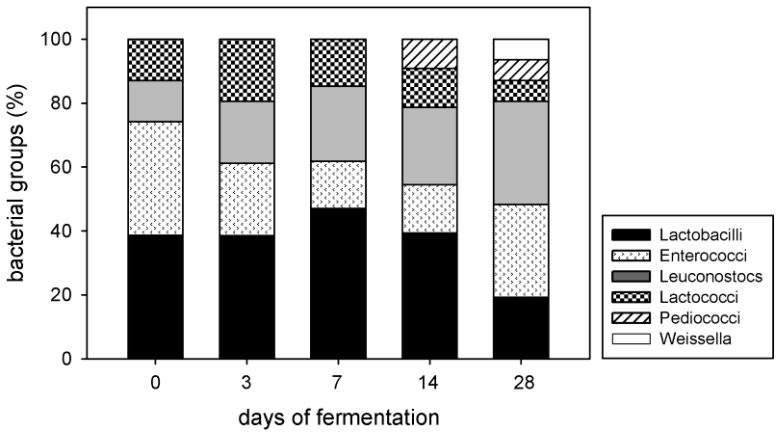

Figure 2. Distribution of lactic acid bacteria during the fermentation process of artisanal Bosnian sudžuk sausage.

Table 4. Distribution of lactic acid bacteria in the samples of Bosnian sudžuk during fermentation

Species identified by 16S rDNA sequencing	Samples of Bosnian sudžuk during fermentation				
	BS0 (31)[a]	BS3 (31)	BS7 (34)	BS14 (33)	BS28 (31)
Lb. plantarum	25.8%	19.4%	23.5%	15.2%	12.9%
Lb. casei	12.9%	16.1%	5.9%	3.0%	0
Lb. brevis	0	0	11.8%	9.1%	0
Lb. sakei	0	3.2%	5.9%	3.0%	3.2%
Lb. curvatus	0	0	0	3.0%	0
Lb. fermentum	0	0	0	6.1%	3.2%
Lc. lactis	12.9%	19.4%	14.7%	12.1%	6.5%
Ln. mesenteroides	12.9%	19.4	20.6%	24.2%	32.2%
Ln. lactis	0	0	2.9%	0	0
Ec. faecalis	6.5%	3.2%	11.8%	6.1%	12.9%
Ec. durans	16.1%	3.2%	2.9%	6.1%	9.7%
Ec. faecium	12.9%	16.1%	0	3.0%	6.5%
P. pentosaceus	0	0	0	9.1%	6.5%
W. confusa	0	0	0	0	3.2%
W. viridescens	0	0	0	0	3.2%

[a] Number of isolates.

Lb. - *Lactobacillus* sp.; *Lc.* - *Lactococcus* sp; *Ln.* - *Leuconostoc.* sp. *Ec.* - *Enterococcus* sp.; *P.* - *Pediococcus* sp.; *W.* - *Weissella* sp..

The second most abundant group of LAB in the Bosnian sudžuk was enterococci (Fig 2), which belonged to *Ec. durans*, *Ec. faecium* and *Ec. faecalis* species (Table 4). The enterococci prevailed immediately after the preparation of the sausage mixture, with 35.5% of the total LAB isolates, and made up 29 % of the total LAB microflora at the end of fermentation (Fig. 2). Together with lactobacilli and enterococci, the leuconostocs were the dominant microflora of the Bosnian sudžuk (Table 4 and Fig. 2). The number of *Ln. mesenteroides* strains increased over the fermentation process and reached a maximum of 32.3% after 28 days of fermentation (Fig. 2). Other heterofermentative species found sporadically are *Ln. lactis* in 3-day-old Bosnian sudžuk, and *W. viridescens* and *W. confusa* in 28-day-old Bosnian sudžuk. The percentage of lactococci in Bosnian sudžuk was 13.1%. A 3-fold decrease in *Lc. lactis* isolates was observed from the third day to the end of fermentation (Fig 2). The correlation among the genera identified in the five-tests of Bosnian sudžuk during fermentation was recorded. The statistical analysis showed a negative correlation between *Weissella* sp. and *Leuconostoc* sp. ($r = 0.892$, $P = 0.021$). In addition, a negative correlation was observed between *Weissella* sp. and *Lactococcus* sp. ($r = 0.829$, $P = 0.041$). These results indicate that the presence of *Weissella* entails the decreased presence of lactobabacilli and lactococci populations in the Bosnian sudžuk.

