Original scientific paper - Izvorni znanstveni rad UDK: 579.678

Biodiversity of microbial consortia isolated from traditional fresh sheep cheese Karakačanski skakutanac

Tomislav Pogačić[1,2,3,5], Maura D'Andrea[2], Dafni-Maria Kagkli[3,4], Viviana Corich[2,3], Alessio Giacomini[2,3], Enrico Baldan[3], Andreja Čanžek Majhenič[5], Tanja Obermajer[5], Irena Rogelj[5], Dubravka Samaržija[1]*

[1]Department of Dairy Science, Faculty of Agriculture, University of Zagreb, Svetošimunska 25, 10000 Zagreb, Croatia
[2]Laboratorio di microbiologia, CIRVE - Centro Interdipartimentale per la Ricerca in Viticoltura ed Enologia, Università degli Studi di Padova, Via XXVIII Aprile 14, 31015 Conegliano (TV), Italy
[3]Dipartimento di Biotecnologie Agrarie, Facoltà di Agraria, Università degli Studi di Padova, Viale dell'Università 16, 35020 Legnaro (PD) Padova, Italy
[4-present address] European Commission - Joint Research Centre, Institute for Health and Consumer Protection, Molecular Biology and Genomics Unit, via E. Fermi 2749, I-21027, Ispra (VA), Italy
[5]Chair of Dairy Science, Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, 1230 Domžale, Slovenia

Received - Prispjelo: 30.03.2011.
Accepted - Prihvaćeno: 19.07.2011.

Summary

The aim of this study was to assess the structure of indigenous microbial community associated with traditional fresh sheep cheese Karakačanski skakutanac and to preserve autochthonous microbial consortia. Eleven cheeses were sampled during production season (April-September) and subjected to microbiological analysis. Bacterial DNA was isolated by Maxwell®16 DNA system from 99 microbial consortia harvested from three culture media (M17, Rogosa, CATC) on the 1st, 2nd and 3rd day of the cheese shelf life. The extracted bulk DNA ($n = 99$) was used as a template for PCR-ARDRA and PCR-DGGE analysis. There were no dramatic shifts in the bacterial number and structure of the microbial consortia harvested on the 1st, 2nd or 3rd day of the cheese shelf life neither during period of sampling. *Lactococcus lactis* subsp. *lactis* reached the number of 10^7-10^8 CFU g^{-1}, while *Leuconostoc pseudomesenteroides*, *Enterococcus faecalis*, and *Lactobacillus versmoldensis* were identified only at lower dilutions (10^{-2} - 10^{-3}). This first polyphasic microbiological-molecular study of the Karakačanski skakutanac indicated the main LAB representatives associated with the cheese. Obtained autochthonous microbial consortia present a valuable pool of strains for further genetic and functional characterizations.

Key words: fresh sheep cheese, microbial consortia, bulk DNA, ARDRA, DGGE

Introduction

Karakačanski skakutanac (KS) is a traditional fresh sheep cheese made of raw milk in a limited area of eastern Croatia and its production is vanishing (Pogačić et al., 2010a). However, there is an intention to revitalize and standardize the manufacture of the Karakačanski skakutanac cheese; as such type of cheese is rarely produced from raw sheep milk. Therefore a project »Functional dairy products made of sheep milk« was managed with the aim to explore the cheese microbial ecology, peculiar technology, structural and textural characteristics. Re-

*Corresponding author/Dopisni autor: Phone/Tel.: +385 1 239 3646; E-mail: tpogacic@agr.hr; tpogacic@gmail.com

vitalization of its production will be an important step towards protection of the traditional cheeses, and a valuable contribution to development of the Vukovar region that has been strongly and violently devastated and forcedly depopulated during war period (1991-1995) in Croatia. According to historical documents the KS cheese has been produced in the Vukovar region since the end of the 10th century when this region was settled by Karakačani, originated from Greece, which introduced the cheese manufacture for the first time in the region. The cheese technology has already been described (Pogačić et al., 2010a) and the whole process has also been literally presented (Pogačić and Samaržija, 2008).

Typical and traditional foods represent an important patrimony for a region and an opportunity for the development of the rural areas (Dolci et al., 2008a). The indigenous microbiota of traditional cheeses produced from raw milk contributes to development of specific aromatic characteristics in such cheeses (Duthoit et al., 2003; Randazzo et al., 2009a; Settanni and Moschetti 2010). Increasing information on the natural microbial population of dairy products can help in preventing the loss of microbial biodiversity and a wide range of traditional cheeses (Fortina et al., 2003; Marino et al., 2003; Scintu et al., 2007). The indigenous lactic acid bacteria (LAB) of artisanal cheeses have been widely studied by culture-dependent and independent approaches (Ercolini et al., 2004, Juste´ et al., 2008; Randazzo et al., 2009b). Culture-independent molecular approaches have become an alternative to culture-based studies (Jany and Barbier, 2008; Randazzo et al., 2009b; Pogačić et al., 2010b). Amplified ribosomal DNA restriction analysis (ARDRA) has also been used to assess structural diversity of the microbial communities from various environmental (Gich et al., 2000; Guthrie et al., 2000; Rodas et al., 2003; Maukonen and Saarela, 2009) and food samples (Kopermsub and Yunchalard, 2010; Pogačić et al., 2010a). The analysis of the cheese microbiota through cultivable microbial community (bulk cells or microbial consortia) can be used as an alternative to traditional tools in order to identify dominant species based on random isolation and identification of the selected single colonies. Identification of the dominant species can be achieved by sequencing of the DGGE bands arising from the patterns corresponding to the appropriate dilutions (Ercolini et al., 2001; Edenborn and Sexstone 2007; Aponte et al., 2008; Van Horde et al., 2008). However, only a combination of culture-dependent and independent approaches can provide more comprehensive overview of microbial diversity of any microbial ecosystem (Juste´ et al., 2008; Martín-Platero et al., 2008; Cocolin et al., 2009). The role of non starter LAB in the cheese manufacturing (Settani et al., 2010) and its impact on specificity of traditional cheeses varieties (Scintu et al., 2007) have recently been reviewed and updated. Moreover, it is obvious there is a strong intention to preserve the indigenous microflora at EU level (Embarc project, 2009), to optimize methods for its characterization and preservation and to create a European microbial DNA network (Lortal, 2010).

A previous culture-independent study (Pogačić et al., 2010a) gave limited information on the KS indigenous microbiota, as only predominant *Lactococcus lactis* population was detected. Therefore in this follow up study the aims were to extend the knowledge on the microbial ecology of the KS cheese, to assess the structure of indigenous viable microbial community and to collect and preserve autochthonous microbial consortia for further genetic and functional characterizations. The focus was on lactobacilli, lactococci and enterococci as the main representatives of the LAB more frequently found in traditional cheeses. The diversity of indigenous microbiota was studied by culture-dependent approach employing PCR-ARDRA, PCR-DGGE and sequencing of 16S rRNA gene.

