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IDENTIFICATION AND CHARACTERIZATION OF LACTIC ACID BACTERIA ISOLATED FROM ARTISANAL WHITE BRINED GOLIJA COWS' MILK CHEESES

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Abstract - The aim of this study was to identify and characterize the lactic acid bacteria (LAB) of artisanal Golija raw and cooked cows' milk cheeses traditionally manufactured without the addition of starter culture. A total of 188 Gram-positive and catalase-negative isolates of Golija cheeses were obtained from seven samples of different ripening time. Phenotype-based assays as well as rep-PCR and 16S rDNA sequence analysis were undertaken for all 188 LAB strains. The most diverse species were isolated from 20-day-old BGGO8 cheese (*Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus casei/paracasei*, *Lactobacillus sucicola*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis, Enterococcus faecium, Enterococcus durans and Leuconostoc mesenteroides*). In other Golija cheeses *Lactobacillus reuteri*, *Lactobacillus curvatus*, *Lactobacillus rhamnosus*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus garvieae*, *Streptococcus thermophilus* and *Leuconostoc pseudomesenteroides* were found. Pronounced antimicrobial properties showed enterococci (13/42) and lactococci (12/31), while the good proteolytic activity demonstrated lactococci (13/31) and lactobacilli (10/29).

Key words: Artisanal white brined Golija cheeses, lactic acid bacteria, rep-PCR, 16S rDNA sequencing

INTRODUCTION

Lactic acid bacteria (LAB) are naturally present in milk and milk products. Due to their metabolism LAB are the main organisms responsible for the acidification of cheese, necessary for the coagulation of the milk. In addition, many LAB species play an important role in the ripening process of cheese, especially to improve the consistency, aroma and flavor (Giraffa, 2002; Hannon et al., 2003; Duan et al., 2008). The nature of fermented dairy products is different from one region to another depending on the local indigenous microflora. Environmental conditions in each geographic region affect the properties of predominant native microflora, limiting the use of some universal starters. The rational solution is the selection of starter cultures from the native flora that could be used successfully in the dairy industry (Menéndez et al., 2004; Abdalla and Hussain, 2010).

Soft white artisanal Golija cheeses in brine are traditionally produced in the rural areas of the Golija Mountain in western Serbia. The specific continental mountain climate and richness of meadows and pastures were favorable for the development of livestock, especially cattle. The Golija cheeses are made from cows' milk without the addition of starter culture.

Autochthonous dairy products represent an important patrimony for each country and a great

opportunity for the development of rural areas. The white cheeses made in the Balkans belong to a group of cheeses ripened in brine (Abd El-Salam and Alichanidis, 2004). Golija cheeses belong to this group of cheeses as well. The cheeses can be consumed immediately after production or during one to six months of the ripening. Previous results have shown that the optimum ripening of white cheeses in brine is about 30 days (Terzic-Vidojevic et al., 2007).

The majority of Serbian cheeses are traditionally manufactured on a small scale. Little is known about their LAB composition. On the other hand, industrial cheese production using pasteurized milk and well established starter cultures has led to a reduction in the biodiversity of the autochthonous microbiota. Thus, the isolation and characterization of LAB involved in the natural fermentation of artisanal cheeses could be useful for the construction of new starter cultures for the dairy industry to produce such cheeses in a controlled way (Beresford et al., 2001; Marino et al., 2003).

The aim of the present study was to identify and characterize the natural LAB population of seven Golija cheeses manufactured from raw and cooked cows' milk. Phenotypic and genotypic characterization of 188 LAB isolates was performed by conventional microbiological and modern molecular techniques, including rep-PCR and 16S rDNA sequencing. Characterization of isolated LAB to the strain level provided an insight into LAB diversity in the artisanal cheese manufactured in this specific region, which is under UNESCO protection. In addition, such an approach could facilitate the selection of appropriate strains with good technological properties that could be eventually used for the preparation of starter culture for standardized Golija cheese production.

MATERIALS AND METHODS

Cheeses manufacturing and sampling

LAB were isolated from seven samples of the soft white brined Golija cheeses which were 1, 5 and 20 days old. These cheeses were made by adding the rennet to raw or cooked cows' milk without the addition of starter cultures. The cows whose milk was used to prepare the cheeses belong to the Domestic Spotted Cattle of Simmental Cattle type. The cows were fed through the winter on hay, corn, oats and barley, while during the summer they were on pasture. For the formation of a curd (coagulation), the commercial rennet strength 1:3000 or 1:5000 was used. The formation of a curd took 20 to 60 min depending on the cheese type. Upon draining the whey the curd was salted and cut into pieces. The cheese pieces were transferred into a plastic container, poured over with brine, covered with a lid and left to ripen at 10-15°C for up to 6 months. The cheeses' labels and general data of the manufacturing process of each cheese are given in Table 1. The cheese samples were taken from households in a sterile plastic container and transported to the laboratory under refrigeration. Microbiological analyses of these samples were performed within the following 24-48 h.

Indicator and reference strains

Sixteen indicator and reference strains were used for the detection of antimicrobial activity and rep-PCR analyses (Table 2). Lactobacilli and leuconostocs were cultivated on MRS medium (pH 5.7) (Merck GmbH, Darmstadt, Germany), while lactococci, enterococci and streptococci were cultivated on M17 medium (pH 7.2) (Merck GmbH) supplemented with 0.5% (w/v) glucose (GM17). The incubation was carried out at the appropriate growth temperature for each bacterial group during 24 to 48 h.

