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ORIGINAL ARTICLE

Preliminary characterization of lactic acid bacteria isolated from Zlatar cheese

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Abstract

Aims: Isolation, characterization and identification of lactic acid bacteria (LAB) from artisanal Zlatar cheese during the ripening process and selection of strains with good technological characteristics.

Methods and Results: Characterization of LAB was performed based on morphological, physiological and biochemical assays, as well as, by determining proteolytic activity and plasmid profile. rep-polymerase chain reaction (PCR) analysis and 16S rDNA sequencing were used for the identification of LAB. PCR analysis was performed with specific primers for detection of the gene encoding nisin production. Strains *Lactobacillus paracasei* subsp. *paracasei, Lactobacillus plantarum, Lactobacillus brevis, Lactococcus lactis* subsp. *lactis, Enterococcus faecium* and *Enterococcus faecalis* were the main groups present in the Zlatar cheese during ripening.

Conclusions: Temporal changes in the species were observed during the Zlatar cheese ripening. Mesophilic lactobacilli are predominant microflora in Zlatar cheese.

Significance and Impact of the Study: In this study we determined that Zlatar cheese up to 30 days old could be used as a source of strains for the preparation of potential starter cultures in the process of industrial cheese production. As the Serbian food market is adjusting to European Union regulations, the standardization of Zlatar cheese production by using starter culture(s) based on autochtonous well-characterized LAB will enable the industrial production of this popular cheese in the future.

Introduction

Large quantities of different cheeses have been produced traditionally for centuries in Serbia from different milk (cow, sheep and goat) as well as from their mixtures. The Zlatar cheese belongs to a group of white brine semi-hard cheeses, which is produced on the highlands of the mountain Zlatar. It is an artisanal cheese manufactured in country households from raw cow's milk without the addition of any known starter culture. Although the consumption of this type of cheese is relatively large, its production has not being standardized. This is because Zlatar cheese can only be found on the open market to which it is brought directly by the producers themselves.

The complexity and varied sensory properties of traditional cheeses manufactured from raw milk depend on their microbial community (Cogan *et al.* 1997). A better understanding of the role that microbial flora plays in the development of certain sensorial qualities of cheeses, requires the description of the microbial ecosystem at the level of strains and species and determination of their biochemical properties (Callon *et al.* 2004). Nonstarter lactic acid bacteria (NSLAB) constitute complex microbial associations that are characterized by the occurrence of

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various species and many biotypes as a result of a number of selective conditions persisting during the manufacturing process and different ecological niches. In cheeses made of raw milk, NSLAB support the cheese-making process in the later phases, being often found as a secondary flora during the ripening (McSweeney *et al.* 1993). The NSLAB composing natural milk microbiota belong to the mesophilic and thermophilic bacterial groups (Beresford *et al.* 2001), and play a significant role in the development of certain properties of cheese made from raw milk. However, most dairy industries employ starter cultures containing strains selected and routinely subcultured in milk for a faster acidification. As a consequence of this technology, the number of strains present in cheese is reduced and a certain uniformity of the products could be observed (Wouters *et al.* 2002).

Conventional cultivation methods, prior to characterization by physiological and biochemical tests, and different molecular techniques can give a significant insight into specific isolates and microbial populations during cheese manufacturing (Randazzo *et al.* 2006).

The Zlatar Mountain represents a specific ecological region in the Western Balkans with a high plant biodiversity. Such specific diversity must have an impact on the formation of specific microflora found in milk. We have selected an isolated household in which the traditional cheese production has a long history. Lactic acid bacteria (LAB) were isolated from cows' milk and cheese made from the same milk during the ripening time. The purpose of this work was to study changes in the population of LAB in an artisanal Zlatar cheese during the ripening process. Identification of isolated LAB to the strain level and their biochemical analysis would be the first step in the selection of proper bacterial strains that could be used for the preparation of starter cultures for the industrial standardized production of Zlatar cheese, while having organoleptic qualities as good as the artisanal cheese.

Materials and methods

Cheese manufacturing and sampling

LAB were isolated from white brine semi-hard Zlatar cheeses at different periods of ripening. The manufacturing of this cheese represents the traditional way in most households in Serbia. Briefly, this cheese is made by adding the rennet to raw nonpasteurized milk immediately after milking in which the formation of a curd (coagulation) takes 1–2 h. Afterwards the curd is cut into smaller pieces and salted. The salting is done by spreading crystal salt (usually 50 g) over each layer of cheese pieces in the barrel. The thickness of slices is between 0·5 and 1·0 cm and no holes inside the cheese body are present. For the longer ripening period, cheese slices are transferred into a small wooden barrel everyday and this process continues until the well-characterized barrel is full. The cheese is then covered over with the brine made of whey created by the self-pressing of the cheese during the manufacturing process. After that, the wooden barrel is covered with a wooden lid which is pressed by a stone weighing about 2 kg and left on the cheese during the ripening process. The ripening time is between 1 and 60 days at 10–15°C in the special chambers.