Materials and methods

The overall strategy of the study is outlined in Fig. 1.

Cheese sampling

Eleven cheeses were collected from eleven independent batches manufactured from the representative traditional cheese farm in Nuštar (Vukovar region, Eastern Croatia) during production period (April-September) and transported to the laboratory in an ice box. The pH of the cheeses was approximately from 4.7 to 5.0. Samples (n = 11) were stored during the shelf life of three days under refrigerated conditions until further analysis.

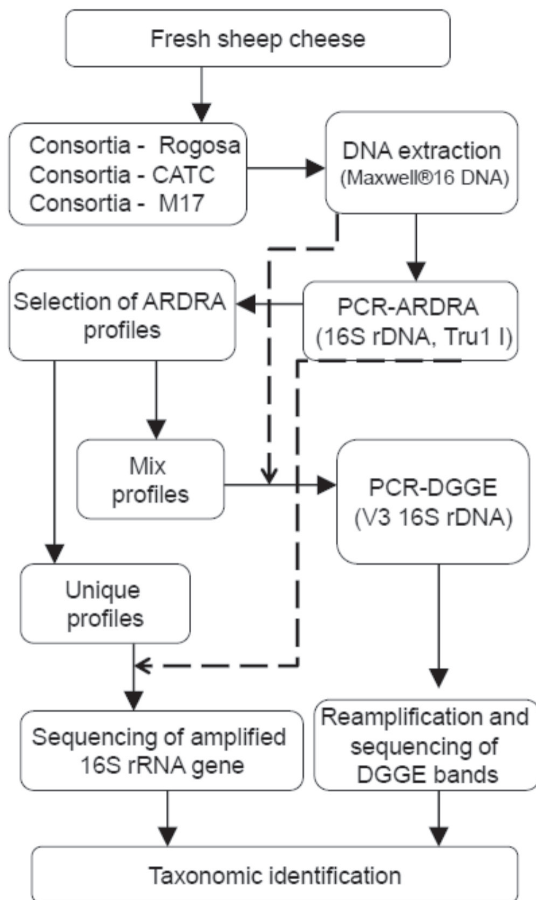

Figure 1. Experimental scheme for identification of microbial consortia isolated from Karakačanski skakutanac cheese

Microbial consortia culturing and harvesting

For culturing and harvesting of the microbial consortia three selective culture media were used: M17 agar (Merck, Germany) for lactococci, CATC agar (Merck, Germany) for enterococci, and Rogosa agar (Merck, Germany) for lactobacilli. Cheeses on the 1st (n = 11), 2nd (n = 11) and 3rd (n = 11) day after manufacturing were aseptically collected and 10 g of samples were homogenized with 90 mL trisodium citrate dihydrate (2 % w/vol) (Kemika, Croatia) in BagMixer® 400 Blender (Interscience, France) for 2 min. Homogenates were serially diluted and appropriate aliquots were spread on M17, CATC and Rogosa agar and incubated at 30 °C for 48 h. Plates with Rogosa agar were incubated in microaerophilic conditions obtained by the Generbox system (Bio-Merieux, France). For every cheese sample (n = 33, 11 cheeses on the 1st, 2nd and 3rd day after manufacturing) and for every culture media (n = 3), 5 plates of the countable dilutions were additionally incubated under conditions already described. After incubation, consortia were harvested by rinsing (2-3 mL Ringer solution) the colonies from the surface of every of the 5 Petri dishes. From each of the 5 plates, 1 mL of consortia was collected in a sterile tube, resulting in a total of 5 mL consortia (bulk cells). Two milliliters of every consortia were subsequently transferred to an ependorf tube and stored at -20 °C until bulk DNA extraction. The remaining consortia (3 mL) were supplemented with glycerol (30 % v/v) and stored at -20 °C for further characterizations. For each of the 33 analyzed cheeses, one consortium was prepared from M17 agar, CATC agar and Rogosa agar, resulting in a total number of 99 consortia for further molecular characterization.

DNA extraction from microbial consortia

Prior DNA extraction microbial consortia (2 mL) were thawed at room temperature. Samples were centrifuged at 3500 g for 10 minutes at 10 °C. After discarding the supernatant, the pellets were resuspended in 400 μL of 1X TE buffer supplemented with 100 μL of lysozyme (25 μg/μL) and 10 μL of mutanolysin (1U/μL) (Sigma, Germany) and incubated at 37 °C for 2 hours. Then entire samples were vortexed and transferred to the first well of predispensed cartridges of Maxwell®16 DNA system (Promega, USA). Before running the Maxwell instrument, 300 μL of a Maxwell elution buffer and 1.5 μL RNAse (4 mg/mL) (Promega, USA) were added to its last cartridge. Further steps were performed according to Maxwell®16 DNA purification kit manual (Promega, USA). The resulting volume of the extracted DNA was ~200 μL. DNA concentration was estimated on agarose gel and it was ~80 ng/μL.

Bacterial strains and preparation of lysed cell suspensions

The bacterial strains from the DSMZ collection (Braunschweig, Germany) and Istituto per la qualità e le tecnologie agroalimentari - Veneto Agricoltura (Thiene, Italy) considered in this study were:

Enterococcus faecalis DSM 20478^T, *Leuconostoc pseudomesenteroides* DSM 20193^T, *Lactobacillus delbrueckii* subsp. *lactis* DSM20072^T, *Lactococcus lactis* subsp. *lactis* F89. Culturing and preparation of lysed cell suspensions was performed as previously described by Pogačić et al., (2010a). The lysed cells suspensions were used as the DNA templates in PCR-ARDRA and PCR-DGGE experiments.

PCR-ARDRA analysis of microbial consortia

Universal bacterial primers pA (5'-AGAGTTTGATCCTGGCT CAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3') were used to amplify 16S rRNA gene (Urlik et al., 1989). The PCR mixture of a final volume 25 μL contained 2.5 μL 10X Taq buffer (Amersham Biosciences, UK), 0.2 mM dNTPs, 0.1 μM of each primer, 1U of Taq polymerase (Applied Biosystems, Italy), 2 μL of the lysed cell suspension or 20 ng of the DNA extracted from microbial consortia. PCR amplifications were carried out in a Bio-Rad cycler (Biorad, Italy) under previously described conditions (Rodas et al., 2003). The PCR products were checked for purity and length on a 1.2 % (w/v) agarose gel containing ethidium bromide (5 μg/mL), for 30 min at 100V in 0.5X TBE buffer (89 mM Tris-acetate, 89 mM boric acid, 1 mM EDTA, pH 8.0), and visualized under UV light. Five hundred nanograms of the amplified 16S rDNA was cleaved with 5 U of *Tru1* I (New England Biolabs, USA) at 65 °C for 2 hours (Pogačić et al., 2010a). The restriction fragments were analyzed on a 2 % (w/v) agarose gel (3 h, 120 V) using a pUC Mix marker (Fermentas) as molecular weight marker. The gel was visualized under UV light and the image was acquired by a digital camera and analyzed by the Kodak EDAS 290 system (Eastman Kodak Company, USA).

