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Characterization of lactic acid bacteria isolated from Bukuljac, a homemade goat's milk cheese

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Abstract

The Bukuljac cheese is traditionally homemade cheese, produced from heat-treated goat's milk without the addition of any bacterial starter culture. The presence of lactic acid bacteria (LAB) in Bukuljac cheese has been analyzed by using a polyphasic approach including microbiological and molecular methods such as rep-PCR with $(GTG)_5$ primer. *Lactobacillus paracasei* subsp. *paracasei* represents a dominant strain in the microflora of analyzed cheese. Out of 55 Gram-positive and catalase-negative isolates, 48 belonged to *L. paracasei* subsp. *paracasei* species. Besides lactobacilli, five *Lactococcus lactis* subsp. *lactis* and two *Enterococcus faecalis* were found. Results of PCR-denaturing gradient gel electrophoresis (DGGE) of DNA extracted directly from the fresh cheese revealed the presence of *Leuconostoc mesenteroides*.

Only lactobacilli showed a high proteolytic activity and hydrolyzed α_{s1}− and β-caseins. They are also producers of diacetyl. In addition, 34 out of 55 isolates, all determined as lactobacilli, showed the ability of auto-aggregation. Among 55 isolates, 50 also exhibited antimicrobial activity.

Keywords: Goat's milk cheese; LAB; DGGE; Rep-PCR; Proteinase; Aggregation; Antimicrobial activity

1. Introduction

The analysis of dominant microorganisms in cheese has been performed by bacterial cultivation, followed by characterization and identification of the microflora by phenotypic, biochemical and physiological tests (Corroler et al., 1998; Stiles and Holzapfel, 1997). Although conventional culture-dependent methods have proven to be useful and indispensable tool, they are regarded as time-consuming and limited in terms of both discriminating ability and accuracy. In the last decade, the profiling of bacterial populations became more precise with the application of molecular methods that enabled direct detection of DNA and RNA in microbial ecosystems. Molecular techniques, including 16S ribosomal DNA (rDNA) sequencing, repetitive bacterial DNA elements (rep-PCR) analysis, and other PCR-based techniques, are necessary for the taxonomic investigation of complex ecosystems (Amann et al., 1995; Schleifer et al., 1995). In particular, denaturing gradient gel electrophoresis (DGGE) of 16S rDNA amplicons has been demonstrated as a suitable tool for the analysis of microbial communities allowing the detection of species and changes in community structure quickly and economically (Randazzo et al., 2002).

Due to the widely appreciated organoleptic characteristics, the production of goat's milk cheese has attracted growing interest over recent years. It has been noticed that the goat milk has more easily digestible fat and protein content than cow milk (Haenlein, 2001). In addition, goat milk has increase content of vitamin A, thiamine and niacin in comparison to cow's milk (Haenlein, 2001). Characterization and identification of the microflora from artisanal goat's milk cheeses have been analyzed in different countries, like Spain (Fontecha et al., 1990; Requena et al., 1992; Olarte et al., 2000; Sanchez et al., 2005), Greece (Litopoulou-Tzanetaki and Tzanetakis, 1992; Xanthopoulos et al., 2000) and Bulgaria (Tserovska et al., 2000–2002).

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The goat's milk cheese (locally called Bukuljac) originated from Serbia. It is a soft white cheese with pleasant flavour made from pure heat-treated goat's milk by the addition of rennet and without the addition of any starter culture. In general, artisanal cheeses with unique characteristics such as goat's milk cheese analyzed in this study are manufactured as a part of historical and cultural heritage in the Balkan region.

In this study, we performed a polyphasic approach, including culture-dependent and independent methods for studying the lactic acid bacteria (LAB) from the goat's milk cheese. Samples were subjected to traditional microbiological analysis, direct DNA extraction, followed by PCR with primers able to detect eubacterial population, and DGGE analysis on the amplified products. Moreover, strains of LAB were isolated, subsequently identified by molecular methods, and characterized by the use of PCR amplification of repetitive bacterial DNA elements (rep-PCR). The aim of this study was the examination of the wild LAB microflora from a homemade goat's milk cheese and technological properties of the isolated strains for the potential inclusion in a starter culture preparation. According to our knowledge, this paper reports the first study regarding the phenotypic, genotypic and physiological characterization of the microflora from goat's milk cheese produced in The Western Balkan region.