A sample of raw cows' milk (500 ml) used for cheese production as well as one sample (500 g) of cheese 1, 10, 20, 30, 45 and 60 days old were collected from October to December 2003. These samples were taken from farmhouses and placed in sterile plastic containers which were transported to the laboratory under refrigeration. Microbiological analyses of these samples were performed within the following 24–48 h.

Microbiological analyses – isolation, characterization and identification of LAB

For microbiological analysis, 20 g of each sample was taken from the cheese interior and homogenized with pastille in sterile mortar and transferred to 180-ml sterile 2% (w/v) sodium citrate solution in a sterile conical flask. Decimal dilutions of homogenates were prepared with sterile 0·85% (w/v) sodium chloride and were plated on media most suitable for isolation of LAB: (i) for presumptive lactobacilli on de Mann Rogosa Sharpe (MRS) agar pH 5·7 (Merck GmbH, Darmstadt, Germany) at 30° and 45°C for 72 h in aerobic conditions and in anaerobic jars with Anaerocult A (Merck GmbH) for 5 days; (ii) for presumptive lactococci on M17 agar pH 7·2 (Merck GmbH) at 30°C for 72 h (Mannu *et al.* 2002). Total LAB counts were determined and the mean of the triplicate plates was noted. Results were expressed as colony forming units (CFU) per millilitre of milk and per gram of cheese.

Thirty to fifty colonies per sample were randomly taken from both MRS (30° and 45°C) and M17 (30°C) agar plates corresponding to the highest dilution at which growth occurred. Cell morphology of all strains of LAB were determined by microscopy (Olympus U-RFL-T, BX51, GmbH, Hamburg, Germany). After microscopic observations, the colonies were subcultured to purity on MRS or M17 medium for rods and cocci, respectively. Gram-positive and catalase-negative isolates were stored in milk at 4°C and also frozen at −80°C in M17 (for cocci) and in MRS (for rods) broth containing 15% of glycerol (Mannu *et al.* 2002).

Gram-positive and catalase-negative isolates were identified to the genus level, by phenotypic tests as follows: morphology, growth at 30° and 45°C, growth in MRS

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and M17 broth with 4% and 6·5% (w/v) sodium chloride, carbon dioxide production from glucose by subculturing the isolates in tubes with MRS broth with Durham's bells, L-arginine and esculin hydrolysis, citrate-utilizing bacteria, activity in milk and test in litmus milk (Hardie 1986; Kandler and Weiss 1986; Mundt 1986a,b; Sneath *et al.* 1986; Prescot *et al.* 1996). The production of diacetyl was performed only qualitatively. Namely, in 1 ml of coagulated milk (duration of coagulation was 16 h) 0·1 g of creatinine and 1 ml of 30% NaOH (w/v) was added. The test was considered to be positive if a red halo around the previously prepared mixture could be detected in 2 h.

The bacterial strains used in this study are listed in Table 1.

DNA and polymerase chain reaction analysis

The total DNA from LAB isolates was purified by the method given by Hopwood *et al.* (1985).

For repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) analysis total DNA from different isolates of LAB was used as a template for PCR amplifications with BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′) and $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) oligonucleotide primers, each with its optimal PCR program (Versalovic *et al.* 1994), using Taq DNA polymerase (Fermentas UAB, Vilnius, Lithuania). Reactions were carried out in a thermal cycler Gene Amp^R PCR System 2700 (Applied Biosystems, Foster City, CA, USA). The PCR products were separated by electrophoresis on 1·5% agarose gel (15 × 20 cm) containing 0·5 μg ml^{-1} ethidium bromide, for 16 h in 1 × TAE buffer and 55 V (constant voltage) at 4°C (Versalovic *et al.* 1994). Electrophoresis was performed using an Electrophoresis Power Supply EPS 301 (Amersham Biosciences, Piscataway, NJ, USA). The rep-PCR profiles were visualized under ultraviolet light, followed by digital image capturing using a CCD camera Biometra BDR2/5/6 (Bio Doc Analyze GmbH, Göttingen, Germany).

For the detection of the gene responsible for nisin production, PCR was performed by using primers designed to amplify a sequence encompassing *nisA* gene and 5′ upstream region of *nisA*, primers Nis1 (5′-CCTCGACGATACCATCAC-3′) and Nis2 (5′-CTCCGTTTATCGTTTGGAG-3′) and primers designed for the amplification of *nisR* gene and 5′sequence of *nisK* gene, Nis3 (5′-CAGTGCCATGGGTAAAAAATATTCAATGCG-3′) and Nis4 (5′-CTTAGAGAA TTCTCTAATGAG-3′). The *nisR* and *nisK* genes encode putative transporter proteins potentially implied in immunity to nisin. PCR amplifications were performed starting with the initial denaturation of DNA at 94°C for 1 min, followed by 30 successive cycles of melting DNA at 94°C for 30 s, annealing at 45°C for 30 s, and elongation at 72°C for 30 s, and the last elongation at 72°C for 5 min.

