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PROBIOTIC POTENTIAL OF *LACTOBACILLUS* AND*LEUCONOSTOC* STRAINS ISOLATED FROM TRADITIONAL SPONTANEOUSLY FERMENTED SHEEP HAM

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ABSTRACT

This paper examined the probiotic characteristics of 29 lactic acid bacteria isolated from traditional sheep ham. The research included isolation and identification of *Lactobacillus* and *Leuconostoc* strains. *In vitro* tests related to their probiotic potential included auto-aggregation and co-aggregation, antibiotic resistance, and hemolysis tests. Auto-aggregation values of the tested strains ranged from 24.5 to 68.1%, co-aggregation values of *Lactobacillus*, *Leuconostoc,* and ATCC strain of *Escherichia coli* ranged from 22.5 to 65.5% after four hours. The isolates showed a significant difference in the hydrophobic ability of the tested hydrocarbons, n-hexadecane, xylene, and chloroform, ranging from 5.25% to 80.9%. All of the isolates have shown high sensitivity to most analyzed antibiotics, while *Lactobacillus* and *Leuconostoc* strains had no positive hemolysis.The zone of inhibition against all tested pathogens was demonstrated by 55.1% of strains. The most significant inhibition zones were exhibited against *Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 14579, while 31.03% of strains did not show a zone of inhibition towards *Listeria monocytogenes* ATCC 19115.

Key words: Lactic acid bacteria, sheep ham, probiotic potential.

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INTRODUCTION

The area of western Balkans is characterized by a large number of autochthonous spontaneously fermented dry-cured meat products. One of these products is dry-cured sheep ham which is produced using a complex production method. Salted and dried sheep meat without bones is called Stelja (Stojković *et al.,* 2015). Fattened male castrates and barren ewes of autochthonous animal species Sjeničko-Pešterskapramenka are used intheproduction of dry-cured sheep ham from the Pešter plateau (Western Serbia). During the ripening period, which lasts at least 5 to 6 months, dry-cured sheep meat develops unique microbiota that directly defines the quality of the product and its organoleptic characteristics. The dominant population of dry-cured sheep ham consists of lactic acid bacteria (LAB) that control the entire flow of fermentation and ripening of the product. By synthesizing antimicrobial components, volatile fatty acids, and chemically modifying bile acids, LAB also create an unfavorable local environment to pathogens, known as "colonization resistance "(Radulović*et al.,* 2012; Wan *et al.,* 2015). The new concept of functional food defines the need to choose LAB strains from indigenous microbiota of traditional products, including fermented meat, based on the most important technological criteria.Another important factor is suitable probiotic properties necessary to obtain strains that are well adapted to the microenvironment and can dominate the microbiota of fermented meat products (Papamanoli*et al.,* 2002). Probiotics in fermented meat products are protected by a layer of fat and meat particles against unfavorable conditions of the human gastrointestinal (GI) tract, and thus in such an environment, they can better exhibit their positive probiotic characteristics (Palamutoğlu and Kasnak, 2014). Defining strains as potential probiotics is complex astheymust meet a great number of conditions to be safe for human consumption. Probiotic strains must have the status of "generally recognized as safe" (GRAS) or "qualified presumption of safety" (QPS).They cannotdemonstrate pathogenic properties or cause infectious GI tract disorders and not dissolve bile salts,andhavegood adherence to the intestinal mucosa (Mitropoulou*et al.,* 2013). This study's aims were isolation and identification of *Lactobacillus* and *Leuconostoc* genera isolated from dry-cured sheep ham, as well as the evaluation of their probiotic characteristics through adhesion, aggregation, hemolysis on blood agar plates,antimicrobial activity and antibiotic resistance ability of the isolated bacteria.

MATERIALS AND METHODS

Nine samples were taken from the three producers (2017/18 production year) from the territory of Sjenica (Western Serbia) andaseptically transported to the microbiology laboratory at the College of Agriculture and Food Technology, Prokuplje, Srbija.

Dry-cured sheep ham -making, manufacture, and sampling: Dry-cured sheep ham or Sjenica sheep ham is produced in a very complex manner. Deboning method for this product is very specific, and it consists of the whole sheep carcasses (except the inner part of the leg) with associated adipose fat and connective tissue being treated in a specified way. All bones, except the distal and forearm bones that are left up to 5 cm in length,are removed and used to hang the product during the dry-curing and fermentation process (Stamenković and Dević, 2004). Sheep meat is cured and cold smoked in beech smoke and is characterized by a long fermentation process of 3 - 6 monthsunder natural environmental conditions (T: 10–15 °C; RH: 80–85%).Spontaneous fermentation of ham is influenced by the indigenous microbiota, which defines the product's quality and sensory properties.