DISCUSSION

Bosnian sudžuk is an artisanal dry fermented sausage produced from meat and spices by traditional techniques. Specific sensory characteristics of Bosnian sudžuk made it the most popular sasage in Bosnia and Herzegovina.

An abundance of LAB species in the microflora of the dry sausage during fermentation caused intensive fermentation of carbohydrates, which led to the accumulation mainly of lactic and acetic acid and thus a reduction of pH (DANILOVIĆ *et al*., 2011; DROSINOS *et al*., 2007). On the other hand, the pH reduction induced by the increase of LAB population during the fermentation and ripening resulted in a reduction of total aerobic bacteria (DROSINOS *et al.*, 2007; PAPAMANOLI *et al*., 2003).

The common dominant group of LAB in the sausages were members of the *Lactobacillus* genera (AMMOR *et al*., 2005; PAPAMANOLI *et al*., 2003).They play an important role in meat preservation and fermentation processes. The prevalence of lactobacilli during the fermentation process is due to their better adaptation to high acidity and salt concentration compared to the other LAB genera (CARIDI *et al*., 2003). The *Lb. plantarum* species was the most frequently isolated species from Bosnian sudžuk, as well as Greek and Croatian traditional sausages (KOZAČINSKI *et al*., 2008). Despite the high number of *Lb. sakei* isolates identified in some fermented sausages (ANDRIGHETTO *et al*., 2001; DANILOVIĆ *et al*., 2011; LANDETA e*t al*., 2013), our result is consistent with data reported by KOZAČINSKI *et al*. (2008), who found 8.7% *Lb. sakei* isolates in Bosnian sudžuk. The most number of lactobacilli isolated from Bosnian sudžuk (they 74.5%) had the ability to grow in MRS broth with 6.5% NaCl. TERZIĆ-VIDOJEVIĆ *et al*. (2009) reported that over 90% of *Lb. plantarum* and *Lb. brevis* strains isolated from artisanal Caucasus cheeses were grown in 6.5% NaCl MRS broth. This ability results from the adaptation of microorganisms to exposure to many stresses, as well as to hostile conditions. Thirty-six lactobacilli produced acetoin and 22 lactobacilli utilized citrate, which is important for the formation of the specific aroma and flavor of the final product. Many studies have reported that the inclusion of various strains of lactobacilli improve flavor development in the product (AWAD *et al*., 2007; HANNON *et al*., 2003). Five lactobacilli from Bosnian sudžuk were EPS producers. EPS-

producing LAB are able to modify the adhesion of probiotics and enteropathogens to human intestinal mucous (MOZZI *et al*., 2009). Moreover, EPS producers can improve the sensory characteristics of the final product. Therefore, EPS production by LAB is relevant for the selection of starter strains (BROADBENT *et al*., 2001). Lactobacilli represent the most common starter cultures used in the production of fermented meat products in Europe (RANTSIOU and COCOLIN, 2006).