For the sequencing of the 16S rRNA region total DNA was used as a template for PCR amplifications with U968-GC (5′-CGCCGGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTAC-3′) and L1401 (5′-GCGTGTGTACA AGACCC-3′) primers (Zoetendal *et al.* 1998; Randazzo *et al.* 2002), using Taq DNA polymerase (Fermentas UAB). Reactions were carried out in a thermal cycler as cited before. The obtained PCR product was purified by QIAquick PCR Purification KIT/250 (Qiagen GmbH, Hilden, Germany), and sequenced by CRIBI-BMR servizio sequenziamento DNA, Univesita di Padova, Italy. The sequence was aligned in

Table 1 Reference strains used in this study

Strain	Source
Lactobacillus plantarum A112*	From laboratory collection
Lactobacillus paracasei subsp. *paracasei* BGBUK2-16/K4*	From laboratory collection
Lactococcus lactis subsp. *lactis* BGMN1-5/MN1-596*	From laboratory collection
Lactococcus lactis subsp. *cremoris* NS1*	From laboratory collection
Lactococcus lactis subsp. *lactis* biovar. *diacetylactis* S50*	From laboratory collection
L. lactis subsp. *lactis* NP45†‡	From laboratory collection
L. lactis subsp. *cremoris* MG1363‡	From laboratory collection
Lact. paracasei subsp. *paracasei* BGBUK2-16†	From laboratory collection§
Lact. plantarum BGHN14†	From laboratory collection§
Lactobacillus brevis BGHI3a†	From laboratory collection§
Enterococcus faecalis BGZLS60-26a†	From laboratory collection§
Enterococcus faecium BGGJ8-3†	From laboratory collection§

*For bacteriocin detection.
†For rep-polymerase chain reaction (PCR).
‡For PCR for nisin-encoding gene.
§Identified in Laboratorium voor Microbiologie, Universitet Gent, Gent, Belgium.

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the NCBI database using the standard nucleotide–nucleotide homology search BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Assays of plasmid profiles and proteolytic activity

Plasmid profiles were determined according to the method of O'Sullivan and Klaenhammer (1993).

Proteolytic activities of the isolates were assayed as previously described (Kojic *et al.* 1991). Collected fresh cells (10 mg approximate density 10^{10} cells ml^{-1}) were resuspended in 0·1 mol l^{-1} of sodium-phosphate buffer, pH 6·5. The cell suspension was mixed with β-casein (5 mg ml^{-1} in 0·1 mol l^{-1} sodium-phosphate buffer, pH 6·5) (Sigma, St Louis, MO, USA) and incubated for 3 h at 30°C. Electrophoresis was carried out on 12·5% polyacrylamide gel. Gels were stained with Coomassie Brilliant Blue G250 (Serva, Heidelberg, Germany) and destained in a mix of methanol (20%) and acetic acid (7%).

Detection of antimicrobial activity

Preliminary production of antimicrobial compounds isolated from LAB was screened by the deferred antagonism method (Harris *et al.* 1989) using various indicator strains. For the detection of antimicrobial activity, agar-well diffusion assay was used (Tagg and McGiven 1971) and indicator strains are represented in Table 1. To confirm the production of antimicrobial compounds, a crystal of proteolytic enzyme pronase E (Sigma Chemie GmbH, Deisenhofen, Germany) was placed close to the edge of the antimicrobial compound-containing well. The plates were incubated overnight at 30°C. A clear zone of inhibition around the well, but not in the vicinity of the pronase E crystal, was taken as an indication of possible antimicrobial compounds production.

Results

Chemical analysis

All cheese samples were subjected to chemical analysis comprising dry matter, pH, total fat, protein and sodium chloride content as described previously (Terzic-Vidojevic *et al.* 2007).

Total count of LAB

Total viable counts of LAB on MRS and M17 medium in aerobic conditions slightly increases from milk to cheese 20 days old. The initial count of LAB in ZLS20 cheese was higher on M17 medium than on MRS medium. After that period the total counts of LAB slightly decreased up to 60 days of cheese ripening, but the ratio of LAB counts on M17 medium and MRS medium retained. Total viable counts of LAB on MRS medium in anaerobic conditions were between $6{\cdot}8 \times 10^4$ CFU ml^{-1} in milk, $3{\cdot}6 \times 10^6$ CFU g^{-1} in cheese 20 days old and $1{\cdot}8 \times 10^5$ CFU g^{-1} in cheese 60 days old (Table 2).

Physiological characteristics of LAB isolated from Zlatar cheese

From all milk and cheese samples used in the study, 306 randomly chosen isolates of LAB were taken for the analysis. Results showed that 253 isolates were gram positive and catalase negative and they were taken for further study.

Results revealed that the percentage of cocci and rods in samples of milk and cheeses of different ripening periods was changing. Raw milk and 1-day-old cheese had a significantly greater number of lactic acid cocci. However, during the ripening period the number of lactic acid cocci had been gradually decreasing while the number of lactic acid rods bacteria was increasing. This trend continued until 30 days of cheese ripening when the number of cocci increased again, resulting in equal percentage of cocci and rods in 60-day-old cheese (Fig. 1).

