UDC 575
https://doi.org/10.2298/GENSR1802465B
Original scientific paper

DIVERSITY OF LACTIC ACID BACTERIA ISOLATED FROM TRADITIONAL MONTENEGRIN DAIRY PRODUCTS

Mirjana BOJANIC RASOVIC[1], Sigrid MAYRHOFER[2]*, Mary A.A. OCHOME[2], Erna AJANOVIC[1], Marija ZUNABOVIC[2], Aleksandra MARTINOVIC[3], Konrad J. DOMIG[2]

[1]University of Montenegro, Biotechnical faculty, Podgorica, Montenegro
[2]BOKU - University of Natural Resources and Life Sciences, Vienna, Department of Food Science and Technology, Institute of Food Science , Vienna, Austria
[3]University of Donja Gorica, Faculty of Food Technology, Food Safety and Ecology, Donja Gorica, Podgorica, Montengro

Bojanic Rasovic M., S. Mayrhofer, M. A.A. Ochome, E. Ajanovic, M. Zunabovic, A. Martinovic, K. J. Domig (2018): *Diversity of lactic acid bacteria isolated from traditional Montenegrin dairy products*.- Genetika, Vol 50, No.2, 465-482.

Traditional production of fermented dairy products in Montenegro is carried out without adding defined starter cultures. This way of production involves lactic acid bacteria (LAB) that are normally present in the raw milk and production environment. This autochthonous ("wild") fermentation microbiota represents a reservoir of unknown strains. In order to study the LAB diversity, 25 indigenous dairy products in Montenegro have been tested. Isolation was performed on microbial media M17 and MRS agar with or without supplementations under aerobic and anaerobic conditions at temperatures of 30°C, 37 °C and 44°C. Identification of these isolates at species level was done by species-specific PCR and gene regions sequencing of representatives of each RAPD-cluster. RAPD-PCR was used to characterize the isolates at strain level. Nine *Lactobacillus* species, five *Leuconostoc* species, four *Enterococcus* species as well as strains of the species *Lactococcus lactis*, *Pediococcus pentosaceus* and *Streptococcus thermophilus* were detected. It can be concluded that a rich lactic acid bacteria diversity existed in the analyzed Montenegrin dairy products. Further examination of the isolates could lead to the development of autochthonous starter cultures that would contribute to

Corresponding author: Sigrid Mayrhofer, BOKU – University of Natural Resources and Life Sciences, Vienna, Department of Food Science and Technology, Institute of Food Science; Muthgasse 18, A-1190 Vienna, Austria, Tel.: +43 1 47654 75455; fax: +43 1 47654 75459.

a product that is characteristic for the geographical area and to which the local population is accustomed.

Key words: lactic acid bacteria, Montenegrin cheese, diversity

INTRODUCTION

Indigenous foods are an important feature of each culture and thus an important wealth of each country. Especially the tradition of producing dairy products on Balkan countries is very old. In Montenegro the majority of these products is home-made, but also several farms and small dairies exist, whose numbers are still increasing. Indigenous Montenegrin cheeses have a versatile taste, aroma and texture when compared to industrially produce ones, for which technologies are strictly defined and production conditions are exactly controlled. These properties mainly originate from the activity of the autochthonous microbiota present in raw milk (MIRECKI *et al*., 2015). Nevertheless, the growth of this microbiota is completely uncontrolled and unpredictable, resulting in less uniform sensory characteristics and compositions (RADULOVIĆ *et al*., 2011). Contrarily, defined starter cultures are used in modern dairies to overcome variable product quality and to allow up-to-date quality assurance for safe products as these cultures work faster and more reliably. Since there is no commercial production of starter cultures for the Montenegrin local dairy industry, universal cultures from international manufacturers are used. Such cultures, however, are tailored to the needs of other markets and are not typical for the Montenegrin products.

Montenegro has a wide range of autochthonous dairy products. The most important ones are white brine cheese, leafy cheese, Njeguši cheese as well as fermented milks and a very specific, creamy product known as skorup (MIRECKI *et al*., 2012). Generally, these products are regionally distributed (DOZET *et al*., 1996). For example, white cheese in brine ('bijeli sir u salamuri'), also known by its generic name 'masni sir' (fat cheese), is the most frequently produced and consumed cheese in the northern and southern area of Montenegro (VESKOVIĆ MORAČANIN *et al*., 2012).

The steamed and leafy cheese 'lisnati sir' is familar for the mountain area in the central and northern part of Montenegro. Due to the main production of this cheese in the town Kolašin, it is better known as Kolašin's cheese. After ripening, this kind of cheese is split up into thin slices of leaves, which gives the cheese its name.

Njeguši cheese ('Njeguški sir') is one of the most famous hard cheeses in Montengro. It is named by the village Njeguši, which is located in the municipality Cetinje of southern Montenegro.

'Skorup' means cream and is a fat layer or crust which is removed from boiled milk. Another name for this dairy product is 'kajmak'. The name 'skorup' is used in the wider area of the mountains Durmitor and Sinjajevina in the northern and northwestern parts of Montenegro, where the cream is ripened in lamb or goat skin bags. On the contrary, 'kajmak' is ripened in tubs and predominantly produced and consumed in the central part of the country (ADŽIĆ and DOZET, 2001).

For producing these local traditional dairy products lactic acid bacteria (LAB) play a significant role (RADULOVIĆ *et al.,* 2011). They do not only represent the fermentation microbiota, but also a reservoir of unknown strains. Insights into the diversity of LAB are the first steps towards creating an authentic collection of dairy microorganisms. After proper investigation, suitable strains may find their application as starter cultures in local dairy

companies for the preservation and production of standardized and safe dairy products with the typical properties of traditional Montenegrin cheese. Moreover, it is important to characterize the LAB microbiota evolving and interacting throughout the manufacturing and ripening process. Understanding the distribution of LAB diversity during this process will make a contribution to the fermentation control, which is required to enhance positive attributes or reduce negative impacts of Montenegrin dairy products. This does not only reflect the relationship between bacterial diversity and the quality of cheese but also between bacterial diversity and human health. Thus, the objective of this study was the isolation and identification of LAB from 25 Montenegrin traditional dairy products for characterizing their LAB microbiota.

MATERIALS AND METHODS

Samples

Twenty-five samples of indigenous fermented dairy products, including cheese, cream and spontaneously fermented milk made of bovine, goat as well as mixed bovine and ovine milk, were taken from manufacturers of the Montenegrin towns Kolašin, Podgorica, Žabljak, Pljevlja, Mojkovac, Danilovgrad, Cetinje and Bar. Except of a small percentage which was picked up from the market, all samples were directly from manufacturers, rural households and mountain huts (Table 1). The samples were placed into sterile plastic bags and transported to the Biotechnical Faculty Laboratory of the University of Montenegro (UCG) at a temperature of 4°C. Samples were also stored at this temperature until their examination within 24 hours. After all investigations were completed, the frozen samples were additionally sent to the Food Microbiology and Hygiene Laboratory of the University of Natural Resources and Life Sciences, Vienna (BOKU) to complement the diversity of LAB with additional isolates by using different media. After their arrival at that laboratory all samples were stored at -20 °C until further analyses.

Table 1. Detected lactic acid bacteria species in autochthonous dairy products of Montenegro

Sample	Locality	Type of cheese	Milk	Producer	Species
ISV	Kolašin	Leafy cheese	Raw bovine milk	1	*Lc. lactis, Lb. paracasei, E. faecium, E. Durans*
IS	Kolašin	Leafy cheese	Raw bovine milk	1	*Lb. plantarum, Le. mesenteroides, E. faecium, E. Faecalis*
IKM	Kolašin	Spontaneously fermented[a]	Raw bovine milk	1	*Lc. lactis, E. Faecium*
ISM	Kolašin	Leafy cheese	Raw bovine milk	1	*Lc. lactis, E. durans, E. faecium*
4B	Podgorica	White cheese	Raw bovine milk	2	*Le. mesenteroides, E. faecium, E. faecalis*
RBS	Podgorica	White cheese	Raw bovine milk	3	*Lc. lactis, Lb. brevis, Lb. plantarum, E. durans, E. Faecium*
RKM	Podgorica	Spontaneously fermented[a]	Raw bovine milk	3	*Lc. lactis, E. faecium*
1Ž	Žabljak	White cheese	Raw bovine milk	4	*Lc.lactis, Lb.paracasei, Lb. curvatus, Lb. delbrueckii, Le. mesenteroides, E. faecium*
23Ž	Žabljak	White cheese	Raw bovine milk	4	*Lb. curvatus, Le. lactis, E. faecium*
894/39	Pljevlja	White cheese	Pasteurized bovine milk	5	*Lb. paracasei, P. pentosaceus, E. faecium, E. hirae*

