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The characterisation of lactic acid bacteria during the fermentation of an artisan Serbian sausage (Petrovská Klobása)

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ABSTRACT

Petrovská Klobása is an artisan Serbian sausage made only from meat and spices without any additives or starter cultures. In order to characterise lactic acid bacteria (LAB) microflora, a total number of 404 LAB strains were isolated from 15 samples collected during 90 days of the fermentation and 120 days of storage of one batch of Petrovská Klobása. The isolates were preliminarily identified by phenotypic tests and subjected to $(GTG)_5$-PCR fingerprinting. Representatives of each group were identified by 16S rDNA sequencing. The results showed that among the isolates, *Lactobacillus sakei* and *Leuconostoc mesenteroides* predominate with 36.4% and 37.1% of total LAB strains, respectively. *Pediococcus pentosaceus* was also isolated in high proportion (18.4%) whereas *Enterococcus durans* and *Enterococcus caseliflavus* made only 1% and 6% of the total isolates, correspondingly. The analysis of vacuum packed and modified atmosphere packed (MAP) samples showed higher presence of *L. mesenteroides* and *L. sakei* in the total microflora.

1. Introduction

Different formulations used in the production of fermented sausages lead to their great variety and diversity in the market nowadays. The main factors affecting the specific organoleptic and physico-chemical characteristics of fermented sausages are: the ingredients, fermentation techniques and manufacturing practises. In general, differentiation can be made on the basis of pH between fermented sausages produced in Northern countries with pH below 5 and sausages produced in the Mediterranean countries with pH of 5.3–6.2 (Talon, Leroy, & Lebert 2007). Although the industrial development requires the use of starter cultures and control of the manufacturing process, artisan products are increasingly appreciated because of their sensory properties and authenticity (Latorre-Moratalla et al., 2008; Talon et al. 2007).

Petrovská Klobása (Petrovac Sausage) is a traditional dry fermented sausage produced in Backi Petrovac (the province of Vojvodina, Serbia). This sausage has been produced for a long time by a traditional technique without any additives and preservatives. It has a specific and recognisable texture, flavour and taste so it is protected by the Serbian law as a designation of origin at the national level (Vaštag, Popović, Popović, Petrović, & Peričin 2010).

Dry fermented sausages are often produced in three phases: formulation, fermentation and ripening/drying (Sanz, Selgas, Parejo, & Ordoñez 1998). A ripening technique affects the constitution of microflora which develops during fermentation. A short ripening time indicates the development of lactobacilli in early stages of fermentation. On the contrary, the sausages exposed to a longer ripening time show higher presence of coagulase-negative cocci (CNC) (Iacumin, Comi, Cantoni, & Cocolin 2006). Lactic acid bacteria (LAB) have great influence on the meat preservation and fermentation processes, so they are considered technologically fundamental. They are able to utilise sugars and other nutrients faster than competitors. They also decrease the oxidation–reduction potential (Eh) of the environment generating the conditions which are inhibiting the growth of aerobic organisms (Hutkins 2006). Besides this, the modification of raw material by LAB provides sensory properties and improves safety, stability and shelf life of the products contributing to the development of the flavour, colour and texture of fermented sausages (Bonomo, Ricciardi, Zotta, Parente, & Salzano 2008).

LAB that have been isolated from fermented sausages belong to the genera *Lactobacillus*, *Weisela*, *Leuconostoc*, *Pediococcus*, *Enterococcus* and *Lactococcus* (Comi et al. 2005, Di Cagno et al. 2008; Rantsiou et al. 2006; Urso, Comi, & Cocolin 2006). Among them lactobacilli are most frequently isolated from sausages produced with different technologies, especially the species *Lactobacillus sakei, Lactobacillus curvatus* and *Lactobacillus plantarum* (Comi et al. 2005; Fontana, Cocconcelli, & Vignolo 2005; Rantsiou et al. 2006; Urso et al. 2006;).

Monitoring of the population changes of microflora during the fermentation depends on the sensitivity and reliability of the applied identification methods. Biochemical profiles rely on subjective evaluations so they cannot always lead to a conclusive identification

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of the species (Rantsiou et al. 2005). Nowadays molecular methods are being employed for the identification of microorganisms (Comi et al. 2005).

The aim of this study was screening the LAB population during the fermentation of an artisan Serbian sausage (Petrovská Klobása). For this purpose, the isolation and identification of LAB from sausages during fermentation was performed. The sausages were produced only from meat and spices in a traditional way in a household in Backi Petrovac.

2. Materials and methods

2.1. Fermented sausage technology and sampling procedures

Fermented sausages were traditionally manufactured by a local producer in Backi Petrovac, whose products showed the best sensory qualities in previous study of sausages (Ikonic et al. 2010). Fresh pork meat (85%) and lard (15%) were firstly mixed and the following gradients were added: red paprika (2.5%), salt (1.8%), garlic (0.2%), caraway seed (0.2%) and sugar (0.15%). The mixture was than stuffed into natural casings. The sausages were ripened in a traditional way which includes smoking by cool procedure for 10 days and then drying in a dry place with high circulation of air. The samples of the sausages for microbiological analysis were taken from the same batch after 0 (sausage mixture), 2, 6, 9, 15, 30, 60 and 90 days of fermentation. After 90 days of fermentation one part of the sausages was stored unpacked, the other was packed in a vacuum packaging and the third part was packed in a modified atmosphere packaging (MAP) consisting of: $CO_2 = 25\%$, $N_2 = 75\%$ and $O_2 = 0\%$ and stored at 15 °C. The packed and unpacked samples of sausages were analysed after 30 and 120 days of storage.

