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Characterization of lactic acid bacteria isolated from artisanal Travnik young cheeses, sweet creams and sweet kajmaks over four seasons

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ABSTRACT

The aim of this study was to investigate the composition of lactic acid bacteria (LAB) in autochthonous young cheeses, sweet creams and sweet kajmaks produced in the Vlašić mountain region of central Bosnia and Herzegovina near the town of Travnik over a four season period. These three products were made from cow's milk by a traditional method without the addition of a starter culture. Preliminary characterization with phenotype-based assays and identification using rep-PCR with a $(GTG)_5$ primer and 16S rDNA sequence analysis were undertaken for 460 LAB isolates obtained from all the examined samples. Fifteen species were identified as follows: *Lactococcus lactis*, *Lactococcus raffinolactis*, *Lactococcus garviae*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus helveticus*, *Enterococcus faecium*, *Enterococcus durans*, *Enterococcus faecalis*, *Enterococcus italicus*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Leuconostoc lactis*, *Streptococcus thermophilus* and *Streptococcus mitis*. A wide genotypic and phenotypic heterogeneity of the species was observed, particularly within the *Lc. lactis* strains. In all of the tested dairy products across four seasons, a significantly positive correlation ($r = 0.690$) between the presence of lactococci and enterococci and a negative correlation ($r = 0.722$) between the presence of lactococci and leuconostocs were recorded. Forty-five percent of the lactobacilli and 54.4% of the lactococci exhibited proteolytic activity, whereas 18.7% of the total LAB isolates exhibited antimicrobial activity.

1. Introduction

Autochthonous cheeses are commonly produced in regions of Bosnia and Herzegovina. The technology of cheese production is simple and adapted to the modest mountain conditions (Sarić and Bijeljac, 2003). Young cheese, sweet cream and sweet kajmak are traditional dairy products produced in rural households in Bosnia and Herzegovina without the addition of commercial starters. These distinct dairy products are recognized in the mountainous area surrounding Travnik. This area has high quality pasturelands, and the climate conditions are optimal for cattle breeding. The milk in these dairy products is produced by the cows of a domestic Bosnian cattle breed, which are fed differently during the year. The cows are typically fed with hay during January, with hay and mown grass in April, and with meadow grass during July and October. The cows drink from the local water supply.

The lactic acid bacteria (LAB) population in traditional Travnik dairy products is the primary factor that contributes to their specific characteristics, such as taste, aroma and texture. Artisanal products, especially those produced from raw milk, are inexhaustible sources of new microbial strains with diverse genetic profiles. These products most likely have notably heterogeneous and distinct microbial populations that are related to environmental conditions, such as the temperature, origin and quality of the milk and the sanitary conditions and specificities of the manufacturing process (Baruzzi et al., 2000; Cappa et al., 2002; Morea et al., 1999; Ouadghiri et al., 2009).

Environmental microbiota have a major role in the fermentation process and significantly contribute to the final quality of dairy products (Poznanski et al., 2004). These authors reported that the sensorial properties of the cheese produced from raw milk collected during summer pastures are better than the sensorial properties of the cheese manufactured during the winter when cows were fed with hay in cattle sheds. Studies on the diversity of LAB in naturally occurring processes are significant for the

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preservation of microbial populations present in traditional food. New bacterial strains could be used for the production of traditional products on an industrial scale or for quality improvement of existing dairy products (Colombo et al., 2009; Leboš Pavunc et al., 2012; Wouters et al., 2002). Consequently, many authors have conducted research investigating the phenotypic and genotypic characteristics of LAB (Franciosi et al., 2009; Garabal et al., 2008; Golić et al., 2013; Poznanski et al., 2004). The classical phenotypical tests employed for LAB identification are frequently time-consuming and can be difficult to interpret. The application of molecular-based techniques offers a rapid and specific alternative. To qualify LAB strains better it is necessary to combine classic physiological and biochemical tests with modern molecular genetics techniques. The development of PCR-based molecular techniques has enabled faster and more reliable identification and characterization of bacterial isolates, and it has significantly increased the knowledge of the biodiversity of the microbial ecosystem. Many authors recommend rep-PCR for the identification of LAB for its high discrimination at the strain level (Berthier et al., 2001; Colombo et al., 2009; Solieri et al., 2012; Golić et al., 2013; Ouadghiri et al., 2005; Tamang et al., 2005). Moreover, 16S rDNA sequencing has become the most valuable tool for investigating the microbial populations in milk and dairy products (Franciosi et al., 2009; Feutry et al., 2012; Golić et al., 2013; Poznanski et al., 2004).

