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Note

Microbiota of Karakačanski skakutanac, an artisanal fresh sheep cheese studied by culture-independent PCR-ARDRA and PCR-DGGE

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Abstract – Karakačanski skakutanac is an artisanal fresh sheep cheese produced on a small scale in a limited area of eastern Croatia. It is manufactured from unrefrigerated raw sheep milk immediately after milking, without the addition of starter culture, and coagulated with industrial rennet. To date, no microbiological or molecular characterization of the biodiversity of the microbiota has been performed. The objective of this study was to obtain an initial insight into the biodiversity of the microbial community associated with this cheese during the production season and shelf life period. Eleven cheeses were obtained from a dairy farm at 14-day intervals during the lactation period of east Friesian sheep in 2007. Bacterial DNA was isolated directly from cheese on the first, second and third day of the cheese shelf life, resulting in a total of 33 DNA samples. Extracted DNA was used as a template for PCR-ARDRA and PCR-DGGE analysis. The use of dual culture-independent approaches revealed similar results and indicated predominance of *Lactococcus lactis*.

microbiota / fresh sheep cheese / ARDRA / DGGE / *Lactococcus lactis*

摘要 – PCR-ARDRA 和 PCR-DGGE 法研究新鲜羊乳干酪 (Karakačanski skakutanac) 中微生物菌群。Karakačanski skakutanac 是一种手工制作的新鲜羊乳干酪，产于克罗地亚东部某些特定地区。采用刚挤下来的羊乳制作，不添加发酵剂而以工业凝乳酶凝乳。目前尚无其微生物菌群生物多态性的微生物特征或分子特征的报道。本研究对该干酪的生产和储存过程中有关微生物菌群的生物多样性变化进行了初步探讨。每隔 14 天对 2007 年东部弗里斯兰产的干酪取一次样，共 11 批。在干酪成熟的第一天、第二天和第三天直接从干酪中提取细菌 DNA，得到 33 个 DNA 样品。提取得到的 DNA 用作 PCR-ARDRA 和 PCR-DGGE 分析的模板。两种方法鉴定的结果一致，并发现乳酸乳球菌 (*Lactococcus lactis*) 是优势菌种。

微生物菌群 / 鲜羊乳干酪 / ARDRA / DGGE / 乳酸乳球菌

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Résumé – Évaluation de la microflore du Karakačanski skakutanac, un fromage frais artisanal au lait de brebis, par les méthodes culture-indépendantes PCR-ARDRA et PCR-DGGE. Le Karakačanski skakutanac est un fromage frais artisanal, produit à petite échelle dans une aire limitée de l'est de la Croatie. Il est fabriqué juste après la traite à partir de lait cru de brebis non réfrigéré, sans addition de levains, et coagulé par de la présure industrielle. La biodiversité de la microflore n'a jusqu'à présent jamais été caractérisée, ni par des méthodes microbiologiques classiques ni par des méthodes moléculaires. L'objectif de cette étude était d'obtenir un premier aperçu de la biodiversité de l'écosystème bactérien associé à ce type de fromage au cours de la période de production et de la durée de conservation. Onze fromages ont été collectés en 2007 d'une ferme laitière tous les 14 jours durant la période de lactation de brebis de race Frisonne. L'ADN bactérien a été isolé directement du fromage les 1er, 2^{e} et 3^{e} jours de stockage, donnant 33 échantillons. L'ADN extrait était utilisé pour analyse par PCR-ARDRA et PCR-DGGE. Les deux méthodes culture-indépendantes mises en œuvre ont conduit à des résultats similaires montrant la prédominance de *Lactococcus lactis*.

microflore / fromage frais de brebis / ARDRA / DGGE / *Lactococcus lactis*

1. INTRODUCTION

Karakačanski skakutanac is an artisanal fresh sheep cheese produced on a small scale in a limited area of eastern Croatia. Throughout centuries, it has been produced from raw sheep milk immediately after milking using lamb rennet, without the addition of starter culture and curd heating. In the past, a wooden vat filled with fresh cheese was stored for three days in water well before consumption. Nowadays, the cheese is stored in the refrigerator and consumed fresh, within three days after production. The pH value of the fresh cheese is around 5.6 (on the first day after manufacturing), 5.0 (on the second) and to 4.8 (on the third), respectively. The milky taste and flavour, lamellar structure and soft texture are the main sensory characteristics of this traditional cheese. Unfortunately, the manufacture of Karakačanski skakutanac is vanishing as it is only produced by a single producer. Nowadays, there is an intention to maintain and further develop its production and to standardize its quality.

The majority of the cheeses produced from raw sheep milk are hard and long ripened and their unique characteristics arise from specific indigenous microbiota which contributes to diversity of taste and flavour of artisanal cheeses [3, 4]. Autochthonous microbiota of such cheeses represent a heritage which has to be protected and preserved, as it contributes to natural microbial biodiversity and typical characteristics of the traditional cheeses [11]. The indigenous microbiota of artisanal cheeses has been widely studied by culture-independent molecular approaches that are based on the amplification of bulk bacterial DNA extracted directly from cheese, targeting a selected gene or variable region(s) of the selected gene [1, 6, 14]. Amplified ribosomal DNA restriction analysis (ARDRA) has also been used to assess putative genotypic changes in the community over time or to compare communities subject to different environmental conditions [9, 10].

