Lactic acid bacteria isolated from sremska sausage using molecular methods

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Abstract. Sremska sausage is a traditionally fermented dry sausage, which is produced in northern Serbia (Vojvodina). It is made from pork with the addition of back fat and natural spices. The entire manufacturing process lasts for 21 days. The goal of this study was to create a collection of lactic acid bacteria isolated during sausage fermentation and identify them using molecular methods. A total of 50 isolates from different stages of fermentation were identified by molecular methods. *Lb. mesenteroides*, *Pediococcus pentosaceus* and *Lb. sakei* are the predominant microorganisms in the sremska sausage studied.

1. Introduction

Sremska sausage is a typical dry fermented sausage produced in northern Serbia (Vojvodina), especially in the area of Srem. It is traditionally produced in households as well as in meat plants. It is made from pork (shoulder), back fat and spices such as sweet and hot ground pepper and garlic and filled into small pork intestine. Sremska sausage is characterized by a recognizable and characteristic flavour of smoke and the added spices.

Traditional fermented sausages with a specific geographical origin have unique sensory characteristics and are generally of high quality [1]. Sensory properties of traditionally fermented sausages depend on the type of meat, salt, spices, additives, smoking intensity, temperature of fermentation, duration of drying and metabolic activity of the microbiota.

During sausage fermentation, different groups of microorganisms possessing specific biochemical potentials contribute to the sensory profile of the final product [2]. The microbial activity and interaction are key factors for the final quality characteristics of fermented sausages [3]. In order to protect the traditional aspect of sausage manufacturing, it is essential to understand the microbial diversity and to select autochthonous starter cultures that could be used in the production of innovative foods with a geographical origin [4]. Fermentation with autochthonous starter cultures allows for controlled production with lower safety risks than in products obtained after natural fermentation [5]. Studies of the microbiota in traditional meat products have revealed considerable microbial diversity [6].

Lactic acid bacteria (LAB) play an important role in meat preservation and fermentation and are considered as a technologically fundamental group of microorganisms. They are able to decrease the pH by lactic acid production, produce bacteriocins which prevent the growth of pathogenic and spoilage microorganisms, provide diversity of sensory properties by modification of raw material,
contribute to the development of flavour, colour and texture and improve the safety, stability and shelf life of meat products [7,8].

The main LAB that have been isolated from fermented sausages belong to the genera Lactobacillus, Pediococcus, Leuconostoc, Weissella and Enterococcus [9,10]. The most commonly identified LAB species in traditional fermented sausages are Lb. sakei, Lb. curvatus and Lb. plantarum [10,11].

In order to create a collection of autochthonous natural isolates of LAB as a future basis for the production of starter cultures with a geographical origin, LAB strains were identified and characterized by molecular methods.

2. Materials and Methods

2.1. Sremska sausage

Sremska sausage was produced by the traditional method in a family household in Srem (northern Serbia). Sremska sausage was composed of pork (shoulder) and back fat in the percentage ratio 70%:30%. Meat and fat were ground to the size of 8 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 pork intestine (diameter of 34-36 mm). Sausages were cold smoked for 3 days. The entire manufacturing process (smoking, fermentation/ripening and drying) in this traditional process 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 samples at each step of sampling were collected and used for analysis. Each 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 new agar plates in order to obtain pure cultures. The LAB isolates from MRS agar were checked 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 1x 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. The DNA was amplified in a thermal cycler (Teche, UK) using primers P1V1 (GCCGCCGTGCAATTACATGC) and P4V3 (ATCTACGCCATTTACCCGCTAC), complementary to the V1-V3 region of the 16S rRNA, 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. Lactic acid bacteria isolated from sremska sausage and identified by molecular methods

| Day of fermentation | 16S rRNA gene sequencing                  |
|---------------------|-----------------------------------------|
| 0                   | Ln. mesenteroides subsp. mesenteroides (5 isolates) |
| **Microorganism**                                      | **Isolates** |
|--------------------------------------------------------|--------------|
| *Pediococcus pentosaceus*                              | (3 isolates) |
| *Lc. lactis subsp. lactis*                             | (2 isolates) |
| *Weissella viridescens*                                |              |
| *Ln. mesenteroides subsp. mesenteroides*               | (3 isolates) |
| *Ln. gasicomitatum*                                   |              |
| *Pediococcus pentosaceus*                              | (2 isolates) |
| *Ln. mesenteroides subsp. mesenteroides*               | (6 isolates) |
| *Pediococcus pentosaceus*                              | (2 isolates) |
| *Ln. gasicomitatum*                                   |              |
| *Ln. mesenteroides subsp. mesenteroides*               | (4 isolates) |
| *Lb. plantarum*                                        |              |
| *Lb. sakei*                                             | (2 isolates) |
| *Ln. mesenteroides subsp. mesenteroides*               | (4 isolates) |
| *Pediococcus pentosaceus*                              | (2 isolates) |
| *Weissella viridescens*                                |              |
| *Lb. curvatus*                                          | (2 isolates) |
| *Lb. sakei*                                             | (2 isolates) |
| *Lb. sakei*                                             |              |
| *Ln. mesenteroides subsp. mesenteroides*               | (2 isolates) |
| *Lb. alimentarius*                                     |              |
| *Weissella viridescens*                                |              |

The most dominant microorganisms in sremska sausage were *Ln. mesenteroides*, *Pediococcus pentosaceus*, *Lb. sakei*, *Lb. curvatus*. Besides them, *Lc. lactis subsp. lactis*, *Weissella viridescens*, *Ln. gasicomitatum*, *Lb. plantarum* and *Lb. alimentarius* were also identified, although in lower numbers.

The dominant presence of *Ln. mesenteroides* found in sremska sausage is in accordance with results obtained for Petrovská klobása [12], in which *Ln. mesenteroides* and *Lactobacillus* constitute the majority of the microbiota. Some of the *Leuconostoc* strains isolated from fermented sausages play an important role in the flavour development and could also exhibit strong antimicrobial activity [13,14].

The prevalence of *Pediococcus pentosaceus* isolated from our sremska sausage is in accordance with results obtained for Iberian dry sausage [15] and Italian fermented sausage [16]. The predominant presence of *Lb. sakei* is in accordance with results obtained by several authors [7,11,17,18]. These species are the most well-adapted lactobacilli to the fermented sausages environment [11].

4. Conclusion
The most dominant LAB during the whole fermentation of this traditionally produced sremska sausage are *Ln. mesenteroides*, *Pediococcus pentosaceus* and *Lb. sakei*. At the beginning of the production, *Ln. mesenteroides* and *Pediococcus pentosaceus* prevail. From day 7 until the end of production, the dominant strain is *Ln. mesenteroides*, followed by *Lb. sakei* and *Lb. curvatus*. The isolated strains of LAB could be used as starter cultures for fermentation of traditional dry sausages.
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