This study is the first to examine LAB in autochthonous dairy products produced in Bosnia and Herzegovina. The aim of this study was to analyze the diversity of LAB in artisanal young cheeses, sweet creams and sweet kajmaks from Travnik during the four seasons. To provide insight into the changes observed in the LAB population during the seasonal changes, conventional microbiological and biochemical tests were combined with molecular genetics techniques, including element palindromic-polymerase chain reaction (rep-PCR) and 16S rDNA sequencing. Additionally, for all of the 460 selected isolates, the antimicrobial activity and β-casein degradation abilities were tested to assess their potential for industrial application.

2. Materials and methods

2.1. Young cheese, sweet cream and sweet kajmak manufacturing and sampling

The analyzed Travnik dairy products were manufactured in one rural household by simple traditional technology without rennet or starter cultures. In general, each household adapts the technology of cheese production according to their possibilities, environmental conditions and given circumstances (Sarić and Bijeljac, 2003). The young cheese and sweet cream were made from raw milk. Immediately after milking, the milk was strained through the gauze, poured into shallow containers and left at 25–30 °C for three days. During that time, the fat rose slowly to the top, and the cream was removed with a spoon and poured into a glass vessel. The product is sweet cream because no salt is added during the production process. The remaining milk turned into curd by the action of the non-starter lactic acid bacteria contained in the milk. The curd is cut with a knife into smaller cubes (3–4 cm) for easier separation of the whey. The sliced curd was subsequently placed in a strainer without pressing for approximately 2 h to allow the whey to drain, and no salt was added to this product. The cheese obtained by this method is a low-fat cheese, called young cheese. It has the shape of the strainer that was used, and it contains the remainder of the whey, which is one of the characteristics of this type of cheese. Three liters of milk produced 1 kg of cheese. Young cheese can be consumed immediately after production or stored in the refrigerator at 4 °C for up to 7 days. The sweet kajmak that does not contain salt was prepared from milk boiled for several seconds. Subsequently, the boiled milk was poured into shallow containers and kept at room temperature for 3 days. On the third day, the "kajmak" formed on the milk surface was collected slowly with a spoon and transferred into the glass vessel. The milk left after removing the kajmak is unpalatable and is primarily used as cattle feed.

During 2009, four samples of young cheese, sweet cream and sweet kajmak were sampled in January, April, July and October from the same household in Kasupovići, a village (altitude 514 m above sea level) near Travnik in central Bosnia and Herzegovina. The designation of the samples is given in Table 1. All the samples of cheese, cream and kajmak were three days old. They were transported to the laboratory under refrigerated conditions (4 °C) and subjected to the analyses.

2.2. Indicator and reference strains

Seventeen indicator and reference strains were used for the detection of antimicrobial activity and the rep-PCR analyses (Table 2). Lactobacilli and leuconostocs were cultivated on MRS broth (pH 5.7) (Merck GmbH, Darmstadt, Germany), whereas lactococci, enterococci and streptococci were cultivated on M17 broth (pH 7.2) (Merck GmbH) supplemented with 0.5% (w/v) glucose (GM17). When a solid medium was required, an agar (2%) (Torlak, Belgrade, Serbia) was added to each medium. The incubation was conducted at an appropriate growth temperature for 24–48 h under anaerobic conditions using the Anaerocult A (Merck GmbH) in anaerobic jars. The species of the *Leuconostoc* and *Lactococcus* genera were incubated at 30 °C, whereas the species of the *Lactobacillus* and *Enterococcus* genera were grown at 37 °C.

2.3. Isolation, enumeration and physiological characterization of LAB

The young cheese, sweet cream and sweet kajmak samples (20 g) were homogenized in 180 ml of sterile 2% (w/v) tri-sodium citrate dehydrate solution serially diluted (10^{-1}–10^{-7}) in a sterile physiological solution and plated onto a standard plate count agar medium (PCA) (Oxoid LTD, Basingstoke, Hampshire, England). Incubation of the inoculated PCA medium was carried out aerobically at 30 °C for 72 h. In parallel, the identical samples were inoculated onto MRS and GM17 agar plates. The MRS and GM17 agar plates were incubated at 30 °C and 45 °C for 72 h under aerobic and anaerobic conditions, respectively. After the incubation period, the plates containing between 30 and 300 colonies were selected for enumeration. The results of three independent experiments were expressed as colony forming units per gram of sample (CFU/g).

Sixty-five colonies were selected randomly from each sample, streaked on new agar plates and examined under a microscope. The pure cultures were stored at −80 °C in GM17 or MRS broth supplemented with 15% (w/v) glycerol.

Seven hundred and eighty colonies were analyzed based on their morphology, Gram staining and catalase test. Upon

Table 1
Sample nomenclature of Travnik young cheese, sweet cream and sweet kajmak sampled during January, April, July and October.

Month	Young cheese	Sweet cream	Sweet kajmak
January	BGTRS1	BGTRM1	BGTRK1
April	BGTRS4	BGTRM4	BGTRK4
July	BGTRS7	BGTRM7	BGTRK7
October	BGTRS10	BGTRM10	BGTRK10