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Quality and autochthonous microbiota of dry-cured sheep ham from western Balkans

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Dry-cured sheep ham is a traditional product of Western Balkans. It is prepared by dry curing specially treated whole sheep carcasses which are smoked for a short time and spontaneously fermented in air under uncontrolled conditions. Lactic acid bacteria (LAB) play a key role in defining the quality and organoleptic characteristics of dry-cured sheep ham. The aim of this research was to investigate the chemical parameters of dry-cured sheep ham quality as well as the isolation and preliminary categorization of LAB. To this end, samples of dry-cured sheep ham were obtained from nine sheep of average age of about five years, from three households from the geographical area Sjenica (Western Serbia). Physicochemical analysis has determined the content of water, protein, fat, mineral matter, water activity and pH values in the product. Phenotypic characterization of LAB isolated from dry-cured sheep ham was based on the general morphology of the cell, physiological tests and sugar fermentation patterns of LAB isolates. 124 isolates of LAB were preliminary identified as *Lactobacillus curvatus* , *Lactobacillus sakei* and *Enterococcus faecium*. Chemical analysis confirmed a harmonious relationship between the quality parameters of dry-cured sheep ham.

KEYWORDS

>> Dry-cured sheep ham
>> Lactic acid bacteria
>> Preliminary categorization
>> Physicochemical parameters

Sheep meat has characteristic smell and taste, light red to dark red color and yellowish fat, densely compacted muscle fibers and is firmly enveloped in, not interwoven, by fat (dos Santos Rodrigues et al., 2014). Dry-cured sheep hamis produced in the territory of Bosnia and Herzegovina (B&H) and during its production process specific spices and garlic are added to give the product specific flavor for which it is famous (Ganić and Smajić, 2013). The former Yugoslav Republic of Macedonia has traditionally produced dry-cured sheep ham by dry salting without smoke and long air fermentation. In Croatia, dry sheep meatcalled kaštradina is produced, a product very similar to dry-cured sheep ham.

There are many factors that affect the quality of dry-cured sheep ham, and the main are proper selection of raw materials and food technology factors (Čaušević et al., 1984). Dry-cured sheep ham or Sjenica (Western Serbia) sheep ham is produced in a very complex manner, and a prerequisite for the production of meat is the sanitary safety of raw materials which meet veterinary and sanitary conditions of production. Dry-cured sheep ham or Sjenica sheep ham is produced in a very complex manner, and a prerequisite for the production of meat is the sanitary safety of raw materials which meet veterinary and sanitary conditions of production. Production of dry-cured sheep ham includes several stages: selection of raw materials, salting and brining, smoking, drying and ripening, in which the product remains sustainable and receives characteristic smell, consistency, and texture (Stamenković and Dević, 2004).

Few authors have dealt with the research of dry-cured sheep ham to Western Balkans, so this type of product has not been explored thoroughly. The research that has been conducted focuses on the traditional production of dry-cured sheep ham from the Sjenica area, during the winter and spring seasons. Physico-chemical analysis of dry-cured sheep ham aims to define the quality of the product and to provide the missing data. Microbiota that develops during the process of fermentation of dry-cured sheep ham consists of lactic acid bacteria (LAB), and coagulase-negative staphylococci (CNS). LAB affects the acidification of the product to a level that inhibits the growth of pathogenic bacteria and allow the solubilization of proteins and gel formation by myofibrillar proteins (MPs) of sarcoplasm, degradation of proteins and lipids, and dehydration (Hammes et al., 2008). The composition of microbial populations originates from microorganisms naturally found in meat or appearing there due to the production process (Žugić Petrović et al., 2016). The aim of this study was the isolation and identification of LAB from dry-cured sheep ham.

Materials and methods

Nine sheep of Sjeničko-pештarska Pramenka were taken from the three producers (A, B, and C) from the territory of Sjenica. On average, the animals were approximately five years old, weighing 47 to 60 kg.

Raw materials and processing

After slaughtering (at 5–10 °C), the carcasses were chilled at 4 °C for 24 h. Then the sheep carcasses were opened by longitudinal cuts in the middle of the pectoral bone and the pelvic joint, and all the bones from the inside of the carcass were removed, taking care not to disturb the outer appearance of the meat (Stamenković and Dević, 2006). Bones remaining on the product are the bones of the shingles and fibula (*Os tibia* et *Os fibula*), which, together with the tendons, serve as a suspension for smoking and drying. Meat from the inside of the leg (šol) is most often used for making dry-cured sheep ham (Stamenković and Dević, 2006).