LDR	Kolašin	Leafy cheese	Raw bovine milk	6	*Lb.kefiri, Lb. plantarum, Lb. paracasei, E. faecium*
LJM	Kolašin	Leafy cheese	Raw bovine milk	7	*Lb. plantarum, Lb. helveticus, Le. gelidum, E. faecium,*
LVR	Kolašin	Leafy cheese	Raw bovine milk	8	*Le. mesenteroides, E. Faecium*
SRD	Kolašin	Cream (skorup)	Pasteurized bovine and ovine milk	9	*Lb. paracasei, Lb. kefiri, E. faecium*
SDR	Kolašin	Cream (skorup)	Pasteurized bovine and ovine milk	10	*Le. citreum, E. Faecium*
6M	Mojkovac	White cheese	Raw bovine milk	11	*Lb. plantarum, Lb. paraplantarum, E. faecium*
906	Danilovgrad	White cheese	Raw bovine milk	12	*Lc. lactis, E. faecium, E. Faecalis*
2115/45	Danilovgrad	White cheese	Raw bovine milk	13	*Lc. lactis, Lb. plantarum, Le. mesenteroides, Le. pseudomesenteroides, E. faecium, E. Faecalis*
9Nj	Cetinje	Semihard cheese	Raw bovine milk	14	*Lc. lactis, Lb. kefiri, Lb. paracasei, E. faecalis*
8K	Bar	White cheese	Raw goat milk	15	*Lb. brevis, Lb. plantarum, E. faecalis*
SKZ	Kolašin	Cream (skorup)	Raw bovine and ovine milk	16	*Lb. paraplantarum, Lb. kefiri, E. faecium*
7K	Podgorica	White cheese	Raw bovine milk	17	*Lb. paraplantarum, Lb. coryniformis, E. faecium, E. Durans*
RS	Podgorica	White cheese	Raw bovine milk	18	*Le. mesenteroides, Le. pseudomesenteroides, E. faecium, E. durans*
CS	Kolašin	Leafy cheese	Raw bovine milk	19	*E. faecium, E. faecalis, S. thermophilus*
JKM	Mojkovac	Spontaneously fermented[a]	Raw bovine milk	20	*Lc. lactis, Le. mesenteroides, Le. pseudomesenteroides, E. faecium, E. faecalis*

[a]Spontaneously fermented raw bovine milk was obtained by incubation of raw milk at room temperature for 24 hours. Within this time the milk coagulated due to the presence of the natural microflora.

Isolation of LAB

At UCG, primary dilutions of cheese and cream samples were made by mixing 20 g samples with 180 ml 2% Na-citrate solution, whereas 20 g of spontaneously fermented bovine milk were primarily diluted in 180 ml 0.9% NaCl solution. These primary dilutions were streaked on MRS or M17 agar (Merck, Darmstadt, Germany). Although M17 agar already contained 0.5% lactose, 1% glucose was supplementary added (M17-G agar). Inoculated plates were aerobically as well as anaerobically incubated at 30°C and 44°C for 2 - 5 days. Anaerobic conditions were provided in anaerobic jars using anaerobic bags (Anaerocult, Merck, Darmstadt, Germany).

After incubation, the grown colonies were observed. Colonies that grew on the medium were randomly selected and transmitted to M17 broth (Merck) supplemented with 1% glucose (M17-G broth). Inoculated broths were incubated as described before. To obtain pure cultures, incubated broths were streaked on M17-G agar and incubated again. After checking the purity of the cultures, colonies were Gram-stained and tested for the presence of catalase. Based on these tests, colonies of Gram-positive, spherical- or rod-shaped, catalase-negative isolates were

inoculated in M17-G broth, incubated and stored at -20°C after adding glycerol (15 %) (RADULOVIĆ *et al*., 2006; MARTINOVIĆ, 2003; MARTINOVIĆ *et al.*, 2005).

At BOKU, 10g of each sample were mixed with 90 ml 2% Na-citrate solution. A loopful of this dilution was streaked on MRS agar supplemented with 0.05% cysteine hydrochloride and 0.01% cycloheximide (MRS-CC agar) as well as on MRS agar additionally containing 0.005% vancomycin (MRS-CCV agar). Inoculated plates were incubated in an anaerobic chamber (80% N_2, 10% CO_2, 10% H_2; ScholzenTechnik, Kriens, Switzerland) for 48 – 72 h at 30°C and 37°C.

Colonies with different morphologies were selected and purified using MRS agar supplemented with 0.05% cysteine hydrochloride. After incubating the plates under the same conditions as described before, one colony of each pure culture was transferred into MRS broth and anaerobically cultivated for 48 h at 30°C or 37°C. The incubated MRS broth of each isolate was mixed with glycerol (15%) and stored at -80°C.

Identification of LAB

The identification of all isolates was performed at BOKU. In this regard all isolates were enriched in M17 broth (Merck) followed by a cell harvest at 6800 x g for 6 min. at 4°C and cell washing with respectively 900 µl NaCl (0.9 %) and 900 µl EDTA (50 mM, pH 8.0). Subsequently, the DNA was extracted from the cell pellets with the Peqgold DNA isolation kit (Peqlab, Erlangen, Germany) according to the manufacturer's instructions.

The extracted DNA was used as template for PCR. Firstly, the Random Amplified Polymorphic DNA (RAPD) typing technique was applied using two different RAPD primers (Table 2). With the obtained fingerprint data of both primers, a combined analysis was performed by calculating the similarity matrices of the individual experiments (Dice coefficient, 1.0% band position tolerance, 1.0% optimization) at first, followed by averaging the matrices of the individual experiments according to their corrected internal weights. Finally, the BioNumerics software (v. 7.5, Applied Maths, Sint-Martens-Latem, Belgium) by the Unweighted Pair Group Method with Arithmetic mean (UPGMA) was used to compare the data and to create a phylogenetic tree for cluster analysis. Representatives of each cluster were subjected to a further PCR, amplifying a region of the 16S rRNA, *rpoB* or *cpn*60 gene. PCR products thereof were purified with the PCRExtract Mini Kit (5 Prime, Hilden, Germany) and sent to commercial sequencing (Eurofins MWG Operon, Germany). Upon receipt of the data, sequence compilation and comparison were performed with the BLASTn program of the National Center for Biotechnology Information (NCBI, http://www.blast.ncbi.nlm.nih.gov). Based on the received results, all isolates of the corresponding cluster were verified by species-specific PCR (Table 2), except of isolates belonging to the species *Lactobacillus* (*Lb.*) *coryniformis*, *Lb. kefiri*, *Leuconostoc* (*Le.*) *gelidum*, *Le. citreum*, *Le. lactis*, and *Le. pseudomesenteroides*, which were only identified by sequencing.

To conduct PCR for typing and sequencing, the PCR mix (25 µl) contained 1 µl DNA solution, 2.5 µl 10 x PCR buffer (Finnzymes, Espoo, Finland), 0.5 µl deoxynucleoside triphosphate (dNTP) mix (10 mM of each dNTP), 0.5 µl DNA polymerase (2U/µl; DynazymeII, Finnzymes, Espoo, Finland), 18.5 µl sterile deionized water and either 2 µl of a RAPD primer (Table 2) or 1 µl of each sequencing primer in concentrations as indicated in Table 3. The thermal cycling program for RAPD PCR consisted of an initial denaturation step at 95°C for 5 min and 45 cycles of 95°C for 1 min, 36°C for 1 min and 72°C for 1 min with a final extension

at 72°C for 8 min. The cycling program for the generation of the PCR products subjected to sequencing was the same as described by the respective authors (Table 3). All PCR reactions were conducted in an Eppendorf Mastercycler.

Table 2. Description of primers used to type and identify LAB isolates

Primer	Specificity	Sequence (5´-3´)	Concentration (nM)	Reference
1283	RAPD PCR	GCGATCCCCA	400	Akopyanz et al. (1992)
M13	RAPD PCR	GAGGGTGGCGGTTCT	400	Morandi et al. (2011)
Dut-F1	*E. faecium*	GCAAGGCTTCTTAGAGA	400	Dutka-Malen et al. (1995)
Dut-F2		CATCGTGTAAGCTAACTTCCT	400	
Dut-E1	*E. faecalis*	ATCAAGTACAGTTAGTCT	400	Dutka-Malen et al. (1995)
DutE2		ACGATTCAAAGCTAACTG	400	
Eh1	*E. hirae*	AAACAATCGAAGAACTACTT	400	Farid et al. (2006)
Eh2		TAAATTCTTCCTTAAATGTTG	400	
Mur-2ed/F	*E. durans*	AACAGCTTACTTGACTGGACGC	400	Arias et al. (2006)
Mur-2ed/R		GTATTGGCGCTACTACCCGTAGG	400	
gadB21_Lc	*Lc. lactis*	CGTTATGGATTTGATGGATATAAAGC	400	Nomura et al. (2002)
Gad7r_Lc		ACTCTTCTTAAGAACAAGTTTAACAGC	400	
St1	*S. thermophilus*	TTATTTGAAAGGGGCAATTGCT	400	Furet et al. (2004)
St2		GTGAACTTTCCACTCTCACAC	400	
Lmes-F	*Le. mesenteroides*	AACTTAGTGTCGCATGAC	500	Lee et al. (2000)
Lmes-R		AGTCGAGTTACAGACTACAA	500	
PPE23S_F	*P. pentosaceus*	CCAGGTTGAAGGTGCAGTAAAAT	400	Pfannebecker and Fröhlich (2008)
P23S_R		CTGTCTCGCAGTCAAGCTC	400	
LdeF	*Lb. delbrueckii*	TACTGTTAAGGTTGGCGACAGC	400	Sheu et al. (2009)
LdeR		TGTAGACTTGGCCCTTGAAAGT	400	
PeCf_helv1	*Lb. helveticus*	CTGTTTTCAATGTTGCAAGTC	400	Fortina et al. (2001)
PeCr_helv2		TTTGCCAGCATTAACAAGTCT	400	
16SrRNA FW	*Lb. paraplantarum*	GCTGGATCACCTCCTTTC	400	Berthier and Ehrlich (1998)
LparaplaR		ATGAGGTATTCAACTTATT	400	
16SrRNA_FW	*Lb. plantarum*	GCTGGATCACCTCCTTTC	400	Berthier and Ehrlich (1998)
LplanR		ATGAGGTATTCAACTTATG	400	
A1c	*Lb. curvatus*	GGAGGGTGTTCAGGAC	400	Berthier and Ehrlich (1999)
A1c’		GGAGGGTGTTGATAGG	400	
Mu1ISRR	*Lb. brevis*	GCCTTGSGAGATGGTCCTC	400	Settanni et al. (2005)
LbreF		TTTGACGATCACGAAGTGACCG	400	
PAR	*Lb. paracasei*	GACGGTTAAGATTGGTGAC	400	Ventura et al. (2003)
CPR		CAANTGGATNGAACCTGGCTTT	400	