The growing ability and some physiological features of these isolates were analysed (data not shown). Preliminary characterization of isolates of LAB has been done according to their growth at 45°C, growth in bouillon with 6·5% sodium chloride, their ability of arginin and esculin hydrolysis, citrate utilization, production of carbon dioxide and diacetyl.

In Zlatar cheese a number of thermophilic lactic acid cocci which grew at 45°C and in media with 6·5%

Table 2 Mean number of total viable lactic acid bacteria in milk and cheese samples during different ripening periods on MRS and M17

Sample	CFU g^{-1} sample*			pH	Sodium chloride content (%)
	MRS†	MRS‡	M17		
ZLM 1	$3{\cdot}2 \times 10^5$	$6{\cdot}8 \times 10^4$	7×10^5	6·1	–
ZLS1	$4{\cdot}1 \times 10^6$	$7{\cdot}5 \times 10^4$	$1{\cdot}13 \times 10^7$	5·2	2·1
ZLS10	$6{\cdot}7 \times 10^7$	$2{\cdot}3 \times 10^6$	$2{\cdot}08 \times 10^8$	4·6	2·3
ZLS20	$1{\cdot}2 \times 10^8$	$3{\cdot}6 \times 10^6$	$1{\cdot}59 \times 10^9$	4·3	2·7
ZLS30	$2{\cdot}1 \times 10^7$	$4{\cdot}8 \times 10^6$	$6{\cdot}3 \times 10^8$	4·0	2·6
ZLS45	$1{\cdot}9 \times 10^6$	$7{\cdot}4 \times 10^4$	$1{\cdot}33 \times 10^7$	3·7	2·4
ZLS60	$7{\cdot}1 \times 10^6$	$1{\cdot}8 \times 10^5$	$3{\cdot}6 \times 10^7$	3·5	2·4

*Average values of three repetition.
†Aerobic conditions.
‡Anaerobic conditions.
§Data reported in Terzic-Vidojevic *et al.* (2007); ZLM1, milk; ZLS1, 1-day-old cheese; ZLS10, 10-day-old cheese; ZLS20, 20-day-old cheese; ZLS30, 30-day-old cheese; ZLS45, 45-day-old cheese; ZLS60, 60-day-old cheese; MRS, de Mann Rogosa Sharpe agar.

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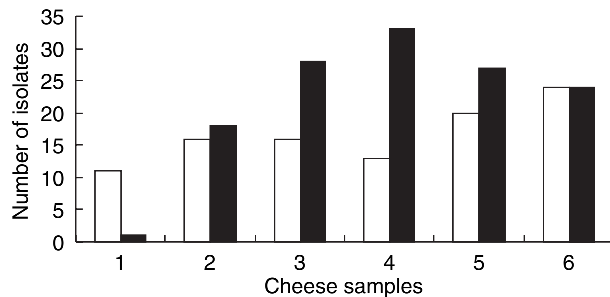

Figure 1 Change in cocci (□) and rod (■) number in cheese samples during different ripening periods: 1, 1 day; 2, 10 days; 3, 20 days; 4, 30 days; 5, 45 days; 6, 60 days.

sodium chloride increased during the ripening period and these cocci also hydrolysed esculin, a characteristic typical for enterococci species. In addition, it was found that 92 out of 115 isolated cocci hydrolyse arginin followed by the production of ammonia, while 68 isolates of cocci used citrate. Determination tests for growth conditions and physiological characteristics have shown that in cheeses older than 30 days enterococci are the most abundant species.

A large number of mesophilic lactobacilli from Zlatar cheese produced diacetyl (66%) but the percentage of diacetyl-producing isolates was higher in older cheeses (45 and 60 days of ripening). The number of cocci which produce diacetyl (21%) is smaller in comparison with rods, but the number of diacetyl-producing cocci also increased during the ripening period.

Test in litmus milk showed that over 90% of isolates produced acid and changed the colour of the indicator from purple to red, created curd and reduced litmus. In addition, the change of the colour of the indicator from purple to white occurred by hydrogen ions. This test also showed that most of the isolates coagulated skimmed milk within 24 h. From this group, eight isolates exhibited good acidification activity in milk. After 5–7 h of incubation the pH reached a value of 4·8 (data not shown).

Identification of LAB by the rep-PCR method

According to morphological and physiological tests, and plasmid profiles, 139 rods and cocci were chosen for molecular identification with rep-PCR (Figs 2a–c and 3a–c). The results revealed that 58 of 75 chosen lactobacilli were identified as *Lactobacillus paracasei* subsp. *paracasei*, 10 as *Lactobacillus brevis* and 7 as *Lactobacillus plantarum*. Based on the same method 64 cocci were identified as follows: 13 belonged to the species *Lactococcus lactis* subsp. *lactis,* 6 were identified as *Enterococcus faecium and* 44 as *Enterococcus faecalis*. Only one isolate designated as BGZLS60-27, was not identified by the rep-PCR method. Five lactobacilli and two enterococci isolates did not exhibit a band pattern of any reference strain from our collection, so these isolates were identified according to 16S rDNA sequencing (Table 3).