Sequencing of the amplified 16S rRNA gene

Representatives of each ARDRA pattern group were chosen for sequence analysis. The PCR products that after restriction analysis exhibited a unique profile were selected to sequencing of the amplified 16S rRNA gene. PCR products were purified using ExoSAP-IT kit (USB Corporation, Italy). To 2 μL of the PCR products 3 μL of milliQ water and 2 μL of ExoSAP-IT were added. The mix was incubated at 37 °C for 15 min, following at 80 °C for 15 min. To the purified PCR products 2 μL of the 3.2 μM forward primer (pA) were added and the mix was incubated at 65 °C for 1 h. Sequencing was performed by BMR Genomics (University of Padova) and sequences were aligned and compared to those in GenBank using the BlastN program (Altschul et al., 1997).

PCR-DGGE fingerprinting of microbial consortia

The consortia which after ARDRA analysis exhibited mixed bacterial profiles and therefore were not identified by sequencing, were subjected to PCR-DGGE analysis. The V3 region of the 16S rRNA gene was amplified using the universal bacterial primer pair V3f (5'-CCT ACG GGA GGC AGC AG-3') and V3r (5'-ATT ACC GCG GCT GCT GG-3'). To the forward primer a GC-clamp (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G) was added according to Muyzer et al., (1993). PCR mixture (final volume 50 μL) contained 5 μL 10X Taq buffer (Amersham Biosciences), 0.2 mM dNTPs, 0.5 μM of each primer, 1.25U of Taq polymerase (Applied Biosystems, Italy) and 20 ng of DNA from consortia. To perform the PCR reaction of control strains, their cell lysate (2 μL) was used as template. PCR amplifications were carried out in a Bio-Rad cycler (Biorad, Italy) under conditions as previously described (Muyzer et al., 1993). PCR products (5 μL) were checked for purity and length by running DNA on a 2 % agarose gel (45 min at 100 V) and visualizing it under UV light. The pUC Mix molecular marker (Fermentas, Italy) was used as standard. Before DGGE analysis, the PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Italy) according to the manufacturer instructions. DGGE experiments were performed using the Dcode system (Biorad, Italy). To facilitate manipulation and handling with DGGE gel, GelBond® PAG Film (0.2 mm) was applied (Lonza, USA). The PCR products (15 μL, ~180 ng) were loaded on a 8 % (w/v) polyacrylamide gel (26 to 58 %, urea - formamide) and separated at the constant temperature of 58 °C for 5 min at 280 V and for 5.5 h at 220 V in 1X TAE buffer. To visualize the DGGE bands, silver staining was applied with a slight modifica-

tion of the method of Sanguinetti et al., (1994). Briefly, the incubation time of the DGGE gels in a 100 mL of the fixing solution (10 % v/v ethanol, 0.5 % v/v acetic acid) containing 0.2 % (w/v) silver nitrate (second step of the coloration protocol) was 15 min instead of 5. The DGGE gels were dried at room temperature and the image was acquired by scanning. The analysis of the consortia fingerprinting profiles obtained with DGGE was performed using GelCompar II software (Applied Maths, Belgium). The similarities between the DGGE patterns were calculated using the Dice coefficient (Giannino et al., 2009), and dendrogram was obtained by the unweighted pair group method with arithmetic averages (UPGMA).

Identification of DGGE bands

Selected bands were excised from the gel with a sterile scalpel 18 hours after DGGE analysis and the DNA solution was prepared as already described by Sanguinetti et al., (1994). Two microliters of the obtained DNA solution were re-amplified using the original pair of universal bacterial primer (V3f, V3r) but without the GC clamp and the identical PCR conditions as already described. PCR amplicons were purified using ExoSAP-IT kit (USB corporation) as already described. To the purified PCR products 2 μL of the 3.2 μM forward primer (V3f) were added and the mix was incubated at 65 °C for 1 h. The prepared samples were delivered for sequencing to BMR Genomics (University of Padova). To determine the closest known relatives of the partial 16S rDNA sequences obtained, sequences were compared to those in GenBank with the BlastN program (Altschul et al., 1997).

Results

PCR-ARDRA analysis of microbial consortia

PCR-ARDRA analysis was performed on the 99 consortia cultured and harvested from the 3 selective culture media: M17 (10^{-7}), Rogosa (10^{-2}), CATC (10^{-3}). The bacterial counts were in the range of 10^{7}-10^{8} cfu/g on M17 agar, 10^{3}-10^{4} cfu/g on CATC agar, and 10^{2}-10^{3} cfu/g on Rogosa agar, respectively. Bulk DNA extraction and purification was performed by Maxwell®16 DNA system and the templates were subjected to PCR-ARDRA analysis. The ARDRA profiles of the consortia originated from the three culture media were unequivocally distinguishable

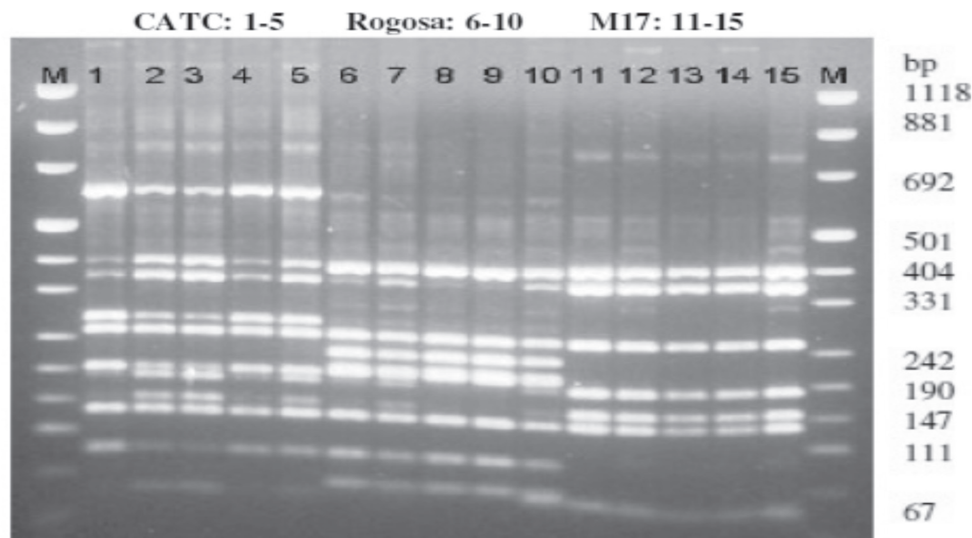

Figure 2. Representative ARDRA profiles of digested 16S rRNA gene of microbial consortia from Karakačanski skakutanac cheese

