Arch. Biol. Sci., Belgrade, 63 (1), 1-10, 2011 DOI:10.2298/ABS1101001J

CHARACTERIZATION OF NON-STARTER LACTIC ACID BACTERIA IN TRADITIONALLY PRODUCED HOME-MADE RADAN CHEESE DURING RIPENING

NATASA JOKOVIC[1], MAJA VUKASINOVIC[2], KATARINA VELJOVIC[3], MAJA TOLINACKI[3] and L. TOPISIROVIC[3]

[1] *Faculty of Technology*, 16000 Leskovac, Serbia
[2] *Faculty of Technology and Metallurgy*, 11000 Belgrade, Serbia
[3] *Institute of Molecular Genetics and Genetic Engineering*, 11000 Belgrade, Serbia

Abstract - Two hundred thirteen non-starter lactic acid bacteria isolated from Radan cheese during ripening were identified with both a classical biochemical test and rep-PCR with $(GTG)_5$ primer. For most isolates, which belong to the *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Enterococcus faecium*, a phenotypic identification was in good agreement with rep-PCR identification. *Lactococeus lactis* subsp. *lactis*, *Enterococcus faecium* and subspecies from the *Lenconostoc mesenteroides* group were the dominant population of lactic acid bacteria in cheese until 10 days of ripening and only one *Streptococcus thermophilus* strain was isolated from the 5-day-old cheese sample. As ripening progressed, *Lactobacillus plantarum* became the predominant species together with the group of heterofermentative species of lactobacilli that could not be precisely identified with rep-PCR.

Keywords: Non-starter lactic acid bacteria, homemade cheese, ripening, $(GTG)_5$-PCR, Radan Mt., Serbia

UDC 577.2:63

INTRODUCTION

Like many Mediterranean countries, Serbia has a long tradition in producing a different variety of cheeses. Today the majority of cheeses are made under controlled conditions in dairy factories but traditional cheese manufacturing in farmhouses is retained in the same style as it was centuries ago. Most traditional home-made cheeses are made from raw or pasteurized cow's milk but in some regions of Serbia cheeses are also produced from ewe and goat milk, as well as from mixtures of all three types of milk. Although at most farmhouses milk coagulation is achieved by using commercial rennet, home-made rennet is still used in restricted mountain areas.

For our research, we chose one variety of artisanal white cheese, which is produced on the mountain Radan located in the south-eastern part of Serbia. The farmhouse where the cheese is produced and ripened is located 800 m above sea-level. The cheese is specific because it has been produced from a mixture of cow and ewe milk and the coagulation is achieved with home-made rennet. In addition, commercial starter cultures or natural whey starters are not used and because of this the cheese could be a source of new strains of lactic acid bacteria (LAB) with specific technological properties.

A common characteristic of raw milk cheeses all over the world is its production without starter cultures. In recent years there have been many reports about the microflora of different raw milk cheeses (Poznanski et al., 2004; Ostlie et al., 2005; Ouadghiri et al., 2005; Casalta et al., 2009). These studies have shown that traditional cheeses have

a complex and unique microflora predominated by LAB, known as non-starter lactic acid bacteria (NSLAB). NSLAB have not only been isolated from traditional cheeses which are produced without the addition of starter cultures, but also from mature cheeses manufactured from pasteurized milk where starter cultures are typically used (Beresford et al., 2001). In contrast to LAB from starter cultures, NSLAB withstand the hostile environment in cheese during ripening and represent the predominant microflora in ripened cheese. Furthermore, proteolytic and lipolytic enzymes from different species of NSLAB are better adapted to the harsh conditions present in cheese where they influence the development of the texture and taste of the final product. Most studies report increased levels of proteolysis and enhanced flavor intensity in cheese where NSLAB have been added as adjunct lactobacilli together with the usual starter cultures. The contribution of NSLAB to flavor development can be species- or even strain-specific (Johnson et al., 1995; Lane and Fox, 1996; Lynch et al., 1996; Ferrazza et al., 2004).

The aim of the present study was to describe the dominant culturable species of natural LAB in traditional Radan cheese made from raw milk during the ripening period. Isolates were identified by using classical phenotypic tests as well as $(GTG)_5$ primed rep-PCR fingerprinting. Such information could help in selecting specific starter cultures for the production of cheese with sensory properties similar to the artisanal cheese from mountain Radan in dairy factories.

MATERIALS AND METHODS

Manufacture and sampling of cheese

The cheese examined in the present study was obtained from a farm which produces cheese traditionally from a mixture of raw cow and ewe milk, ratio 60:40. This farm and cheese were selected because of the good sensorial qualities of the cheese. For practical reasons (ewe's milk is available only in the period between December and July), this cheese is produced in a short period of the year from the milk taken at evening and morning milking. A bulk of the milk was coagulated for 3-4 h at 30°C after the addition of homemade rennet. The rennet was prepared by salting and sun-drying the stomach of a calf, after which it was mixed with salted whey and kept in the refrigerator until use. Clotting time was established visually by the cheese maker. The coagulum was cut, placed in a gauze cloth and drained for 2-3 h under pressure (2-3 kg). After drainage, the curd was cut, salted and put in batches with salted whey. The cheese was ripened for four months at about 15°C. Since no starter cultures were used, the fermentation and ripening process was entirely performed by the indigenous microflora present in the raw milk or the cheese manufacturing environment.