As microbiota of Karakačanski skakutanac had never been studied, the aim of this study was to obtain an initial insight into its biodiversity by culture-independent PCR-ARDRA and PCR-DGGE.

2. MATERIALS AND METHODS

2.1. Cheese samples

Cheese samples (n = 11) were collected from a dairy farm in Slavonia (eastern Croatia) at 14-day intervals from April to September 2007. The cheeses were

manufactured from unrefrigerated raw sheep milk immediately after morning milking without the addition of starter culture. Milk coagulation was achieved using commercial lamb rennet. Following coagulation at 32 °C for about 45 min, thick curd slices were transferred to cheesecloth for the first drainage of the whey for 30 min. After the whey drainage without pressing, the curd was subsequently pressed into colander for 6 h. After pressing, the fresh cheese was transported to the laboratory in an ice box and stored at +4 °C for three days. During storage, on the first, second and third day of shelf life, samples of the cheeses were taken, resulting in a total of 33 cheese samples for the DNA extraction. The samples were stored at −80 °C until DNA extraction.

2.2. DNA extraction from cheese samples

Cheese samples (10 g) were homogenized in 90 mL trisodium citrate dihydrate (2% w/v) with Bagmixer (Interscience, France). Cheese homogenates (10 mL) were stored at −20 °C till DNA extraction. Prior to extraction, cheese homogenates were thawed at room temperature and centrifuged (6000 rpm, 10 min, 10 °C). Supernatants were decanted, and the pellets were resuspended in 2 mL of milli-Q water and additionally centrifuged (3500× *g*, 10 min, 10 °C). Finally, the pellets were resuspended in 400 μL of 1 X TE buffer supplemented with 100 μL of lysozyme (25 $\mu g \cdot \mu L^{-1}$) and 10 μL of mutanolysin (1 $U \cdot \mu L^{-1}$) (Sigma, Germany), and incubated at 37 °C for 2 h. After incubation, samples were vortexed and transferred to cartridge of a Maxwell 16 DNA system (Promega, USA). The procedure described by the Maxwell 16 DNA system was followed from this step onwards. The extracted DNA was quantified on 0.5% agarose gel and it was ~ 100 $ng \cdot \mu L^{-1}$.

2.3. DNA extraction from type and reference strains

The reference and type strains from the DSMZ collection (Braunschweig, Germany) and Istituto per la qualità e le tecnologie agroalimentari – Veneto Agricoltura (Thiene, Vicenza) were used as follows: *Enterococcus faecalis* DSM20478^T, *Lactobacillus gasseri* DSM20243^T, *Lactobacillus acidophilus* DSM20079^T, *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM20081^T, *Lb. delbrueckii* subsp. *lactis* DSM20072^T, *Lactococcus lactis* subsp. *lactis* F89 and *Lactobacillus fermentum* A85. Lactococci and enterococci were cultured in M17 agar (Oxoid), while lactobacilli were cultured in MRS agar (Oxoid). The plates were incubated at the appropriate temperatures under anaerobic conditions. One colony for each strain was picked from the plate and transferred into 50 μL of a freshly prepared lysis solution (SDS 0.25% w/v, NaOH 0.05 $mol \cdot L^{-1}$), resuspended and vortexed (1 min). After incubation (95 °C, 15 min) and centrifugation (10 000× *g*, 4 °C, 10 min), the supernatant was 10-fold diluted in distilled H_2O, vortexed and stored at −20 °C. Cell lysis suspensions were used as the DNA templates.

2.4. PCR-ARDRA analysis

Universal bacterial primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′) were used to amplify 16S rDNA [19]. The PCR mixture (25 μL) contained 10 X Taq buffer (Amersham Biosciences), 200 $\mu mol \cdot L^{-1}$ dNTPs, 1 $\mu mol \cdot L^{-1}$ of each primer, 1 U of Taq polymerase, 2 μL of the cell lysis suspension or 20 ng of the DNA extracted from cheese. PCR amplifications were carried out in a Bio-Rad cycler (Hercules, California) under conditions as previously described [17], with a modified denaturation step (2 min). The PCR products were visualized on a 1.2% (w/v)

agarose gel containing ethidium bromide. The amplified fragments were digested (65 °C, 2 h) in 20 μL of incubation buffer containing 5 U of *Tru1* I (New England Biolabs) and enough PCR product (6–10 μL) to give 500 ng of the rDNA. The restriction fragments were analysed on a 2% (w/v) agarose gel (3 h, 120 V) using a pUC Mix marker (Fermentas) as a ladder. The gel was visualized under UV light, and the image was acquired by a Kodak EDAS 290 system (Kodak).

2.5. Sequencing of the amplified 16S rDNA used for ARDRA digestion

PCR products were purified using ExoSAP-IT kit (USB Corporation). To 2 μL of the purified PCR products, 3.2 μmol·L^{-1} of 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). Sequences were aligned and compared to those in GenBank database with the BlastN program.

