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Evaluation of lactic acid bacteria and yeast diversity in traditional white pickled and fresh soft cheeses from the mountain regions of Serbia and lowland regions of Croatia

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ABSTRACT

The goal of this study was the characterisation of indigenous lactic acid bacteria (LAB) and yeasts isolated from nine white pickled (BG) and nine fresh soft (ZG) artisanal cheeses collected in Serbia and Croatia. While LAB were present in all of the cheeses collected, yeasts were found in all BG cheeses but only in three ZG cheese samples. High LAB and yeast species diversity was determined (average $H'_L = 0.4$ and $H'_Y = 0.8$, respectively). The predominant LAB species in white pickled (BG) cheeses were *Lactococcus lactis*, *Lactobacillus plantarum*, and *Leuconostoc mesenteroides*, while in fresh soft (ZG) cheeses the most dominant LAB species were *L. lactis*, *Enterococcus faecalis*, and *Leuconostoc pseudomesenteroides*. Among the 20 yeast species found, *Debaryomyces hansenii*, *Candida zeylanoides*, and *Torulaspora delbrueckii* were found to be predominant in BG cheeses, while *Yarrowia lipolytica* was predominant in ZG cheeses. The characterisation of metabolic and technological potentials revealed that 53.4% of LAB isolates produced antimicrobial compounds, 44.3% of LAB strains showed proteolytic activity, while most of the yeast species possessed either lipolytic or proteolytic activity. In conclusion, the results obtained in this study showed that the composition of LAB and yeast populations in white pickled and fresh soft cheeses is region specific. The knowledge gained in this study could eventually be used to select region specific LAB and yeast strains for the production of white pickled and fresh soft artisanal cheeses with geographically specific origins under controlled conditions.

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1. Introduction

The Western Balkan Countries (WBC) constitute a distinct geographical region in Europe with long experience in the production of traditional dairy foods by spontaneous or controlled fermentation of cow's, ewe's and goat's milk. In this region, various spontaneously fermented dairy products, such as white pickled and fresh soft cheeses, are manufactured in households without the use of commercial starter cultures. Most of these products are made from unpasteurised milk and the composition of the "natural starter" depends solely on the microbiota present in the raw milk or in the local environment. These microorganisms contribute to preservation, flavour, aroma and texture, determining unique product characteristics.

Preliminary characterisation of the dominant microbiota in these artisanal dairy products revealed that they contain a considerable diversity of lactic acid bacteria (LAB) and yeasts. LAB isolated from autochthonous cheeses have considerable genetic, metabolic and technological potential: good milk protein coagulation, accelerated acidification, production of proteinases, exopolysaccharides, aroma and taste precursors, as well as health-promoting properties (Topisirovic et al., 2006). The dominant LAB are represented by various lactobacillus species: leuconostocs, enterococci, *Streptococcus thermophilus* and *Lactococcus lactis* (Randazzo et al., 2002; Terzic-Vidojevic et al., 2007; Nikolic et al., 2008; Jokovic et al., 2008; Chebeňová-Turcovská et al., 2011). Yeast species usually represent secondary microbiota in dairy products and among them *Kluyveromyces marxianus*, *Debaryomyces hansenii* and *Saccharomyces cerevisiae* are usually found to be predominant (Frolich-Wyder, 2003;

Abbreviations: L, *Lactococcus*; Lb, *Lactobacillus*; Ln, *Leuconostoc*; E, *Enterococcus*; Strep, *Streptococcus*; Staph, *Staphylococcus*; D, *Debaryomyces*; C, *Candida*; T, *Torulaspora*; Y, *Yarrowia*; Tr, *Trichosporon*.

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Chebeňová-Turcovská et al., 2011). Moreover, recent studies have indicated that food borne yeasts *D. hansenii* and *Yarrowia lipolytica* could successfully be used as part of starter cultures for cheese manufacturing, enhancing flavour development during cheese maturation (Ferreira and Viljoen, 2003; Sørensen et al., 2011).

Considering the significant role of autochthonous microbiota in the determination of the main features of a cheese, the goal of this study was the identification and characterisation of LAB and yeasts associated with the production of artisanal white pickled and fresh soft cheeses traditionally performed in rural households in the mountain regions of Serbia and lowland regions of Croatia. To our knowledge, this is the most complete study highlighting the phenotypic and genotypic diversity of LAB and natural yeast isolates from traditional cheeses in Serbia and Croatia.

2. Material and methods

2.1. Cheese samples

The cheese sampling regions were chosen based on the history of traditional dairy product manufacturing from different kinds of raw milk without the addition of any starter culture. Nine white pickled artisanal cheeses (designated as BG) were collected from three regions in Serbia: the South Morava region (one-day-old BGAL2, two-day-old BGLE1, and 10-day-old BGAL3), Golija Mountain (10-day-old BGGO5 and BGGO11, and 60-day-old BGGO7) and the mountainous region of Eastern Serbia (three-day-old BGVL1 and BGBU1, and 10-day-old BGRE2). Nine one-day-old fresh soft artisanal cheeses (designated as ZG) were collected from three regions in Croatia: Prigorje (ZGPR1, ZGPR2 and ZGPR3), the Bilogorsko-Podravska region (ZGBP4, ZGBP5, and ZGBP6) and Zagorje (ZGZA7, ZGZA8 and ZGZA9). The cheeses were taken from the households where they were originally produced. All samples were placed in sterile plastic containers that were transported to the laboratory immediately, under refrigeration. Microbiological analysis of each sample was performed within 24–48 h.