Salting process is conducted by rubbing table salt in an amount of 3–3.5% and kept in containers for 5–8 days (4–7 °C, RH 80–90%). After the salting process is finished, squeezing of extra liquid and air drying are carried out After that, dry-cured sheep ham is smoked in cold beech smoke at the temperatures of 16–18 °C, RH 65–80% for a period of 15 days. During the smoking process, it is important that the hanging carcasses do not touch each other in order to get evenly cured.

Curing of the product is carried out in the temperature range of 4–10 °C, RH 60–70%, depending on weather conditions. In order to determine physicochemical quality parameters and microbiota of dry-cured sheep ham samples from the anatomical positions on the product, ham and shoulder, were taken for the examination. The sampling method of dry-cured sheep ham was conducted according to the regulation of

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general and specific food hygiene requirements at any stage of production, processing, and transport ("Sl. glasnik RS", br. 72/2010).

Chemical analysis of the products

Water content in dry-cured sheep ham was determined by using the reference method SRPS ISO 1442: 1998. Fat content in dry-cured sheep ham was determined by using the reference method SRPS ISO 1443: 1992. Protein content in dry-cured sheep ham was determined by using the reference method SRP ISO 937:1992. The total ash content in dry-cured sheep ham was determined by the reference method SRPS ISO 936: 1999.

Determination of the pH value of dry-cured sheep ham was performed by pH meter (Testo 205; Lenzkirh, Germany). Determination of a_w value of the product was carried out using a mobile device LabSwift – a_w (Novasina, Lachen, Switzerland).

Enumeration and isolation of LAB

Samples of dry-cured sheep ham (10 g) were aseptically transferred to a 90 ml of sterile (sterilization for 20 min at 121 °C) peptone saline solution (NaCl 0.8 g/ l and 1 g of peptone/l) and stirred for 15 minutes. The number of microorganisms was determined by the indirect method of successive dilutions, which consists in making and transmitting the appropriate dilution into solid substrates: De Man, Rogosa, Sharpe, Agar (MRS, Torlak, Belgrade, Serbia) in accordance with the standard method for sample preparation (SRPS EN ISO 6887-1: 2008). The number of LAB was determined on MRS agar plates which, after application of 1 cm^3 dilution and solidification were poured an additional layer of the substrate to achieve microaerophilic conditions. After 5 days of incubation at 37 °C and counting of the grown colonies, seemingly disparate colonies were transferred into De Man, Rogosa, Sharpe, broth (MRS, Torlak, Belgrade, Serbia) and incubated for 48 h at 37 °C. After incubation, bacterial cultures were transferred from the broth to the Petri dishes and triple purification of the isolates was carried out by successive transfer of single colonies on new Petri dishes with MRS agar and incubated for 48 h at 37 °C (Radulović et al., 2004).

Phenotypic characterisation

Preliminary characterization of isolates was performed by Gram-staining, as well as by examining the ability of catalase synthesis by dripping 30% H_2O_2 on the smear slide of a pure culture on the glass slide. All Gram-positive (G+) and catalase-negative isolates were subjected to further examination. Cell morphology of isolates, as well as their motility, was determined using a phase contrast microscope (Olympus, Japan). There were 13 tests for the identification of isolates: morphological characteristics, arginine hydrolysis, the ability to grow 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, growth on the esculin bile agar, lipolytic activity, proteolytic activity, exopolysaccharide synthesis, sugar fermentation patterns of LAB isolates.

Arginine hydrolysis

Hydrolysis of arginine was analysed by growing the culture in arginine broth (HiMedia Laboratories, Mumbai, India). After incubation, a few drops of phenol red were added to arginine broth (occurrence of red color indicates a positive reaction, and yellow negative) (Phalakornkule and Tanasupawat, 2006–2007).

Growth ability at different temperatures (15 °C and 45 °C)

The growth ability was examined using the method described by Kloos, Tornabene and Schleifer (1974), with certain modifications. Bacteria were seeded on MRS agar (Torlak, Belgrade, Serbia), and then incubated at temperatures of 15 and 45 °C/24h. After the incubation, the growth of colonies was examined at various temperatures.

Growth ability in the presence of 4 and 8% NaCl

The growth ability was examined using the method described Phalakornkule and Tanasupawat, (2006–2007). Bacteria were seeded in MRS broth (Torlak, Beograd, Serbia) with varying concentrations of salt (4 and 8% NaCl), then incubated at temperatures of 37 °C/24h. After the incubation, the growth of colonies in broths with certain concentrations of NaCl was examined.