Species-specific PCR was performed as described above with minor modifications: instead of the previously mentioned amounts, 0.25µl of Dynazyme II (2 U/µl) and 18.75 µl of sterile deionized water were used. PCR thermocycling conditions were applied according to the references (Table 2). The PCR products were examined by electrophoresis using a 2% agarose gel and visualized after staining with ethidiumbromide.

The performance of PCR was checked during the study by including DNA of the corresponding type strains (e.g. *Enterococcus* (*E.*) *faecalis* LMG 7937, *E. faecium* LMG 11423, *E. durans* LMG 10746, *E. hirae* LMG 14198, *Lactococcus* (*Lc.*) *lactis* LMG 6890, *Lb. brevis* LMG 7944, *Lb. curvatus* LMG 9198, *Lb. delbrueckii* LMG 13086, *Lb. helveticus* LMG 13555, *Lb. paracasei* LMG 13087, *Lb. paraplantarum* LMG 16673, *Lb. plantarum* LMG 6907, *Le. mesenteroides* LMG 6893, *Pediococcus* (*P.*) *pentosaceus* LMG 11488, *Streptococcus* (*S.*) *thermophilus* LMG 6896.

Table 3.Description of primers used to sequence representative isolates

Primer	Gene	Sequence (5´-3´)	Concentration (nM)	Reference
P0	16S rRNA	GAGAGTTTGATCCTGGCTCAG	300	Di Cello et al. (1997)
P6		CTACGGCTACCTTGTTACGA	300	
bak 4	16S rRNA	AGGAGGTGATCCARCCGCA	300	Dasen et al. (1998)
bak 11w		AGTTTGATCMTGGCTCAG	300	
rpoB1	*rpoB*	ATTGACCACTTGGGTAACCGTCG	400	Renouf et al. (2006)
rpoB2		ACGATCACGGGTCAAACCACC	400	
H279A	*cpn*60	GAIIIIGCIGGIGAYGGIACIACIAC	500	Goh et al. (1997)
H280A		YKIYKITCICCRAAICCIGGIGCYTT	500	

RESULTS AND DISCUSSION

Isolation and identification of LAB at species level

A total of 248 isolates was obtained by investigating indigenous fermented dairy products using MRS and M17-G media and an anaerobic or aerobic incubation at 30°C or 44°C for 2 to 5 days. Based on molecular biological methods, these isolates were identified as *E. faecium* (n=170), *Lc. lactis* (n=40), *E. faecalis* (n= 22), *E. durans* (n=8), *E. hirae* (n=4), *Le. mesenteroides* (n=2), *Lb. paracasei* (n=1) and *S. thermophilus* (n=1).

However, the high percentage of *Enterococcus* spp. isolates (82.3%) has to be considered carefully. M17 agar containing lactose is usually a standard medium recommended for isolating lactic streptococci (e.g. *Lactococcus*) and *S. thermophilus*. Due to the addition of glucose, the selectivity of this medium may have been reduced, facilitating the advantaged growth of enterococci, which were formerly known as faecal streptococci (FACKLAM, 2002). Next to the close relationship of these genera (FACKLAM, 2002), the ability of enterococci to grow wide-spread due to their metabolic versatility and intrinsic resistance to inhospitable conditions (RAMSEY *et al.*, 2014) may explain the reduced detectability of lactococci and *S. thermophilus* on M17 agar. Regarding this genus and species 40 *Lactococcus*-isolates and one *Streptococcus* isolate were found. Furthermore, the growth of lactobacilli on M17 agar is partially suppressed, as the ingredient disodium-ß-glycerophoshate inhibits some *Lactobacillus* species (SHANKAR and DAVIES, 1977). Hence, MRS medium, especially designed for the

cultivation of lactobacilli (DE MAN *et al*., 1960), was also used within this study. This medium contains sodium acetate, which suppresses the growth of many competing bacteria (STILES *et al*., 2002), although other LAB such as the related genera *Leuconostoc* and *Pediococcus* as well as species of the former *Streptococcus* genus may also grow (TEMMERMAN *et al.*, 2006). Despite the fact that lactobacilli tolerate lower pH levels than streptococci, only one *Lactobacillus* strain and many enterococci were recovered from the investigated dairy products on MRS agar. Additionally, two *Le. mesenteroides* strains were found on MRS agar.

To obtain a higher diversity of LAB, isolation was repeated on MRS agar additionally containing cysteine-hydrochloride, cycloheximide and/or vancomycin using only anaerobic conditions for incubation. The addition of cysteine-hydrochloride, a reducing agent, should especially improve the cultivation of oxygen susceptible species, whereas vancomycin makes MRS agar more selective for almost all lactobacilli excluding the vancomycin-susceptible *Lb. delbrueckii*-group (HAMILTON-MILLER and SHAH, 1998). Cycloheximide was added to prevent the growth of undesired yeasts. Although the samples were not fresh anymore and frozen in the meantime, the LAB species *Lb. plantarum* (n=13), *Lb. paracasei* (n=11), *Le. mesenteroides* (n=8), *Lb. kefiri* (n=6), *Le. pseudomesenteroides* (n=5), *Lb. paraplantarum* (n=4), *Lb. brevis* (n=3), *Lb. curvatus* (n=2), *Lb. delbrueckii* (n=2), *Le. citreum* (n=2), *Le. gelidum* (n=1), *Le. lactis* (n=1), *Lb. coryniformis* (n=1), *Lb. helveticus* (n=1), *Lc. lactis* (n=1), *P. pentosaceus* (n=1) and *E. faecalis* (n=1) could be detected.