LAB isolation:The isolation procedure was conducted using serial 10-fold dilution technique by mixing 1 gram of meat sample (taken aseptically from deeper inside) in 9 ml of quarter strength ringer solution (Hemofarm, Vršac, Serbia) and further serially diluted. After this, 0.1 ml of sample was placed on non-selective medium de Man Rogosa Sharpe (MRS) agar (Torlak, Beograd, Serbia). The incubation period lasted 48-72 hours at a temperature of 37°C, after which individual colonies were randomly selected and pure colonies obtained.The purity of these isolates was tested using a microscope, and Gram-positive, catalase-negative, non-spore-forming isolates were considered LAB. Twenty-nine isolated LAB-s from nine samples of dry-cured sheep ham were selected for further investigation. After isolation, a series of biochemical tests were conducted, which included: arginine hydrolysis, growth ability on MRS agar at different temperatures (15°C and 45°C), growth ability on MRS agar in the presence of 4 and 8% NaCl, CO_2 production from glucose, lipolytic activity, proteolytic activity, and exopolysaccharide synthesis. LAB isolates were then identified using API 50 CH research strips for bacterial identification (BioMerieux, S.A., France). The API strips were prepared as recommended by the kit supplier and read after 24 and 48 hours of incubation at 37ºC. Cultivation was conducted in MRS broth using 15% glycerol and stored at -18ºC in a deep-freezer in the laboratory at the Department of Biology and Ecology, University of Kragujevac.MALDI-TOF mass spectrometry was used to identify LAB. MALDI-TOF protein identification was conducted using Microflex LT/SH BioTyper spectrometer (BrukerDaltonics, Bremen, Germany), equipped with a nitrogen laser (337 nm),and uses Flexcontrol software ver. 3.1 (Bruker Daltonics). Collected spectra ranged from 2 to 20 kDa. Spectra acquisition in the mass range were collected using the Auto Execute option by accumulating 240 laser shots (laser frequency, 60 Hz; ion source one voltage, 19.9 kV; ion source two voltage, 18.53 kV; lens voltage, 6 kV) acquired at 30–40% of maximum laser power. Sample preparation was performed using the standard method of protein extraction with minor modifications. Overnight cultures (500 µL) were grown in MRS broth and then centrifuged (Rotofix 32A, Hettich, Germany) at 12000 rpm / 5 min / 4°C. After centrifugation, the supernatant was discarded, and 300 µL of distilled water and 900 µL of absolute ethanol were added to the pellet. The resulting suspension was vortexed for 1 minute and centrifuged at 13000 rpm / 2 min / 4°C. Following that, the supernatant was discarded, and the pellet dried at 55°C for 30 min, while adding 50 µL of 70% formic acid and thoroughly mixing it by pipetting. The final step was adding fifty microliters of 50% acetonitrile and centrifuging the samples again at 13000 rpm / 2 min / 4°C. Then, 1 µL of supernatant was placed directly onto a 96-spot MALDI target plate (BrukerDaltonics), allowed to dry for 10 min, and immediately overlaid with 1 µL of the matrix solution (Bruker Matrix HCCA; α-Cyano-4-hydroxycinnamic acid). The measurement results for each isolate were expressed in terms of MALDI-bioTiper (from 0,000 to 3,000), whereby a comparison of similarities with the unknown bacterial profiles available on the MALDI-bioTiper software database was performed. The corresponding values ≥ 2.00 were taken as the correct isolation level of the isolates (Muruzović*et al.,* 2018).

In vitro probiotic tests

Auto-aggregation and co-aggregation: Auto-aggregation of the tested isolates was monitored in phosphate buffer solution (PBS; Merck, Darmstadt, Germany). *Lactobacillus* and *Leuconostoc mesenteroides* strains were grown at 37°C for 18 h in MRS broth after which they were centrifuged at 5000 rpm for 15 minutes and washed twice in the PBS buffer and then resuspended in 4 ml of the same buffer so that the number of cells was approximately 10^8 CFU/ ml (absorbance of the suspension at 600 nm was around 1.0). The resulting suspension was mixed in the vortex, and then 100 µl from the suspension surface was transferred to the microtuber with 900 µl of PBS buffer, and A600 (A_0) was measured. The procedure was repeated after four hours. Auto-aggregation was calculated using the following form (Ocañaand Nader-Macías, 2002).

Auto-aggregation % = $(A_0 - A_t)/A_0$ x100, where At-A_{600nm} after 1h and4h.

Examined microorganisms and *Escherichia coli* ATCC 25922 were identically prepared forthe co-aggregation test as in the previous test. The difference was that 2 ml of each suspension of both types of bacteria were mixed in the PBS buffer. 100 µl from the suspension surface was transferred to the microtuber with 900 µl of PBS buffer and A600 (Ai) was measured(Ocaña and Nader-Macías, 2002).

Co-aggregation % = $(A_i-A_f)/A_i \times 100$, where Af represents the absorbance of supernatant A_{600nm} after 2h and 4h.