The samples of the cow, ewe milk and cheese after 5, 10, 20, 30, 60 and 120 days of ripening were taken for isolation and further characterization of the LAB.

Enumeration and isolation of LAB

The samples of milk and cheeses (10 ml or 10 g) were homogenized in 90 ml of sterile 2% (w/v) sodium citrate solution (pH 7.5) preheated to 45°C. Afterwards serial dilutions were made in 0.85% sterile saline and plated on specific media to enumerate microbial groups. Aerobic mesophilic bacteria were enumerated on nutrition agar (NA, Torlak, Belgrade, Serbia), lactococci on M17 agar supplemented with glucose, referred to below as M1730 (Merck, Darmstadt, Germany), lactobacilli on MRS agar, referred to below as MRS30 (Torlak, Belgrade, Serbia), leuconostocs on MSE agar (Mayeux et al., 1962) and yeasts on SMA agar (Torlak, Belgrade, Serbia). All the plates were incubated at 30°C for 5 days. Presumptive streptococci and thermophilic lactobacilli were enumerated on M17 agar supplemented with glucose and MRS agar referred to below as M1745 and MRS45, respectively. Both media were incubated for 3 days at 45°C. Enterococci were enumerated on KF agar (Torlak, Belgrade, Serbia) after incubation at 37°C for 48 h.

After the incubation period, the plates containing between 10 and 300 colonies were selected for enumeration and isolation. The number of colonies grown on each medium was expressed as logcfu g^{-1}.

From M17 agar, MRS agar and MSE agar at least five single colonies were randomly picked and subcultivated in the appropriate broth. The cells were Gram-stained and the catalase activity was determined. Gram-positive and catalase-negative isolates were sub-cultivated to purity at least twice on M17 for cocci and MRS agar for lactobacilli and coccobacilli. The pure cultures (designated BGGJ cultures) were maintained as frozen stocks at –80°C in M17 or MRS broth supplemented with 15% glycerol as a cryoprotective agent. When required, cultures were revitalized by two consecutive transfers in M17 or MRS broth at 30°C for mesophilic LAB or 37°C for thermophilic LAB, respectively.

Characterization of LAB
Phenotypic identification of isolates

Gram-positive, catalase-negative isolates were further characterized by physiological tests. These included the growth at different temperatures (10, 15 and 45°C) in MRS (bacilli and coccobacilli) or M17 (cocci) broth for 5 days and the growth in M17 or MRS broth with 4% and 6.5 % (w/v) NaCl for 5 days, the gas production from glucose (determined in MRS broth lacking beef extract and containing inverted Durham tubes); hydrolysis of arginine, hydrolysis of esculin in esculin broth (Torlak, Belgrade, Serbia); growth in 10% skimmed-milk medium with litmus, and citrate utilization (according to Kempler and Mc Kay (1981)). Bile esculin agar (Himedia, India) was used for the presumptive identification of enterococci.

Further characterization of isolates was performed on the basis of their sugar fermentation profiles, as were determined in the modified MRS broth without glucose and beef extract containing bromocresol purple (0.17 gL^{-1}) as a pH indicator and supplemented with 2% of the following carbohydrates: lactose, sucrose, xylose, arabinose, sorbitol, fructose, galactose, mannose, cellobiose, raffinose, melibiose, mannitol, trehalose, maltose, glucose, rhamnose, ribose, salicin, and sorbose.

$(GTG)_5$ primed rep-PCR fingerprinting

After clustering the isolates with similar biochemical characteristics, a set of 129 isolates was chosen for genotypic identification with rep-PCR fingerprinting using the $(GTG)_5$ oligonucleotide primer (5'-GTGGTGGTGGTGGTG-3'). Total DNA extraction, PCR amplification and electrophoresis were performed as previously described (Gevers et al., 2001). Normalization and numerical comparison of rep-PCR profiles were performed using the BioNumerics v 4.0 software package (Applied Maths, Ghent, Belgium). Strains were grouped by using the Pearson product moment correlation coefficient expressed for convenience as a percentage of similarity and dendrograms were constructed based on the unweighted pair group method using arithmetic averages (UPGMA).

RESULTS

Changing of microflora during ripening

The changes in the viable counts of different microbial populations in the cows and ewe milk and in the cheese samples after 5, 10, 20, 30, 60 and 120 days of ripening are presented in Fig. 1, 2 and 3. The counts of aerobic mesophilic flora, like the presence of yeasts, peaked at the beginning of the ripening (5-day-old and 10-day-old cheeses, respectively) and decreased around 2 log cfu g^{-1} in later phases of ripening (Fig. 1).