Ec. faecium and *Ec. faecalis* are the most common enterococci isolated from different types of the fermented sausages (HUGAS *et al*., 2003; LANDETA *et al*., 2013; MARTY *et al*., 2012; RANTSIOU *et al*., 2006). Numerous enterococci isolated from Bosnian sudžuk were able to utilize citrate. Citrate utilization leads to the production of diacetyl, which is considered a main flavor compound of fermented products (HEMME and FOUCAUD-SCHEUNMANN, 2004). These features contributed greatly to the taste and flavor of Bosnian sudžuk. Enterococci, including *Ec. faecium* and *Ec. faecalis*, have the ability to produce bactericidal peptides, so-called enterocins, which inhibit the growth of certain pathogens and spoilage microorganisms (DE VUYST and VANDAMME, 1994; GOMES *et al*., 2008). The contribution of enterococci to the sensorial properties of fermented food-stuffs (cheeses, sausages, vegetables and olives), as well as their ability to produce enterocins, are important characteristics for their application in food technology (BEN OMAR *et al*., 2004; FOULQUIÉ MORENO *et al*., 2006). However, the latest findings on biogenic amine production, antibiotic resistance and virulence factors point to the need for detailed phenotypic and genotypic characterization of each enterococcal strain before their application as a potential starter culture (FRANZ *et al*., 2011; GIRAFFA, 2002; GOMES *et al*., 2008).

With 22.5 % of total isolated LAB leuconostocs make, together with lactobacilli and enterococci, the dominant microflora of Bosnian sudžuk. Our result is consistent with data reported by DANILOVIĆ *et al*. (2011) and KOZAČINSKI *et al*. (2008), who found a similar percentage of *Ln. mesenteroides* in the Serbian sausage Petrovská Klobása, a Hungarian sausage and Serbian Sremska sausage. The role of leuconostocs in fermented sausages has not been thoroughly studied and their presence is highly controversial. Leuconostocs, as heterofermentative strains, produce CO_2, which leads to the formation of holes in the meat products that are considered an undesirable characteristic (AMMOR and MAYO, 2007). On the other hand, leuconostocs contribute to the characteristic taste and aroma of fermented sausages because of citrate utilization and the production of acetic acid, acetaldehyde, diacetyl and ethanol (LEE *et al*., 2006).

Similar to lactobacilli some lactococci isolated from Bosnian sudžuk showed the ability to growth in broth with 6.5% NaCl. Previous results showed that certain natural lactococci isolated from raw milk could have the ability to grow at pH 9.6 and in the presence of 6.5% NaCl (CORROLER *et al*.,1998). Lactococci are not always observed in the distinct dry fermented sausages (DANILOVIĆ *et al*., 2011, KOZAČINSKI *et al*., 2008). Remarkably, we detected that the *Lc. lactis* strain made up 13.1% of total LAB isolates from Bosnian sudžuk. The reduction of lactococci during the fermentation period was caused by their sensitivity to lower pH values and higher salt concentrations (MANGIA *et al*., 2008). In addition to their acidifying activity (BALLESTEROS *et al*., 2006), lactococci participate in the formation of the flavor and aroma of meat products.

In the later stages of the Bosnian sudžuk fermentation the presence of *Pc. pentosaceus* strains was observed. Pediococci made up 3.1% of total LAB, which is consistent with the results reported earlier by KOZAČINSKI *et al*. (2008). On the contrary, the pediococci in Serbian Petrovská Klobása sausage were one of the dominant LAB groups (DANILOVIĆ *et al*., 2011). Pediococci do not play a major role in the formation of aroma compounds because they display low catabolism of

branched-chain amino acids (LEROY *et al*., 2006). However, certain strains can produce EPS (SEMJONOVS and ZIKMANIS, 2008) as well as bacteriocins that inhibit the growth of *Listeria monocytogenes* and *Listeria innocua* (ALBANO *et al*., 2007). For that reason certain pediococci strains are occasionally used as starter cultures for the production of fermented sausages in the USA (RANTSIOU and COCOLIN, 2006).