Bacterial adhesion to hydrocarbons (hydrophobicity): Bacterial adhesion to hydrocarbons (hydrophobicity) was determined by the modified method was described by Kotzamanidis *et al.* (2010).Overnight cultures were centrifuged at 5000 rpm for 15 minutes, the pellets were washed twice with PBS buffer, and optical densities were measured at 540 nm and adjusted to an optical density of A_{540}=1.0. Then 1 ml of bacterial suspension was added to 1 ml of each of the hydrocarbons and vortexed vigorously for 30 seconds. After phase separation, which lasted for 30 min, the optical density of the aqueous phase was again measured and compared with the initial value. Hydrophobic percentage was calculated based on the equation: Absorbance % = $(A_0 - At) / A_0 \times 100$

Hemolysis on blood agar plates: The presence of hemolysis in selected LAB strains was performed by inoculation onto blood agar plates with 5% sheep blood (Torlak, Belgrade, Serbia) and incubation at 37°C for 24 h. A strain of *Staphylococcus aureus* was used as a positive control of α- and β-hemolysis (Olufemi *et al.,* 2018).

Antibiotic resistance: The occurrence of resistance in investigated isolates was performed by using BBL discs impregnated with:ofloxacin (ofx5), azithromycin (azm15), clindamycin (da2), trimethoprim (sxt25), erythromycin (e15), tetracycline (te30)(BBL Test Discs, Becton, Dickinson & Company). Several identical colonies of *Lactobacillus* and *Ln. mesenteroides* strains were resuspended in 5 ml of 0.9% physiological solution, and the standardization of suspension was achieved by comparison with McFarland standard No 1. (Ledina *et al.,* 2013). Plates were incubated for 24 h at 37°C.

Statistical analysis. Auto-aggregation, co-aggregation, and hydrophobicity were analyzed using Microsoft Excel, and results presented as the mean value ± SD. Statistical analysis was performed on the data using SPSS 11.0 Bivariate Correlation Analysis (SPSS Inc., Chicago, IL, USA). The experiment was done in triplicate.

Antimicrobial activity: The antimicrobial activity of the tested isolates was tested by diffusion method in small wells, 5 ml of soft (0.7%) nutrient agar (Torlak, Belgrade, Serbia) was poured on solid MRS medium inoculated with 10^5-10^6 cells indicator culture / ml medium (Vesković-Moračanin *et al.* 2010). Small wells with a diameter of 5 mm were formed in agar into which 100 µl of partially purified bacteriocin was poured. Partial purification of bacteriocins was performed after 18 h of growth. The cultures were spin-dried at 10000 rpm for 30 minutes at a temperature of 4 °C. The separated supernatant was neutralized to pH = 6.5 - 7.0 with 10 M NaOH. Deposition of bacteriocin was performed with ammonium sulfate (472.2 g / l) until a saturated solution was obtained.Separated bacteriocin in the form of a white deposit was dissolved in 25 ml of 0.05 M sodium phosphate buffer with pH 7. Sterilization of bacteriocin was performed by filtration through a microfilter of 0.22 µm (Acrodisc, Germany). Antimicrobial activity was identifiedby the appearance of bright zones around small wells due to growth inhibition in susceptible strains of bacteria. Tested microorganisms were:*E. coli* ATCC 25922, *St. aureus* ATCC 25923, *Listeria monocytogenes* ATCC 19115, *Pseudomonas aeruginosa* ATCC 27853, and *Bacillus cereus* ATCC 14579.

RESULTS AND DISCUSSION

Twenty-nine isolates from 9 samples of sheep ham from Sjenica were preliminarily characterized and grouped using biochemical tests (Tab. 1). Homofermentative rods that had a negative reaction to arginine were classified in 26 isolates and identified as lactobacilli. According to the sugar fermentation pattern, these isolates were identified as *Lb. curvatus* (11) and *Lb. sakei* (15) as they did not ferment esculin, mannitol, melezitose, raffinose, sorbitol, and xylose. All of the *Lb. curvatus* isolates had a positive fermentation reaction of sucrose and cellobiose. *Lb. curvatus* strains did not ferment gluconate and melibiose, while *Lb. sakei* strains showed positive fermentation tests of the abovementioned carbohydrates. Heterofermentative ovoid cocci characterized by proteolysis and a negative reaction to arginine and EPS synthesis were identified as *Leuconostoc* sp. (3), and according to the sugar fermentation patternas *Ln. mesenteroides.* Sjenica sheep ham is as of yet an unexplored fermented meat product whose autochthonous microbiota is made up of wild strains of LAB. This product is not publicized in papers, and there are no published works. Authors who studied autochthonous microbiota of similar dry-cured meat from Eastern Himalayas emphasize the dominance of *Lb. sakei* and *Lb. curvatus* in such products, while the presence of *Ln. mesenteroides* was considerably less recorded (Rai *et al.,* 2010). Pavli *et al.* (2016) have identified the microbiota, which for the more significant part consisted of isolates identified as *Lb. sakei,* with good probiotic characteristicsby researching Greek fermented meat products. Todorov *et al.* (2017) have investigated the safety and technological aspects of *Lb. plantarum*, *Lb. brevis,* and *Lb. sakei* isolates from"Lukanka" salami