During ripening, the viable counts of LAB evolved differently for each of the cultivation media used (Fig. 2 and 3). The highest number of thermophilic LAB was detected after 10 days of ripening and from that point onwards their numbers decreased, reaching values of about 4 log cfu g^{-1} after 120 days (Fig. 3). Almost all strains isolated from the KF, M1745 and MRS45 agar plates were later identified as *Enterococcus* sp. (see below).

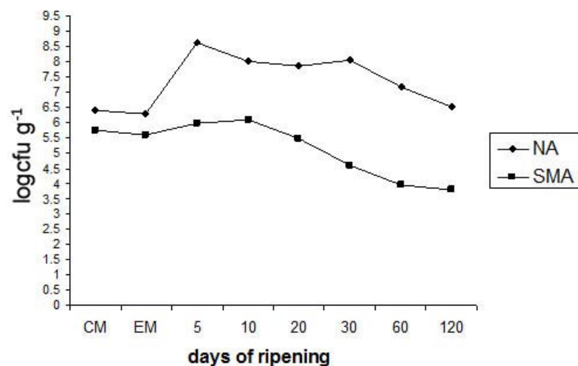

Figure 1. Change of aerobic mesophilic bacteria (NA) and yeast (SMA) in the CM (cow) and EM (ewe milk) and cheese during 5, 10, 20, 30, 60 and 120 days of ripening.

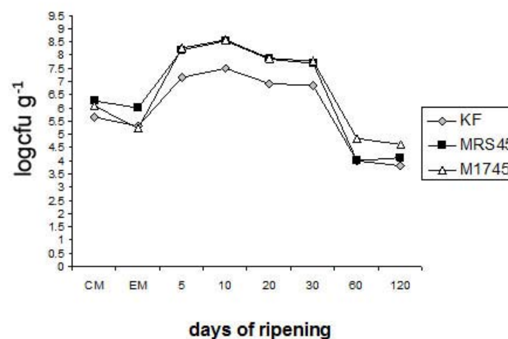

Figure 3. Viable counts of thermophilic lactic acid bacteria in the CM (cow) and EM (ewe milk) and cheese during 5, 10, 20, 30, 60 and 120 days of ripening.

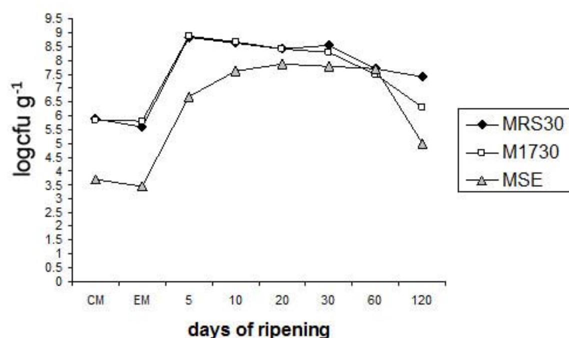

Figure 2. Viable counts of mesophilic lactic acid bacteria in the CM (cow) and EM (ewe milk) and cheese during 5, 10, 20, 30, 60 and 120 days of ripening.

The numbers of bacteria growing on MRS30 and M1730 media reached maximum counts in the 5-day-old cheese, slightly declined in the first month of ripening and subsequently decreased to 7.40 and 6.25 log cfu g^{-1}, respectively, after 120 days of ripening (Fig. 2). In contrast to MRS30 and M1730 media, viable numbers of cells on MSE medium (specific for leuconostoc) were the lowest in 5-day-old cheese and increased to about 7.86 log cfu g^{-1} after 10 days of ripening. Between 10 days and two months of ripening the viable number on MSE media remained stable, after which it rapidly decreased near the end of the ripening process (Fig. 2). In all media used, the viable counts in the cow and ewe milk were about 1-2 log cfu g^{-1} below those of the cheese samples.

Identification of isolates

Two hundred thirteen lactic acid bacteria were isolated from cow and ewe milk and cheese after 5, 10, 20 30, 60 and 120 days of ripening. These isolates were differentiated into preliminary groups based on their morphology studies, physiological tests and sugar fermentation assays. About 129 isolates belonging to the different phenotypic preliminary groups were further identified by rep-PCR methods with $(GTG)_5$ oligonucleotide primer.

Phenotypic identification suggested that the isolates belonged to the genera *Lactococcus*, *Enterococcus*, *Leuconostoc, Lactobacillus* and *Pediococcus.* The results of biochemical and PCR identification are presented in Table 1. Digitally reproduced $(GTG)_5$ fingerprints and a dendrogram reflecting the pattern similarity for identified isolates as well as the well-characterized reference strains available from the BCCM/LMG Bacteria Collection (http://bccm.belspo.be/) are presented in Fig. 4.