Lanes: M - molecular marker; 1-5, CATC consortium; 6-10, Rogosa consortium; 11-15, M17 consortium. Identified by sequencing of amplified 16S rRNA gene: *Leuconostoc pseudomesenteroides* (lanes 8, 9); *Lactococcus lactis* subsp. *lactis* (lanes 11-15). Lanes 1-5; 6,7,10: mix profiles.

based on the number and weight of the fragments highlighting dominant LAB species on every medium. There were no dramatic shifts in the bacterial number and structure of the microbial consortia harvested on the 1st, 2nd or 3rd day of the cheese shelf life neither during period of sampling; indicating homogenous structure of the microbial community and a stable ecosystem of the cheese. The representative ARDRA profiles of the consortia considered in this study are shown in Fig. 2.

The majority of the consortia (n = 28) harvested from the M17 culture media exhibited identical ARDRA profiles as presented in Fig. 2, lanes 11 to 15; matching reference strain *Lactococcus lactis* subsp. *lactis*. Unequivocal attribution was also confirmed by sequencing of the amplified 16S rRNA gene used for the restriction analysis. The sequences showed 100 % similarity to *L. lactis* subsp. *lactis* (Acc. No EU483103). The ARDRA profiles of the consortia harvested from M17 culture media which did not unequivocally present one bacterial fingerprint profile (ARDRA profiles not shown), were subjected to further PCR-DGGE analysis to assess the structure of microbial community by separation of the amplified fragments.

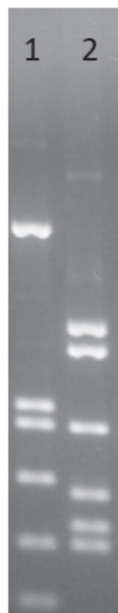

Figure 3. ARDRA profiles of (1) *Ec. faecalis* DSM 20478^T and (2) *Lc. lactis subsp. lactis* DSM20072^T

Among the consortia obtained from the Rogosa culture medium, 25 of them exhibited ARDRA patterns corresponding to *Leuconostoc pseudomesenteroides* (Fig. 2, lanes 8 and 9) that was also confirmed by sequencing of amplified 16S rRNA gene (100 % similarity with *Leuconostoc pseudomesenteroides*, Acc. No. AB326299). However, mixed bacterial profiles were also observed (Fig. 2, lanes 6, 7 and 10) and its structure was further assessed by DGGE. All of the consortia (n = 33) harvested from the CATC agar showed mix ARDRA patterns (Fig. 2, lanes 1-5) of 2 LAB species matching mix of *Enterococcus faecalis* and *Lactococcus lactis* subsp. *lactis* reference strains (Fig. 3). Unequivocal structure of the CATC consortia was further assessed by PCR-DGGE.

PCR-DGGE fingerprinting of microbial consortia

The consortia which microbial structure was not unequivocally assessed by PCR-ARDRA were subjected to PCR-DGGE analysis. Bulk bacterial DNA was amplified by universal bacterial primer (V3f-GC, V3r) targeting V3-16S rRNA gene. Representative DGGE fingerprints of the microbial consortia are shown in Fig. 4. The selected DGGE bands were excised from the gel, reamplified, purified and sequenced. The identified species are reported in Table 1.

The DGGE profile of the microbial consortia demonstrated that *Lactococcus lactis* was present on the all culture media indicating non selectivity of the media and prevalence of this species in the cheese. The DGGE profiles of the all consortia (n = 33) harvested from the CATC media consisted of 2 species: *Enterococus faecalis* and *Lactococcus lactis*. Higher diversity was observed in consortia harvested from M17 media. A multiple copy of the same band (band 2 and 3, Fig. 4) was also detected and identified as *Lactococcus lactis*. At the lower part of the gel few more bands were observed identified as *Citrobacter freundii*, *Pantoea sp.* and *Klebsiella oxitoca*. Consortia harvested from Rogosa medium also demonstrated presence of *Lactococcus lactis* and in only one consortia *Lactobacillus versmoldensis* (band 8, Fig. 4). Several other faint bands obtained from the DGGE gel unfortunately did not reamplify and its identification was not possible.

Table 1. Bacterial species identification after sequencing of the V3-16S rDNA fragments from DGGE gel of Karakačanski skakutanac microbial consortia

Band[a]	Species	Identity %	Genbank Acc. No.
1	*Enterococcus faecalis*	99	AF477496
2, 7,9	*Lactococcus lactis*	100	AB375867
3	*Lactococcus lactis*	100	AB375867
4	*Citrobacter freundii*	100	EU420953
5	*Klebsiella oxitoca*	100	EU420947
6	*Pantoea spp.*	99	EU784085
8	*Lactobacillus versmoldensis*	99	AF502292

[a]The numbers of the bands correspond to the bands in Fig. 4

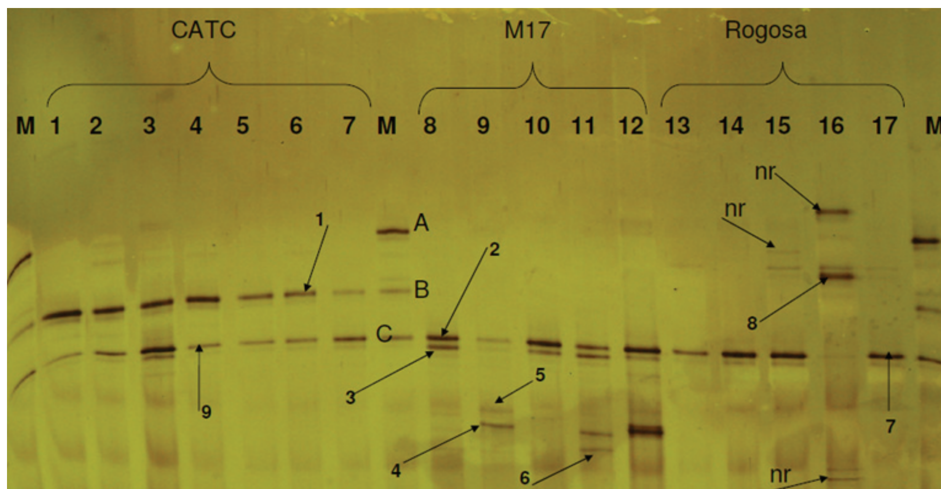

Figure 4. Representative DGGE profiles of V3-16S rRNA gene amplicons of bulk DNA isolated from microbial consortia of Karakačanski skakutanac cheese harvested from 3 selective agar media

