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Influence of processing conditions on the lactic acid bacteria population of a traditional sausage

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Abstract

The constitution of the microbiota of lactic acid bacteria (LAB) during fermentation of sausages is under great influence of the technique utilized during ripening. In order to investigate the influence of ripening technique on the microbiota of a Serbian sausage which is usually produced only in a traditional way, ripening was performed under both traditional and controlled conditions. During 90 days of processing 296 LAB strains were isolated. Preliminary identification and characterisation of the isolates was performed by phenotypic tests and (GTG)5-PCR fingerprinting, while molecular identification of the representatives was done by 16S rDNA sequencing. Among isolated LAB strains Lactobacillus sakei and Leuconostoc mesenteroides predominate in both processes. Enterococcus durans, Pediococcus pentosaceus and Lactobacillus curvatus were also isolated but in much lower percentage. Controlled fermentation resulted in a higher number of Ln. mesenteroides and the reduction of frequency of isolation of Lb. sakei during fermentation. On the other hand, uncontrolled conditions during ripening led to higher presence of Lb. sakei and a reduced presence of Ln. mesenteroides.

Keywords: fermented sausages, lactic acid bacteria, molecular identification, controlled ripening, traditional ripening

1. Introduction

Petrovac sausage (Petrovská Klobása), an artisan Serbian dry fermented sausage, is made only in Bački Petrovac (the province of Vojvodina, Serbia) among population of Slovakia minority. In village households, this sausage has been made at the end of November and during December when the outer air temperatures are around 0°C or lower. Because of its specific and recognizable texture, flavour and taste, the Petrovac sausage is protected by Serbian law as designation of origin at national level.

Significant changes in the characteristics of sausages are the result of complex biochemical and physical reactions during the fermentation (CASABURI & al. [1]). The knowledge and control of the bacteria present in the meat batter and involved in the fermentation are essential in terms of the microbiological quality, sensory characteristics, and food safety (COCONCELLI and FONTANA [2]). The main constitutes of the microbiota involved in the fermentation of sausages are lactic acid bacteria (LAB), coagulase-negative cocci (CNC), yeasts and moulds (AMMOR and MAYO [3]). LAB causes the reduction of pH value due to the production of lactic and acetic acid during the sausage fermentation and the inhibition of unwanted microbiota (RANTSIOU and COCOLIN [4]).

Traditional fermented sausages have a great significance and economic effect. Since they are mainly produced without the addition of starter cultures, the fermentation relies on wild type strains and the process favors the growth of the characteristic in-house microbiota (BAKA &

al. [5]). The constitution of the microbiota during fermentation is affected by the ripening technique utilised (IACUMIN & al. [6]). In order to investigate possibility of industrializing the production of Petrovac sausages, the effects of drying and ripening conditions on the sensory properties, as well as physicochemical characteristics, proteolysis and biogenic amines formations have been studied (IKONIĆ & al. [7], JOKANOVIĆ & al. [8]). The present study evaluated the impact of the technology of ripening on the species diversity and community dynamics of LAB.

2. Materials and Methods

The meat batter was manufactured in Bački Petrovac (Serbia) by a following recipe: cold pork meat (24 h post mortem) (80%) and lard (20%) were grounded to a 10 mm particle size and then mixed with the following ingredients: red paprika (2.5%), salt (1.8%), garlic (0.2%), caraway seed (0.2%) and sugar (0.15%). The mixture was then stuffed into synthetic collagen casings 500 mm long and 55 mm in diameter. The prepared sausages were divided in two groups and ripened in traditional and industrial conditions. The traditional procedure took place in household and included smoking of the sausages by cool procedure for 10 days (with pauses) followed by drying in naturally ventilated storerooms. According to the data obtained from the local Weather station, the air temperature during the fermentation was in the range of 2 to12°C, relative air humidity (RH) in the range of 43.3% to 93.0%, and air velocity ranged from 0 to 0.5 m/s. The samples for the industrial procedure were cold smoked for 6 h during 3 days (10 °C, 90% RH and 0.5-0.6 m/s), dried in the same room for 27 days (10 °C, 75% RH and 0.5-0.6 m/s) and after that ripened in an industrial ripening chamber under the same conditions, but with minimal air circulation. The sampling was performed until the moisture content in all the analysed sausages got down under 35% according to Serbian legislation quality requirements (Serbian Regulations, 2004). For the analysis, the samples of the three randomly selected sausages were taken during 45 and 90 days of processing in the controlled and traditional conditions, respectively.