CO_2 production from glucose

The production of gas from D-glucose was examined by using MRS broth with a Durham tubes (Phalakornkule and Tanasupawat, 2006–2007).

Growth ability on the esculin bile agar

For preliminary identification of enterococci isolates were grown on esculin bile agar (Torlak, Belgrade, Serbia). The appearance of red colonies indicated a positive reaction, that is, the presence of enterococci.

Proteolytic activity of bacteria

Proteolytic activity of the bacteria was examined according to the method described by Harrigan and McCance (1976) with some modifications. The substrate was made by mixing nutrient agar and milk (1.6% fat) in 1:1 ratio. Bacteria were aseptically transferred to the medium and left to incubate at 37 °C/24-48 h. Thereafter, proteolytic solution (Lugol's iodine) was poured on the nutrient medium, the occurrence of transparent zones around the cultured bacteria was tracked. If that zone was present, proteolytic activity of bacteria was present, if not, there was no proteolytic activity. As a positive control, we used the strain of *Bacillus subtilis*.

Lipolytic activity

Lipolytic activity of bacteria was examined according to the method described by (Harrigan and McCance, 1976) with some modifications on the medium which is made by adding of egg yolk (4%) to the nutrient agar. Bacteria were then aseptically transferred to the surface and left to incubate at 37 °C/72 h. After that, the occurrence of transparent zones around the cultured bacteria, which must be greater than 1 mm (Škrinjar, 2001), was tracked. If the zone was present, bacteria exhibited lipolytic activity, if not, and then there was no lipolytic activity. As a positive control, we used the strain of *Bacillus subtilis*.

Exopolysaccharide synthesis

Exopolysaccharide synthesis is detected visually (appearance of gelatinous colonies) after incubation of isolates on modified nutrient agar with (10%) of sucrose, fructose, lactose and glucose (Torlak, Belgrade, Serbia) at a temperature of 37 °C /48 h (Drosinos et al., 2005).

Sugar fermentation patterns of LAB isolates

Sugar fermentation profiles were determined by using the API 50CH bacterial identification system (BioMerieux, S.A., France). The API test strips were prepared as recommended by the kit supplier and scored after incubation for 24 and 48 hours at 37 °C.

Statistical analysis

Allthe tests have been performed in triplicate. The results represent the mean ± standards deviations. Statistical analysis was conducted with SPSS 11.0 Bivariate Correlation Analysis (SPSS, Chicago, Illinois, USA).

Results and Discussion

Chemical composition of dry-cured sheep ham samples is relatively uniform for all manufacturers and is presented in Table 1. The average water content in the samples taken from the household A had an average value of 43.7%. In the samples taken from household B, the average moisture content was 42.9%. The water percentage in samples taken from household C had an average value of 44.2%. The amount of protein was also uniform and ranged in an interval of 32.9–34.2%. While researching dry-cured sheep ham, Gajić et al. (2000) determined that protein percentage in the product had a value of 29% at 5.3% NaCl. Čaušević et al. (1984) present the results for manufactured dry-cured

Quality indicators

Tab 1: Average values of physic-ochemical quality indicators of dry-cured sheep ham

Test group	Examined properties (%)						
	Water	Proteins	Fat	Ash	NaCl	a_w	pH
Household A	43.7±1.1*	33.9±1.6*	10.7±0.1*	9.8±0.5*	5.3±0.2*	0.82±0.01*	5.47±0.23*
Household B	42.9±0.1*	34.2±0.7*	11.6±0.1*	9.1±0.0*	5.1±0.1*	0.80±0.01*	5.58±0.03*
Household C	44.2±1.3*	32.9±2.5*	10.9±0.5*	9.3±1.3*	5.2±0.2*	0.83±0.03*	5.6±0.1*

There is no statistically significant difference in the controlled parameters (P>0,05) mean value±standard deviacion.

Source: Petrovic

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Attributes

Tab. 2: Biochemical properties of LAB isolates isolated from dry-cured sheep ham

Character	*Lb. curvatus* os (88)	*En. faecim* os (8)	*Lb. sakei* os (28)
Cell morphology	rods	cocco	rods
Growth at:			
15 °C	+	+	+
45 °C	+	+	+
Growth in:			
4% NaCl	+	+	+
8% NaCl	+	+	+
Gas from glucose	–	–	–
NH_3 from arginine	–	+	–
Lipolytic activity	–	+	–
Proteolytic activity	–	+	–
EPS	–	+	–
Bile esculin agar	–	+	–
Biochemical (API)			
L-arabinose	–	–	–
Cellobiose	+	+	+
Ribose	+	+	+
Esculin	+	+	+
Galactose	+	+	+
Lactose	+	+	–
D-mannose	+	+	+
Melezitose	–	–	–
Melibiose	–	+	+
D-raffinose	–	–	–
Saccharose	+	+	+
Trehalose	–	–	+
D-xylose	–	+	–
Rhamnose	–	+	–
Mannitol	–	+	–
Maltose	+	+	+
Sorbitol	–	–	–
Salicin	+	+	+