M - marker composed of 5 μL of PCR products of each type strain: A - *Lb. delbrueckii* subsp. *lactis*, B - *Ec. faecalis*, C - *Lc. lactis* subsp. *lactis*. nr - not reamplified. Bands indicated by arrows were cut, reamplified and sequenced as reported in materials and methods. The identified species are reported in Table 1. DGGE gel: 26-58 % denaturant gradient; 5.5 h, 220 V; 15 μL ~180 ng of PCR products; silver stained - 15 min

Cluster analysis

DGGE profiles of the microbial consortia were subjected to cluster analysis according to Dice coefficient and the dendogram obtained by UPGMA is presented in Fig. 5. Three main clusters were obtained. The consortia harvested from CATC agar clustered at a similarity level of 65 %, M17 agar at 70 % and Rogosa (except Rogosa-16) at 50 %, respectively.

Discussion

In the present study, structural diversity of microbial communities associated with the traditional fresh sheep cheese Karakačanski skakutanac (KS) was studied by polyphasic approach. Microbial consortia were harvested from the CATC, M17 and Rogosa agar, subjected to extraction of bulk DNA and further characterized by PCR-ARDRA, PCR-DGGE and sequencing. A polyphasic microbiological-molecular approach allowes us to understand better the microbial ecology of the specific traditional fresh sheep cheese. Considering the fact that

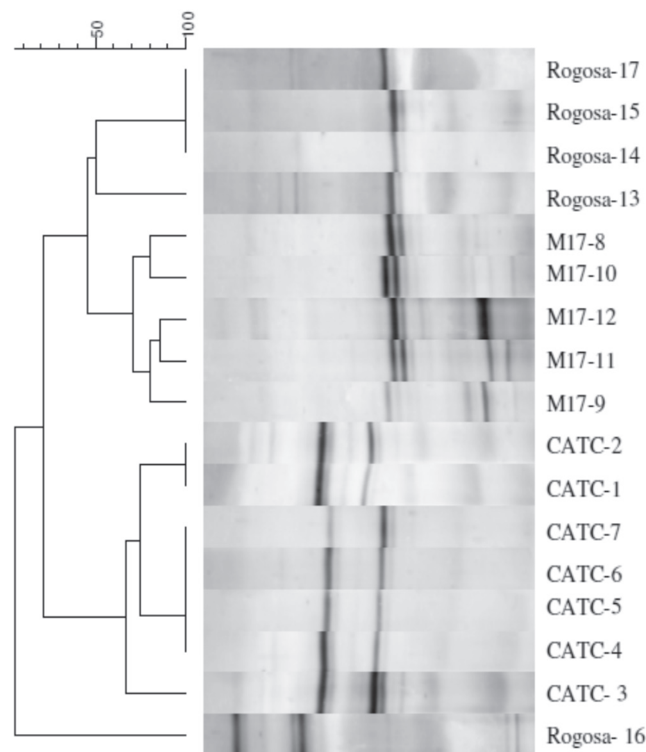

Figure 5. Cluster analysis according to Dice coefficient showing the degree of similarity between DGGE profiles of microbial consortia isolated from Karakačanski skakutanac cheese. CATC consortia clustered at similarity level of 65 %, M17 at 70 % and Rogosa (except Rogosa-16) at 50 %

KS cheese was produced from raw fresh milk immediately after milking, and that no heating of the curd was applied, mesophilic LAB were targeted by incubating the plates at 30 °C. Harvesting colonies and consortia preparation has already been reported as an alternative to randomly picking up and identification of single isolates (Ercolini et al., 2001; Edenborn and Sexstone, 2007; Aponte et al., 2008; Van-Horde et al., 2008; Roth et al., 2010). Additionally, microbial consortia are becoming topic of interest as it is well known that consortia can perform more complicated task and endure more changeable environments than individual strains (Brenner et al., 2008).

The present study demonstrated that the main LAB representatives of the microbial community associated with Karakačanski skakutanac are: *Lactococcus lactis* subsp. *lactis*, *Leuconostoc pseudomesenteroides* and *Enterococcus faecalis*. Several other representatives of the C*itrobacter/Pantoea/Klebsiella* group were also identified in few samples and their presence might be associated to the lower hygienic conditions during milking. The presence of *Lactobacillus versmoldensis* was also observed in only one consortium isolated from the Rogosa agar. There is no information that *Lb. versmoldensis* has been frequently associated with traditional cheeses. Considering available resources *Lb. versmoldensis* was identified by culture-independent PCR-TTGE targeting V3-16S rRNA gene in Egyptian Domiati cheese (El-Baradei et al., 2007), and it was for the first time isolated from raw fermented sausage and identified by BOX-rep-APD, RAPD-PCR and DNA-DNA hybridization (Krockel et al., 2003).

Leuconostoc psudomesenteroides was identified only by PCR-ARDRA in majority of consortia collected from Rogosa medium, while PCR-DGGE allowed separation of mixed communities. It was already reported (Ercolini et al., 2003; Aponte et al., 2008; Mohar-Lorbeg et al., 2009) that different target in PCR (gene, region) might yield different results. In this study the target in PCR-ARDRA was 16S rRNA gene, while in PCR-DGGE V-3 16S rRNA gene. However, there is no method and no

optimal approach that can identify the entire LAB associated with any ecosystem, and just the combination of molecular approaches might provide more objective results (Dolci et al., 2008b; Dolci et al., 2008c; Martín-Platero et al., 2008; Pogačić et al., 2010b). Furthermore, in recent period several studies demonstrated differences in the results obtained by analyzing microbiota of traditional cheeses by culture-dependent and independent approaches, as both approaches demonstrated drawbacks (Rantsiou et al., 2008; Van Horde et al., 2008; Cocolin et al., 2009; Martín-Platero et al., 2009; Alessandria et al., 2010).

The sequencing of DGGE bands did not allow further discrimination of *Lc. lactis* at subspecies level that was also reported by Rantsiou et al., (2008), Van Horde et al., (2008), Giannino et al., (2009) and Serhan et al., (2009). It is interesting to note that subspecies *cremoris* was identified in KS cheese in the previous culture-independent study (Pogačić et al., 2010a). The discrepancy of the results in both studies might be explained by the fact that subspecies *cremoris* was present in the cheese, but it was not cultivated or it was overgrown by subspecies *lactis*. Viable but not cultivable state (VBNC) has already been reported for lactococci (Martín-Platero et al., 2009). Therefore its DNA abundance in bulk DNA isolated from the consortia might be too low to amplify due to preferential amplification of the predominant DNA. Another bias might be the target. Bonetta et al., (2008) successfully discriminated subspecies *lactis* and *cremoris* targeting V1-16 S rRNA gene, while Ercolini et al., (2001b) just demonstrated it was not possible by V3-16S rRNA gene.