Isolation and physiological characterization of LAB

Twenty grams from the interior of each cheese sample were homogenized in 180 ml of sterile 2% (w/v) trisodium citrate dehydrate solution for first dilution (10^{-1}). Next dilutions (10^{-2} to 10^{-7}) were made in 0.85% sterile saline. For LAB isolation, 1 ml of the appropriate dilutions was plated on MRS and GM17 agar medium, respectively. The plates were incu-

bated at 30°C and 45°C for 72 h under aerobic and anaerobic conditions using the Anaerocult A (Merck GmbH) in anaerobic jars.

Forty colonies from each cheese sample were randomly picked and tested for catalase activity, gram staining and cell morphology. After microscopic examination, pure cultures were stored at -80°C in their respective media, GM17 or MRS broth with 15% (w/v) glycerol.

One hundred and eighty eight Gram-positive and catalase-negative isolates were tested for: (i) growth at 15°C, 30°C and 45°C; (ii) growth in broth with 2%, 4%, 6.5% and 8% (w/v) NaCl; (iii) hydrolysis of arginine; (iv) gas production from glucose in reconstitute MRS broth with inverted Durham bells; (v) survival at 63.5°C for 30 min; (vi) creation of the black zone on bile-esculin agar (Himedia, Mumbai, India) for the presumptive identification of enterococci; (vii) the time required for curd formation in 11% reconstituted skimmed milk (RSM) and litmus milk test. The production of diacetyl was assessed qualitatively. The LAB were inoculated in 11% RSM for 16 h. To 1 ml of coagulated milk, 0.1 g of creatinine and 1 ml 30% NaOH (w/v) were added. Diacetyl generation was indicated by the formation of a red ring at the top of the tubes after 2 h. Exopolysaccharides (EPS) production was detected visually as long strands when colonies were extended with an inoculation loop.

The preliminary identification of LAB isolates based on phenotypic characteristics was performed according to the criteria of Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

rep-PCR analysis and 16S rDNA sequencing of LAB isolates

Total DNA from all 188 LAB isolates was extracted as described previously (Singh and Ramesh, 2009). For repetitive element palindromic-polymerase chain reaction (rep-PCR) analysis, $(GTG)_5$ oligonucleotide (5′-GTGGTGGTGGTGGTG-3′) was used (Gevers et al., 2001). Briefly, the 30 µl reaction contained 500 ng/µl of DNA, 1 U of KAPA Taq DNA Polymerase (Kapa Biosystems, Woburn, U.S.A.), 200 µM of each deoxynucleoside triphosphate, 0.4 µM of $(GTG)_5$ oligonucleotide and 1 x Kappa buffer with Mg^{2+}. Conditions for DNA amplification were 95°C for 7 min; 33 cycles of 94°C for 1 min, 40°C for 1 min, and 65°C for 8 min; and 65°C for 16 min. The PCR products were analyzed by 1% agarose gel electrophoresis (9 V/cm for 5 h) and visualized by CCD camera Biometra BDR2/5/6 (Bio Doc Analyze GmbH, Göttingen, Germany).

PCR amplification of 16S rDNA, with UNI16SF (5`GAGAGTTTGATCCTGGC-3`) and UNI16SR (5`- AGG AGGTGATCCAGCCG-3`) oligonucleotides was performed as described by Jovcic et al. (2009) with some modifications. The PCR reaction mixture (30µl) contained 500 ng/µl of DNA, 1 U of KAPA Taq DNA Polymerase, 200 µM dNTPs each, 0.1 µM oligonucleotide each and 1 x KAPA buffer with Mg^2. PCR conditions were 95°C for 5 min; 30 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec; and 72°C for 7 min. Obtained PCR amplicons were purified (Qiagen, GmbH, Hilden, Germany) and sequenced (Macrogen, Amsterdam, Netherlands and Seoul, South Korea). The BLAST algorithm was used to determine the most related sequence relatives in the NCBI nucleotide sequence database (http://www.ncbi.nlm.nih.gov/BLAST).

Detection of antimicrobial compounds

Preliminary screening of 188 LAB isolates from Golija cheeses for the production of antimicrobial compounds was done by the deferred antagonism method using overnight cultures of isolates and various indicator strains (Table 2). Briefly, soft GM17 and MRS agars (0.7% w/v) containing lactococci, enterococci or lactobacilli indicator strains were overlaid onto GM17 and MRS plates, respectively. The plates were incubated overnight at the appropriate temperature (30°C or 37°C), depending on the LAB strain. A clear zone of inhibition of indicator strain growth around the well was taken as a positive signal for production of antimicrobial compound.