Lactoccocal isolates weren't able to grow in broth at 45°C and in the presence of 6.5% NaCl (Table 1). They had similar fermentation profiles and differences were obtained only for the fermentation of

Table 1. Differentiation of LAB isolates based on phenotypic tests and the identification of representative strains from each group by $(GTG)_5$-PCR

Cell morphology and genus[1]	Production of CO_2	Growth on			Growth in broth with NaCl		Milk clotting	Hydrolysis of		Production of dextran	BSA[2]	No isolates	Identified by	
		45ºC	15 ºC	10 ºC	4%	6.5%		arginine	esculin				Sugar fermentation as	$(GTG)_5$-PCR as
Cocci														
Lactococcus sp.	-	-	nd	+	+	-	+	+	+	-	-	19	*Lc. lactis* subsp. lactis (15)[3]	*Lc. lactis* (15) [3]
Enterococcus sp.	-	+	nd	+	+	+	+	+	+	-	+	83	*En. faecium* (32) *En. faecalis* (2)	*En. faecium* (34)
Unclassified	-	+v	nd	+v	-	-	+	-	-	-	-	11	ni (11)	St. thermophilus (1) ni (10)
Cocoid														
Leuconostoc sp. I	+	-	+	nd	+	+v	+v	-	+v	+	-	16	*Ln. mesenteroides subsp. mesenteroides* (2) *Ln. mesenteroides subsp. dextranicum* (11)	*Ln. mesenteroides* (13)
Leuconostoc sp. II	+	-	+	nd	-	-	+	-	-	-	-	1	*Ln. mesenteroides subsp. cremoris* (1)	*Ln. mesenteroides* (1)
Rods														
Lactobacillus sp. I	-	-	+	nd	+	-	+	-	-	-	-	3	*Lb. paracasei* (3)	*Lb. paracasei* (2) *Lb. plantarum* (1)
Lactobacillus sp. II	-	-	+	nd	+	+	+	-	+	-	-	60	*Lb. plantarum* (37) *Lb. paraplantarum*. (3)	*Lb. plantarum*(37) *Lb. paraplantarum (3)*
Lactobacillus sp. III	+	-	+	nd	+	-	-	-	-	-	-	17	ni (10)	ni (10)
Cocci tetrade.														
Pediococcus sp.	-	-	nd	+	+	+	-	-	+/-	-	-	3	ni (2)	ni (2)

+ positive; – negative; +/- weakly positive; +v reaction positive for some isolates; nd – not determined; ni – not identified
[1]-classification based on morphological and physiological characteristics
[2]-Black colonies on BSA (bile esculin agar)
[3]-numbers in parenthesis represent the number of isolates that underwent identification

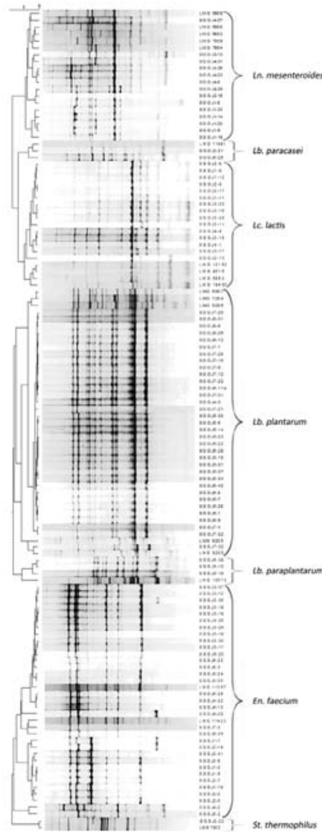

Figure 4. Dendrogram based on statistical analysis of the $(GTG)_5$–PCR fingerprints.

ribose, mannitol, sucrose and arabinose. All isolates had a blue appearance on Kempler and McKey agar for the differentiation of the *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, so they were identified all as this biovariety by phenotypic identification. In the $(GTG)_5$-PCR analysis they were identified as *L. lactis* (Fig. 4).

A great number of Radan cheese isolates (n = 83) was identified as enterococci. They formed black colonies on bile esculin agar and could grow in broth at 10°C and 45°C. A biochemical analysis discovered that some enterococci isolates belonged to *Enterococcus faecium* while a secondary group had biochemical characteristics closer to *Enterococcus faecalis*. However, their $(GTG)_5$-PCR analysis showed that all of them had high similarity in $(GTG)_5$-PCR fingerprint with the reference strains *E. faecium* LMG 11397 and LMG 11423 (Fig. 4). One coccal isolate derived from 5-day-old cheese, which could not be identified with sugar fermentation profiles, was identified as *Streptococcus thermophilus* with rep-PCR.