However, ARDRA profiles of the consortia, demonstrated that *Lactococcus lactis* subsp. *lactis* was the prevailing LAB in the KS cheese. Additionally, cultivation of bacteria demonstrated that lactococci on M17 agar were present in the relatively high number of 10^7-10^8 cfu/g, while less than 10 countable colonies were observed on the CATC and Rogosa on higher dilutions than 10^{-4}. In Spanish Cueva de la Magaha cheese, enterococci were also detected only at dilutions 10^{-2} to 10^{-4} (Martin-Platero et al., 2008), while in Slovenian Karst cheese low level of lactobacilli (10^2-10^4 cfu/g) was also observed at the beginning of ripening period (Čanžek Majhenič et al., 2007). The dominance of lactococi has recently been reported for several traditional cheeses: Raschera (Dolci et al., 2008a), Robiola di Roccaverano (Bonetta et al., 2008), Casin (Alegría et al., 2009), Istrian cheese (Mrkonjić Fuka et al., 2010). The prevalence of lactococci in the cheese underlines that just indigenous lactococci play a crucial role during fermentation period of the KS cheese. Considering the short shelf life period of the cheese, of only three days, the protective role of lactococci and its antagonistic potential towards pathogenic bacteria (Trmčić et al., 2008; Cretenet et al., 2009; Even et al., 2009; Trmčić et al., 2010) should be considered in further studies as well. However, low level of the other bacterial groups, especially leuconostocs, should not be neglected. They may also play an important role in the fermentation process as they are able to ferment citrate and may also be involved in proteolysis, while *Lc. lactis* is mainly acidifying bacteria thus preventing both alteration and growth of potentially pathogenic bacteria (Casalta et al., 2009). The presence of lactococci on the all culture medium highlighted non-selectivity of the medium and ability of predominant species to adapt to the environmental conditions and outgrow the less abundant LAB (Dolci et al., 2008a; Dolci et al., 2008b; Van Horde et al., 2008). Considering the non-selectivity of the culture medium, differential plate counting of LAB on selective medium, without further molecular identification, might be a tricky aim since it comprises the risk of a false quantification of bacterial groups (Abriouel et al., 2008; Giannino et al., 2009). Van Horde et al., (2008) did not identify enterococci on the KAA agar, neither leuconostocs on the MSE agar. Just the opposite, Dolci et al., (2008a) reported that KAA medium showed a high selectivity level towards enterococci. The reasons for such different results might the strain variability which more or less prefers the "selective" medium. It just highlights that culturing without further molecular identification might provide serious data misinterpretation. Therefore, further molecular approaches are "a must" to obtain reliable taxonomic data about cultivated community.

Conclusion

The present study is contribution to protection of microbial biodiversity and an effort towards revitalization of the manufacture of the vanishing KS cheese. It is the first polyphasic microbiological ap-

proach in the analysis of the KS bacterial community. The pool of the representative viable community, grown up on the medium, were analyzed and also preserved for further studies. The fact that no dramatic shifts were observed in the structure of the indigenous LAB community, demonstrates that the cheese ecogenome is dominated by *Lactococcus lactis* subsp. *lactis*, *Enterococcus faecalis* and *Leuconostoc pseudomesenteroides* as the main LAB representatives of the KS cheese which contribute to its taste and flavour. ARDRA, DGGE and sequencing provided complementary results and just confirmed that combination of methods and approaches provide more objective picture of the cheese microbiota. Further studies should also include genetic and functional characterization of the indigenous isolates as they might posses interesting flavour and protective capabilities.

Bioraznolikost mikrobnih konzorcija izoliranih iz tradicionalnog svježeg ovčjeg sira Karakačanski skakutanac

Sažetak

Cilj ovog rada bio je analizirati strukturu mikrobnih konzorcija tradicionalnog svježeg ovčjeg sira Karakačanski skakutanac. Jedanaest sireva sakupljeno je tijekom sezone proizvodnje od travnja do rujna. Mikrobni konzorciji sakupljeni su sa 3 hranjive podloge (M17, Rogosa, CATC) od 11 sireva nakon prvog, drugog, i trećeg dana proizvodnje. Mikrobna DNA je izolirana iz 99 konzorcija, te korištena u PCR-ARDRA i PCR-DGGE analizi. Nije bilo promjene u strukturi mikrobnih konzorcija sakupljenih prvog, drugog i trećeg dana nakon proizvodnje, niti tijekom sezone. Utvrđena je dominantnost populacije *Lactococcus lactis* subsp. *lactis* (10^7-10^8 CFU g^{-1}), dok su ostale vrste bakterija mliječne kiseline, *Leuconostoc pseudomesenteroides*, *Enterococcus faecalis* i *Lactobacillus versmoldensis*, identificirane samo na nižim razrjeđenjima (10^{-2}-10^{-3}). Ova prva mikrobiološko-molekularna analiza tradicionalnog sira Karakačanski skakutanac omogućila je uvid u strukturu njegove specifične mikrobne populacije. Sakupljeni mikrobni konzorciji predstavljaju značajan izvor sojeva za daljnju genetsku i funkcionalnu karakterizaciju.

Ključne riječi: svježi ovčji sir, mikrobni konzorciji, DNA, ARDRA, DGGE

Acknowledgement:

The molecular approach presented in this study was carried out during PhD student Pogačić Tomislav's research training period at University of Padova (May - July / 2008), while the microbiological approach was carried out at University of Ljubljana (March - April / 2008). Tomislav Pogačić would like to thank: Professor Viviana Corich (ex-supervisor) and Professor Alessio Giacomini for unconditional direct provision of the research training period at UNIPD research facilities and for unequivocal direct support provided, being the crucial elements at the given moments for possibility and feasibility of the research in a limited period of time; all the colleagues at both Universities for all kind of help, valuable advices, constructive discussions and kind hospitality; University of Padova and Coimbra Group for scholarship granted in the frame of Coimbra Group Hospitality Scheme; Professor Irena Rogelj for permission to carry out microbiological approach. The authors are grateful to the cheese producer for collaboration in the project. The study was partially supported by Croatian Ministry of Science (Project No. 178-1782128-2124) and by LATSIER project (Regione Veneto).

Disclaimer

The views expressed in this publication are the sole responsibility of the author(s) and do not necessarily reflect the views of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use which might be made of the information contained herein. This disclaimer is also valid for paper: Pogačić, T. et al., *Mljekarstvo* 61 (1) 3-14 (2011).