Proteolytic activity and plasmid profiles

According to phenotypic characterization and rep-PCR results, 43 LAB isolates were chosen for further analysis of their plasmid profiles and proteolytic activity. Examination of their proteolytic activity revealed that 9 of the isolates identified as *Lact. paracasei* subsp. *paracasei*, exhibited strong proteolytic activity, while 7 strains were not able to degrade β-casein at all (data not shown). Representatives of isolated strains, identified as *Lact. plantarum*, *Lact. brevis*, *L. lactis* subsp. *lactis*, *Ent. faecalis* and *Ent. faecium*, degraded β-casein poorly, or not at all. All the isolates from genus *Lactococcus* and *Enterococcus* contained plasmids and the number of plasmid bands in lactococci isolates varied from 2 to 7, while in enterococci isolates was from 1 to 7. The number of plasmid bands of *Lactobacillus* spp. was from 1 to 12 (data not shown).

Antimicrobial activity of LAB

It was confirmed that 87 out of 253 analysed isolates produced antimicrobial compounds. Experiments with pronase E revealed a proteinous nature of antimicrobial compounds, indicating the possibility that they could be a bacteriocin-like substance. Isolates exhibited a clear, turbid zone or both zones at 1, 2, 3 or all 4 indicator strains used in the test (Table 4). The majority of antimicrobial compound producers were isolated from milk and 1-day-old cheese. It was determined that the isolates which produced antimicrobial compounds belonged to all of the three genera detected in the cheese.

Strain BGZLM1-24, identified as *L. lactis* subsp. *lactis*, gave zones of inhibition on all four indicator strains. Cross-immunity bacteriocin test with *L. lactis* subsp. *lactis* NP45, a nisin producer, showed that the strain BGZLM1-24 was resistant to nisin. The results indicated a possibility for the presence of the gene responsible for nisin production in this strain, which was confirmed by using PCR (Fig. 4).

Discussion

A better understanding of the role which natural LAB have in the production of traditional cheeses requires a detailed study of the microbial community involved in

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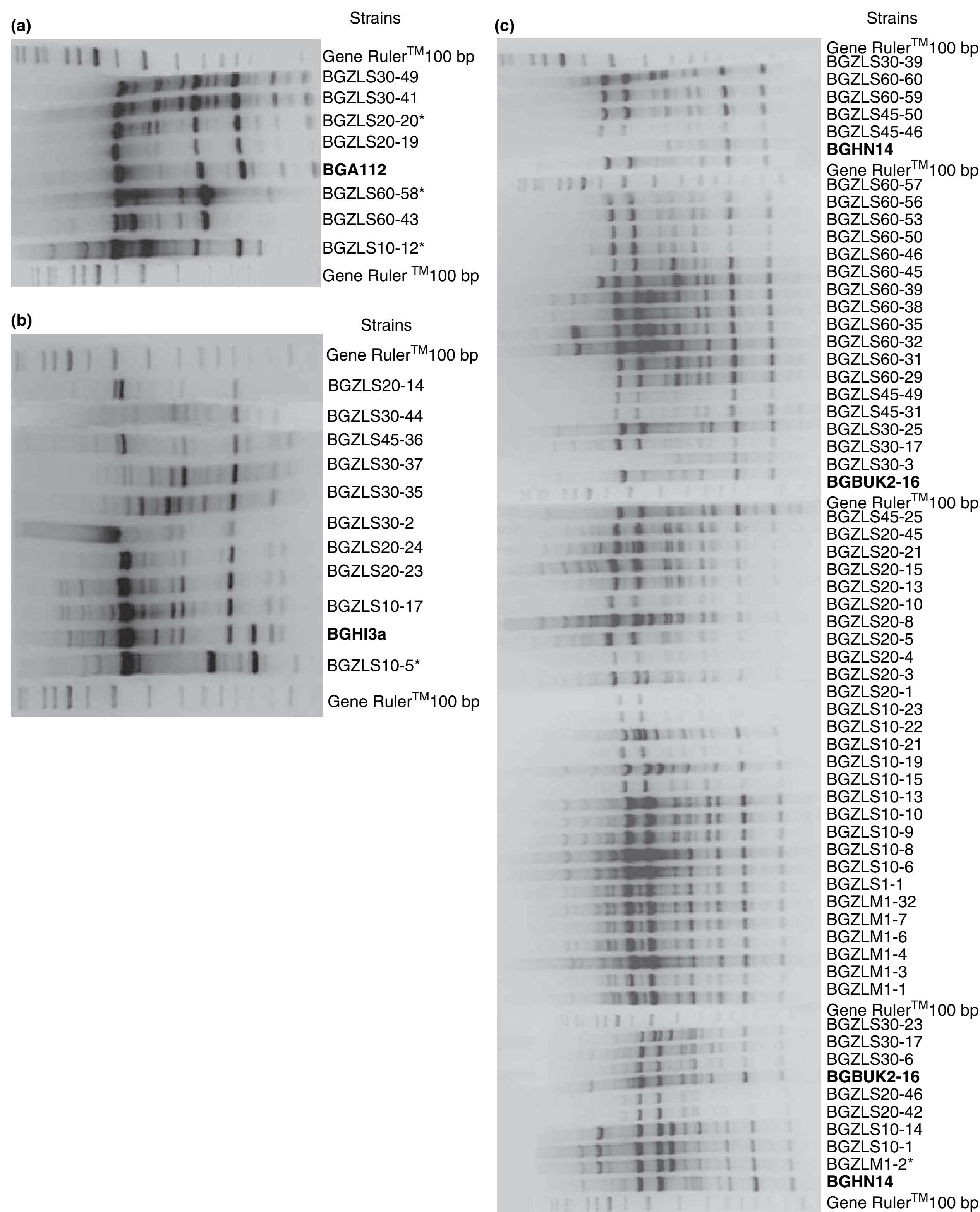