unique to Bulgarian cuisine. In traditional fermented meat products such as sausages, Lactobacilli are the most commonly investigated strain,with particular emphasis on *Lb. sakei*, *Lb. curvatus* and *Lb. plantarum* (Talon *et al.,* 2007). Using MALDI-TOF, the study confirmed the preliminary identification of LAB strains, with almost all LAB strains having high–confidence identification ranging from 2.00 to 3.00. The isolate marked IIIos4 was identified as *Ln. mesenteroides*spp. *mesenteroides* had a slightly smaller but acceptable low–confidence identification rating of 1.70 to 1.99. MALDI-TOF identification is nowadays successfully applied in food microbiology to identify and classify lactic acid bacteria in fermented foods and probiotics to detect pathogens in milk and meat (Singhal *et al.,* 2015).

Table 1. Isolated species of LAB from the surface of sheep ham.

Identification MALDI TOF	***Lb. curvaus* (11)**	***Ln. mesenteroides* (3)**	***Lb. sakei* (15)**
Growth at:			
15ºC	+	+	+
45ºC	+	+	+
Growth in:			
4.0% NaCl	+	+	+
8.0% NaCl	+	+	+
Gas from glucose	-	+	-
NH_3 from arginine	-	-	-
Lipolytic activity	-	-	-
Proteolytic activity	-	+	-
EPS	-	+	-
Biochemical (API)			
L-arabinose	-	+	-
Cellobiose	+	±	+
Ribose	+	+	+
Esculin	+	+	+
Galactose	+	+	+
Lactose	+	+	-
D-mannose	+	+	+
Melezitose	-	±	-
Melibiose	-	-	+
D-raffinose	-	+	-
Sucrose	+	+	+
Trehalose	-	-	+
D-xylose	-	-	-
Rhamnose	-	+	-
Mannitol	-	-	-
Maltose	+	±	+
Sorbitol	-	+	-
Salicin	+	+	+

In vitro tests related to probiotic potential:

Auto-aggregation and co-aggregation. The ability of auto-aggregation is an essential feature of probiotic strains. It is defined as a measure in which cells can bind to the intestinal epithelium in the GI tract. At the same time, co-aggregation represents the grouping of cells of different strains to the intestinal epithelium. Probiotic strains adhere to the intestinal epithelial cells by co-aggregating with pathogens to prevent their reproduction (Aslim *et al.,* 2007). The results of the auto-aggregation and co-aggregation study of the tested LAB and *E. coli* strains are shown in Tab. 2.

The values of co-aggregation demonstrated by the tested lactobacilli and *Leuconostoc* isolates ranged from 22.5 to 65.5%. The best auto-aggregation ability was observed in isolates labeled *Lb. sakei*Ia9 and *Lb. sakei*Ia8 (68.1% after 4h), while the lowest level of auto-aggregation was demonstrated by the isolate marked as *Lb. sakei*IIb3 (24.5% after 4h).Very similar results were also observed in co-aggregation between investigated lactobacilli, *Leuconostoc,* and ATCC strain of *E. coli* which is in accordance with the results of (Mojgani *et al.,* 2015),which emphasize the association of co-aggregation with the auto-aggregation phenotype. Isolates *Lb. sakei*Ia9 and *Ln. mesenteroides*IIIos4 showed the highest degree of co-aggregation, while *Lb. sakei*IIb1a (22.5% after 4h) had the lowest degree of co-aggregation with enteropathogens. Based on the results presented, we can say that all tested strains possessed a good ability of auto and co-aggregation. While representing the results of auto and co-aggregation of LAB strains,Tuo*et al.* (2013) point out that this property may be species-specific. Kos *et al.* (2008) indicate the importance of co-aggregation of LAB strains with enteropathogenic *E. coli*, pointing to the importance of creating co-aggregates as a defense mechanism against GI and human urogenital tract infections.

Bacterial adhesion to hydrocarbons (hydrophobicity): Hydrophobicity of indigenous bacterial probiotics to different hydrocarbons is presented in Figure 1. In our study, 29 isolates showed a significant difference in hydrophobicity ranging from 5.25% to 80.9%. As a probiotic characteristic, cell surface hydrophobicity affects the adhesion and proliferation of microorganisms on the intestinal epithelial cells, which can help in adhering but is not a requirement for colonization of the intestine by probiotic bacteria (Ramiah *et al.,* 2008; Sourabh *et al.,*2010). Hydrophobicity can vary significantly in different microorganisms and even within the strains of the same species (Schär-Zammaretti and Ubbink, 2003). Hydrophobic values regarding chloroform we obtained in the study were much lower for these autochthonous isolates than the values obtained for xylene and n-hexadecane. Hydrophobicity of bacterial cells to chloroform was the most pronounced in isolate *Lb. sakei*IIos15 (28.55%), while the lowest hydrophobic capacity of 5.25% showed the isolate elabeled *Lb.sakei*IIb1a. The highest affinity for n-hexadecane was shown by strains *Lb. curvatus*IIos25, *Lb. sakei*IIb11, *Lb. sakei*IIb3 and *Lb. sakei*IIa14,whose hydrophobicity exceeded 79%. Results by Sourabh *et al.* (2010) point to high hydrophobicity with n-hexadecane ranging from