In conclusion, in this study we investigated the microbiological diversity of natural populations of LAB in an artisanal dry-fermented sausage from Bosnia, sudžuk, during fermentation. Our results demonstrate that combining classical and modern molecular techniques is an effective way to differentiate wild lactic acid bacteria isolated from artisanal Bosnian sudžuk. Identification of LAB strains from traditionally produced Bosnian sausage at the species level revealed the presence of six genera: *Lactococcus* sp., *Enterococcus* sp., *Leuconostoc* sp., *Lactobacillus* sp., *Pediococcus* sp. and *Weissella* sp. Among the 15 distinct species identified, the dominant species were *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Enterococcus faecalis* and *Enterococcu durans.* These species were present throughout the entire process of fermentation. LAB characterization of meat products is important because the LAB are responsible for the formation of favorable sensory characteristics during the ripening of dry sausages. *Lb. plantarum*, *Ec. durans* and *Ln. mesenteroides* were the best producers of aromogenic compounds. The ability to produce EPS was shown by 32.3% of *Lb. plantarum* and 28.6% of *Ln. mesenteroides*. The industrial production of Bosnian sudžuk requires further examination of the technological properties of certain sudžuk LAB isolates to be potentially used as a starter culture.

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KARAKTERIZACIJA MLEČNOKISELINSKIH BAKTERIJA IZOLOVANIH IZ ZANATSKI IZRAĐENE SUŠENE FERMENTISANE KOBASICE BOSANSKI SUDŽUK TOKOM FERMENTACIJE

Josip ČOLO[1], Sanja MIHAJLOVIĆ[2], Maja TOLINAČKI[2], Mersiha ALKIĆ[1], Dušanka POPOVIĆ[2], Milan KOJIĆ[2], Amarela TERZIĆ-VIDOJEVIĆ[2*]

[1]Poljoprivredno-prehrambeni fakultet, Univerzitet u Sarajevu, Sarajevo, Bosna i Hercegovina
[2]Institut za molekularnu genetiku i genetičko inženjerstvo, Univerzitet u Beogradu, Beograd, Srbija

Izvod

Bosanski sudžuk je sušena fermentisana kobasica proizvedena u jednom seoskom domaćinstvu u blizini grada Visoko u centralnoj Bosni i Hercegovini. Ova vrsta kobasice je proizvedena na tradicionalan način od goveđeg mesa i začina, bez dodatka starter kultura. Za identifikaciju mlečnokiselinskih bakterija iz pet uzoraka bosanskog sudžuka je izolovano ukupno 160 bakterijskih sojeva u toku 28 dana fermentacije. Preliminarna identifikacija svih 160 mlečnokiselinskih bakterijskih izolata je rađena fenotipskim testovima i 16S rDNK sekvenciranjem. U ispitivanoj tradicionalno proizvedenoj bosanskoj kobasici je utvrđeno prisustvo šest rodova mlečnokiselinskih bakterija: *Lactococcus* sp., *Enterococcus* sp., *Leuconostoc* sp., *Lactobacillus* sp., *Pediococcus* sp. i *Weissella* sp.. Od identifikovanih 15 različitih vrsta, vrste *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Enterococcus faecalis* i *Enterococcus durans* su bile prisutne svo vreme fermentacionog procesa. Najveća procentualna zastupljenost je utvrđena kod vrsta *Leuconostoc mesenteroides*, *Lactobacillus plantarum* i *Lactococcus lactis* i iznosila je 21.8%, 19.3% i 13.1% pojedinačno, u odnosu na ukupan broj izolovanih mlečnokiselinskih bakterija. Statistički značajna negativna korelacija je primećena između prisustva *Weissella* sp. i *Lactobacillus* sp., kao i između prisustva *Weissella* sp. i *Lactococcus* sp. ($r = 0.892$ i $r = 0.829$, pojedinačno). *Lactobacillus plantarum*, *Enterococcus durans* i *Leuconostoc mesenteroides* su bili najbolji proizvođači aromatskih komponenti, dok je 32.3% *Lactobacillus plantarum* i 28.6% *Leuconostoc mesenteroides* vrsta proizvodilo egzopolisaharide.

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