2. Materials and methods

2.1. Cheese manufacture and sampling

Bukuljac cheese was made in a household using traditional cheese-making procedure, located in Central Serbia, near town of Arandjelovac, at approximate altitude of 500 m. The cheese Bukuljac was made on the only Sana breed goat farm in this region. The milk was obtained by mixing refrigerated evening and fresh morning milk. The milk was heated until boiling and the temperature of milk was adjusted to approximately 30 °C prior to addition of the commercial rennet ("Sirela", Cacak, Serbia) for milk coagulation, without the addition of a starter culture. The formation of a curd took 2 to 3 h. The curd was cut into pieces, approximately 12×6×3 cm weighed 200–350 g and salted by dry salt (10 g kg^{-1} of cheese). In order to remove whey, the curd was sieved 2 to 3 h without pressure. Afterward, the cheese was left to ripen. The usual ripening time was between 1 and 10 days at 10 to 15 °C. Cheese samples (one sample per batch) from three different batches (300 g in weight per sample), with the same ripening period (5 days) were taken sterile and kept refrigerated at 4 °C until the arrival to the laboratory for the analysis. Microbiological analyses of these samples were performed within the following 24 to 48 h.

2.2. Microbiological analyses–isolation, characterization and identification of lactic acid bacteria

For microbiological analysis, 20 g of each cheese sample was taken from the cheese interior, homogenized with pestle in sterile mortar, and transferred to 180 ml sterile 2% (w/v) sodium citrate solution. Decimal dilutions of the homogenates were prepared with sterile 0.85% (w/v) sodium chloride and were plated on media most suitable for isolation of LAB: a) for presumptive lactobacilli, on MRS agar pH 5.7 (Merck GmbH, Darmstadt, Germany); b) for presumptive lactococci on M17 agar pH 7.2 (Merck GmbH, Darmstadt, Germany). Incubation of inoculated media was performed at 30 °C and 45 °C for 72 h in aerobic conditions and in anaerobic conditions (Mannu et al., 2002). For incubation in anaerobic conditions, jars with Anaerocult A (Merck GmbH, Darmstadt, Germany) were used.

Thirty to fifty colonies per sample were randomly taken from both M17 and MRS (30 °C and 45 °C) agar plates corresponding to the highest dilution at which growth occurred. Cell morphology of all isolates 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 MRS or M17 medium for rods and cocci, respectively. Gram-positive and catalase-negative isolates were frozen at −20 °C and −80 °C in M17 (for cocci) and in MRS (for rods) broth containing 15% of glycerol (v/v) (Mannu et al., 2002).

Overall, 131 pure cultures were isolated and 88 of them were Gram-positive and catalase-negative. According to the morphology of bacterial colonies and cells, 55 isolates out of 88 were chosen for further analysis and they were identified to the genus or species level, by phenotypic tests as follows: (a) colony morphology and pigmentation, (b) growth at 15 °C, 30 °C and 45 °C in MRS broth for rods and in M17 broth for cocci (the tests were performed three times), (c) salt tolerance: growth with 4%, 6.5% and 8% NaCl in MRS and M17 broth for rods or cocci, respectively (the tests were performed three times), (d) production of carbon dioxide from glucose by sub-culturing the isolates in tubes with MRS broth containing Durham's tubes, (e) L-arginin and esculin hydrolysis, (f) only for cocci-test of forming black zone on bile esculin agar (HiMedia, Mumbai, India), (g) citrate-utilization, (h) activity in milk and test in litmus milk, (i) diacetyl production-only for LAB which coagulated skimmed milk (Kandler and Weiss 1986; Mundt, 1986a,b; Prescot et al., 1996).

The bacterial strains used in this study are listed in Table 1. *Lactobacillus* strains were generally grown in MRS (Merck GmbH, Darmstadt, Germany) at 30 °C or 37 °C, depending on the strain. *Lactococcus lactis* and *Enterococcus* strains were grown in M17 (Merck GmbH, Darmstadt, Germany) with 10 g l^{-1} of glucose, at 30 °C and 37 °C, respectively.

2.3. DNA isolation

Total DNA from cheese was extracted as described by Randazzo et al. (2002). Total DNA from pure cultures was extracted as described by Hopwood et al. (1985). Plasmid DNA from the isolates was extracted as described by O' Sullivan and Klaenhammer (1993). Plasmid profiles were observed in 1% agarose gels and visualized in ethidium bromide by CCD camera Biometra BDR2/5/6 (Bio Doc Analyze GmbH, Göttingen, Germany).