

The characterization of lactic acid bacteria isolated during the traditional production of Užička sausage

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Abstract. Užička sausage is a traditionally fermented dry sausage that is produced in western Serbia. It is made of beef and pork with the addition of solid fat and natural spices. The whole manufacturing process lasted for 21 days. The goal of this study was to create a collection of lactic acid bacteria isolated during the ripening and identify them using molecular methods. A total of 50 isolates from different stages of ripening (fermentation and drying) were identified by molecular methods. *Leuconococcus mesenteroides*, *Lactobacillus brevis*, and *Lactobacillus sakei* were the predominant microorganisms in Užička sausage.

1. Introduction

Modern manufacturing procedures employ technologies for contemporary meat production that are significantly faster than traditional procedures. However, the resulting meat products, especially dried meat and dry fermented sausages, often fail to acquire the sensory characteristics typical for traditionally manufactured products. Basic differences occur due to usage of smoke produced in smoke generators, while traditional procedures employ smoke obtained from open fire beds. Next, neither starter cultures, nor glucono delta-lactone are used in traditional procedures. Ripening (fermentation and drying) processes, rather, are influenced by proteolytic and lipolytic enzymes from muscle and fat tissue as well as the epiphytic microbiota present. The dynamics of these processes, which are mainly directed by activity of the microbiota, are not under control in traditional manufacturing, and therefore, dry fermented sausages manufactured in traditional ways often fail to meet uniform quality standards.

The production of fermented sausages is an area of meat processing that has been the subject of very intensive scientific research in recent decades. Due to the desirable and recognizable sensory properties of traditionally produced dry sausages, demand for them is constantly growing. The production of these products is still based on local customs and traditional production methods.

The microorganisms that are most often responsible for these transformations are lactic acid bacteria (LAB), coagulase negative cocci (CNC) and yeasts [1]. LAB play an essential role in the production of fermented meat products. The products of their metabolism affect the process of ripening, development of desired sensory and nutritive characteristics of the products and, at the same time, inhibit the development of undesired microbiota [2]. They contribute to the development of flavor, color and texture of meat products. Reduction of pH as a result of lactic acid synthesis by LAB is an important effect of fermentation, which ensures the safety, stability and shelf life of meat products [3,4,5]. Traditional fermented sausages with a specific geographical origin have unique sensory characteristics and are generally of high quality [6].



There is growing interest in being able to isolate and describe the LAB population in traditional fermented foods, especially with the advancement of methods in molecular microbiology [7,8,9]. Molecular methods are all reliable, simple to interpret and their application avoids subjective interpretation by the user. In order to protect the traditional approach to sausage manufacturing, it is essential to understand the microbial diversity and to select autochthonous starter cultures that can be used in the production of innovative foods with a geographical origin [8]. LAB that have been isolated from fermented sausages belong to the genera *Lactobacillus*, *Weissella*, *Leuconostoc*, *Pediococcus*, *Enterococcus* and *Lactococcus* [8,9,10]. Among them, lactobacilli are most frequently isolated from sausages produced with different technologies, especially the species *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum* [3,9,10]. Other lactobacilli that can be found in lower numbers are *Lactobacillus brevis*, *Lactobacillus buchneri* and *Lactobacillus paracasei* [11,12].

One of the traditionally fermented dry sausages in Serbia is Užička sausage, which is produced in western Serbia. The goal of this study was to create a collection of LAB isolated during the ripening of Užička sausage and complete their molecular identification.

2. Materials and Methods

2.1 Užička sausage

Užička sausage was traditionally produced in an artisanal household in Zlatibor district, Serbia. Užička sausage was composed of beef, pork and fat in the ratio of 50:20:20. Meat and fat were ground to the size of 3 mm and mixed with nitrite curing salt (2.5%), sucrose (0.33%) and spice mixture (0.25%; composed of sweet and hot red peppers, black pepper and garlic). Prepared stuffing was filled into small beef intestine casings (diameter of 40 mm) to form sausages. Sausages were cold smoked for 4 days. The whole manufacturing process (smoking, fermentation and drying) lasted for 21 days.