Leuconostoc isolates were differentiated from the other LAB genera by their coccobacillus cell morphology and the ability to produce CO_2 from glucose. With the exception of one isolate, all isolates produced dextran from sucrose and were identified according to their sugar fermentation pattern as *Leuconostoc mesenteroides*. Two of these isolates fermented arabinose and were therefore identified as *L. mesenteroides* subsp. *mesenteroides:* all remaining isolates were identified as *L. mesenteroides* subsp. *dextranicum* (Table 1). One isolate did not produce dextran and was identified as *L. mesenteroides* subsp. *cremoris*, although it was able to ferment mannose. All *Leuconostoc* isolates were identified as *L. mesenteroides* by $(GTG)_5$-PCR identification (Fig. 4).

Rod-shaped isolates were divided into three different phenotypic groups (Table 1). The fermentation pattern for most *Lactobacillus* isolates from Radan cheese matched the fermentation pattern of *Lactobacillus plantarum.* Three isolates that fermented rhamnose were identified as *Lactobacillus paraplantarum.* The $(GTG)_5$-PCR identification confirmed the phenotypic identification for all of these isolates. Three isolates were identified as *Lactobacillus paracasei* using the phenotypic test. Nevertheless, the rep-PCR profile of one isolate (BGGJ6-1) resembled that of *L. plantarum* (Fig. 4). One group of heterofermentative bacilli could not be identified by either sugar fermentation profiles or by rep-PCR.

Finally, one group of coccus-shaped isolates that formed tetrads and thus probably belonged to the genus *Pediococcus*, was not identified to the species level by using physiological tests or $(GTG)_5$-PCR identification.

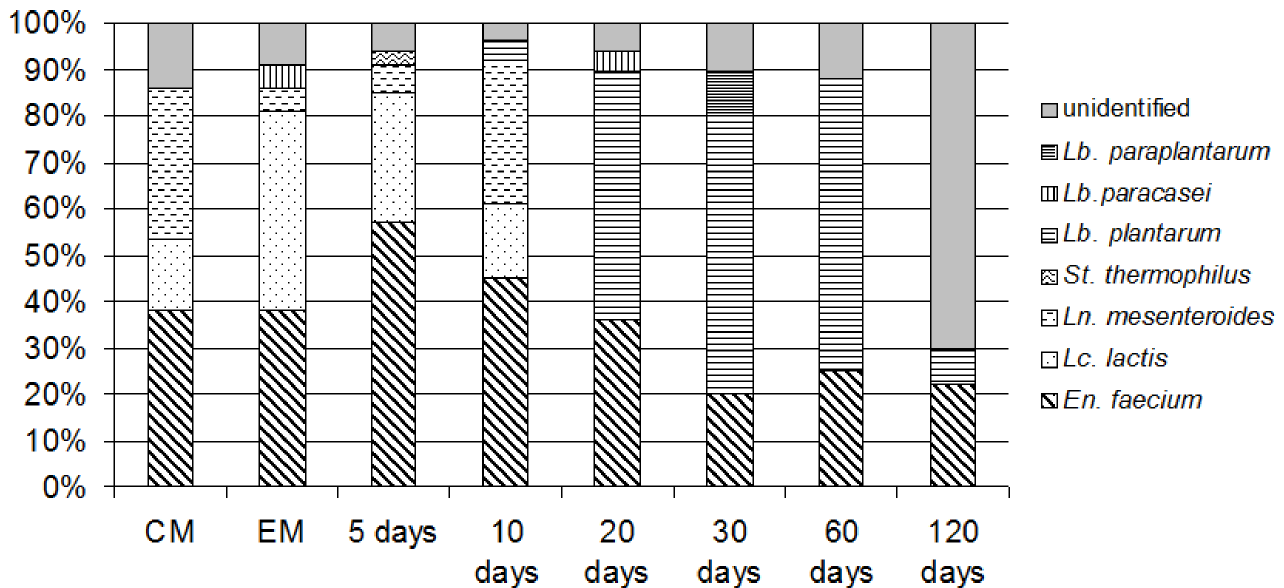

Figure 5. Change of different species of lactic acid bacteria during the cheese ripening period.

Species dynamics during ripening

The composition of LAB in cow and ewe milk and in different cheese samples is presented in Fig. 5. The cow and ewe milk comprised different LAB species. *E. faecium* and *L. mesenteroides* were predominant in cow's milk (38% and 33% of the isolates, respectively) whereas *L. lactis* subsp. *lactis* biovar. *diacetylactis* accounted for 15%. Contrary to this, the predominant species in ewe's milk were *E. faecium* and *L. lactis* subsp. *lactis* biovar. *diacetylactis*, which accounted for 38% and 43%, respectively, while *L. mesenteroides* was present in lower numbers (5%). In both milk types about 10% of the isolates could not be completely identified to the species or subspecies level. All these isolates formed a common cluster closer to *Leuconostoc* (data not shown).