52.66% to 79.69%. *Lb. sakei*IIa14 showed the highest hydrophobicity to xylene (69.9%), while *Lb. curvatus*Ios18 showed the lowest hydrophobicity (30.55%). The results showed that in 93.11% of tested strains, hydrophobicity to xylene exceeded 40%. Strains whose hydrophobicity exceeds 40% are considered hydrophobic (Abdulla *et al.,* 2014).The degree of hydrophobicity depends on the composition and structure of bacterial cell walls and the presence of hydrophobic proteins (Pan *et al.,* 2006). Abdulla *et al.* (2014) presented the results of hydrophobicity of tested *Lactobacillus* spp. strains which ranged between 77.4% and 29.5%.

Hemolysis on blood agar plates: The research results showed no hemolysis on the blood agar was detected in autochthonous isolates, which excludes pathogenicity and confirms their safe use in the meat industry. Results presented by Pavli *et al.,* (2016) showed on noticeable hemolysis on the blood agar was observed in the hemolytics test of LAB.Results by Maragkoudakis *et al.* (2009) show that none of the tested LAB strains exhibited β-hemolytic activity, while four strains exhibited α-hemolysis. Miranda *et al.* (2014) observed that the strains of lactobacilli isolated from meat usually show no hemolytic reaction.

Table 2. Auto-aggregation and co-aggregation of studied LABValues are means of 3 replicates ± average,SD.

Strains	Auto-aggregation OD at 600 nm		Co-aggregation % OD at 600 nm	
	after 1h	after 4h	after 2h	after 4h
*Lb. curvatus*Ios1	40.0±2	45.0±3	32.3±5	36.0±4
*Lb. curvatus*Ios6i	58.3±3	50.6±3	48.0±1	49.1±1
Lb. curvatus I7a	41.0±2	45.5±2	50.9±5	46.0±2
*Lb. curvatus*Ios18	57.3±2	56.6±4	42.3±1	35.0±2
*Lb. curvatus*IIb4	62.5±2	53.3±5	55.3±0	50.8±5
*Lb. curvatus*IIosv6	56.9±3	52.7±2	51.9±3	56.9±1
*Lb. curvatus*IIa8	30.0±2	39.3±5	46.5±0	25.5±0.1
*Lb. curvatus*IIa18	51.3±1	58.6±5	33.5±4	40.9±0
*Lb. curvatus*IIa19	21.6±3	40.7±6	43.6±0	51.9±3
*Lb. curvatus*IIos21	45.5±0	52.0±2	30.8±1	29.6±1
*Lb. curvatus*IIos25	50.3±4	55.4±3	48.5±2	41.6±0
*Lb. sakei*Ia8	60.9±2	68.1±7	57.0±3	58.5±0
*Lb. sakei*Ia9	52.5±0	68.1±2	39.5±5	65.5±4
*Lb. sakei*Ia22a	45.5±4	51.5±1	35.9±9	38.2±1
*Lb. sakei*IIb1	47.8±1	50.4±1	51.5±0	50.5±2
*Lb. sakei*IIb1a	28.0±5	39.0±5	46.1±0	22.5±1
*Lb. sakei*IIb2	52.0±0	55.2±5	44.8±2	47.5±5
*Lb. sakei*IIa2a	42.1±2	40.5±3	38.0±5	40.5±5
*Lb. sakei*IIb3	62.1±2	24.5±3	29.0±4	41.0±4
*Lb. sakei*IIa6	37.1±2	32.9±5	32.6±0	38.0±5
*Lb. sakei*IIb11	41.5±5	45.5±2	31.9±5	40.5±2
*Lb. sakei*IIa13	60.5±5	53.6±5	44.5±4	46.5±2
Lb. sakei IIa14	45.3±4	59.0±9	37.0±3	38.5±2
*Lb. sakei*IIos15	47.2±1	47.1±5	44±1	48±4
*Lb. sakei*IIb21	38.2±1	41.9±5	51.0±6	41.9±5
*Lb. sakei*IIa22	37.5±2	49.0±5	41.0±8	46.0±5
*Ln. mesenteroides*Ios11	41.9±2	50.5±5	40.2±4	33.5±0
*Ln. mesenteroides*IIos4i	52.0±1	65.2±3	32.5±2	45.1±4
*Ln. mesenteroides*IIIos4	55.5±4	63.8±5	50.5±4	65.2±2