2.2 Microbiological investigation

Sausage samples for microbiology examinations were taken on days 0, 2, 4, 7, 14 and 21. The experiment was repeated three times. Three replicate sausage samples were collected on each sampling day and used for analysis. Each sausage sample weighing 25 g was homogenized in 225 mL of MRD (Oxoid, UK) in a stomacher (AES, France) for 90 s. Serial dilutions (10-fold) were plated onto MRS agar (Oxoid, UK) in duplicate, and incubated for 48 h at 30°C under microaerophilic conditions. From each plate, single colonies were randomly picked and streaked on fresh agar plates in order to obtain pure cultures. Suspect LAB isolates from MRS agar were examined by Gram staining and catalase reaction. A total of 50 Gram-positive and catalase-negative isolates were further identified and characterized by molecular methods. Total DNA from LAB was extracted from a single colony by using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Germany) according to the manufacturer's protocol for Gram-positive bacteria. PCR was performed in a final volume of 50 µL containing 1× PCR buffer (10× PCR buffer: 500 mM KCl, 100 mM Tris-HCl, 0.8% Nonidet P40), 2.5 mM MgCl₂, 10 µM dNTP, 200 nM of each primer, 1 U of Taq polymerase (Fermentas, Lithuania) and 100 ng of DNA template. DNA in PCR tubes was amplified in a thermal cycler (Techne, UK) using primers P1V1 (GCGGCGTGCCTAATACATGC) and P4V3 (ATCTACGCATTTACACGCTAC), complementary to the V1-V3 region of the 16S rRNA, by 5 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 42°C, 2 min at 72°C and the final extension of 5 min at 72°C. PCR products were purified by QIAquick PCR purification kit (Qiagen, Germany) and sent for sequencing to IIT Biotech (Bielefeld, Germany). The BLAST algorithm was used to determine the most related sequence relatives in the NCBI nucleotide sequence database (<http://blast.ncbi.nlm.nih.gov>).

3. Results and Discussion

Table 1. LAB species identified by molecular methods during production of Užička sausage using traditional techniques.

Day of ripening	Strain identification by 16S rRNA gene sequencing
0	<i>Ln. mesenteroides</i> subsp. <i>mesenteroides</i> (7 isolates) <i>Lb. curvatus</i> <i>Lb. brevis</i> <i>Pediococcus parvulus</i> <i>Lc. lactis</i> subsp. <i>cremoris</i> (2 isolates)
2	<i>Ln. mesenteroides</i> subsp. <i>mesenteroides</i> (2 isolates) <i>Ln. inhae</i> <i>Weissella hellenica</i> <i>Lb. brevis</i> (2 isolates)
4	<i>Lb. sakei</i> (3 isolates) <i>Lb. brevis</i> (4 isolates) <i>Weissella hellenica</i>
7	<i>Ln. mesenteroides</i> subsp. <i>mesenteroides</i> (2 isolates) <i>Pediococcus pentosaceus</i> <i>Lb. curvatus</i> <i>Lb. brevis</i> (2 isolates)
14	<i>Ln. mesenteroides</i> subsp. <i>mesenteroides</i> (5 isolates) <i>Lb. sakei</i> <i>Weissella hellenica</i>
21	<i>Lb. brevis</i> (2 isolates) <i>Lb. sakei</i> subsp. <i>sakei</i> (4 isolates) <i>Ln. mesenteroides</i> subsp. <i>mesenteroides</i> (5 isolates)

The most dominant microorganisms in Užička sausage were *Leuconostoc mesenteroides*, *Lb. brevis*, and *Lb. sakei* (Table 1). Besides them, *Lactococcus lactis* subsp. *cremoris*, *Lb. curvatus*, *Weissella hellenica*, *Pediococcus pentosaceus*, *Pediococcus parvulus* and *Leuconostoc inhae* were also identified, although in lower levels.

The prevalence of *Ln. mesenteroides* found in Užička sausage is not in agreement with findings of authors who studied traditionally fermented sausages [9,10]. However, it was in accordance with results obtained for another type of sausage produced in Serbia, Petrovska klobasa [13], in which *Ln. mesenteroides* and *Lactobacillus* constituted the majority of the microbiota. Some of the *Leuconostoc* isolates from fermented sausages play an important role in sausage flavor development and could also exhibit strong antimicrobial activity [14,15]. However, the role of *Leuconostoc* species in the sausage ripening is not yet well understood. The finding of high levels of *Lb. brevis* is in agreement with other authors [16,17], while the predominant presence of *Lb. sakei* is in accordance with other authors [3,9,10,18]. These species are the most adapted of the lactobacilli to the fermented sausage environment [9].

4. Conclusion

Results of this study showed that during the ripening of Užička sausage, *Ln. mesenteroides*, *Lb. brevis*, and *Lb. sakei* were the most dominant species. At the beginning of the production, *Ln. mesenteroides* prevailed. From day 4 until the end of fermentation, the dominant lactobacilli were *Lb. brevis* and *Lb. sakei*. Isolates of LAB from this study could be used as starter cultures in the production of traditional dry sausages.

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