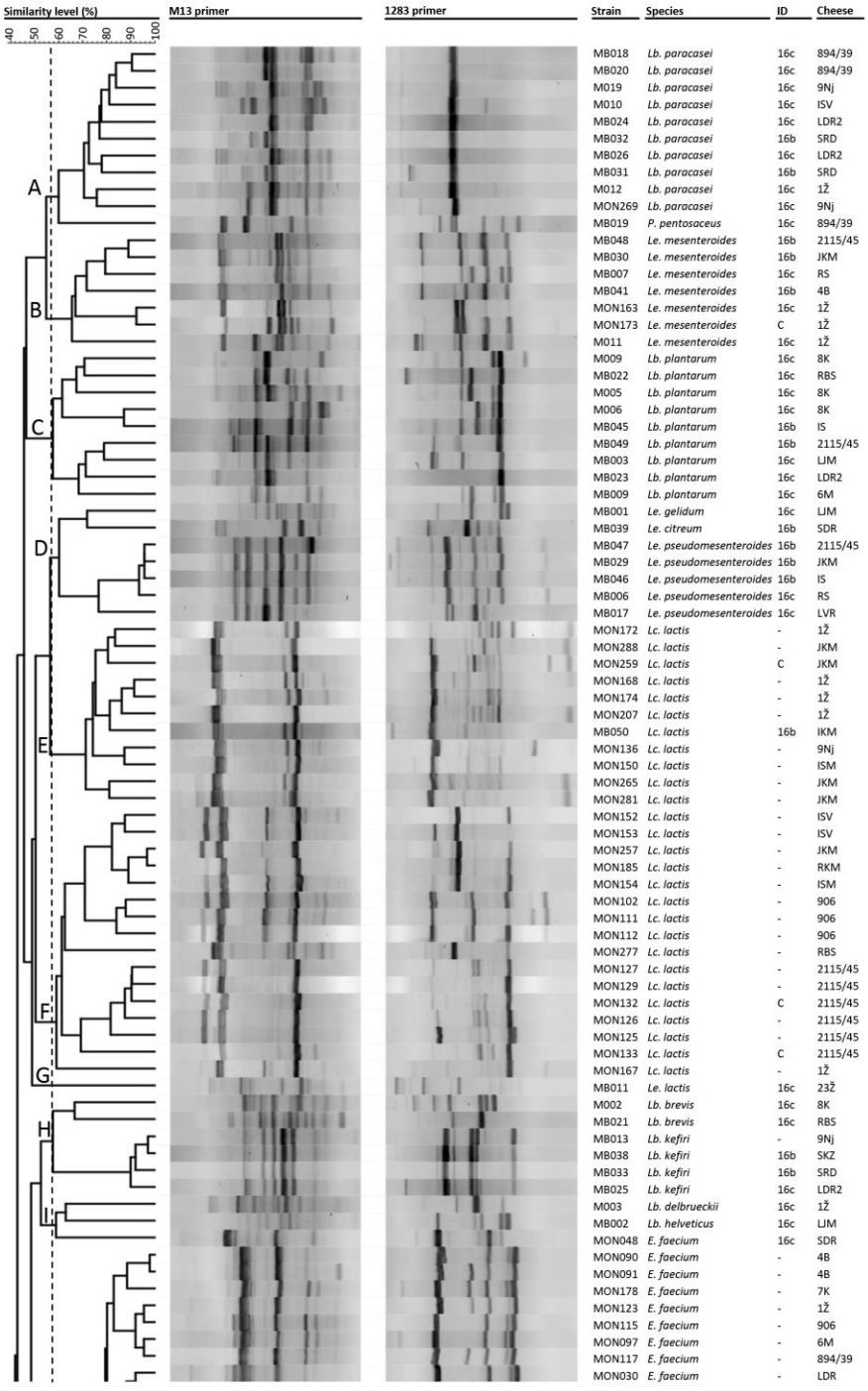
Identification of LAB at strain level

As many isolates showed the same or similar RAPD patterns, only strains with different fingerprints or the same fingerprint and different origins (dairy products) were considered for further evaluation. Thus, the reproducibility of the composite data set was calculated to define single strains. For this purpose, duplicate testing of 15 isolates was performed and the corresponding reproducibility was expressed by determining the average of the similarity between the two patterns of each isolate. Hence, a similarity value of 91.9 % was obtained. Consequently, all isolates with a similarity level higher than or equal to 91.9 % and the same origin were regarded as multiple isolates representing a single strain. Choosing only one strain for multiple isolates, the 311 LAB isolates were reduced to 57 *E. faecium*, 27 *Lc. lactis*, 12 *E. faecalis*, 10 *Lb. paracasei*, nine *Lb. plantarum*, seven *Le. mesenteroides*, six *E. durans*, five *Le. pseudomesenteroides*, four *Lb. kefiri*, four *Lb. paraplantarum*, two *Lb. brevis*, two *Lb. curvatus* and two *E. hirae* strains as well as respectively one strain of the species *Lb. delbrueckii*, *Lb. coryniformis*, *Lb. helveticus*, *Le. citreum*, *Le. gelidum*, *Le. lactis*, *P. pentosaceus* and *S. thermophilus* resulting in a total of 155 LAB strains.

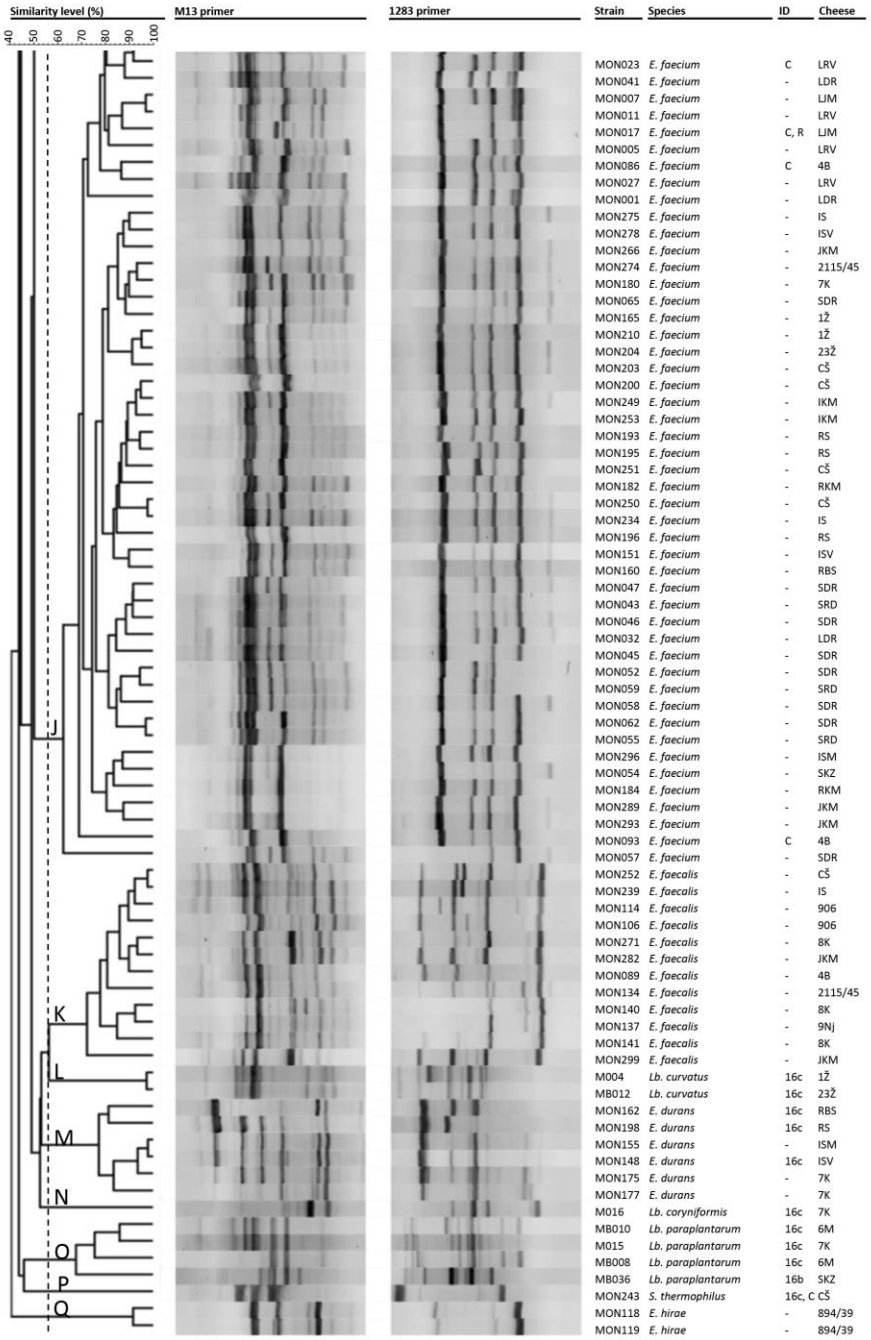
Figure 1 shows the dendrogram calculated using the results of cluster analysis for the combined M13- and 1283-patterns of the 155 LAB strains. All 155 strains produced different or similar patterns. At a similarity level of 57 %, 14 clusters and three single genotypes were gained. The size of the 14 clusters was very different containing two to 56 strains. The three single genotypes G, N and P depicted the species *Le. lactis*, *Lb. coryniformis* and *S. thermophilus*. All other species, which were also only represented by one strain, formed major clusters with some other species. Thus, cluster D and I were composed of three species, respectively: *Le. gelidum*, *Le. citreum*, *Le. pseudomesenteroides* and *Lb. delbrueckii*, *Lb. helveticus*, *E. faecium* (Figure 1). The two first mentioned species of cluster I are members of the *Lb. delbrueckii* group, representing one of three phylogenetic groups into which the

Lactobacillus genus has been originally subdivided (POT *et al.*, 2014). The assignment of one *E. faecium* strain to this cluster is rather opaque and may be due to misidentification as it was very difficult to identify the isolated enterococci at species-level. Especially the sequencing of 16S rRNA gene fragments of enterococcal isolates displayed results that couldn't be verified by species-specific PCR. The limited discriminating power of 16S rRNA gene sequences for several closely related *Enterococcus* species has already been described elsewhere (NASER *et al.*, 2005). In contrast, *Enterococcus* isolates were distinguished clearly by partial sequencing of the *cpn*60 or *rpo*B gene. Furthermore, a single *P. pentosaceus* strain could be found in cluster A along with ten *Lb. paracasei* strains. Both species belong to the originally described *Lb. casei – Pediococcus* group, which also includes species of the *Lb. buchneri* group such as *Lb. brevis* and *Lb. kefiri* (POT *et al*., 2014). Representatives of the two last species were found in cluster H. Each of the clusters B, C, K, L, M, O and Q described several strains of a single species (e.g. *Le. mesenteroides*, *Lb. plantarum*, *E. faecalis*, *Lb. curvatus*, *E. durans*, *Lb. paraplantarum*, *E. hirae*). The species *Lc. lactis* was even split into cluster E and F. With the exception of the *E. faecium* strain mentioned above, all other *E. faecium* strains formed the largest cluster named J.