The composition of LAB in Radan cheese changed drastically during ripening and only *E. faecium* was found throughout the whole ripening stage. *E. faecium* (57%), *L. lactis* subsp. *lactis* biovar. *diacetylactis* (28%), *L. mesenteroides* (6%) and *S. thermophilus* (3%) were the dominant LAB at the beginning of the ripening (5-day-old cheese).

During ripening the relative numbers of *E. faecium*, *L. mesenteroides* and *L. lactis* subsp. *lactis* biovar. *diacetylactis* (45%, 31% and 16% respectively after 10 days of ripening) decreased and after 20 days of ripening, lactococci and leuconostocs species were no longer isolated. *L. mesenteroides* and *E. faecium* isolates were dominant in the 10-day-old sample and at this stage of ripening *L. plantarum* (4%) was isolated for the first time. The latter species became the most dominant LAB species (54%) in cheese after 20 days of ripening, and it remained at relatively high percentages up to 60 days of ripening. In addition, a small fraction of *L. paraplantarum* was detected in 30-day-old cheese as only one isolate of *L. paracasei* in 20-day-old cheese.

After 120 days of ripening we detected a considerable number of isolates that could not be identified to the species level. These isolates represented two clusters of heterofermentative lactobacilli and one

group of coccus-shaped isolates which may belong to the genus *Pediococcus.*

DISCUSSION

Dominant LAB species in cheese direct the ripening of cheeses by enzymatic activities and influence the organoleptic quality of the product. The diversity of LAB in cheeses, especially in traditional cheeses, depends on adventitious microflora present in the milk and milk environment as well as on a number of selective conditions persisting during the manufacturing process and ripening (Beresford et al., 2001; Caridi et al., 2003). Therefore, knowledge about the diversity and dynamics of LAB species throughout the ripening of cheese is an important way to control the production of artisanal cheeses.

The evaluation of the viable counts on different selective media throughout the ripening of the Radan cheese showed that all presumptive microbial groups reached their maximum values at the beginning of ripening and decreased in the later stages of ripening. In comparison with some other traditional cheeses produced from raw cow and ewe milk, the changes in counts of aerobic mesophilic microflora and LAB in Radan cheese reveal similar trends (Elortondo et al., 1998; Estepar et al., 1999; Mannu et al., 2000; Fontan et al. 2001; Prodromou et al., 2001; Caridi et al., 2003; Poznanski et al., 2004; Ostlie et al., 2005; Ouadghiri et al., 2005).

In order to explain the role of a predominant group of LAB throughout the ripening of Radan cheese, 213 gram-positive and catalase-negative isolates were isolated from ewe and cow milk and cheese after 5, 10, 20 30, 60 and 120 days of ripening. Firstly, those isolates were identified by phenotypic tests. For a long time, phenotypic, biochemical and physiological tests have been used for the identification of microbial flora in cheeses. However, it is well-known that phenotypic tests are not sufficiently reliable for the identification of natural LAB isolates because of the considerable phenotypic variability between strains of the same species. The introduction of molecular techniques has enabled the accurate identification of phenotypically aberrant strains (Manolopoulou et al., 2003; Ostlie et al., 2004). To confirm the identification obtained by phenotypic analyses, we used $(GTG)_5$ primed rep-PCR (Gevers et al., 2001) to examine a subset of 129 isolates which represented all the different phenotypic groups.

In comparison with some other results published earlier where the phenotypic identification did not correspond to applied molecular identification fingerprinting (Fortina et al., 2003; Ostlie et al., 2005; Ouadghiri et al., 2005), a phenotypic identification for isolates from Radan cheese was in good correlation with the results obtained with rep-PCR. In general, a phenotypic identification was in good agreement with rep-PCR identification for most isolates belonging to *L. lactis* subsp. *lactis* biovar. *diacetylactis*, *L. mesenteroides*, *L. plantarum*, *L. paraplantarum* and *E. faecium*. Phenotypic tests appeared to be especially useful for the identification of *L. lactis* subsp. *lactis* biovar. *diacetylactis* strains and for the differentiation of *Leuconostoc* isolates at subspecies level. On the contrary, rep-PCR did not allow discrimination between *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* biovar. *diacetylactis* or differentiation of the subspecies within *L. mesenteroides* (subspecies *mesenteroides, dextranicum* and *cremoris*).

Phenotypic and rep-PCR identification of isolates enabled the characterization of microflora during the ripening of Radan cheese. At the earlier stages of ripening, the species *L. lactis* subsp. *lactis* biovar. *diacetylactis*, *L. mesenteroides* and *E. faecium* were the dominant groups of LAB found in the cheese samples. They probably originated from the ewe and cow milk where they were found in great amount. The same species have frequently been isolated from other types of cheeses made from raw cow and ewe milk (Menendez et al., 2001; Prodromou et al., 2001; Fortina et al., 2003; Callon et al., 2004; Poznanski et al., 2004). In cheese *L. lactis* subsp. *lactis* is responsible for acid development at the beginning of the ripening and for the proteolysis and conversion of amino acids into flavor compounds, while enterococci have a positive role in the flavor development of artisanal cheese due to their high proteolytic and

lipolytic activities (Casalta and Montel, 2008; Ogier and Serror, 2008). The role of the *Leuconostoc* species in the ripening process is unknown, but they may contribute to the ripening process due to their proteolytic and lipolytic activities, the production of lactic acid and reduction of the oxidation-reduction potential (Manolopoulou et al., 2003).