Table 1. Basic information about soft white Golija cheeses and technological process of cheese manufacturing

Basic information and technological process	Labels of cheese samples						
	BGGO1	BGGO2	BGGO3	BGGO6	BGGO8	BGGO9	BGGO10
Sampling location (village) and altitude	Katići, 1000 m	Katići, 1000 m	Katići, 1000 m	Katići-Brezova, 1000 m	Maće-Priboj, 1000 m	Maće-Priboj, 1000 m	Maće, 1000 m
Type of cheese according to fat content	full-fat	semi-fat	semi-fat	semi-fat	semi-fat	semi-fat	semi-fat
Kind of cow's milk for cheese manufacturing	skimmed, cooked evening + full-fat, raw morning	skimmed, cooked evening + full-fat, raw morning	evening or morning cooked and skimmed	evening or morning cooked and skimmed	evening or morning cooked and skimmed	evening or morning cooked and skimmed	evening or morning cooked and skimmed
Amount of added commercial rennet, temperature of curdling and duration	no data, 18-20°C, 60-90 min	no data, 18-20°C, 60-90 min	4 tablespoons of rennet per 7 l of milk at 4-6°C, 4 h	no data, 30°C, 60 min	3 tablespoons of rennet per 17 l of milk at 25-30°C in the beginning of curdling, after which the milk is heated up on the stove, 20 min	3 tablespoons of rennet per 17 l of milk at 25-30°C in the beginning of curdling, after which the milk is heated up on the stove, 20 min	no data, 30°C, 60-90 min
Cutting the curd	wooden spoon	wooden spoon	no cutting	no cutting	no cutting	no cutting	no cutting
Duration of curd draining	5 min	5 min	5 min	15 min	10 min	10 min	40 min
Using pressure during curd draining	no pressure	no pressure	stone slab during 5 h	no pressure	1 kg stone	1 kg stone	1 kg stone
The pressure of the cheese base	stone 0.5-1 kg during 60 min	stone 0.5-1 kg during 60 min	stone 0.5-1 kg during few hours	stone 2 kg during 6-8 h	no data, about 2 h	no data, about 2 h	no data, about 6 h
Size of cheese base	30-35 cm x 4 cm	30-35 cm x 4 cm	30-35 cm x 4 cm	30-35 cm x 4 cm	40 cm x 5 cm	40 cm x 5 cm	40 cm x 5 cm
Salting	randomly with dry salt	randomly with dry salt	randomly with dry salt	randomly with dry salt	randomly with dry salt	randomly with dry salt	randomly with dry salt
Drying cheese	10 min	10 min	no drying	no drying	no drying	no drying	no drying
Type of brine	boiled salted whey	boiled salted whey	no data	boiled salted water	cold unsalted whey	cold unsalted whey	no data
Cheese ripening	10 d	10 d	20-30 d	10-15 d	30 d	30 d	15 d
Cheese storage	storeroom at 10°C, 30 d	storeroom at 10°C, 30 d	cooler room at 4-6°C, 30-35 d	the special room so-called mlekar, 30 d	the special room so-called mlekar, 2 months	the special room so-called mlekar, 2 months	the special room so-called mlekar, 5-6 months
For 1 kg of cheese needed	5-5.5 l of milk	5-5.5 l of milk	7 l of milk	5.5-6 l of milk	6-7 l of milk	6-7 l of milk	5 l of milk

Proteolytic activity

The proteolytic activity of all 188 LAB isolates was tested as previously described (Kojic et al., 1991). LAB strains were grown on milk-citrate agar (MCA) containing 4.4% RSM, 0.8% sodium citrate, 0.1% yeast extract, 0.5% glucose and 1.5% agar for 48 h at 30°C. Collected fresh cells (10 mg approximate density 10^{10} cells/ml) from the MCA plates were re-suspended in 0.1 M sodium phosphate buffer, pH 6.8 and mixed in the ratio 1:1 with 5 mg/ml of β-casein (Sigma, St. Louis, MO, USA) dissolved in the same buffer. The mixtures were incubated for 3 h at 30°C or 37°C, depending on the LAB strain. Degradation of β-casein was analyzed on 12.5 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

RESULTS

Presumptive identification of LAB based on cell morphology and phenotypic characteristics (Table 3) showed the presence of five genera in Golija cheeses: *Leuconostoc* sp. (45.2%), *Enterococcus* sp. (22.3%), *Lactococcus* sp. (16.5%), *Lactobacillus* sp. (15.5%) and *Streptococcus* sp. (0.5%). In contrast to leuconostocs that were detected in all seven cheese samples, streptococci were found only in the BGGO3 cheese. Interestingly, the absence of enterococci was found in the BGGO3 cheese, lactobacilli were absent in the BGGO1 and BGGO10 cheeses, while lactococci were lacking in the BGGO6 cheese.

Identification of LAB by rep-PCR analysis was performed for all 188 isolates (Table 3; Figs 1, 2 and 3). The rep-PCR band patterns revealed that enterococci, previously identified from BGGO3 cheese based on their physiological characteristics, are actually lactococci. Furthermore, some lactococcal and lactobacilli strains could not be completely identified by rep-PCR. Thus, the sequencing of 16S rDNA was performed. Data obtained from rep-PCR and 16S rDNA analyses revealed differences in the composition of LAB population among seven Golija cheeses. Although leuconostocs were the predominant group of LAB in all Golija cheeses (45.2 %), only two dis-