For microbial analysis, 10 g of each sample were mixed with 90 ml of saline/peptone water (8 g/l NaCl, 1 g/l peptone) and homogenized (URSO & al. [9]). After preparing serial dilutions, higher dilutions were plated onto nutrition agar (Torlak, Belgrade, Serbia) for enumeration of aerobic mesophilic bacteria, and MRS agar (Torlak, Belgrade, Serbia) for enumeration of LAB. After solidification, MRS agar plates were covered with a thin layer of the same medium to establish microaerophilic conditions. Incubation was performed at 30°C for 5 days and after enumeration, colonies showing different appearance on MRS agar plates were selected (HARRIGAN and McCANCE [10]) and streaked on new agar plates for purification. All data are presented as mean values of three enumerations.

All Gram positive, catalase-negative purified LAB isolates were subjected to physiological tests: morphology under microscopic examination, the production of gas from glucose in MRS broth, the growth at 15°C and 45°C on MRS agar plates for 72 h, the growth on MRS agar plates containing 4%, 6.5% and 8% of NaCl for 72 h, hydrolysis of arginine in arginine broth, hydrolysis of esculin in esculin broth (Torlak, Belgrade, Serbia), the production of proteases on skimmed milk agar plates and the production of lipase on tributyrin agar plates (Merck GmbH, Darmstadt, Germany). The production of exopolisaccharides was analysed by the method of SMITINONT & al. [11]. Presumptive identification of enterococci was performed on bile esculin agar (Himedia, Mubai, India).

The total DNA extraction from pure cultures, PCR amplification with (GTG)5-primer and electrophoresis were performed as previously described (NIKOLIC & al. [12]). Clustering was carried out in Statistica 7.0 for Windows (StatSoft Inc. USA) using the algorithm “Unweighed Pair-Group Average Linkage Analysis”. Distances between the clusters were

performed by using “Percent of disagreement”. For the sequencing of the 16S rRNA gene, total DNA was used as a template for PCR amplifications with primers UNI16SF (5'-GAG AGT TTG ATC CTG GC-3) and UNI16SR (5'-AGG AGG TGA TCC AGC CG-3') according to the procedure previously described (DANILOVIĆ & al. [13]).

3. Results and Conclusions

Petrovac Sausage (Petrovská Klobása), as an indigenous artisan product, is produced only in Bački Petrovac, Serbia, and it is traditionally fermented under uncontrolled conditions in the households. Naturally fermented sausages are appreciated for their unique aroma and taste but variations in the characteristics of the final product are present. On the other hand, industrial manufacturing allows shorter fermentation period and the production of the safe product with defined characteristics. This fact favours the trend to industrialise the production of artisanal sausages. Therefore, the dynamics of the LAB population during traditional ripening as well as ripening under controlled conditions of Petrovac sausages was analysed.
The results showed that the viable numbers of aerobic mesophilic bacteria and LAB during the first 12 days of fermentation are much higher in the samples ripened under controlled conditions (Fig. 1).