At later stages of ripening, *L. plantarum* was a dominant group of LAB found in Radan cheese, while *L. paraplantarum* and *L. paracasei* were isolated in low amounts. As a single *L. paracasei* isolate was also obtained from the ewe's milk, this species probably remained present in the ripening cheese in low numbers. *L. plantarum* and *L. paraplantarum* were found only in the cheese samples but not in either kind of milk. Therefore, the origin of these two species in cheese could be the specific environment in which the Radan cheese was manufactured. Alternatively, they could have been present in very low numbers in the milk samples and were omitted during the sampling process. *L. plantarum* and *L. paracasei* are most frequently found to be the dominant NSLAB population in mature cheeses (Elortondo et al., 1998; Mannu et al., 2000; De Angelis et al., 2001; Veljovic et al., 2007). Lactobacillus isolates have a lower metabolic rate than lactococci and leuconostoc, they grow slower in cheese, but they have the ability to grow under the highly selective conditions of the later stages of ripening (Wouters et al., 2002).

At the end of the ripening heterofermentative lactobacilli were detected in high proportion. Although the presence of heterofermentative bacilli in cheese could be considered negative because they can influence its organoleptic characteristics, this group of lactobacilli were also found in other cheeses produced from raw milk (Estepar et al., 1999; Menendez et al., 2001; Coton et al., 2008).

This study described for the first time the dynamics of LAB species during the ripening of Radan cheese, which is manufactured in a traditional way and, therefore, contains the LAB that exist in that environment. The results of this study offer information about the diversity of LAB in this region. Moreover, isolated and characterized LAB could be eventually used for the construction of starter cultures for the production of Radan cheese under the controlled conditions of a dairy factory.

Acknowledgments - M.V. would like to thank the Belgian Office for Scientific, Technical and Cultural Affairs for a fellowship for researchers from Central and Eastern Europe. The research was carried out under financial support of the Ministry of Science and Technological Development of Serbia (Project no. 143036).

REFERENCES

Beresford, T. P., Fitzsimons, N. A., Brennan, N.L. and *T.M. Cogan* (2001). Recent advances in cheese microbiology. *Int. Dairy J.* **11**, 259-274.

Callon, C., Millet, L. and *M. C. Montel* (2004). Diversity of lactic acid bacteria isolated from AOC Salers cheese. *J. Dairy Res.* **71**, 231-244.

Caridi, A., Micari, P., Caparra, P., Cufari, A. and *V. Sarullo* (2003). Ripening and seasonal changes in microbial groups and in physico-chemical properties of the ewe's cheese Pecorino del Poro. *Int. Dairy J.* **13**, 191-200.

Casalta, E. and *M. C. Montel* (2008). Safety assessment of dairy microorganisms: The *Lactococcus* genus. *Int. J. Food. Microbiol.* **126**, 271-273.

Casalta, E., Sorba, J. M., Aigle, M. and *J. C. Ogier* (2009). Diversity and dynamics of the microbial community during the manufacture of Calenzana, an artisanal Corsican cheese. *Int. J. Food Microbiol.* **133**, 243-251.

Coton, M., Berthier, F. and *E. Coton* (2008). Rapid identification of the three major species of dairy obligate heterofermenters *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus parabuchneri* by species-specific duplex PCR. FEMS Microbiol. Lett. **284**, 150-157.

De Angelis, M., Corsetti, A., Tosti, N., Rossi, J., Corbo, M. R. and *M. Gobbetti* (2001). Characterization of Non-Starter Lactic Acid Bacteria from Italian Ewe Cheeses Based on Phenotypic, Genotypic and Cell Wall Protein Analyses. *Appl. Environ. Microbiol.* **67**, 2011-2020.

Elortondo, F. J .P., Echobarria, P. A., Albisu, M. and *Y. Barcina* (1998). Indigenous Lactic Acid Bacteria in Idiazabal Ewes' Milk Cheese. *Int. Dairy J.* **8**, 725-732.

Estepar, J., Del Mar Sanchez, M., Alonso, L. and *B. Mayo* (1999). Biochemical and microbiological characterization of artisanal Penamellera cheese: analysis of its indigenous lactic acid bacteria. *Int. Dairy J.* **9**, 737-746.

Ferrazza, R. E., Fresno, J. M., Ribeiro, J. I., Tornadijo, M. E. and *M. Mansur Furtado* (2004). Changes in the microbial flora of Zamorano cheese (P.D.O.) by accelerated ripening process. *Food Res. Int.* **37**, 149-155.