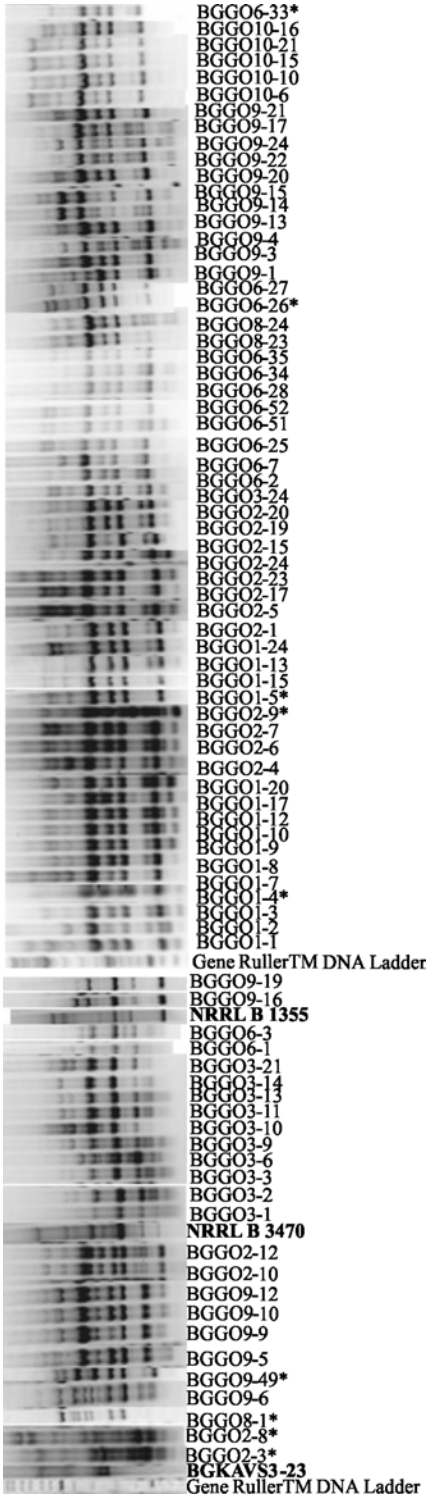

Fig. 1. The rep-PCR patterns of *Leuconostoc* sp. obtained with $(GTG)_5$. The strains marked with asterisks (*) were characterized using the 16S rDNA sequencing. Reference strains used in the test are given in bold letters.

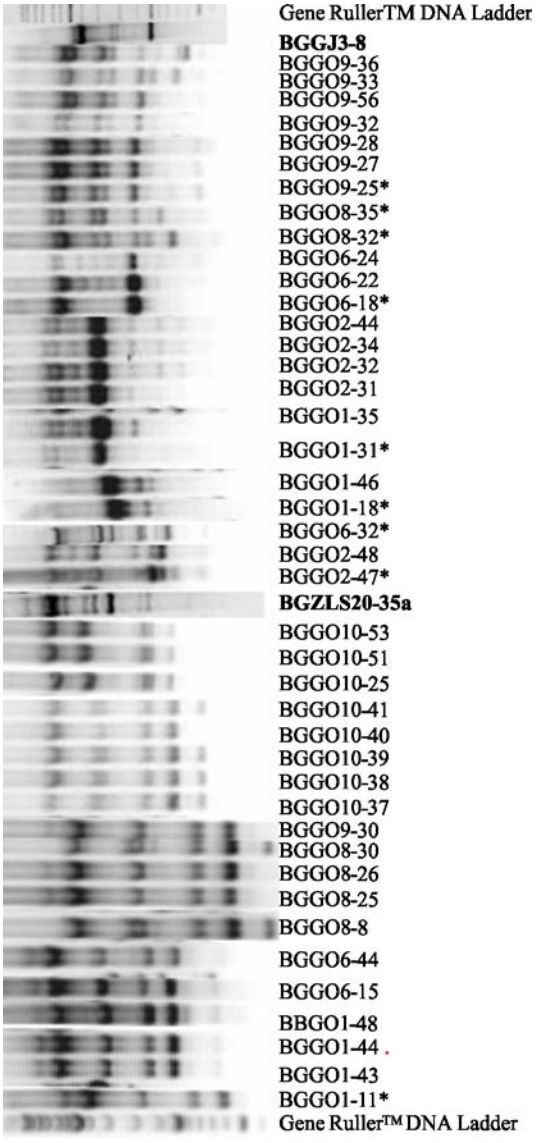

Fig. 2. The rep-PCR patterns of *Enterococcus* sp. obtained with $(GTG)_5$ The strains marked with asterisks (*) were characterized using the 16S rDNA sequencing. Reference strains used in the test are given in bold letters.

tinct species, *Ln. mesenteroides* and *Ln. pseudomesenteroides*, were identified (Table 3, Fig. 1). Twenty *Ec. faecium* and 22 *Ec. durans* were isolated from Golija cheeses (Table 3, Fig. 2). All enterococci from BGGO10 belonged to the species *Ec. durans* while the species *Ec. faecium* was predominant in BGGO9 cheese. Despite the presence of the same band pattern in *Lc. lactis* subsp. *lactis, Lc. lactis* subsp. *cremoris, Lc. lactis* subsp. *lactis* biovar. *diacetylactis* (Table 3, Fig. 3A), the differences in cell morphology, hydrolysis of arginine and production of acetoin were determined by phenotypic tests (data not shown). The LAB group of the greatest diversity in the Golija cheeses was lactobacilli. Among a total of 29 isolated lactobacilli, seven different species were identified: *Lb. curvatus*, *Lb. casei*, *Lb. rhamnosus*, *Lb. sucicola*, *Lb. reuteri*, *Lb. plantarum* and *Lb. fermentum* (Table 3, Fig.3C). The most diverse lactobacilli composition was shown in 20-day- old BGGO8 cheese.