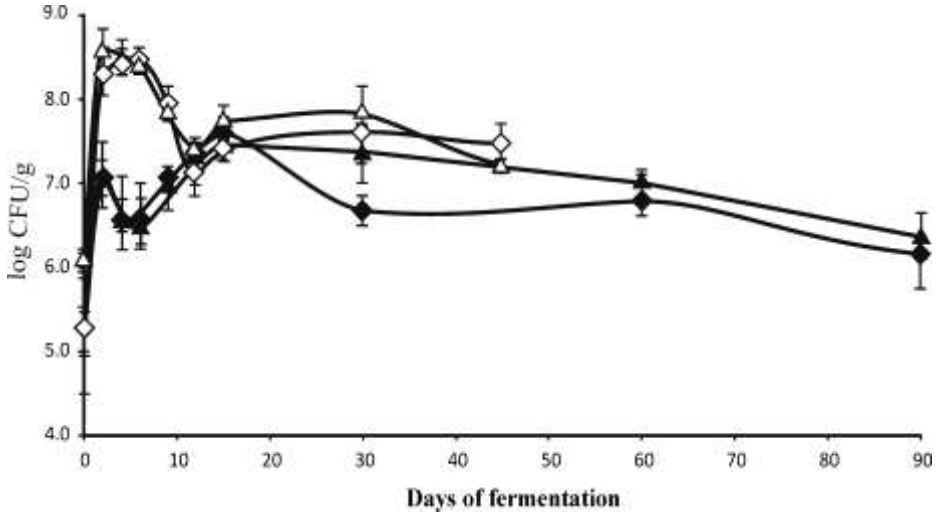

Figure 1.Changes of the bacterial number during the ripening of the Petrovská Klobása under traditional (filled symbols) and controlled (open symbols) conditions (▲ - aerobic mesophilic bacteria; ♦ - LAB) Vertical error bars: standard deviation

After only two days of fermentation the total number of aerobic mesophilic bacteria increased rapidly from the beginning of fermentation reaching the value of 7.0 log CFU/g for the samples ripened under traditional conditions and the value of 8.5 log CFU/g for the samples ripened under controlled conditions. After 9 days of fermentation the number of mesophylic bacteria in the samples ripened in the industrial chamber remained in the range of 7-8 log CFU/g, which is higher than already presented for some fermented sausages produced in Serbia (KOZACINSKI & al. [14]), but in agreement for Petrovac sausages produced from hot meat (DANILOVIĆ & al. [13]). The number of presumable LAB detected on MRS agar plates rapidly increased after 2 days of fermentation and became almost the same as the total number of aerobic mesophilic bacteria suggesting that LAB were dominant microbes in both processes. The final numbers of LAB enumerated on MRS agar plates and aerobic mesophilic

bacteria were in the range of 6-6.5 log CFU/g and 7-7.5 log CFU/g for the sausages fermented under traditional and controlled conditions, respectively.
A total number of 296 LAB were isolated from 18 samples of the Petrovac sausages ripened in different conditions. Preliminary identification and grouping of isolates were performed by physiological tests (Table 1). Isolates were further subjected to (GTG)5-PCR (Fig. 2 and 3) and the representatives of each group were identified by 16S rDNA sequencing (Table 1). All isolates were divided into 5 groups based on their morphological and biochemical characteristics (Table 1).

Table 1. The characterization of LAB isolated during processing of Petrovská Klobása ripened under traditional and controlled conditions

Group	I	II	III	IV	V
No of isolates	175	3	106	7	5
Cell morphology	rods	rods	coccoid	cocci	cocci
CO_2 formation	-	-	+	-	-
Grow at 45°C	-	-	-	+	+
15°C	+	+	+	+	+
Grow on NaCl 4%	+	+	+	+	+
6.5%	+	-	+	-	+
8%	-	-	-	-	-
Hydrolysis of arginine	-	-	-	+	-
Hydrolysis of esculin	-	-	+	+	+
Production of protease	-	-	-	-	-
Production of lipase	-	-	-	-	-
Black colonies on Bile esculin agar (BSA)	-	-	-	+	-
Production of EPS	-	-	+	-	-
Identified by 16S rDNA sequencing	***Lb. sakei***	***Lb. curvatus***	***Ln. mesenteroides***	***En. durans***	***Pd. pentosaceus***

More than half of the isolated LAB species (60%) had rod-shaped cells, grew well at 15°C, did not produced CO2 from glucose and had no ability to hydrolyse esculin or arginine, so they were classified as lactobacilli. On the basis of morphological characteristics, two groups of lactobacilli were distinguished and this was confirmed by (GTG)5-PCR fingerprinting. Molecular identification of the representatives showed that isolated lactobacilli belonged to *Lactobacillus curvatus* and *Lactobacillus sakei* species. Among them, *Lb. sakei* represented the majority, while *Lb. curvatus* was detected in only two samples. Domination of lactobacilli in the Petrovac sausage has already been shown (DANILOVIĆ & al. [13]). The prevalence of lactobacilli, especially *Lb. sakei*, in fermented sausages has been confirmed for many different types of sausages and fermentation techniques. Thus, *Lb. sakei* was found to be dominant in spontaneously fermented Belgian-type salami and Boulogne sausages (JANSSENS & al. [15]), Swiss meat products (MARTY & al. [16]), French dry sausages (AMMOR & al. [17]) and the Spanish traditional pork sausage (GARCIA FONTÁN & al. [18]).
Heterofermentative coccoid forms with the ability to produce slimy colonies on modified MRS agar plates with sucrose (Table 1) represented 36% of LAB isolates and were preliminary classified as Leuconostoc spp. The representatives of this group were identified by molecular methods as Leuconostoc mesenteroides (Fig. 2 and Fig. 3).