2.2. pH measurements

For the pH measurements 10 g of the sample was diluted in 90 ml of distilled water and was homogenised. pH values were determined with digital pH-metre (Hanna Instruments HI 9052, Lisbon, Portugal). The shown values are the average of six readings for each sample.

2.3. Enumeration and isolation of LAB

10 g of each sample was mixed with 90 ml of saline/ peptone water (8 g/l NaCl, 1 g/l peptone) and was homogenised (Urso et al. 2006). Serial dilutions were made and higher dilutions were plated onto nutrition agar (NA, Torlak, Belgrade, Serbia), MRS agar (Torlak, Belgrade, Serbia) and Mayeux, Sandine, Elliker (MSE) agar (Mayeux, Sandine, & Elliker 1962). Nutrition agar was used for the enumeration of aerobic mesophilic bacteria, and MRS and MSE agar for enumeration of LAB. After solidification, MRS and MSE agar plates were covered with a thin layer of the same medium to establish microaerophilic conditions. After incubation of the plates at 30 °C for 5 days and enumeration, five to ten colonies from MRS and MSE agar plates for each sample were randomly selected (Harrigan & Mc Cance 1976) and streaked on new agar plates for purification.

2.4. Phenotypic characterisation

Gramme positive, catalase negative purified isolates were characterised according to the following 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 containing 4%, 6.5% and 8% of NaCl for 72 h, hydrolysis of arginine, hydrolysis of esculin in esculin broth (Torlak, Belgrade, Serbia), the production of protease on skimmed milk agar plates. The production of exopolisaccharides was analysed on modified MRS agar plates with maltose, sucrose, galactose, lactose, fructose and glucose as previously described (Smitinont et al. 1999). Bile esculin agar (Himedia, Mubai, India) was used for the presumptive identification of enterococci.

2.5. $(GTG)_5$-PCR identification

The total DNA extraction from pure cultures, PCR amplification with $(GTG)_5$-primer and electrophoresis were performed as previously described (Nikolic et al. 2008). 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 using "Percent of disagreement".

2.6. Sequencing of 16S rDNA amplicons

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′). Amplification of the variable region of the 16S rRNK was done by using Taq DNA polymerase kit (Fermentas UAB, Vilnius, Lithuania). The reactions were carried out in GeneAmp PCR System 2700 (Applied Biosystems) programmed as follows: the initial denaturation of DNA for 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C; and the extension of incomplete products for 7 min at 72 °C. PCR products were quantified by electrophoresis on a 1% agarose gel containing ethidiumbromide and visualised by the CCD camera Biometra BDR2/5/6 (Bio Doc Analyze). The amplified fragments were purified by QIAquick PCR Purification Kit/250 (QIAGEN GmbH, Hilden, Germany), and sequenced at Macrogen in Seoul, South Korea. The BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST) was used to determine the most related sequence relatives in the NCBI nucleotide sequence database.

3. Results

3.1. pH measurements

The determination of pH value of unpacked fermented sausages (Fig. 1) showed that a decrease in the pH value occurs during the fermentation. At the end of fermentation pH value in the sausage samples decreased from the value of 5.67 to 5.27.

3.2. Enumeration of bacteria

The results of viable counts of bacteria are shown in Fig. 1. For the first 15 days, a continuous increase of the total number of aerobic mesophilic bacteria occurs during the fermentation. The value rose from 4 to 7.07 log CFU/g. Afterwards, the number remained mostly stable till the end of fermentation.

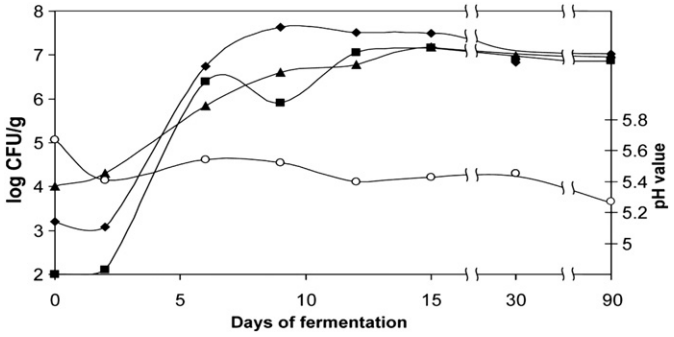

Fig. 1. Changes of pH value (○) and population dynamics of aerobic mesophilic bacteria (▲) and LAB enumerated on MRS agar plates (♦) and MSE agar plates (■), during spontaneous fermentation of sausages Petrovská Klobása.