For this rich diversity of LAB in traditional Montenegrin cheeses the raw milk microbiota may be an important part. According to MONTEL *et al.*, (2014), more than 400 microbial species have been detected in raw milk, including about 60 LAB species. Also most *Lactobacillus* (e.g. *Lb. brevis*, *Lb. delbrueckii*, *Lb. coryniformis*, *Lb. curvatus*, *Lb. helveticus*, *Lb. paraplantarum*, *Lb. plantarum*), *Leuconostoc* (e.g. *Le. gelidum*, *Le. lactis*, *Le. mesenteroides*, *Le. pseudomesenteroides*) as well as all *Lc. lactis* strains could be detected in dairy products made of raw milk within this study. Even strains of the species *E. durans* and *E. faecalis* were exclusively found in these products (during cheese making by traditional equipment and practices (MONTEL *et al.*, 2014). Next to this, the occurrence of LAB in pasteurized milk and dairy products thereof may be due to their partial resistance to heat treatment (BERESFORD *et al.*, 2001). Hence, it is known that *Lb. curvatus*, *Lb. delbrueckii*, *Lb. helveticus*, *Lb. paracasei*, *Lb. plantarum*, *P. pentosaceus* and *S. thermophilus* are thermotolerant and thus able to survive pasteurization temperatures (DE LOURDES PERÉZ-CHABELA *et al*., 2008; DELCOUR *et al.*, 2000). Likewise heat resistant strains are found among *Enterococcus* spp. (MCAULEY *et al*. 2012), whereas *E. faecium* strains should be more heat tolerant than those of *E. faecalis* (AHMAD *et al.*, 2002). This may explain the exclusive appearance of *E. faecalis* in raw milk cheese samples in this study (Table 1). The species *E. hirae*, *Le.citreum* and one *Pd. pentosaceus* strain were also only identified in dairy products made of pasteurized milk, whereas strains of the species *E. faecium*, *Lb. kefiri* and *Lb. paracasei* were part of the LAB flora of cheese either made of raw milk or pasteurized milk. Molecular analyses of QUIGLEY *et al*. (2013a) indicate that the bacterial population of pasteurized milk is more diverse than previously assumed, but non-thermoduric bacteria, which are still present in pasteurized milk, are damaged and consequently non-culturable. Moreover, only three of the 25 cheeses samples tested were produced with pasteurized milk, which may additionally account for the low number of LAB species found in pasteurized milk products.



Strain	Species	ID	Cheese
MB018	*Lb. paracasei*	16c	894/39
MB020	*Lb. paracasei*	16c	894/39
M019	*Lb. paracasei*	16c	9Nj
M010	*Lb. paracasei*	16c	ISV
MB024	*Lb. paracasei*	16c	LDR2
MB032	*Lb. paracasei*	16b	SRD
MB026	*Lb. paracasei*	16c	LDR2
MB031	*Lb. paracasei*	16b	SRD
M012	*Lb. paracasei*	16c	1Ž
MON269	*Lb. paracasei*	16c	9Nj
MB019	*P. pentosaceus*	16c	894/39
MB048	*Le. mesenteroides*	16b	2115/45
MB030	*Le. mesenteroides*	16b	JKM
MB007	*Le. mesenteroides*	16c	RS
MB041	*Le. mesenteroides*	16b	4B
MON163	*Le. mesenteroides*	16c	1Ž
MON173	*Le. mesenteroides*	C	1Ž
M011	*Le. mesenteroides*	16c	1Ž
M009	*Lb. plantarum*	16c	8K
MB022	*Lb. plantarum*	16c	RBS
M005	*Lb. plantarum*	16c	8K
M006	*Lb. plantarum*	16c	8K
MB045	*Lb. plantarum*	16b	IS
MB049	*Lb. plantarum*	16b	2115/45
MB003	*Lb. plantarum*	16c	LJM
MB023	*Lb. plantarum*	16c	LDR2
MB009	*Lb. plantarum*	16c	6M
MB001	*Le. gelidum*	16c	LJM
MB039	*Le. citreum*	16b	SDR
MB047	*Le. pseudomesenteroides*	16b	2115/45
MB029	*Le. pseudomesenteroides*	16b	JKM
MB046	*Le. pseudomesenteroides*	16b	IS
MB006	*Le. pseudomesenteroides*	16c	RS
MB017	*Le. pseudomesenteroides*	16c	LVR
MON172	*Lc. lactis*	-	1Ž
MON288	*Lc. lactis*	-	JKM
MON259	*Lc. lactis*	C	JKM
MON168	*Lc. lactis*	-	1Ž
MON174	*Lc. lactis*	-	1Ž
MON207	*Lc. lactis*	-	1Ž
MB050	*Lc. lactis*	16b	IKM
MON136	*Lc. lactis*	-	9Nj
MON150	*Lc. lactis*	-	ISM
MON265	*Lc. lactis*	-	JKM
MON281	*Lc. lactis*	-	JKM
MON152	*Lc. lactis*	-	ISV
MON153	*Lc. lactis*	-	ISV
MON257	*Lc. lactis*	-	JKM
MON185	*Lc. lactis*	-	RKM
MON154	*Lc. lactis*	-	ISM
MON102	*Lc. lactis*	-	906
MON111	*Lc. lactis*	-	906
MON112	*Lc. lactis*	-	906
MON277	*Lc. lactis*	-	RBS
MON127	*Lc. lactis*	-	2115/45
MON129	*Lc. lactis*	-	2115/45
MON132	*Lc. lactis*	C	2115/45
MON126	*Lc. lactis*	-	2115/45
MON125	*Lc. lactis*	-	2115/45
MON133	*Lc. lactis*	C	2115/45
MON167	*Lc. lactis*	-	1Ž
MB011	*Le. lactis*	16c	23Ž
M002	*Lb. brevis*	16c	8K
MB021	*Lb. brevis*	16c	RBS
MB013	*Lb. kefiri*	-	9Nj
MB038	*Lb. kefiri*	16b	SKZ
MB033	*Lb. kefiri*	16b	SRD
MB025	*Lb. kefiri*	16c	LDR2
M003	*Lb. delbrueckii*	16c	1Ž
MB002	*Lb. helveticus*	16c	LJM
MON048	*E. faecium*	16c	SDR
MON090	*E. faecium*	-	4B
MON091	*E. faecium*	-	4B
MON178	*E. faecium*	-	7K
MON123	*E. faecium*	-	1Ž
MON115	*E. faecium*	-	906
MON097	*E. faecium*	-	6M
MON117	*E. faecium*	-	894/39
MON030	*E. faecium*	-	LDR



Strain	Species	ID	Cheese
MON023	*E. faecium*	C	LRV
MON041	*E. faecium*	-	LDR
MON007	*E. faecium*	-	LJM
MON011	*E. faecium*	-	LRV
MON017	*E. faecium*	C, R	LJM
MON005	*E. faecium*	-	LRV
MON086	*E. faecium*	C	4B
MON027	*E. faecium*	-	LRV
MON001	*E. faecium*	-	LDR
MON275	*E. faecium*	-	IS
MON278	*E. faecium*	-	ISV
MON266	*E. faecium*	-	JKM
MON274	*E. faecium*	-	2115/45
MON180	*E. faecium*	-	7K
MON065	*E. faecium*	-	SDR
MON165	*E. faecium*	-	1Ž
MON210	*E. faecium*	-	1Ž
MON204	*E. faecium*	-	23Ž
MON203	*E. faecium*	-	CŠ
MON200	*E. faecium*	-	CŠ
MON249	*E. faecium*	-	IKM
MON253	*E. faecium*	-	IKM
MON193	*E. faecium*	-	RS
MON195	*E. faecium*	-	RS
MON251	*E. faecium*	-	CŠ
MON182	*E. faecium*	-	RKM
MON250	*E. faecium*	-	CŠ
MON234	*E. faecium*	-	IS
MON196	*E. faecium*	-	RS
MON151	*E. faecium*	-	ISV
MON160	*E. faecium*	-	RBS
MON047	*E. faecium*	-	SDR
MON043	*E. faecium*	-	SRD
MON046	*E. faecium*	-	SDR
MON032	*E. faecium*	-	LDR
MON045	*E. faecium*	-	SDR
MON052	*E. faecium*	-	SDR
MON059	*E. faecium*	-	SRD
MON058	*E. faecium*	-	SDR
MON062	*E. faecium*	-	SDR
MON055	*E. faecium*	-	SRD
MON296	*E. faecium*	-	ISM
MON054	*E. faecium*	-	SKZ
MON184	*E. faecium*	-	RKM
MON289	*E. faecium*	-	JKM
MON293	*E. faecium*	-	JKM
MON093	*E. faecium*	C	4B
MON057	*E. faecium*	-	SDR
MON252	*E. faecalis*	-	CŠ
MON239	*E. faecalis*	-	IS
MON114	*E. faecalis*	-	906
MON106	*E. faecalis*	-	906
MON271	*E. faecalis*	-	8K
MON282	*E. faecalis*	-	JKM
MON089	*E. faecalis*	-	4B
MON134	*E. faecalis*	-	2115/45
MON140	*E. faecalis*	-	8K
MON137	*E. faecalis*	-	9Nj
MON141	*E. faecalis*	-	8K
MON299	*E. faecalis*	-	JKM
M004	*Lb. curvatus*	16c	1Ž
MB012	*Lb. curvatus*	16c	23Ž
MON162	*E. durans*	16c	RBS
MON198	*E. durans*	16c	RS
MON155	*E. durans*	-	ISM
MON148	*E. durans*	16c	ISV
MON175	*E. durans*	-	7K
MON177	*E. durans*	-	7K
M016	*Lb. coryniformis*	16c	7K
MB010	*Lb. paraplantarum*	16c	6M
M015	*Lb. paraplantarum*	16c	7K
MB008	*Lb. paraplantarum*	16c	6M
MB036	*Lb. paraplantarum*	16b	SKZ
MON243	*S. thermophilus*	16c, C	CŠ
MON118	*E. hirae*	-	894/39
MON119	*E. hirae*	-	894/39