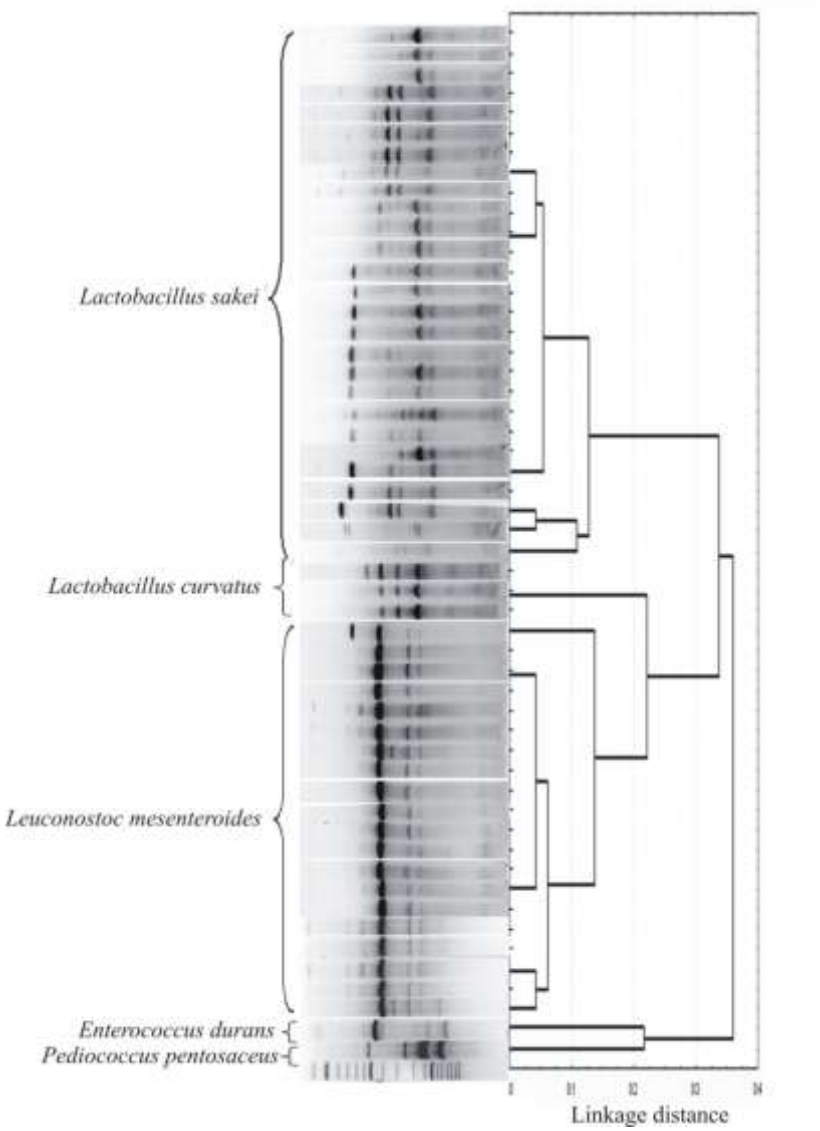

Figure 2.Dendrogram based on a statistical analysis of the (GTG)5-PCR fingerprints of the strains isolated during ripening of the Petrovská Klobása under traditional conditions

Only 4 % of the isolated bacteria were esculin positive cocci divided into two groups (Table 1). The isolates of the first group formed black colonies on bile esculin agar, while the second group had cells in pairs or tetrads so it was assumed they belong to *Enterococcus* spp. and *Pediococcus* spp., respectively. 16S rDNA sequencing has shown that the representatives of the first group belong to *Enterococcus durans* and the representatives of the second group to *Pediococcus pentosaceus* (Fig. 2 and Fig. 3). The presence of pediococci (2%) was much lower in the analysed samples comparing to the Petrovac sausages produced of hot meat where it represented 18,3 % of total isolated LAB (DANILOVIĆ & al. [13]). Low frequency of isolation of enterococci and pediococci is in correspondence with some previous studies of fermented sausages produced in Southern Europe (PAPAMANOLLI & al. [19], RANTSIOU & al. [20], COMI & al. [21], KOZACINSKI & al. [14]).