Figure 2 The BOXA1R rep-polymerase chain reaction (PCR) analysis of *Lactobacillus plantarum* group isolates (a), of *Lactobacillus brevis* group isolates (b) and of *Lactobacillus paracasei* subsp. *paracasei* group isolates (c). Reference strains used in the test are given in bold letters. *Isolates also identified by 16S rDNA sequencing.

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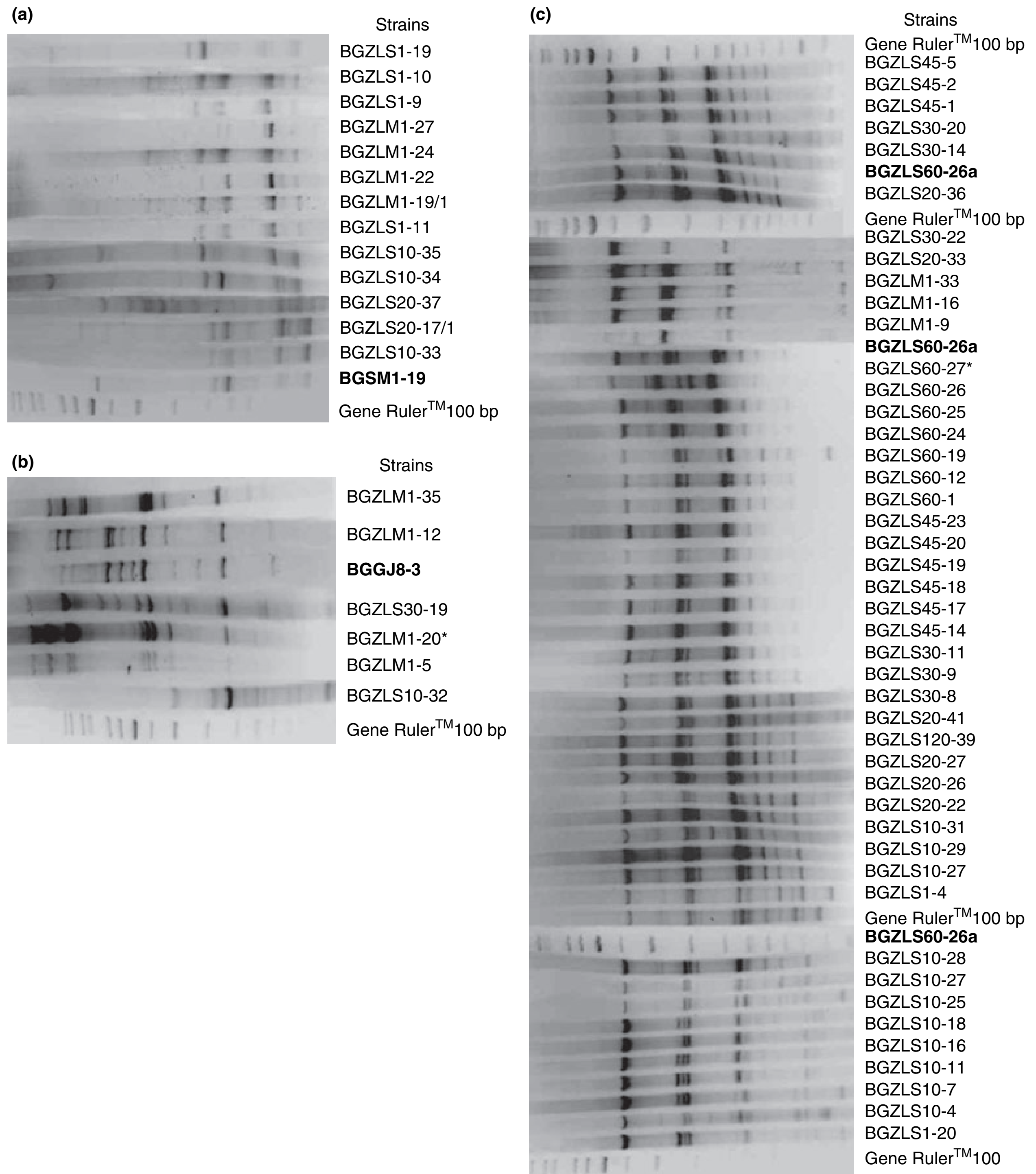