Antibiotic resistance: Antibiotic resistanceof microorganisms has increased recently and is a significant problem in the contemporary world, so resistance to clinically relevant antibiotics should be examined when exploring potential starters and probiotic cultures, as these microorganisms can reach high growth rates in the final product (Babic *et al.,*2011). Resistant strains of LAB bacteria in human and animal nutrition pose a health threat because LAB can transfer its resistant genes to antibiotics as a horizontal transfer to pathogenic bacterial species (Hummel *et al.,* 2007). Tested isolates from dry-cured sheep ham showed significant sensitivity in the commercial antibiotic disc test (Tab. 3).No investigated lactobacilli and leuconostoc isolates have

exhibited absolute resistance to antibiotics. Sensitivity to all investigated antibiotics was noticed in 65.51% of isolates, and such high antibiotic sensitivity is justified as animals, the Sjenica-Pešter sheep, whose meat was used for the production of dry-cured sheep ham, are predominantly held at the grazing alpine elevation of about 1150 meters and rarely treated using antibiotic therapy. Antibiotic to which most strains have shown resistance was erythromycin (20.68%). Similar results were presented by Dias *et al.* (2015), indicating a high percentage of LAB strains resistance (94%) to erythromycin. *Lb.curvatus*Ios18 and *Lb.sakei*IIb21 showed resistance to ofloxacin and erythromycin while *Lb. sakei*Ia22a, and *Lb. sakei*IIa2a showed resistance to ofloxacin and azithromycin but were susceptible to all other antibiotics.The results obtained in this study show a good sensitivity of the tested strains of LAB to commercial antibiotics and present them as potential probiotics. Babic *et al.* (2011) research on microbiota from Slavonskikulen showed that most of the staphylococci and all of the lactobacilli showed sensitivity to all antibiotics tested.

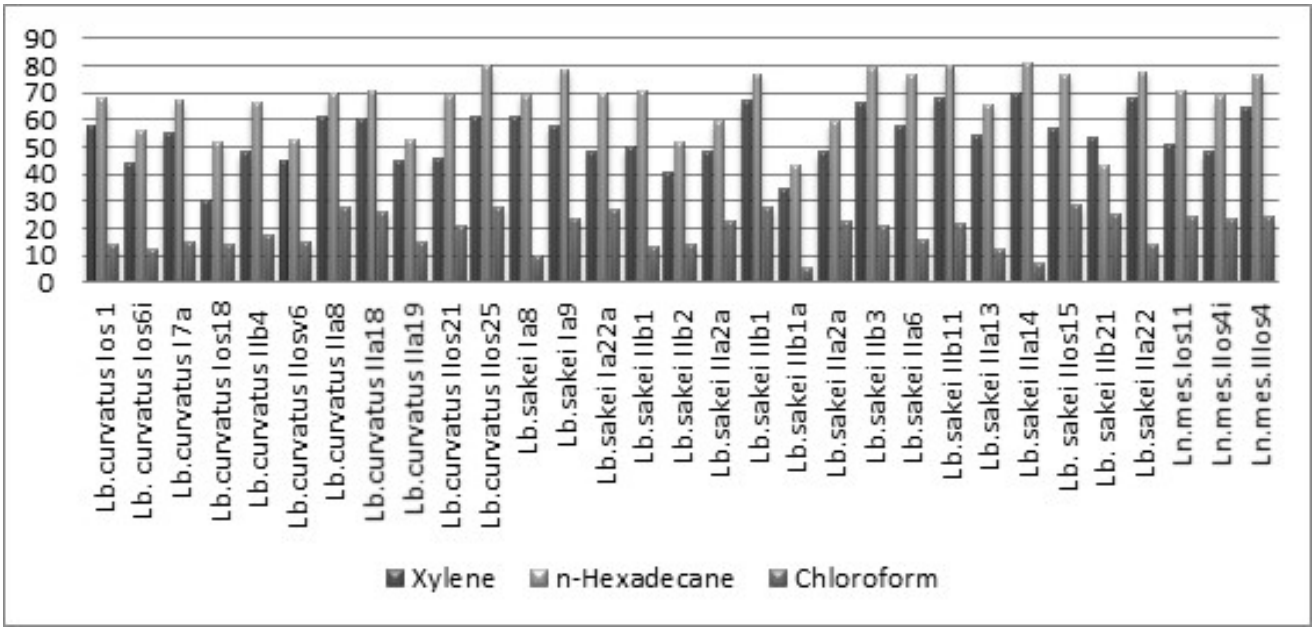

Fig. 1.Hydrophobicity of indigenous bacterial probiotics to different hydrocarbons.