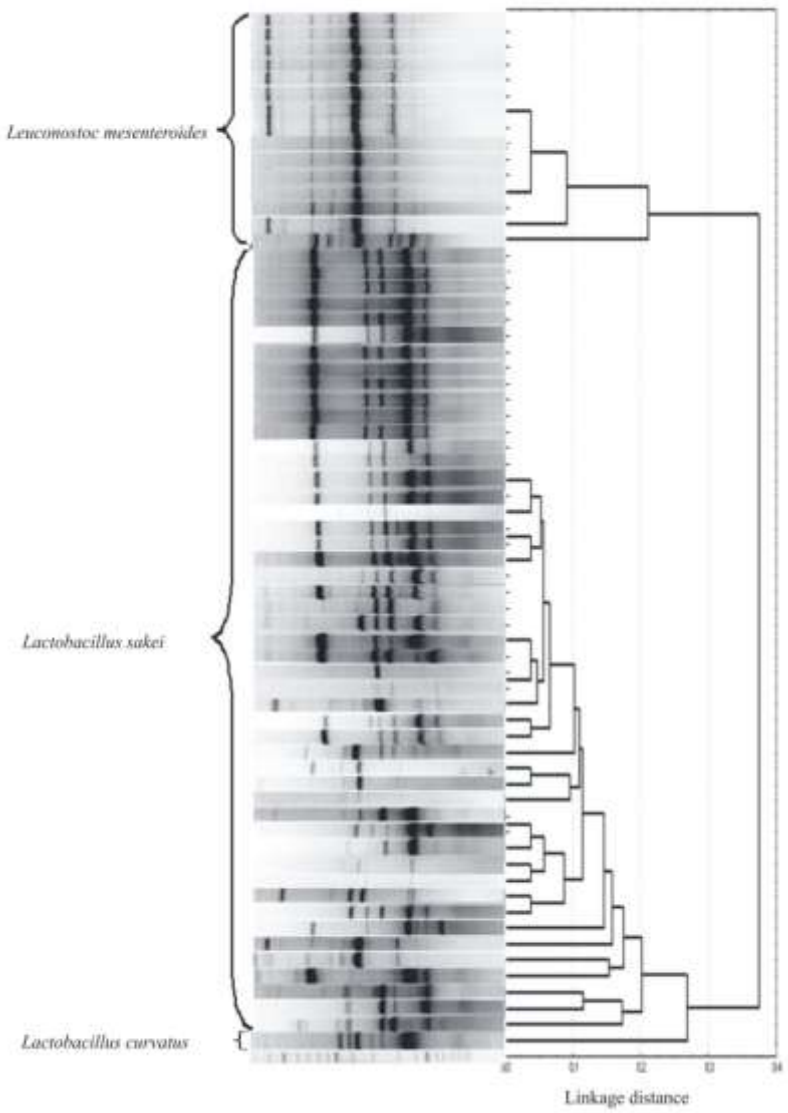

Figure 3.Dendrogram based on a statistical analysis of the (GTG)5-PCR fingerprints of the strains isolated during the fermentation of Petrovská Klobása under controlled conditions

The conditions that prevailed through the ripening process had the influence on the number of bacteria and on the composition of LAB microbiota. Sausages were produced from the same meat batter with the LAB microbiota constituted of pediococci (33%), enterococci (47%) and leuconostocs (20%) (Fig. 4). The initial stages of the traditional fermentation (Fig. 4a) showed almost equal distribution of *Ln. mesenteroides* and *Lb.sakei*. After 9 days of fermentation, the presence of *Lb.sakei* rapidly increased up to 75%. This value remained almost stable until the 30th day when a little deviation was noticed as the presence of *Ln. mesenteroides* increased to 44%. Nevertheless, this fluctuation had no influence on mature samples in which the significant domination of *Lb. sakei* occurred. *Lb. curvatus* was least present species during fermentation in traditional conditions and was found in one sample only.