In order to assess the industrial potentials of LAB isolated from Golija cheeses, their activity in milk and other technological characteristics were monitored. Three lactococci from BGGO9 cheese and one lactococci from BGGO2 cheese, as well as one streptococcus isolated from BGGO3 cheese, curdled the milk after about 5.5-6 h. However, other isolates, mainly leuconostocs and enterococci, needed a significantly longer time (24 to 48 h) to curdle the milk or did not curdle the milk at all. Enterococci were the best producer of volatile compounds as diacetyl (18 out of 42 enterococci). In addition, two *Lc. garvie* and one *Str. thermophilus* of BGGO3 cheese, as well as one *Lb. casei* isolate of BGGO8 cheese produced diacetyl. *Lb. reuteri* that was isolated from BGGO6 cheese with EPS as producer. Interestingly, most of the BGGO9 isolates showed an aggregation ability (six enterococci, two leuconostocs and one isolate identified as *Lb. sucicola*). Moreover, five enterococci of BGGO10 cheese and one leuconostoc of BGGO1 have the same aggregation ability (data not shown).

All Golija cheeses except BGGO3 contain bacteriocin producers. Thirteen enterococci and twelve lactococci produced bacteriocins inhibiting the growth of indicator strains BG221, NS1, BGZLM1-24 and BGMN1-596, as could be observed by the appearance of a clear or diffusion inhibitory zone around the applied supernatant samples from overnight cultures of the tested strains (data not shown).

Results of the proteolytic activity of LAB isolates from Golija cheeses showed that lactobacilli and lactococci are the most numerous LAB strains that

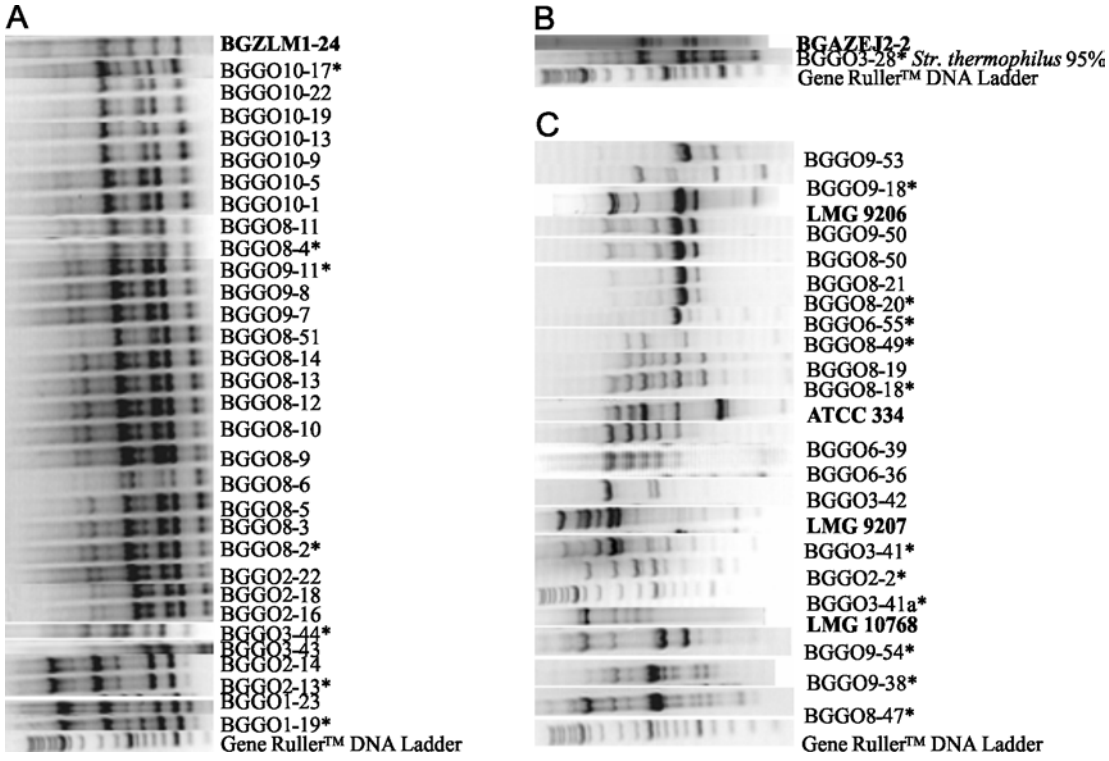

Fig. 3. The rep-PCR patterns of *Lactococcus* sp. (A), *Streptococcus* sp. (B) and *Lactobacillus* sp. (C) obtained with $(GTG)_5$ The strains marked with asterisks (*) were characterized using the 16S rDNA sequencing. Reference strains used in the test are given in bold letters.

possessed the ability to hydrolyze β-casein (Table 4). Among them, the higher number of proteolytic active strains, four lactobacilli and ten lactococci, were isolated from BGGO8 cheese. In contrast, there were no LAB showing proteolytic activity amongst isolates from BGGO1 cheese.

DISCUSSION

Characterization of LAB isolated from traditional Golija cheeses produced in households on Golija Mountain from raw and cocked milk fermented spontaneously without a starter culture is a first study of its kind. The microbiological characteristics of these cheeses were unknown. Identification and characterization of LAB were performed by combining the classical microbiological and modern molecular methods. The combined methodology approach provided a better insight into the LAB composition from Golija cheeses, as well as knowledge about their physiological and technological characteristics, their proteolytic activity and their abilities to produce volatile compounds, EPS and antimicrobial substances. The results of the LAB identification demonstrated a complex LAB population of Golija cheeses. The presence of 16 different LAB species was revealed in all samples of Golija cheeses. The well-known characteristic of artisanal cheeses analyzed so far worldwide is the great richness of LAB species. Many authors have reported about the significant diversity of LAB obtained from raw milk dairy products (Ayad et al., 2004; Ouadghiri et al., 2005; Abriouel et al., 2008; Mas et al., 2002; Terzic-Vidojevic et al., 2009; Jokovic et al., 2011).