Figure 3 The $(GTG)_5$ rep-polymerase chain reaction (PCR) analysis of *Lactococcus lactis* subsp. *lactis* group isolates (a), of *Enterococcus faecium* group isolates (b) and of *Enterococcus faecalis* group isolates (c). Reference strains used in the test are given in bold letters. *Isolates also identified by 16S rDNA sequencing.

their production and ripening. Cheese ripening is a complex process involving a range of biochemical reactions, which can influence the presence of different species of LAB in cheese. The composition of LAB in cheese can fluctuate significantly depending on the period of the ripening process. Recent studies have shown that artisanal

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Table 3 Isolates of lactic acid bacteria identified by 16S rDNA sequencing

Isolate	Identification by 16S rDNA sequencing
BGZLS10-12	*Lactobacillus plantarum*
BGZLS20-20	*Lact. plantarum*
BGZLS60-58	*Lact. plantarum*
BGZLM1-2	*Lactobacillus paracasei* subsp. *paracasei*
BGZLS10-5	*Lactobacillus brevis*
BGZLM1-20	*Enterococcus faecium*
BGZLS60-27	*Enterococcus avium*

cheeses have a distinct and typical microbial population dynamics related to the local production technology and geographic area of origin (Gobbetti *et al.* 2002; Mannu *et al.* 2002).

The total count of viable LAB was the highest in 20-day-old cheese, although a peak of sodium chloride concetration (2·7%) was reached in this cheese. It is possible that the pH value of this cheese (pH 4·3) was not too low and did not influence dramatically a bacterial growth. On the other hand, in samples of cheeses 45- and 60-day-old, containing 2·4% sodium chloride, a total viable count

Table 4 Antimicrobial activity among isolates tested in agar diffusion assay

		Indicator strains			
Genus	Isolates	*Lactococcus lactis* subsp. *lactis* BGMN1-596	*Lactococcus lactis* subsp. *cremoris* NS1	*Lactobacillus plantarum* A112	*Lactobacillus paracasei* subsp. *paracasei* BGBUK2-16/K4
Lactobacillus spp.	18 isolates				t
	7 isolates				c
	5 isolates	c			
	5 isolates			c	c
	2 isolates	c			
	2 isolates		c	t	
	1 isolate	c + t			
	1 isolate	t	t		c
	1 isolate	c			
	1 isolate		c		
	1 isolate			c	
Lactococcus spp.	5 isolates	c	c	t	t
	4 isolates	c	c + t	t	t
	2 isolates				t
	1 isolate	c	c	t	c
	1 isolate	c	c	t	
	1 isolate	c	c + t	t	c
	1 isolate	c	c + t	t	
Enterococcus spp.	4 isolates	c	c + t	t	
	3 isolates	c + t	c + t	c + t	t
	2 isolates	c	c + t	t	c
	2 isolates	c	c		c
	2 isolates				t
	2 isolates	c	c		
	2 isolates	c + t	c + t		
	2 isolates		t		
	1 isolate	c			
	1 isolate				c
	1 isolate	c			c
	1 isolate	c	t		t
	1 isolate	t	c		
	1 isolate	c	c		c
	1 isolate	c	c	t	c
	1 isolate	c	c + t		
	1 isolate	c	c + t	t	t

c, clear zone of inhibition; t, turbid zone of inhibition.

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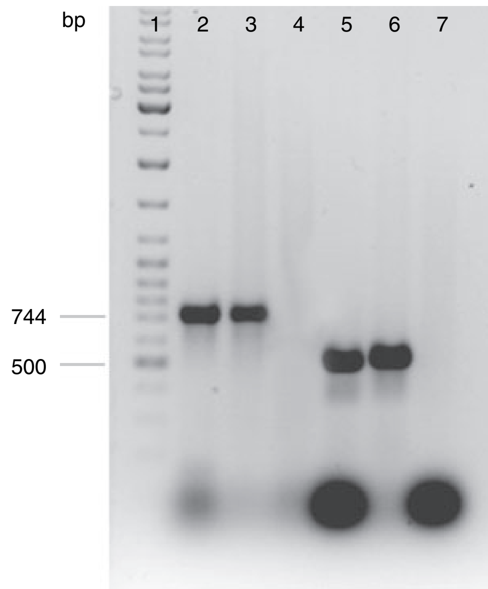

Figure 4 Detection of the *nis* gene in the strain *Lactococcus lactis* subsp. *lactis* BGZLM1-24. Amplification products were obtained with Nis1 and Nis2 primers (lanes 2–4) and Nis3 and Nis4 primers (lanes 5–7). Lane 1, Gene Ruler^{TM} DNA Ladder Mix (Fermentas); lanes 2 and 5, *L. lactis* subsp. *lactis* NP45 (nisin producer – positive control); lanes 3 and 6 isolate BGZLM1-24; lanes 4 and 7, *Lactococcus lactis* subsp. *cremoris* MG1363 (nisin nonproducer – negative control).

of LAB slightly decreased. The lower pH values of these cheeses (pH 3·7 and 3·5, respectively) compared with 20-day-old cheese, suggests that pH has a greater influence on bacterial growth than salt concentration. Similar data were reported previously (Aboola and Radovanovic-Tesic 2002).