On the other hand, the samples ripened under industrial conditions showed a different trend of the changes in the microbial population (Fig. 4b). The presence of *Ln. mesenteroides*, which was also found in the meat batter, increased during the whole fermentation period. In the sample after 12 days of fermentation, it represented 29% of total LAB population but in the sample after 30 days, this value increased to 82% (Fig. 4b).

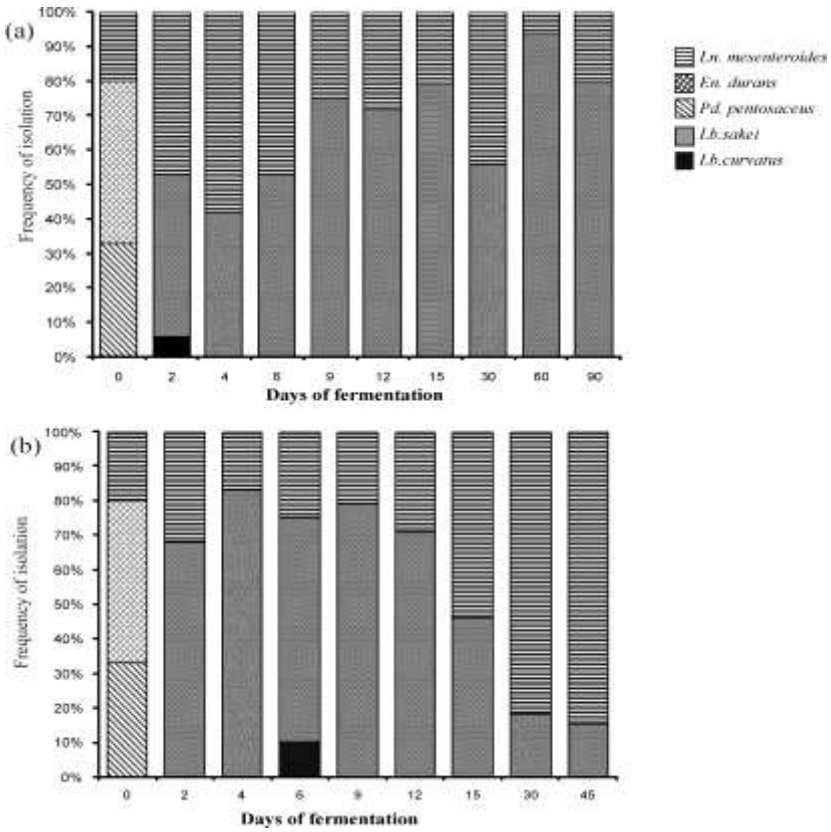

Figure 4. Changes of LAB population during the fermentation of Petrovská Klobása under traditional (a) and controlled (b) conditions

The population of lactobacilli decreased during ripening in an industrial chamber, even though they were dominant species during the first 12 days of fermentation with the frequency of isolation up to 83% (Fig. 4b). *Lb. curvatus* was the least frequently isolated during the fermentation and was detected only in the 6-day-old sample with the contribution to the LAB microbiota with 10%. Although *Ln. mesenteroides* was not frequently isolated from various types of fermented sausages (COMI & al. [21], RANTSIOU & al. [20], DROSINOS & al. [22], PAPAMANOLLI & al. [19]), its presence in Petrovac sausages was already stated (DANILOVIĆ & al. [13]).

The two applied ripening processes differed mostly in ambient temperature and humidity. In the fermentation chamber these parameter were maintained at constant level while in the traditional fermentation the temperature and relative humidity fluctuated depending on outdoor weather conditions. So, the difference in the LAB profile during ripening was, probably, due to the changes in the ambient temperature and relative humidity. Sensory analyses at the end of ripening processes showed that sausages produced in a traditional way had better score than those fermented in industrial conditions (JOKANOVIĆ & al. [8]). In order to industrialize the production of Petrovac sausage, a further research needs to be done and the implementation of autochthonous starter cultures should be taken into consideration.

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