Fig. 1. Composite dataset of RAPD-patterns obtained for LAB strains isolated from Montenegrin dairy products. Dashed line = similarity level of 57 %; MB, M = BOKU strains; MON = UCG strains; ID = identification by sequencing; 16c = P0/P6 primerset; 16b = bak4/bak11w primerset; C = H279A/H280A primerset, R = rpoB1/rpoB2 primerset

The primary role of LAB species during fermentation is the production of lactic acid from lactose (SAMARŽIJA *et al.,* 2001). Referring to this, particularly the detected species *Lc. lactis* is of importance. Strains of this species are frequently used as starters for the manufacture of cheeses (KOJIĆ *et al.,* 2007). The species *S. thermophilus*, also found in this study, is often regarded as the second most important industrial dairy starter after *Lc. lactis* (QUIGLEY *et al.,* 2013b). Both species also contribute to proteolysis, the conversion of amino acids into flavor compounds, citrate utilization and/or fat metabolism (QUIGLEY *et al*., 2013b; MOREA *et al.,* 1999). However, they have a less important role in the breakdown of large and small peptides (CROW *et al*., 1995).

Next to *S. thermophilus*, *Lb. delbrueckii* and *Lb. helveticus* belong to the main thermophilic, homofermentative LAB (MONTEL *et al.,* 2014). Respectively one strain was isolated for both species from Montenegrin cheeses. These thermophilic, homofermentative lactobacilli are important in the early part of cheese-ripening to readily degrade proteins, previously hydrolysed by starter and milk proteinases as well as enzymes released upon autolysis of lactococci (CROW *et al.,* 1995; GRAPPIN *et al.,* 1999). Next to proteolysis, these species continue to decompose lactose, decrease the redox potential in cheese cores, produce aromatic compounds and provide substrates that can be further degraded by other microbial populations such as non-starter LAB (NSLAB) (MONTEL *et al*., 2014).

As NSLAB are tolerant to selective values of pH, salt, moisture and a wide range of temperature (MONTEL *et al*., 2014), they are well adapted to the conditions of cheese ripening, where many nutrients are depleted, the pH is reduced, and the moisture content is low (QUIGLEY *et al.,* 2013b). In general, NSLAB reach significant levels in the later ripening process, where they are decisive for cheese flavor and texture development by carrying out proteolysis and lipolysis (SMIT *et al.,* 2005; MONTEL *et al.,* 2014; CROW *et al.,* 1995). Mesophilic lactobacilli are among the most common NSLAB. Their relative abundance varies according to the type of cheese technology and the length of ripening (MONTEL *et al.,* 2014). Of the species isolated from Montenegrin dairy products, *Lb. brevis*, *Lb. curvatus*, *Lb. paracasei*, *Lb. plantarum* and *Lb. paraplantarum* are known as NSLAB (PARENTE and COGAN, 2004, ROBINSON, 2002; QUIGLEY *et al*., 2013b). Next to these species, *Lb. kefiri* and *Lb. coryniformis* were either found in the present study, which may also be relevant as NSLAB as strains of the first species were able to produce NH_3 as well as citrate (BARUZZI *et al.,* 2000) and representatives of the second species exhibited strong peptidase and α-glucosidase activities (LITOPOULOU-TZANETAKI and TZANETAKIS, 2014).

Leuconostoc spp. require amino acids or peptides for growth (HEMME and FOUCAUD-SCHEUNEMANN, 2004), which are abundant during the stage of ripening (QUIGLEY *et al*., 2013b). Thus, strains of the *Leuconostoc* genus are also more prevalent in the later stage of cheese production, contributing to organoleptic properties due to their metabolisms of lactose and citrate to lactate, acetate, CO_2, ethanol, acetaldehyde, diacetyl, acetoin and 2,3-butanediol (VEDAMUTHU, 1994; SANCHEZ *et al.,* 2005). Of this genus, the species *Le. citreum*, *Le. gelidum*, *Le. lactis*, *Le. mesenteroides* and *Le. pseudomesenteroides* were found within this study.

Pediococci are only occasionally found in cheese with *P. acidilactici* and *P. pentosaceus* being the most frequent species. This may explain the only detection of one *P. pentosaceus* strain from a white cheese. Next to *Lb. plantarum* and *Lb. brevis*, this species belongs to the NSLAB capable of oxygen consumption. As the redox potential is likely to be

important in the production of flavor compounds in cheese, these species are also essential for the development of flavor compounds in cheese (CROW *et al.*, 1995).

The large number of enterococci found in Montenegrin dairy products is typical for Mediterranean cheeses. *E. faecium* and *E. faecalis* are the predominant species (SARANTINOPOULOS *et al.* 2002; GOMES *et al.* 2008). They contribute to ripening due to proteolysis, lipolysis, citrate breakdown and the production of aromatic volatile compounds (FRANZ *et al.*, 1999), resulting in the typical taste and flavor of dairy products in these regions. Irrespective of their desirable use as starter cultures in food production, they give rise to concern because of the possible presence of virulence factors and the potential transfer of antibiotic resistances (FRANZ and HOLZAPFEL, 2006).

Due to different methods applied, it is difficult to compare the obtained results with other studies. Nonetheless, referring to two recent papers describing the LAB diversity in artisanal buffalo's milk cheese (SILVA *et al.*, 2015) and raw cow's milk cheese (DOMINGOS-LOPES *et al.*, 2017) including MRS and M17 for isolation, a similar LAB microbiota was reported. Hence, lactobacilli (e.g. *Lb. paracasei*, *Lb. plantarum*, *Lb. paraplantarum*, *Lb. otakiensis*), lactococci (e.g. *Lc. lactis*, *Lc. garviae*), leuconstocs (e.g. *Le. mesenteroides*, *Le. citreum*) and enterococci (e.g. *E. faecalis*, *E. italicus*, *E. pseudoavium*) were found by DOMINGOS-LOPES *et al.* (2017), whereas SILVA *et al.* (2015) determined *S. thermophilus*, *E. faecium*, *E. durans*, *Le. mesenteroides*, *Lb. fermentum*, *Lb. delbrueckii*, *Lb. helveticus* and *Lb. casei*.

CONCLUSION

The preservation of a high taxonomic diversity in the LAB microbiota of traditional raw milk cheeses is of upmost importance. Due to this heterogeneous microbiota, raw artisanal cheeses acquire richer and more intense flavors than pasteurized ones, which need to be sustained. Additionally, the defense of biodiversity stimulates the production of foods that provide benefits for the consumers as stated by PANIZZON *et al.* (2015). In this regard it is known that an increased diversity has been linked to a favorable metabolism and immune system response, while a reduced variation in microbiota has been associated with health problems (MCINTOSH, 2014). A high LAB diversity could be observed in traditional Montenegrin dairy products representing nine *Lactobacillus* species, five *Leuconostoc* species, four *Enterococcus* species as well as strains of the species *Lc. lactis*, *P. pentosaceus* and *S. thermophilus*, which is comparable to those of similar studies. However, just the occurrence of different LAB species was observed within this study and not the corresponding concentrations. Furthermore, the influence of the composition of different culture media and cultivation conditions on the microbial diversity could be demonstrated within this study. Since these limitations should be overcome by culture-independent analyses (ERCOLINI, 2013), methods such as high-throughput sequencing would be more suitable for future investigations. To develop autochthonous starter cultures that would contribute to the production of Montenegrin cheese with flavors characteristic for this area, further examination of the received strains are needed.

ACKNOWLEDGEMENTS

The study was funded by the Ministry of Science of Montenegro (national project:"Isolation and characterization of autochthonous lactic acid bacteria to be used for the production of specific cheeses in Montenegro"- No 49/2008).We are also thankful to Dr Ljubisa

Topisirović for his help in the preparation and application of the national project. The Science and Technology Cooperation (WTZ) of the Austrian agency for international mobility and cooperation in education, science and research (OeAD) is thanked for the support given (bilateral project: „Identity and basic characterization of potential lactic acid bacteria starter cultures isolated from traditionally fermented milk in Montenegro" – ME02/2011).

Received, December 19th, 2017
Accepted April 18th, 2018

REFERENCES

ADŽIĆ, N., N. DOZET (2001): Crnogorski skorup, Univerzitet Crne Gore, Biotehnički institut, Podgorica.

AHMAD, M., D.G. SMITH, S. MAHBOOB (2002): Effect of heat treatment on stationary phase cells of *Enterococcus faecium* and *E. faecalis*. Int. J. Agric. Biol., *2*:234-236.

AKOPYANZ, N, N.O. BUKANOV, T.U. WESTBLOM, S. KRESOVICH, D.E. BERG (1992): DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. Nuc, Acids Res., *20*:5137–5142.