The unusual distribution of LAB population was observed for the BGGO8 cheese. The presence of lactobacilli was significantly higher (40% out of a total 35 LAB isolates), while the presence of leuconostocs was less pronounced (8.6% out of 35 LAB iso-

Table 2. The list of strains used in this study

Bacterial strains	Reference
Lactobacillus plantarum A112[a]	Laboratory collection
Lactobacillus plantarum LMG 9026[b]	BCCM/LMG[d]
Lactobacillus casei BGHN14 [a]	Laboratory collection
Lactobacillus casei ATCC 334[b]	ATCC[e]
Lactobacillus paracasei subsp. *paracasei* LMG 9207[b]	BCCM/LMG
Lactobacillus rhamnosus LMG 10768[b]	BCCM/LMG
Lactococcus lactis subsp. *lactis* BGMN1-596[a]	Laboratory collection
Lactococcus lactis subsp. *lactis* BGZLM1-24 [a, b]	Laboratory collection
Lactococcus lactis subsp. *cremoris* NS1[a]	Laboratory collection
Leuconostoc pseudomesenteroides BGKAVS3-23	Laboratory collection
Leuconostoc mesenteroides subsp. *mesenteroides* NRRL B–3470	NRRL[f]
Leuconostoc mesenteroides subsp. *mesenteroides* NRRL B–1355	NRRL
Enterococcus faecalis BG221[a]	Laboratory collection
Enterococcus durans BGZLS20–35b[b]	Laboratory collection
Enterococcus faecium BGGJ8–3[b]	Laboratory collection
Streptococcus thermophilus BGAZEJ2-2[b]	Laboratory collection

[a] Used for antimicrobial-activity detection.
[b] Used for rep-PCR.
[c] These strains were identified by AFLP, SDS-PAGE and rep-PCR with $(GTG)_5$ primer in the Laboratorium voor Microbiologie, Universitet Gent, Gent, Belgium.
[d] BCCM/LMG–Bacteria Collection, Laboratorium voor Microbiologie, Universitet Gent, Gent, Belgium.
[e] ATCC–American Type Culture Collection, Manassas, VA, USA.
[f] NRRL– Agricultural Research Service Culture Collection, Peoria, IL, USA.

lates) compared to the other cheeses. It is noteworthy that the BGGO8 was 20 days old while the other examined cheeses were between 1 to 5 days old. A high presence of lactobacilli in 14-day-old raw milk Serra da Estrela cheese as well as in 20-day-old Zlatar cheese has been previously reported (Macedo et al., 2004; Terzic-Vidojevic et al., 2007). The increase of lactobacilli during cheese ripening is due to their better ability to resist higher acidity than lactococci and leuconostocs (Caridi et al., 2003). Their most important role is to create the specific flavor of a cheese during ripening by the acting of their proteolytic system on cheese proteins (Lopez and Mayo, 1997).

The strains belonging to *Lb. plantarum*, *Lb. casei* and *Lb. paracasei* species that were found in Golija cheeses are the common isolates from Serbian artisanal raw milk cheeses (Terzic-Vidojevic et al., 2007; Nikolic et al., 2008; Jokovic et al., 2011). Moreover, the other lactobacilli from Golija cheese, *Lb. rhamnosus*, *Lb. curvatus* and *Lb. fermentum* species were also found in cheeses made from raw milk in other geographical locations (Ayad et al., 2004; Ouadghiri et al., 2005; Colombo et al., 2009). However, the presence of *Lb. reuteri* and *Lb. sucicola* in Golija cheeses is an unusual characteristic of artisanal raw milk cheeses.

Table 3. Identification and characterization of LAB isolated from Golija cheeses

Cheese[a] Age of the cheese	Cell shape[a]	Phenotypic characteristics				Preliminary identification	Identification by rep-PCR[a]	Sequencing of 16S rDNA (max. % of identity)[b]
		CO_2 from glucose	Arginine hydrolysis	Growth at 45 °C	Growth in 6.5% NaCl			
BGGO1 (26) 2 day old	Cocci/round (2)	–	+	–	–	Lactococci	*Lactococcus sp.* (2)	*Lc. garvieae* (BGGO1-19) 97%
	Cocci/round (8)	–	+	+	+	Enterococci	*Ec. faecium* (4) *Ec. durans* (4)	*Ec. faecium* (BGGO1-31) 97% *Ec. durans* (BGGO1-11) 96%
	Cocci/ovoid (16)	+	–	–	–	Leuconostocs	*Ln. mesenteroides* (16)	*Ln. mesenteroides*[c] (BGGO1-5) 96%
BGGO2 (28) 5 day old	Rods (1)	–	–	–	–	Homofer. lactobacilli	*Lactobacillus* sp. (1)	*Lb. curvatus* (BGGO2-2) 96%
	Cocci/round (2)	–	+	–	–	Lactococci	*Lactococcus sp.* (2)	*Lc. garviae* (BGGO2-13) 95%
	Cocci/round (3)	–	+	–	–	Lactococci	*Lc. lactis* (3)	
	Cocci/ovoid (16)	+	–	–	–	Leuconostocs	*Ln. mesenteroides* (14) *Ln. pseudomesent.* (2)	*Ln. mesenteroides* (BGGO2-9) 96% *Ln. pseudomesent.* (BGGO2-3) 97%
	Cocci/round (6)	–	+	+	+	Enterococci	*Ec. faecium* (4) *Ec. durans* (2)	*Ec. durans* (BGGO2-47) 96%
BGGO3 (18) 1 day old	Rods (3)	–	+	–	–	Homof. lactobacilli	*Lb. casei/paracasei* (2) *Lb. rhamnosus* (1)	*Lc. casei*[d] (BGGO3-41) 94% *Lb. rhamnosus* (BGGO3-41a) 93%
	Cocci/round (2)	–	+	+	+	Enterococci	*Lactococcus* sp.	*Lc. lactis subsp. lactis* (BGGO3-44) 95%
	Cocci/round/ chains (1)	–	–	–	–	Streptococci	*Str. thermophilus*	*Str. thermophilus* (BGGO3-28) 95%
	Cocci/ovoid (12)	+	–	–	–	Leuconostocs	*Ln. mesenteroides* (12)	