Source: Petrovic

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sheep ham where the amount of protein in the sample (sampled from the ribs and kidneys) was 21.2%. Fat content of dry-cured sheep ham in samples taken from producer A had a mean value of 10.7%, B 11.6% and C 10.9%. The quantity of ash was uniform in all tested samples, with the mean value ranging from 9.1–9.8%. The averages value of aw and pH of the product at all manufacturers is very similar. Such results were expected given that the samples were of manufactured dry-cured sheep ham with identical technological and microclimatic conditions of production (Tab. 1).

Phenotypic characterization of LAB

Testing results of 124 strains isolated from 9 samples of dry-cured sheep ham showed that all isolates were G+ and catalase negative, and the results of morphological and biochemical tests, as well as sugar fermentation using the API system, are shown in Table 2. Rod-shaped cells were observed in 116 isolates (93.5%) and classified as lactobacilli. Present homofermentative lactobacilli years did not show hydrolysis reaction to arginine. Lipolytic, proteolytic and exopolysaccharides synthesis tests were negative, and a slight difference in colony morphology and shape, as well as the fermentation of carbohydrates using API system, allowed us to identify isolates as *Lb. curvatus* and *Lb. sakei* species (Tab. 2).

The isolates identified as *Lb. curvatus* and *Lb. sakei* did not ferment esculin, mannitol, melezitose, raffinose, sorbitol, and xylose. *Lb. curvatus* strains did not ferment gluconate and melibiose, while *Lb. sakei* strains showed positive fermentation tests of the aforementioned carbohydrates in this study. All of the *Lb. curvatus* strains showed positive fermentation of sucrose and cellobiose. Lactobacilli are dominant in the studied samples of dry-cured sheep ham due to the formation of lactic acid and they play a key role in the maturation of this traditional meat product. The study of microbiota in fermented sausages by Drosinos et al. (2005) found isolates of *Lb. curvatus* which did not ferment sucrose and a large number of isolates had a positive reaction of cellobiose fermentation. In this study, *Lb. curvatus* was identified in 70.9% of the samples, while *L. sakei* was the second dominant flora of dry-cured sheep ham with 22.5%.

Preliminary identification of nine isolates characterized by the appearance of black colonies on bile esculin agar identified them as *Enterococcus* sp. Enterococci were homofermentative, with good growth in the temperature range of 15 and 45 °C, successfully grew in ambient conditions with 4 and 8% NaCl, and also showed the hydrolysis of arginine as well as the synthesis of exopolysaccharides. *Enterococcus* sp. samples were identified based on the carbohydrates fermentation by means of API system as *Enterococcus faecium* . *En. faecium* strains fermented esculin, cellobiose, and sucrose, but did not ferment sorbitol, raffinose and xylose. The composition of the microbial population of Sjenica sheep ham comes from microorganisms naturally found in meat or appearing there due to the production process. Various factors condition favoring the growth of useful microbial population and prevent the growth of pathogenic and harmful microorganisms that can be found in the product. Aksu and Kaya (2001) present the results of the identification of

microorganisms in pastirma where LAB, in addition to *Micrococcus* sp. and *Staphylococcus* sp. play a major role in the fermentation of dry-cured sheep ham. Dincer and Kivanc (2012) in their work emphasize the dominance of LAB in Turkish pastirma (a salted and dry-cured meat product), the authors isolated 92 isolates which were identified using API system as *Lactobacillus plantarum* , *Lb. sakei*, *En. faecium* and *Pediococcus acidilactici*.

Conclusion

Dry-cured sheep ham is a fermented meat product whose quality and organoleptic properties depend on microorganisms. In the traditional production of Sjenica dry-cured sheep ham, microorganisms originate from raw materials and the environment in which the products are made. Thus, the fermentation process is not controlled, so in this case, only wild strains of microorganisms that make up the microbiota of the product were examined. Lactobacilli proved dominant in the investigated samples and it can clearly be stated that due to the formation of lactic acid they play a crucial role in the maturation of this traditional product, while the *Enterococcus* are represented in a very small percentage as microbiota in Sjenica dry-cured sheep ham.

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