Fontan, M. C. G., Franco, I., Prieto, B., Tornadijo, M. E. and *J. Carballo* (2001). Microbiological changes in 'San Simón' cheese throughout ripening and its relationship with physico-chemical parameters. *Food Microbiol.* **18**, 25-33.

Fortina, M. G., Ricci, G., Acquati, A., Zeppa, G., Gandini, A. and *P.vL. Manachini* (2003). Genetic characterization of some lactic acid bacteria occurring in an artisanal protected denomination origin (PDO) Italian cheese, the Toma piemontese. *Food Microbiol.* **20**, 397-404.

Gevers, D., Huys, G. and *J. Swings* (2001). Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol. Lett.* **205**, 31–36.

Johnson, J. A. C., Etzel, M. R., Chen, C. M. and *M. E. Johnson* (1995). Accelerated ripening of reduced-fat Cheddar cheese using four attenuated *Lactobacillus helveticus* CNRZ-32 adjuncts. *J. Dairy Sci.* **78**, 769-776.

Kempler, G. M. and *L. L. McKay* (1981). Biochemistry and Genetics of Citrate Utilization in *Streptococcus lactis* ssp. *diacetylactis*. *J. Dairy Sci.* **64**, 1527-1539.

Lane, C. N. and *P. F. Fox* (1996). Contribution of starter and adjunct lactobacilli to proteolysis in Cheddar cheese during ripening. *Int. Dairy J.* **6**, 715-728.

Lynch, C. M., McSweeney, P. L. H., Fox, P. F., Cogan, T. M. and *F. D. Drinan* (1996). Manufacture of Cheddar cheese with and without adjunct lactobacilli under controlled microbiological conditions. *Int. Dairy J.* **6**, 851-867.

Mannu, L., Comunian, R. and *M. F. Scintu* (2000). Mesophilic lactobacilli in Fiore Sardo cheese: PCR-identification and evolution during cheese ripening. *Int. Dairy J.* **10**, 383–389.

Manolopoulou, E., Sarantinopoulos, P., Zoidou, E., Aktypis, A., Moschopoulou, E., Kandarakis, I.G. and *E. M. Anifantakis* (2003). Evolution of microbial populations during traditional Feta cheese manufacture and ripening. *Int. J. Food Microbiol.* **82**, 153-161.

Mayeux, J. V., Sandine, W. E. and *P. R. Elliker* (1962). A selective medium for detecting Leuconostoc organisms in mixed-strain starter cultures. *J. Dairy Sci.* **45**, 655-660.

Menendez, S., Godinez, R., Centeno, J. A. and *J. L. Rodriguez-Otero* (2001). Microbiological, chemical and biochemical characteristics of 'Tetilla' raw cows-milk cheese. *Food Microbiol.* **18**, 151-158.

Ogier, J. C. and *P. Serror* (2008). Safety assessment of dairy microorganisms: the *Enterococcus* genus. *Int. J. Food. Microbiol.* **126**, 291-301.

Ostlie, H. M., Eliassen, L., Florvaag, A. and *S. Skeie* (2004). Phenotypic and PCR-based characterization of the microflora in Norvegia cheese during ripening. *Int. J. Food Microbiol.* **94**, 287-299.

Ostlie, H .M., Eliassen, L., Florvaag, A. and *S. Skeie* (2005). Phenotypic and PCR-based characterization of the microflora in Prast cheese during ripening. *Int. Dairy J.* **15**, 911-920.

Ouadghiri, M., Amar, M., Vancanneyt, M. and *J. Swings* (2005). Biodiversity of lactic acid bacteria in Moroccan soft white cheese (Jben). FEMS Microbiol. Lett. **251**, 267-271.

Poznanski, E., Cavazza, A., Cappa, F. and *P. S. Cocconcelli* (2004). Indigenous raw milk microbiota influences the bacterial development in traditional cheese from an alpine natural park. *Int. J. Food Microbiol.* **92**, 141-151.

Prodromou, K., Thasitou, P., Haritonidou, E., Tzanetakis, N. and *E. Litopoulou-Tzanetaki* (2001). Microbiology of "Orinotyri", a ewe's milk cheese from the Greek mountains. *Food Microbiol.* **18**, 319-328.

Veljovic, K., Terzic-Vidojevic, A., Vukasinovic, M., Strahinic, I., Begovic, J., Lozo, J., Ostojic, M. and *Lj. Topisirovic* (2007). Preliminary characterization of lactic acid bacteria isolated from Zlatar cheese. *J. Appl. Microbiol.* **103**, 2142-2152.

Wouters, J. T. M., Ayad, E. H. E., Hugenholtz, J. and *G. Smit* (2002). Microbes from raw milk for fermented dairy products. *Int. Dairy J.* **12**, 91-109.