Table 3. Continued

Cheese[a] Age of the cheese	Cell shape[a]	Phenotypic characteristics				Preliminary identification	Identification by rep-PCR[a]	Sequencing of 16S rDNA (max. % of identity)[b]
		CO_2 from glucose	Arginine hydrolysis	Growth at 45 °C	Growth in 6.5% NaCl			
BGGO6 (23) 1 day old	Rods (2)	+(1) – (1)	±(1) – (1)	+	+	Lactobacilli	*Lb. casei* (2)	
	Rods (1)	–	–	±	–	Lactobacilli	Unidentified (1)	*Lb. sucicola* (BGGO6-41) 95%
	Rods (1)	–	±	+	+	Lactobacilli	Unidentified (1)	*Lb. reuteri* (BGGO6-55) 95%
	Cocci/round (6)	–	+	+	+	Enterococci	*Ec. faecium* (3) *Ec. durans* (3)	*Ec. faecium* (BGGO6-18) 94% *Ec. durans* (BGGO6-32) 96%
	Cocci/ovoid (13)	+	± (10) +(3)	–(10) +(3)	+(12) –(1)	Leuconostocs	*Ln. mesenteroides* (13)	*Ln. mesenteroides* (BGGO6-33) 96%
BGGO8 (35) 20 day old	Rods (7)	–	–	–	+	Lactobacilli	*Lb. casei/paracasei* (3) *Lb. plantarum* (4)	*Lc. casei*[d] (BGGO8-49) 94% *Lb. plantarum* (BGGO8-20) 97%
	Rods (6)	+	+	+	+	Lactobacilli	Unidentified (6)	*Lb. fermentum* (BGGO8-47) 97%
	Rods (1)	–	–	+	–	Lactobacilli	*Lb. sucicola* (1)	
	Cocci/round (12)	–	+	–	–	Lactococci	*Lc. lactis* (12).	*Lc. lactis subsp. lactis*[e] (BGGO8-2) 95%
	Cocci/round (6)	–	+	+	+	Enterococci	*Ec. faecium* (2) *Ec. durans* (4)	*Ec. faecium* (BGGO8-36) 96%
	Cocci/ovoid (3)	+	±	–	±(1) +(2)	Leuconostocs	*Ln. mesenteroides* (3)	*Ln. mesenteroides* (BGGO8-1) 96%

Table 3. Continued

Cheese[a] Age of the cheese	Cell shape[a]	Pphenotypic characteristics				Preliminary identification	Identification by rep-PCR[a]	Sequencing of 16S rDNA (max. % of identity)[b]
		CO_2 from glucose	Arginine hydrolysis	Growth at 45 °C	Growth in 6.5% NaCl			
BGGO9 (37) 1 day old	Rods (2)	–	±(10 –(1)	–	–	Lactobacilli	*Unidentified* (2)	*Lb. sucicola* (BGGO9-18) 95%
	Rods (1)	–	–	–	+	Lactobacilli	*Lb. plantarum* (1)	
	Rods (4)	+	±(1) +(3)	–	+(2) –(2)	Lactobacilli	Unidentified (4)	*Lb. fermentum* (BGGO9-38) 96%
	Cocci/round (3)	–	+	–	–	Lactococci	*Lc. lactis* (3)	*Lc. lactis subsp. lactis*[f] (BGGO9-11) 97%
	Cocci/round (8)	–	+	+	+	Enterococci	*Ec. faecium* (7) *Ec. durans* (1)	*Ec. faecium* (BGGO9-25) 97%
	Cocci/ovoid (19)	+	± (16) – (3)	–	± (6) –(13)	Leuconostocs	*Ln. mesenteroides* (19)	*Ln. mesenteroides*[c] (BGGO9-49) 98%
BGGO10 (21) 1 day old	Cocci/round (7)	–	+	–	–	Lactococci	*Lc. lactis* (7).	*Lc. lactis subsp. lactis* (BGGO10-17) 96%
	Cocci/round (8)	–	+	+	+	Enterococci	*Ec. durans* (8)	
	Cocci/ovoid (6)	+	±	–	±(2) –(4)	Leuconostocs	*Ln. mesenteroides* (6)	

[a] Number of isolates is given in brackets.
[b] Demonstrate identity with 16S rDNA sequences of relevant species deposited in GenBank database (NCBI).
[c] *Ln. mesenteroides* subsp. *mesenteroides/dextranicum*.
[d] *Lb. casei/paracasei*.
[e] *Lc. lactis subsp. lactis/ Lc. lactis subsp. lactis* biovar. *diacetylacti*.
[f] *Lc. lactis subsp. lactis/cremoris*.