References

1. Abriouel, H., Martín-Platero, A., Maqueda, M., Valdivia, E., Martínez-Bueno, M. (2008): Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. *International Journal of Food Microbiology 127*, 200-208.
2. Altschul, S.F., Madden, T.L., Shaffer, A.A., Zhang, Z., Miller, W., Lipman, D.J., (1997): Gapped BLAST and PSI-BLAST: A new generation of protein database database search programs. *Nucleic Acids Research 25*, 3389-3402.

3. Allessandria, V., Dolci, P., Rantsiou, K., Pattono, D., Dalmasso, A., Civera, T., Cocolin, L. (2010): Microbiota of Planalto de Bolona: an artisanal cheese produced in uncommon environmental conditions in the Cape Verde Islands. *World Journal of Microbiology and Biotechnology 26*, 2211-2221.
4. Alegría, A., Álvarez-Martín, P., Sacristán, N., Fernández, E., Delgado, S., Mayo, B. (2009): Diversity and evolution of the microbial populations during manufacture and ripening of Casin, a traditional Spanish, starter-free cheese made from cow's milk. *International Journal of Food Microbiology 136*, 44-51.
5. Aponte, M., Fusco, V., Andolfi, R., Coppola, S. (2008): Lactic acid bacteria occurring during manufacture and ripening of Provolone del Monaco cheese: Detection by different analytical approaches. *International Dairy Journal 18*, 403-413.
6. Bonetta, S., Bonetta, S., Carraro, E., Rantsiou, K., Cocolin, L. (2008): Microbiological characterization of Robiola di Roccaverano cheese using PCR-DGGE. *Food Microbiology 25*, 786-792.
7. Brenner, K, You, L., Arnold, H.F. (2008): Engineering microbial consortia: a new frontier in synthetic biology. *Trends in Biotechnology 9*, 483-489.
8. Casalta, E., Sorba, J.M., Aigle, M., Ogier, J.C. (2009): Diversity and dynamic of the microbial community during the manufacture of Calenzana, an artisanal Corsican cheese. *International Journal of Food Microbiology 133*, 243-251.
9. Cocolin, L., Nucera, D., Alessandria, V., Rantsiou, K., Dolci, P., Grass, M.A., Lomonaco, S., Civera, T. (2009): Microbial ecology of Grogonzola rinds and occurence of different biotypes of Listeria monocytogenes. *International Journal of Food Microbiology 133*, 200-205.
10. Cretenet, M., Even, S., Charlier, C., Nouille, S., Cocaign-Bousquet, M., Loubiere, P., Le Loir, Y. (2009): *Lactococcus lactis* antagonistic potential on *Staphylococcus aureus* in cheese matrix. 4th IDF Dairy Science and Technology Week, Book of Abstract, 34.
11. Čanžek Majhenič, A., Mohar-Lorbeg, P., Rogelj, I. (2007): Characterisation of the *Lactobacillus* community in traditional Karst ewe's cheese. *International Journal of Dairy Technology 60*, 182-190.
12. Dolci, P., Alessandria, V., Zeppa, G., Rantsiou, K., Cocolin, L. (2008a): Microbiological characterization of artisanal Rashera PDO cheese: analysis of its indigenous lactic acid bacteria. *Food Microbiology 25*, 392-399.
13. Dolci, P., Allessandria. V., Rantsiou, K., Rolle, L., Zeppa, G., Cocolin, L. (2008b): Microbial dynamic of Castelmango PDO, a traditional Italian cheese, with a focus on lactic acid bacteria ecology. *International Journal of Food Microbiology 122*, 302-311.
14. Dolci, P., Barmaz, A., Zenato, S., Pramotton, R., Alessandria, V., Cocolin, L., Rantsiou, K., Ambrosoli, R. (2008c): Maturing dynamics of surface microflora in Fontina PDO cheese studied by culture-dependent and - independent methods. *Journal of Applied Microbiology 106*, 278-287.
15. Duthoit, F., Godon, J.J., Montel, M.C. (2003): Bacterial community dynamics during production of registered designation of origin Salers cheese as evaluated by 16S rRNA Gene Single-Strand Conformation Polymorphism Analysis. *Applied and Environmental Microbiology 69*, 3840-3848.
16. Edenborn, S.L., Sexstone, A.J. (2007): DGGE fingerprinting of culturable soil bacterial communities complements culture-independent analyses. *Soil Biology & Biochemistry 39*, 1570-1579.
17. El-Baradei, G., Delacroix-Buchet, A., Ogier, J.C. (2007): Biodiversity of bacterial ecosystem in traditional Egyptian cheese. *Applied and Environmental Microbiology 73*, 1248-1255.
18. Embarc Project (2009): www.embarc.eu (accessed 15.3.2011.).
19. Ercolini, D., Moshetti. G., Blaiotta, G., Coppola, S. (2001): The potential of a polyphasic PCR-DGGE approach in evaluating microbial diversity of natural whey cultures for water-buffalo mozzarella cheese production: Bias of culture - dependent and culture-independent analyses. *Systematic and Applied Microbiology 24*, 610-617.
20. Ercolini, D., Moscheti, G., Blaiotta, G., Coppola, S. (2001b): Behaviour of variable V3 region from 16S rDNA of lactic acid bactria in denaturing gradient gel electrophoresis. *Current Microbiology 42*, 199-202.
21. Ercolini, D., Hill, P.J., Dodd, C. (2003): Bacterial community structure and location in Stillton cheese. *Applied and Environmental Microbiology 69*, 3540-3548.
22. Ercolini, D., Mauriello, G., Blaiotta, G., Moschetti, G., Coppola, S. (2004): PCR-DGGE fingerprints of microbial succession during a manufacture of traditional water buffalo mozzarela cheese. *Journal of Applied Microbiology 96*, 263-270.
23. Even, S., Charlier, C., Nouaille, S., Ben Zakour, N.L., Cretenet, M., Cousin, J.F., Gautier, M., Cocaign-Bousquet, M., Loubiere, P., Le Loir, Y. (2009): *Staphylococcus aureus* Virulence Expression Is Impaired by *Lactococcus lactis* in Mixed Cultures**.** *Applied and Environmental Microbiology 75*, 4459-4472.
24. Fortina, M.G., Ricci, G., Acquati, A., Zeppa, G., Gandini., A., Manachini., P.L. (2003): Genetic characterization of some lactic acid bacteria occurring in an artisanal protected denomination origin (PDO) Italian cheese, the Toma piemontese. *Food Microbiology 20*, 397-404.
25. Giannino, M.L., Marzotto, M., Dellaglio, F., Feligni, M. (2009): Study of microbial diversity in raw milk and fresh curd used for Fontina cheese production by culture-independent methods. *International Journal of Food Microbiology 130*, 188-195.
26. Gich, F.B., Amer, E., Figueras, J.B., Abella, C.A., Balaguer, D.M., Poch, M. (2000): Assessment of microbial community structure changes by amplified ribosomal DNA restriction analysis (ARDRA). *International Microbiology 3*, 103-106.
27. Guthrie, J., Moriarty, D., Blackall, L. (2000): DNA extraction from coral reef sediment for the PCR. *Journal of Microbiological Methods 43*, 73-80.