ARIAS, A.C., B. ROBREDO, V.K. SINGH, C. TORRES, D. PANESSO, E.B. MURRAY (2006): Rapid identification of *Enterococcus hirae* and *Enterococcus durans* by PCR and detection of a homologue of the *E. hiraemur*-2 gene in *E. durans*. J. Clin. Microbiol., *44*:1567-1570.

BARUZZI, F., M. MOREA, A. MATARANTE, P.S. COCCONCELLI (2000): Changes in the *Lactobacillus* community during Ricotta fortecheese natural fermentation. J. Appl. Microbiol., *89*:807-814.

BERESFORD,T.P., N.A. FITZSIMONS, N.L. BRENNAN, T.M. COGAN (2001): Recent advances in cheese microbiology. Int. Dairy J., *11*:259-274.

BERTHIER, F., S.D. EHRLICH (1998): Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16S/23S rRNA spacer region. FEMS Microbiol. Lett., *161*:97-106.

BERTHIER, F., S.D. EHRLICH (1999): Genetic diversity within *Lactobacillus sakei* and *Lactobacillus curvatus* and design of PCR primers for its detection using randomly amplified polymorphic DNA. Int. J. Syst.Bacteriol., *49*:997-1007.

CROW, V.L., T. COOLBEAR, P.K. GOPAL, F.G. MARTLEY, L.L. MCKAY, H. RIEPE (1995): The role of autolysis of lactic acid bacteria in ripening of cheese. Int. Dairy J., *5*:855-875.

DASEN, G., J. SMUTNY, M. TEUBER, L. MEILE (1998): Classification and identification of propionibacteria based on ribosomal RNA genes and PCR. Syst. Appl. Microbiol., *21*:251-259.

PERÉZ-CHABELA, M.L., A. TOTOSAUS, I. GUERRERO (2008): Evaluation of thermotolerant capacity of lactic acid bacteria isolated from commercial sausages and the effects of their addition on the quality of cooked sausages. Cienc. Techn. Aliment., *28*:132-138.

DELCOUR, J., T. FERAIN, P. HOLS (2000): Advances in the genetics of thermophilic lactic acid bacteria. Curr.Opin.Biotech.,*11*: 497–504.

DE MAN, J.C., M. ROGOSA, M.E. SHARPE (1960): A medium for the cultivation of lactobacilli. J. Appl. Microbiol., *23*:130-135.

DI CELLO, F., A. BEVIVINO, L. CHIARINI, R. FANI, D. PAFFETTI, S. TABACCHIONI, C. DALMASTRI (1997): Biodiversity of a *Burkholderia cepacia* population isolated from the maize rhizosphere at different plant growth stages. Appl. Environ. Microbiol., *63*:4485–4493.

DOMINGOS-LOPES M.F.P., C. STANTON, P.R. ROSS, M. L.E. DAPKEVICIUS, C.C.G. SILVA (2017): Genetic diversity, safety and technological characterization of lactic acid bacteria isolated from artisanal Pico cheese. Food Microbiol., *63*:178-190.

DOZET N., N. ADŽIĆ, M. STANIŠIĆ, N. ŽIVIĆ (1996): Autohtoni mlječni proizvodi. Poljoprivredni institut, Podgorica.

DUTKA-MALEN, S.,S. EVERS, P. COURVALIN (1995): Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol., *33*:24-27.

ERCOLINI, D. (2013): High-throughput sequencing and metagenomics: moving forward in the culture-independent analysis of food microbial ecology. Appl. Environ. Microbiol., *79*:3148–3155.

FACKLAM, R. (2002): What happened to the streptococci: overview of taxonomic and nomenclature changes. Clin. Microbiol. Rev., *15*:613-630.

FARID, B., M.J. FLORES, A. MOUATS (2006): A rapid PCR based method to distinguish between *Enterococcus* species by using degenerate and species-specific *sod*A gene primers. Afr. J. Biotechnol., *5*:697-702.

FORTINA, M.G., G. RICCI, D. MORA, C. PARINI, PL. MANACHINI (2001): Specific identification of *Lactobacillus helveticus* by PCR with *pep*C, *pep*N and *htr*A targeted primers. FEMS Microbiol. Lett., *198*:85-89.

FRANZ, C.M., W.H. HOLZAPFEL, M.E. STILES (1999): Enterococci at the crossroads of food safety? Int. J. Food Microbiol., *47*:1–24.

FRANZ C.M., W.H. HOLZAPFEL (2006): Enterococci. In: Motarjemi Y., Adams M. (eds) Emerging foodborne pathogens. CRC Press, Boca Raton, Florida, USA, pp. 557-613.

FURET, J.P., P. QUÉNÉE, P. TAILLIEZ (2004): Molecular quantification of lactic acidbacteria in fermented milk products using real-time quantitative PCR. Int. J. Food Microbiol., *97*:197-207.

GOH S.H., Z. SANTUCCI, W.E. KLOOS, M. FALTYN, C.G. GEORGE, D. DRIEDGER, S.M. HEMMINGSEN (1997): Identification of *Staphylococcus* species and subspecies by the chaperonin 60 gene identification method and reverse checkerboard hybridization. J. Clin. Microbiol., *35*:3116-3121.

GOMES, B.C., C.T. ESTEVES, I.C. PALAZZO, A.L. DARINI, G.E. FELIS, L.A. SECHI, B.D. FRANCO, E.C. DE MARTINIS (2008): Prevalence and characterization of *Enterococcus* spp. isolated from Brazilian foods. Food Microbiol., *25*:668-675.

GRAPPIN, R., É. BEUVIE, Y. BOUTON, S. POCHER (1999): Advances in the biochemistry and microbiology of Swiss-type cheeses. Lait, *79*:3-22.

HAMILTON-MILLER, J.M., S. SHAH (1998): Vancomycin susceptibility as an aid to the identification of lactobacilli. Lett. Appl. Microbiol., *26*:153–154.

HEMME, D., C. FOUCAUD-SCHEUNEMANN (2004): *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. Int. Dairy J., *14*:467-494.

KOJIĆ, M., J. LOZO, J. BEGOVIĆ, B. JOVČIĆ, L.J. TOPISIROVIĆ (2007): Characterization of lactococci isolated from homemade kefir. Arch. Biol. Sci., *59*:13-22.

LEE, H.-J., PARK, S.Y., KIM, J. (2000): Multiplex PCR-based detection and identification of *Leuconostoc* species. FEMS Microbiol. Lett., *193*:243-247.

LITOPOULOU-TZANETAKI, E., N. TZANETAKIS (2014): The microfloras of traditional Greek cheeses. Microbiol. Spectrum. *2*: CM-0009-2012. doi:10.1128/microbiolspec.CM-0009-2012

MARTINOVIĆ, A. (2003): Classification of lactic acid bacteria isolated from autochthonous fermented dairy products (Master Thesis). Belgrade, Serbia: University of Belgrade.

MARTINOVIĆ, A., Z. RADULOVIĆ, A. WIND, T. JANZEN, D. OBRADOVIĆ (2005): Isolation and characterization of bacterial flora from farmhouse fermented milk products of Serbia and Montenegro, Acta Vet., (Belgrade) *55*:307-318.

MCAULEY, C.M., K.S. GOBIUS, M.L. BRITZ, H.M. CRAVEN (2012): Heat resistance of thermoduric enterococci isolated from milk. Int. J. Food Microbiol., *154:*162-168.

MCINTOSH, J. (2014): Gut bacteria diversity improves with exercise, study shows. Medical News Today.https://www.medicalnewstoday.com/articles/277960.php

MIRECKI, S., I. IVANOVIĆ, N. NIKOLIĆ (2012): Characteristics of Montenegrian autochtonous "lisnati cheese" JHED, *1*:320-324.

MIRECKI, S., N. POPOVIĆ, N. ANTUNAC, N. MIKULEC, D. PLAVLJANIĆ (2015): Production technology and some quality parameters of Njeguši cheese. Mljekarstvo, *65*:280-286.

MONTEL, M.C., S. BUCHIN, A. MALLET, C. DELBES-PAUS, D.A. VUITTON, N. DESMASURES, F.BERTHIER (2014): Traditional cheeses: Rich and diverse microbiota with associated benefits. Int. J. Food Microbiol., *177*:136–154.

MORANDI, S., M. BRASCA, R. LODI (2011): Technological, phenotypic and genotypic characterisation of wild lactic acid bacteria involved in the production of Bitto PDO Italian cheese. Dairy Sci. Technol., *91*:341-359.

MOREA, M., F. BARUZZI, P.S. COCCONCELLI (1999): Molecular and physiological characterization of dominant bacterial populations in traditional Mozzarella cheese processing. J. Appl. Microbiol., *87*:574–582.

NASER, S.M., F.L. THOMPSON, B. HOSTE, D. GEVERS, P. DAWYNDT, M. VANCANNEYT, J. SWINGS (2005). Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpo*A and *phe*S genes. Microbiology, *151*:2141–2150.

NOMURA, M., M. KOBAYASHI, T. OKAMOTO (2002): Rapid PCR-based method which candetermine both phenotype and genotype of *Lactococcus lactis* subspecies. Appl. Environ. Microbiol., *68*:2209-2213.