Table 4. β-casein degradation ability of LAB from Golija cheeses

Cheese	Ability of LAB to hydrolyze β-casein
BBGO1	no ability
BBGO2	± ability (one *Lc. garviae*); + ability (one *Lb. curvatus*)
BBGO3	+ ability (one *Lb. rhamnosus*)
BBGO6	+ ability (one *Lb. sucicola*)
BBGO8	± ability (10 *Lc. lactis*; two *Lb. casei/paracasei*; one *Lb. sucicola*) + ability (one *Lb. casei*; one *Lb. plantarum*)
BBGO9	± ability (one *Lb. sucicola*); + ability (one *Lb. plantarum*)
BBGO10	+ ability (two *Lc. lactis*)

+ complete hydrolysis of β-casein; ± partial hydrolysis of β-casein.

On the other hand, leuconostocs were present in large number in all younger Golija cheeses and *Ln. mesenteroides* was the most frequently isolated species. This group of LAB is commonly detected in many varieties (Ostlie et al., 2004; Randazzo et al., 2006; Duan et al., 2008; Terzic-Vidojevic et al. 2009). Generally, heterofermentative LAB of the genus *Leuconostoc* are very important for cheese quality. Although their proteolytic activity is weak, they contribute to the formation of the taste and aroma of final dairy products. Carbon dioxide production is responsible for eye formation in cheeses, while lactose fermentation and citrate utilization leads to the production of diacetyl that is considered a main flavor compound of fermented milk products (Hemme and Foucaud-Scheunmann, 2004).

The important LAB group of Golija cheeses was enterococci that were represented by two species: *Ec. faecium* and *Ec. durans*. Enterococcal strains are widely distributed in nature (Ayad et al., 2004; Psoni et al., 2006). They are commonly isolated from dairy products, especially from Southern Europe (Giraffa, 2003), where they are considered to play an important role in the developing of the specific cheese taste and texture owing to their metabolic and technologic traits. *Ec. faecium*, *Ec. durans* and *Ec. faecalis* are the species most frequently found in cheeses and other dairy products (Cosentino et al., 2004; Psoni et al., 2006; Veljovic et al., 2007). The ability of bacteriocinogenic enterococci to inhibit the growth of certain pathogens and spoilage microorganisms shows their great potential to be used in food preservation (Andrighetto et al., 2001; Giraffa, 2002; Foulquié-Moreno et al., 2006). According to our results, two *Ec. faecium* strains isolated from BGGO1, BGGO2 and BGGO6 cheeses and one *Ec. durans* strain from BGGO9 cheese showed antimicrobial activity toward the BG221 indicator strain as well as one *Ec. durans* strain from BGGO10 cheese that inhibited the growth of the NS1 indicator strain (data not shown). Two *Ec. durans* strains from BBGO8 cheese (BGGO8-25 and BGGO0-26) and one *Ec. durans* strain from BGGO10 cheese (BGGO10-25) inhibited the growth of NS1 and BGZLM1-24 indicator strains. In addition, *Ec. durans* strains BGGO10-51 and BGGO10-53 were bacteriocin producers that inhibited the growth of three indicator strains, BG221, BGZLM1-24 and NS1 (data not shown), indicating a possibility that more than one agent is produced by these LAB (Lozo et al., 2004)

The presence of *Lc. lactis* strains was most frequent in raw milk cheeses (Terzic-Vidojevic et al.,

2007; Nikolic et al., 2008; Ouadghiri et al., 2009; Jokovic et al., 2011; Feutry et al., 2012). Lactococci were found in all Golija cheeses except the BGGO10 cheese. This is a very important group of LAB because they are responsible for acid development at the beginning of cheese ripening (Casalta and Montel, 2008). In addition, lactococci, like lactobacilli, have a good proteolytic activity. The possibility of LAB to hydrolyze ß-casein is essential for bacterial growth in milk (Ayad et al., 2004). Moreover, the hydrolysis of β-casein is very important for the development of the specific sensory properties of different fermented milk products (Christensen et al., 1999). The highest number of proteolytic active lactococci was isolated from BGGO8 cheese (10 out of 12 lactococci).

In conclusion, our results show that combined classical and modern molecular techniques are very good tools for differentiating wild-type LAB isolated from artisanal dairy products. Characterization of LAB strains from traditionally manufactured dairy products to species level enables us to assess the microbiological diversity of the LAB natural population. It is noteworthy that the natural LAB microflora of artisanal fermented foods consists of microorganisms adapted to the particular product, specific environmental conditions and production technology. Our examinations of LAB populations in raw milk products from Golija Mountain revealed 16 distinct species, some of which could be used in the dairy industry. Numerous lactococci (45.2%) were producers of antimicrobial compounds, and 38.7% among them were proteolytically active. One third of lactobacilli showed proteolytic activity that is the most important feature in the formation of the specific sensory characteristics of Golija dairy products. The best producers of aromogenic compounds were enterococci (42.8%), while 28.6% of them produced antimicrobial compound(s).

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