28. Jany, J.L., Barbier, G. (2008): Culture-independent methods for identifying microbial communities in cheese. *Food Microbiology 25*, 839-848.

29. Juste´, A., Thomma, B.P.H.J., Lievens, B. (2008): Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes. *Food Microbiology 25*, 745-761.

30. Krockel, L., Schillinger, U., Franz, C.M.A.P., Bantleon., A., Ludwig, W. (2003): *Lactobacillus versmoldensis* sp. nov., isolated from raw fermented sausage. *International Journal of Systematic and Evolutionary Biology 53*, 513-517.

31. Lortal, S. (2010): European consortium of microbial resources centers http://www.embarc.eu/1-SLortal.pdf.

32. Marino, M., Maifreni, M., Rondini, G. (2003): Microbiological characterization of artisanal Montasio cheese. *FEMS Microbiology Letters 229*, 133-140.

33. Martín-Platero, A.M., Valdivia, E., Maqueda, M., Martín-Sánchez, I., Martínez-Bueno, M. (2008): Polyphasic approach to bacterial dynamics during the ripening of Spanish farmhouse cheese, using culture-dependent and - independent methods. A*pplied and Environmental Microbiology 74*, 5662-5673.

34. Martín-Platero, A.M., Maqueda, M., Valdivia, E., Purswani, J., Bueno, M.M. (2009): Polyphasic study of microbial communities of two Spanish farmhouse goats milk cheeses from Sierra de Aracena. *Food Microbiology 26*, 294-304.

35. Maukonen, J, Saarela, M. (2009): Microbial communities in industrial environment. C*urrent Opinion in Biotechnology 12*, 238-243.

36. Mohar Lorbeg, P., Čanžek Majhenič, A., Rogelj, I. (2009): Evaluation of different primers pairs for PCR-DGGE analysis of cheese associated enterococci. *Journal of Dairy Research 76*, 265-271.

37. Mrkonjić Fuka, M., Engel, M., Skelin, A., Redžepović, S., Schloter, M. (2010): Bacterial communities associated with the production of artisanal Istrian cheese. *International Journal of Food Microbiology 142*, 19-24.

38. Muyzer, G., De Wall, E., Uitterlinden, A.G. (1993): Profiling of complex microbial populations by Denaturating Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology 59*, 695-700.

39. Pogačić, T., Samaržija, D. (2008): Traditional fresh sheep cheese Karakačanski skakutanac. http://bib.irb.hr/datoteka/477811.Karakacanski_skakutanac_-_traditional_fresh_sheep_cheese.pdf (accessed 29.3.2011.).

40. Pogačić, T., Samaržija, D., Corich, V., D'Andrea, M., Kagkli, D.M., Giacomini, A., Čanžek-Majhenič, A., Rogelj, I. (2010a): Microbiota of Karakačanski skakutanac, an artisanal fresh sheep cheese studied by culture-independent PCR-ARDRA and PCR-DGGE. *Dairy Science & Technology 90*, 641-648.

41. Pogačić, T., Kelava, N., Zamberlin, Š., Dolenčić-Špehar, I., Samaržija, D. (2010b): Methods for culture-independent identification of lactic acid bacteria in dairy products. *Food Technology and Biotechnology 48*, 3-10.

42. Randazzo, C.L., Pitino, I., Ribbera, A., Caggia, C. (2009a): Pecorino Crotonese cheese: Study of bacterial population and flavour compounds. *Food Microbiology 27*, 363-374.

43. Randazzo, C.L., Caggia, C., Neviani, E. (2009b): Application of molecular approaches to study lactic acid bacteria in artisanal cheeses. *Journal of Microbiological Methods 78*, 1-9.

44. Rantsiou, K., Urso, R., Dolci, P., Comi, G., Cocolin, L. (2008): Microflora of Feta cheese from four Greek manufacturers. *International Journal of Food Microbiology 126*, 36-42.

45. Rodas, A., Ferrer Sergi, M., Isabel, P. (2003): 16S-ARDRA, a Tool for Identification of Lactic Acid Bacteria Isolated from Grape Must and Wine. *Systematic and Applied Microbiology 26*, 412-422.

46. Roth, E., Miescher Schwenninger, S., Hasler, M., Eugster-Meier, M., Lacroix, C. (2010): Population dynamics of two antilisterial cheese surface consortia revealed by temporal temperature gradient gel electrophoresis. *BMC Microbiology 10*, 1-14.

47. Sanguinetti, C.J., Dias Neto, E., Simpson, A.J. (1994): Rapid Silver staining and recovery of PCR products separated on polyacrylamide gels. *Bio Techniques 17*, 914-921.

48. Serhan, M., Cailliez-Grimal, C., Borges, F., Revol-Junelles, A-M. (2009): Bacterial diversity of Darfiyeh, a Lebanese artisanal raw goat's milk cheese. *Food Microbiology 26*, 645-652.

49. Settani, L., Moschetti, G. (2010): Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. *Food Microbiology 27*, 691-697.

50. Scintu, M.F., Piredda, G. (2007): Typicity and biodiversity of goat and sheep milk products. *Small Ruminant Research 68*, 221-231.

51. Trmčić, A., Obermajer, T., Rogelj, I., Bogovič Matijašić, B. (2008): Short Communication: Culture-independent detection of lactic acid bacteriocin genes in two traditional Slovenian raw milk cheeses and their microbial consortia. *Journal of Dairy Science 91*, 4535-4541.

52. Trmčić, A., Obermajer, T., Čanžek Majhenič, A., Rogelj, I., Bogovič Matijašić, B. (2010): In-situ inhibition of Staphylococcus aureus by lactic acid bacteria consortia from two traditional Slovenian raw milk cheeses. M*ljekarstvo 60*, 183-190.

53. Urlik, E., Rogall, T., Blocker, H., Emde, M., Bottger, E.C. (1989): Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Research 17*, 7843-7853.

54. Van Horde, K., Verstraete, T.,Vandamme, P., Huys, G. (2008) Diversity of lactic acid bacteria in two Flemish artisan raw milk Goude-type cheeses. *Food Microbiology 25*, 929-935.