During the ripening time of cheese the proportion between lactic acid cocci and rods was constantly changing. Lactococci constitute the dominant bacterial population during the first ripening phase of the artisanal Zlatar cheese, which is in accordance with previously published results by Ayad (2001). During subsequent ripening phases (20 and 30 days) the number of lactic acid rods bacteria increases, most probably because of their ability to grow under low pH conditions (Caridi *et al.* 2003). Physiological tests showed that in Zlatar cheese, mesophilic lactobacilli were largely present as a part of NSLAB. Mesophilic lactobacilli play a very important role in the process of the maturation of cheeses as they have the ability to ferment citrate. Mesophilic lactobacilli could be involved in proteolysis as well as in different enzymatic processes that occur during cheese ripening (Palles *et al.* 1998). The presence of mesophilic lactobacilli, particularly *Lact. paracasei* subsp. *paracasei*, in 30-day-old cheese samples, could be the reason why consumers prefer the taste and aroma of older cheeses (Albenzio *et al.* 2001). Physiological tests also confirmed that a large number of enterococci were present in Zlatar cheese, particularly in 45- and 60 day-old cheeses, reflecting the resistance of enterococci to a variety of adverse conditions such as low pH, high salt concentrations and low temperature.

The taste of cheese older than 30 days was not acceptable for consumption as it was too bitter and lacked its specific organoleptic characteristics. According to the findings from this paper, future research should be focused on the analysis of cheeses up to 30 days old, as the older cheese becomes saturated with enterococci.

Plasmid profiles of LAB may be used for the determination of their genetical diversity and identification of bacteria to the strain level (Prescot *et al.* 1996; Leisner *et al.* 2001; Gevers *et al.* 2003). In contrast, Mannu *et al.* (2000) did not find any correlation between plasmid profiles and phenotypic characteristics of bacteria. Results from the plasmid profiles analysis of bacteria isolated from Zlatar cheese were inconclusive but could serve as a criterion for species grouping.

LAB identification according to the results obtained with rep-PCR showed that *Lact. paracasei* subsp. *paracasei*, *Lact. plantarum*, *Lact. brevis*, *L. lactis subsp. lactis*, *Ent. faecium* and *Ent. faecalis* represent the dominant LAB in Zlatar cheese. The observed intraspecies differences of band patterns are most probably the result of genetic variability within the species.

Because of increased public interest in food safety, including demands for less artificial additives, research attention has been focused on the use of naturally occurring metabolites produced by food-grade bacteria. Bacteriocin produced by LAB may be a very promising source of biological food preservatives (Piard and Desmazeaud 1992). A large number of LAB isolated from the Zlatar cheese produces antimicrobial compounds. The species *Lact. paracasei* subsp. *paracasei*, *L. lactis* subsp. *lactis* and *Ent. faecalis* showed antimicrobial activity and often created inhibition zones on 2, 3 or 4 different indicator strains. For further elucidation of the nature and activity of these compounds, more detailed analysis is required. Many authors have studied and classified bacteriocins produced from LAB in detail (Messi *et al.* 2001; Atanassova *et al.* 2003; Lozo *et al.* 2004).

Presently only nisin and pediocin PA1/AcH are commercially used as food preservers and approved for the use in the alimentary industry in over 50 countries (Henning *et al.* 1986; Delves-Broughton 1990). Nisin is produced by the species *L. lactis* subsp. *lactis*, and performs a large action range against gram-positive bacteria like *Listeria monocytogenes*, *Staphylococcus aureus* and bacteria from genus *Bacillus* and *Clostridium*. The

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results of the research revealed that the isolate BGZLM1-24, identified as *L. lactis* subsp. *lactis* produced a nisin-like antimicrobial substance.

The experiments performed in our study demonstrated that Zlatar cheese up to 30 days old may be used as a source of potential strains that could constitute a starter culture for the industrial cheese production. From 253 LAB strains isolated and tested in this study, 10 isolates have been chosen for further detailed analysis and a trial production of Zlatar cheese with the defined starter culture. We have taken into consideration some important bacterial properties for cheese production such as the ability of lactic acid production in a short period of time, good proteolytic activity, production of antimicrobial compounds and production of diacetyl. As our food market is adjusting to European Union standards and consumers also demand the increment both in food production as well as in quality, standardization of Zlatar cheese production will enable the future industrial production of this very popular cheese.

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