Antimicrobial activity: Probiotics act as a first line of defense against disease-causing pathogens, improving the intestinal microbiota (Kailasapathy and Chin, 2000). Identification and characterization of microorganisms as potential probiotics should demonstrate the strain's ability to be sustained and metabolically active in the GI tract and biologically active against identified pathogens (Kailasapathy and Chin, 2000). Table 4 presents the obtained values of antimicrobial activity of the analyzed isolates of lactobacilli and leuconostoc species. The study indicates that more than 13% of the strains do not possess antimicrobial activity against *St. aureus* and *B. cereus,* while 55.1% showed antimicrobial activity against all investigated pathogens.The inhibition zone diameters against *E. coli* ATCC 25922 ranged from 10.2 to 25.5 mm. *Lb. curvatus*IIa8, *Lb. sakei*IIb1a, *Lb. sakei*IIa2a, *Lb. sakei*IIa6, and *Lb. sakei*IIos15 isolates did not inhibit *E. coli* ATCC 25922 while *Lb. curvatus*Ios6 and IIv6, *Lb.sakei*IIb1 and IIa13 showed zones of inhibition greater than 15 mm against all pathogens, while 31.03% of strains did not show zones of inhibition to *L.monocytogenes.*The inhibition zone diameters ranged from 8 to 24 mmagainst *P. aeruginosa* ATCC 27853. The obtained results are in accordance with the investigations of other researchers. While exploring traditional sausages,Erkaya *et al*. (2020) presented the diversity of the indigenous microbiota and the ability of isolates to produce bacteriocins, emphasizing tha *Ln. mesenteroides*and *Lb. sakei* carried the mecentericin and sacacin genes.LAB strains from meat as starter culture can influence improved safety and health benefits in fermented meat products(Ajao *et al.,*2018).The ability of *Lb. sakei*, *Lb. curvatus* and *Lb. plantarum* strains to inhibit *L. monocytogenes* and *St. aureus* is of great importance for improving the hygienic quality of Greek sausage (Papamanoli *et al.,*2003).LAB strains isolated from raw beef exhibited strong and broad antimicrobial activities, inhibiting *E. coli*, *L. monocitogenes*, *Klebsiella pneumoniae,* and *St.aureus* (Ajao *et al.,*2018).

Table 3.Antibiotic susceptibilityofLAB isolates from dry-cured sheep ham.

Strain	Antibiotics [a,b]					
	OFX5	AZM15	DA2	SXT25	E15	TE30
*Lb. curvatus*Ios1	S (23)	S (28.4)	S (31)	S (29)	S (24)	S (24.5)
*Lb. curvatus*Ios6i	S (25.5)	S (28)	S (33)	S (31.5)	S (29)	S (24)
Lb. curvatus I7a	S (30)	S (29.5)	S (24)	S (27.5)	S (30)	S (13)
*Lb. curvatus*Ios18	R (19.5)	S (23)	S (22)	S (28.5)	R (17)	S (28)
Lb. curvatus IIb4	S (24)	S (28)	S(26.5)	S (22)	R (18)	S (22.5)
*Lb. curvatus*IIv6	S (25)	S (25)	S (24)	S (22.5)	S (26)	S (29)
Lb. curvatus IIa8	S (20)	S (22.5)	S (25)	S (20)	S (21)	S (20.5)
Lb. curvatus IIa18	S (30.5)	S (24)	S (23)	S (24)	S (25.5)	S (25.5)
Lb. curvatus IIa19	S (22)	S (21.5)	S (22)	S (25)	S (25)	S (27)
*Lb. curvatus*IIos21	S (30)	S (27)	R (16)	S (22.5)	S (25)	S (22)
*Lb. curvatus*IIos25	S (30)	S (26)	S (30)	S (27.2)	S (26.5)	S (25.5)
Lb. sakei Ia8	S (22.5)	S (24)	S (30)	S (29.5)	S (30)	S (22)
Lb. sakei Ia9	S (29.5)	S (26)	S (29.5)	S (30)	S (24)	S (24)
Lb. sakei Ia22a	R (15.5)	R (18)	S (26)	S (30)	S (26)	S (26)
Lb. sakei IIb1	S (24.6)	S (23)	S (25.5)	S (24.5)	S (28)	S (28.5)
Lb. sakei IIb1a	S (24)	S (23)	S (26.5)	S (21)	R (16)	S (21)
Lb. sakei IIb2	R (18.6)	S (22)	S (26)	S (30)	S (23)	S (30)
Lb. sakei IIa2a	R (16)	R (16)	S (20)	S (20.5)	S (21.5)	S (21)
Lb. sakei IIb3	S (29.5)	S (25)	S (25.5)	S (23)	S (26)	S (25)
Lb. sakei IIa6	S (21)	S (24.5)	S (23)	S (25)	R (17.5)	S (23)
Lb. sakei IIb11	S (25)	S (30.5)	S (25)	S (28.5)	S (29)	S (25)
Lb. sakei IIa13	S (25)	S (29)	S (31)	S (30)	S (30)	S (27)
Lb. sakei IIa14	S (26)	S (26.5)	S (29.5)	S (30)	S (30)	S (25)
*Lb.sakei*IIos15	S (29)	S (28)	S (26)	S (30)	S (26)	S (26.6)
Lb. sakei IIb21	R (17.5)	S (24)	S (28.5)	S (26)	R (18)	S (26)
Lb. sakei IIa22	S (24)	S (28.5)	S (30)	S (28.5)	S (24.5)	S (23)
*Ln. mesenteroides*Ios11	S (25.4)	S (24)	S (22)	S (21)	S (22)	S (20.5)
*Ln. mesenteroides*IIos4i	S (25)	S (22)	S (30)	S (28)	R (15)	S (23)
*Ln. mesenteroides*IIIos4	S (26)	S (25)	S (23)	S (28)	S (28)	S (21)

*Zones of inhibition given in mm; R resistant, S susceptible.
ofloxacin (ofx5), azithromycin (azm15), clindamycin (da2), trimethoprim (sxt25), erythromycin (e15), tetracycline (te30).