PANIZZON J.P., H.L.P. JÚNIOR, N. KNAAK, R.C. RAMOS, D.R. ZIEGLER, L.M. FIUZA (2015): Microbial diversity: relevance and relationship between environmental conservation and human health. Braz. Arch. Biol. Technol., doi: http://dx.doi.org/10.1590/S1516-8913201502821

PARENTE, E., T.M. COGAN (2004): Starter cultures: general aspects. In: Fox P.F., McSweeney P.L.H., Cogan T.M., Guinee T.P. (eds) Cheese: chemistry, physics and microbiology, vol.1.General aspects. Elsevier, Amsterdam, The Netherlands, pp. 123–147.

PFANNEBECKER, J., J. FRÖHLICH (2008): Use of a species-specific multiplex PCR for the identification of pediococci. Int. J. Food Microbiol., *128*:288-296.

POT, B., G.E. FELIS, K. DE BRUYNE, E. TSAKALIDOU, K. PAPDIMITROU, J. LEISNER, P. VANDAMME (2014): The genus *Lactobacillus*. In: Holzapfel W.H., Wood B.J.B. (eds) Lactic acid bacteria - biodiversity and taxonomy.Wiley Blackwell, New Jersey, USA,pp.249-353.

QUIGLEY, L., O. O'SULLIVAN, T.P. BERESFORD, R.P. ROSS, G.F. FITZGERALD, P.D. COTTER (2013a). The microbial content of raw and pasteurized cow's milk as determined by molecular approaches. J. Dairy Sci., *96*:1–10.

QUIGLEY, L, O. O'SULLIVAN, C. STANTON, T.P. BERESFORD, RP. ROSS, G.F. FITZGERALD, P.D COTTER (2013b): The complex microbiota of raw milk. FEMS Microbiol. Rev., *37*:664–698.

RADULOVIĆ, Z., D. RADIN, D. OBRADOVIĆ (2006): Autohtona microflora sjeničkog sira. Prehrambena Ind., *1-2*:48-51.

RADULOVIĆ, Z., J. MIOČINOVIĆ, P. PUDJA, M. BARAĆ, Z. MILORADOVIĆ, D. PAUNOVIĆ, D. OBRADOVIĆ (2011): The application of autochthonous lactic acid bacteria in white brined cheese production. Mljekarstvo, *61*:15-25.

RAMSEY, M., A. HARTKE, M. HUYCKE (2014): The physiology and metabolism of enterococci. In: Gilmore M.S., Clewell D.B., Ike Y., Shankar N (eds) Enterococci – from commensals to leading causes of drug resistant infection. Eye and Ear Infirmary, Boston, Massachusetts, pp. 581-635.

RENOUF V., O. CLAISSE, A. LONVAUD-FUNEL (2006): *rpoB* gene: a target for identification of LAB cocci by PCR-DGGE and melting curves analyses in real time PCR. J.Microbiol. Methods, *67*:162-170.

ROBINSON, K.R. (2002): Dairy microbiology handbook, Wiley-Interscience, New York, USA.

SAMARŽIJA, D., N. ANTUNAC, J. LUKAČ HAVRANEK (2001): Taxonomy, physiology and growth of *Lactococcus lactis*: a review. Mljekarstvo, *51*:35-48.

SÁNCHEZ, J.I., B. MARTÍNEZ, A. RODRÍGUEZ (2005): Rational selection of *Leuconostoc* strains for mixed starters based on the physiological biodiversity found in raw milk fermentations. Int. J. Food Microbiol., *105*:377–387.

SARANTINOPOULOS, P., G. KALANTZOPOULOS, E. TSAKALIDOU (2002): Effect of *Enterococcus faecium* on microbiological, physicochemical and sensory characteristics of Greek Feta cheese. Int. J. Food Microbiol., *76*:93–105.

SETTANNI, L., D. VAN SINDEREN, J. ROSSI, A. CORSETTI (2005): Rapid differentiation and in situ detection of 16 sourdough *Lactobacillus* species by multiplex PCR. Appl. Environ.Microbiol., *71*:3049-3059.

SHANKAR, P.A., F.L. DAVIES (1977): Recent developments in yoghurt starters: a note on the suppression of *Lactobacillus bulgaricus* in media containing ß-glycerophosphate and application of such media to selective isolation of *Streptococcus thermophilus* from yoghurt. Int. J. Dairy Technol., *30*:28-30.

SHEU, S.J., W.Z. HWANG, H.C. CHEN, Y.C. CHIANG, H.Y. TSEN (2009): Development and use of *tuf* gene-based primers for the multiplex PCR detection of *Lactobacillus acidophilus*, *Lactobacillus casei* group, *Lactobacillus delbrueckii*, and *Bifidobacterium longum* in commercial dairy products. J. Food Prot., *72*: 93-100**.**

SILVA L.F., T. CASELLA, E.S. GOMES, M.C.L. NOGUEIRA, J.DE DEA LINDNER, A.L.B. PENNA (2015): Diversity of lactic acid bacteria isolated from Brazilian water buffalo Mozzarella cheese. J. Food Sci., *80*:411-417.

SMIT, G., B.A. SMIT, W.J.M. ENGELS (2005): Flavour formation by lactic acid bacteriaand biochemical flavour profiling of cheese products. FEMS Microbiol. Rev., *29*:591–610.

STILES, J., S. PENKAR, M. PLOCKOVÁ, J. CHUMCHALOVA, L.B. BULLERMANN (2002): Antifungal activity of sodium acetate and *Lactobacillus rhamnosus*. J. Food Prot., *65*:1188-1191.

TEMMERMAN, R., L. MASCO, G. HUYS, J. SWINGS (2006): Application of repetitive element sequence-based (rep-) PCR and Denaturing Gradient Gel Electrophoresis for the identification of lactic acid bacteria in probiotic products. In: Goktepe I, Juneja V.K., Ahmedna M. (eds) Probiotics in food safety and human health. CRC Press, Boca Raton, Florida, USA, pp.207-228.

VEDAMUTHU, E.R. (1994): The dairy *Leuconostoc:* use in dairy products. J. Dairy Sci., *77*:2725–2737.

VENTURA, M., C. CANCHAYA, V. MEYLAN, T.R. KLAENHAMMER, R. ZINK (2003): Analysis, characterization, and loci of the *tuf*genes in *Lactobacillus* and *Bifidobacterium* species and their direct application for species identification. Appl. Environ. Microbiol., *69*:6908–6922.

VESKOVIĆ MORAČANIN, S.M, S. MIRECKI, D.K. TRBOVIĆ, L.R. TURUBATOVIĆ, V.S. KURĆUBIĆ, P.Z. MAŠKOVIĆ (2012): Traditional manufacturing of white cheese in brine in Serbia and Montenegro – similarities and differences. APTEFF,*43:*107-113.

DIVERZITET BAKTERIJA MLEČNE KISELINE IZOLOVANIH IZ TRADICIONALNIH CRNOGORSKIH MLEČNIH PROIZVODA

Mirjana BOJANIĆ RAŠOVIĆ[1], Sigrid MAYRHOFER[2*], Mary A.A. OCHOME[2], Erna AJANOVIC[1], Marija ZUNABOVIC[2], Aleksandra MARTINOVIC[3], Konrad J. DOMIG[2]

[1]Univerzitet Crne Gore, Biotehnički fakultet, Podgorica, Crna Gora
[2]BOKU – Univerzitet za prirodne resurse i nauke, Beč, Departman za hranu i tehnologiju, Institut za hranu i tehnologiju, Beč, Austrija
[3]Univerzitet Donja Gorica, Fakultet za prehrambenu tehnologiju, bezbednost hrane i ekologiju, Donja Gorica, Podgorica, Crna Gora

Izvod

Tradicionalna proizvodnja fermentisanih mlečnih proizvoda u Crnoj Gori sprovodi se bez dodavanja definisanih starter kultura. Ovaj način proizvodnje uključuje bakterije mlečne kiseline (LAB) koje su normalno prisutne u proizvodnom okruženju. Ovaautohtona ("divlja") fermentacijska mikroflora predstavlja rezervoar nepoznatih sojeva. Kako bi se proučio diverzitet LAB-a, ispitano je 25 autohtonih mlečnihproizvoda u Crnoj Gori. Izolacija je sprovedena n amikrobiološkim medijumima M17 i MRS agaru sa ili bez dodataka pod aerobnim i anaerobnim uslovima n atemperaturama od 30°C, 37°C i 44°C. Identifikacija izolata na nivou vrsta obavljena je specifičnim PCR i 16S rDNA sekvenciranjem predstavnika svakog RAPD klastera. RAPD-PCR metoda je korištena za karakterizaciju izolata na nivou soja. Utvrđeno je devet vrsta *Lactobacillus*, pet vrsta *Leuconostoc*, četiri vrste *Enterococcus*, kao i vrste *Lactococcus lactis*, *Pediococcus pentosaceus* i *Streptococcus thermophilus*. Može se zaključiti da je u analiziranim crnogorskim mlečnim proizvodima postojao bogat biodiverzitet bakterija mlečne kiseline. Dalje ispitivanje izolata moglo bi dovesti do razvoja autohtonih starter kultura koje bi dovele do proizvodnje proizvoda karakterističnih za geografsko područje na koje je lokalno stanovništvo naviklo.

Primljeno 19.XII.2017.
Odobreno 18. IV. 2018.