In general, white pickled cheeses manufactured in the rural regions of Serbia are characterised by a mild salty and sour taste; the curd has a soft consistency with a number of bigger or smaller cheese holes and a porcelain shine. Briefly, the cheeses are made from fresh milk by the addition of rennet. The curd forms after 1 to 2 h. After the whey forms, the curd is drained and then pressed with a weight for 2 h. The resulting pressed curd is cut into rectangular pieces, salted, and covered with brine containing 2% (w/v) NaCl. The cheese ripens from 1 to 60 days at 15–18 °C, kept permanently under the brine.

The traditional procedure of fresh soft cheese production in rural lowland regions of Croatia is carried in a similar fashion to that described above, although without the ripening and without the addition of salt and brine (Kirin, 2006; Leboš Pavunc et al., 2012). The cheese is kept in a cold place until consumption or sale.

2.2. Isolation and identification of lactic acid bacteria

LAB isolation from the cheese samples was performed essentially as described by Terzic-Vidojevic et al. (2007). For microbiological analysis, 20 g of each sample was taken from the cheese interior, homogenized with a pestle in a sterile mortar, and transferred to 180 ml of sterile 2% (w/v) sodium citrate solution in a sterile conical flask. Decimal dilutions of the homogenates were prepared with sterile 0.85% (w/v) sodium chloride and were plated on the media most suitable for the isolation of each LAB: a) for presumptive lactobacilli, on MRS agar pH 5.7 (Merck GmbH, Darmstadt, Germany) at 30 °C and 45 °C for 72 h in aerobic conditions, and in anaerobic conditions in anaerobic jars with Anaerocult A (Merck, GmbH, Darmstadt, Germany) for 5 days; b) for presumptive lactococci on M17 agar pH 7.2 (Merck, GmbH, Darmstadt, Germany) at 30 °C for 72 h (Mannu et al., 2002). Thirty to fifty colonies per sample were taken at random from both MRS (30 °C and 45 °C) and M17 (30 °C) agar plates corresponding to the highest dilution at which growth occurred. The cell morphology of all strains of LAB was determined by microscopy (Olympus U-RFL-T, BX51, GmbH, Hamburg, Germany). After microscopic observations, the colonies were sub-cultured to purity on an MRS or M17 plate, and stored at −80 °C in an appropriate medium (GM17 or MRS) supplemented by 15% (v/v) glycerol. Identification of LAB isolates based on their phenotypic characteristics and 16S rDNA sequencing, as well as rep-PCR analysis were performed as described previously (Veljovic et al., 2007; Golic et al., 2012). The 16S rDNA region was sequenced (Macrogen, Amsterdam, The Netherlands), by using primers UNI16SF and UNI16SR (Jovcic et al., 2009). Rep-PCR analysis was performed by using $(GTG)_5$ primers (Versalovic et al., 1994).

2.3. Isolation and identification of yeasts

For the isolation of yeasts, 10 g of each cheese was diluted in 90 ml of phosphate buffer solution and homogenized in a Stomacher blender for 60 s. Each cheese was sampled in triplicate. The cheese suspensions were serially diluted (10^{-1} to 10^{-6}) in sterile PBS. From each dilution 100 μl of suspension was transferred to YPD agar plates (Sigma-Aldrich, St. Louis, MO, USA) containing 10 mg/ml of ampicillin (Roche, Mannheim, Germany). The plates were incubated at 26 °C for 3–5 days. From each countable plate, the yeast colonies were inspected by naked eye and grouped according to their macromorphology (nine plates per cheese sample). To obtain an abundance of each species the morphotypes were enumerated and representative colonies from every countable plate were streaked to obtain a pure culture for further identification. The yeast isolates were preserved in 10% (v/v) glycerol at −80 °C.

Genomic DNA from pure cultures was isolated using a MasterPure Yeast Isolation kit (Epicentre, Madison, USA) according to the manufacturer's instructions. The ITS (ITS1 and ITS2) and 5.8S rRNA gene regions were amplified using ITS1 and ITS4 primers (White et al., 1990) as described previously (Cadez et al., 2002) and were digested with restriction enzymes HaeIII, CfoI and HinfI. Additionally, for separation between *D. hansenii* and *Candida zeylanoides*, restriction enzymes DdeI and MseI were used. The digests were separated on 2.5% agarose gels. The isolates sharing identical restriction patterns were grouped by computer using BioNumerics ver. 6.6 (Applied Maths, Sint-Martens-Latem, Belgium).

The final identification of the yeast species was determined by sequencing the D1/D2 domain of the large subunit rRNA gene or ITS using primer pairs NL1/NL4 and ITS1/NL4, respectively, (Macrogen, Amsterdam, The Netherlands) as described by Cadez et al. (2003). Identified strains were deposited in the ZIM Collection of Industrial Microorganisms and their nucleotide sequences were deposited in the GenBank/EMBL/DDBJ database.

2.4. Physiological and technological characterisation of the isolates

Gram-positive and catalase-negative isolates of LAB were characterised by physiological tests such as growth at 4.0 and 6.5% (w/w) NaCl in MRS and M17 broth, production of carbon dioxide from glucose by sub-culturing the isolates in Durham tubes with MRS broth, L-arginine and esculin hydrolysis, citrate utilisation, diacetyl production (only for LAB which coagulated casein), activity in milk and testing in litmus milk (Veljovic et al., 2007).

Antimicrobial activity of LAB isolates was detected by an agar-well diffusion assay using *L. lactis* subsp. *cremoris* NS1, *Lb. plantarum* A112 and *L. lactis* subsp. *lactis* BGMN1-596 as indicator strains, as described previously (Lozo et al., 2004). Proteolytic activity of LAB isolates was determined by incubating washed cells of the bacteria with casein and by measuring protein degradation by SDS PAGE, according to Kojic et al. (1991). Lipolytic activity of yeasts was examined on Tributyrin agar

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