Table 4. Antimicrobial activity of the isolated LAB fromdry-cured sheep ham.

Strains	Diameter of inhibitory zone				
	***Escherichia coli* ATCC 25922**	***Staphylococcus aureus* ATCC 25923**	***Listeria monocytogenes* ATCC 19115**	***Pseudomonas aeruginosa* ATCC 27853**	***Bacillus cereus* ATCC 14579**
*Lb. curvatus*Ios1	10.5±0.5	18.7±1.0	10.14±0.3	8.4±0.4	15.5±0.4
*Lb. curvatus*Ios6i	25.1±0.8	27.0±0.5	15.5±1.7	24.7±.4	28.2±0.5
*Lb. curvatus*Ios18	13.0±0.1	10.3±0.1	12.6±0.3	20.6±4.2	12.5±0.2
*Lb. curvatus*IIv6	25.2±0.2	27.0±1.5	16.0±0.5	20.5±0.0	23.1±0.1
*Lb. curvatus*IIa8	/	/	/	10.5±0.6	/
Lb. curvatus I7a	20.1±0.1	25.4±0.6	/	10.3±0.3	25.0±0.9
*Lb. curvatus*IIa18	16.5±0.5	20.5±1.6	10.3±0.5	20.6±0.8	20.6±1.1
*Lb. curvatus*IIa19	20.4±0.4	25.2±0.9	10.0±0.9	24.0±1.9	25.25±0,1
*Lb. curvatus*IIos21	10.3±0.2	12.0±1.1	10.2±0.1	10.0±0.1	14.5±0.2
*Lb. curvatus*IIos25	15.9±0.6	18.5±1.4	10.2±0.4	8.0±0.7	14.4±0.9
*Lb. sakei*Ia8	14.3±0.1	20.5±0.5	/	15.3±0.2	18.3±0.1
*Lb. sakei*Ia9	20.3±0.2	22.6±2.0	8.3±0.1	18.3±0.2	20.3±0.2
*Lb. sakei*Ia22a	12.3±0.1	25.8±1.0	/	10.3±0.1	18.6±1.1
*Lb. sakei*IIb1	22.2±0.2	26.7±0,1	17.7±0.9	20.7±0.2	25.9±0.0

*Lb. sakei*IIb1a	/	8.2±0,9	/	/	/
*Lb. sakei*IIb2	22.0±0.0	23.0±0.4	10.0±0.0	19.5±0.5	25.5±0.2
*Lb. sakei*IIa2a	/	20.4±1.0	/	/	/
*Lb. sakei*IIb3	12.3±0.2	25.6±0.1	8.2±0.2	8.7±0.0	25.6±0.2
*Lb. curvatus*IIb4	10.2±0.0	20.5±0.2	16.2±0.0	/	20.4±0.2
*Lb. sakei*IIa6	/	/	/	/	10.3±0.2
*Lb. sakei*IIb11	25.5±0.0	15.3±1.9	18.2±0.3	10.2±0.2	8.5±0.5
*Lb. sakei*IIa13	23.1±0.5	28.0±0.0	18.0±0.1	22.5±0.1	25.6±0.0
Lb. sakei IIa14	22.3±0.8	/	10.3±0.2	10.5±0.0	/
*Lb. sakei*IIos15	/	22.3±1.7	8.0±0.0	/	20.6±0.1
*Lb. sakei*IIb21	14.0±0.8	16.5±0.8	/	13.5±0.2	15.2±0.1
*Lb. sakei*IIa22	10.6±0.2	/	14.4±0.6	12.6±0.0	12.0±0.1
*Ln. mesenteroides*Ios11	11.5±0.8	25.8±1.4	/	10.5±0.4	23.5±0.4
*Ln. mesenteroides*IIos4i	15.7±0.4	15.4±1.0	10.0±0.0	14.3±0.6	15.4±0.1
*Ln. mesenteroides*IIIos4	13.0±2.7	18.0±2.2	12.4±0.1	10.0±0.0	19.3±0.4

zone of growth inhibition given in mm (millimeter); / - no zone of inhibition

Conclusions: This paper presented the results of isolation and identification of indigenous strains of LAB from spontaneously fermented dry-cured sheepmeatproduct for the first time. As wild and previously untreated LAB strains were examined, in vitro tests were used to screen their most important probiotic features and confirmed their potential. Good ability of auto and co-aggregation, high degree of hydrophobicity, absence of hemolysis, presence of antibiotic sensitivity to most commercial antibiotics,and antimicrobial activityopen new fields of research for tested strains of *Lb. curvatus,Lb. sakei* and *Ln.mesenteroides* as potential probiotics and starter cultures for the meat industry and for the product itself as a functional food.

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