2.6. PCR-DGGE analysis

The V3 region of the 16S rDNA was amplified using the universal bacterial primers, V3f (5′-CCT ACG GGA GGC AGC AG-3′) and V3r (5′-ATT ACC GCG GCT GCT GG-3′). A GC clump was added according to Muyzer et al. [13]. PCR mixture (50 μL) contained 10 X Taq buffer (Amersham Biosciences), 200 μmol·L^{-1} dNTPs, 0.5 μmol·L^{-1} of each primer, 1.25 U of Taq polymerase, 20 ng of DNA isolated directly from cheese samples or 2 μL of cell lysis suspension. PCR amplifications were carried out in a Bio-Rad cycler (Hercules, California) under the following conditions: initial denaturation at 95 °C for 7 min, 20 cycles at 94 °C for 1 min, 65 °C for 1 min and 72 °C for 1 min with a 1 °C touchdown every second cycle, followed by 10 cycles with an annealing temperature of 55 °C, and extension for 10 min at 72 °C. PCR products (5 μL) were visualized on 2% agarose gel. Before DGGE analysis, the PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). DGGE experiments were performed using the Dcode system (Bio-Rad) as previously described [13]. PCR products (15 μL) were loaded on an 8% (w/v) polyacrylamide gel (26–58% urea – formamide) and electrophoresed at the constant temperature of 58 °C for 5 min at 280 V and for 4 h at 220 V. The marker used for DGGE analysis was constructed by mixing PCR products (5 μL) of three strains in one tube and applied to DGGE gel. To visualize the DGGE bands, silver staining was applied [18], with a modified incubation time (15 min) of the gel in the fixing solution. The DGGE gel image was acquired by scanning.

2.7. Sequencing of the DGGE bands

The DGGE bands to be sequenced were prepared according to Sanguinetti et al. [18]. Two microliters of the extracted DNA solution were reamplified as previously described (Sect. 2.6) using the same primers but without the GC clump. PCR amplicons were purified as previously described (Sect. 2.5). Sequencing was performed by BMR Genomics (University of Padova). Sequences were aligned and compared to those in GenBank database with the BlastN program.

3. RESULTS AND DISCUSSION

In the present study, the DNA extracted from Karakačanski skakutanac cheese was subjected to PCR-ARDRA and PCR-DGGE to obtain an initial insight into the biodiversity of the cheese microbiota. PCR-ARDRA was initially applied to

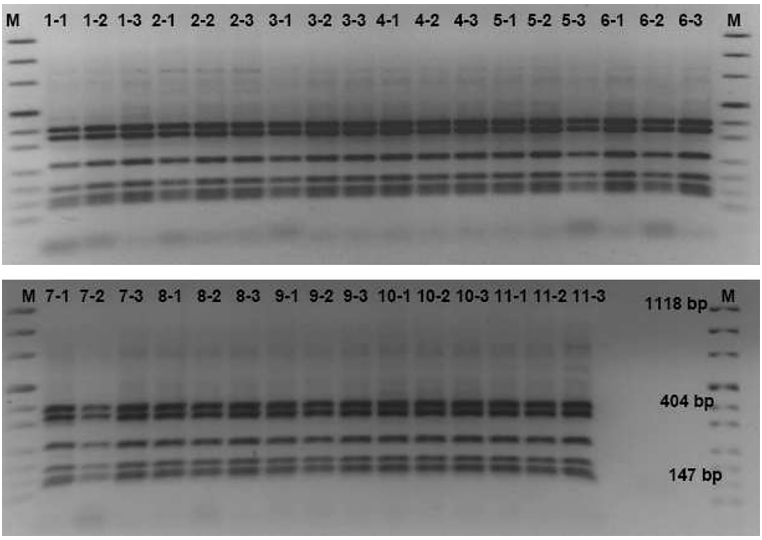

Figure 1. ARDRA profiles of digested (*Tru1* I) 16S rDNA amplicons of total DNA extracts from 11 Karakačanski skakutanac cheeses. The samples were collected from a dairy farm at 14-day intervals from April (1) to September (11). First sample – first day (1-1), first sample – second day (1-2), first sample – third day (1-3), 1 – first sample, 11 – last sample, M – molecular marker. Sequences of amplified 16S rDNA used for ARDRA experiments were 100% homologous to *Lc. lactis* subsp. *lactis* (Accession No. EU483103).

reference and type strains (data not shown). According to Rodas et al. [17], *Tru1* I restriction enzyme is highly discriminative for LAB differentiation and is considered a reliable choice for enzymatic digestion of the amplified 16S rDNA. ARDRA profiles of the digested 16S rDNA of the cheese samples revealed identical profiles (Fig. 1). Sequencing of the amplified 16S rDNA, used for the ARDRA digestions, showed 100% identity with *Lc. lactis* subsp. *lactis*. The fact that all cheese ARDRA profiles were identical demonstrates that there were no significant changes of the microbiota throughout the sampling period. Nevertheless, a culture-independent PCR might as well be biased as the amplification of bulk bacterial DNA by universal bacterial primers might not result in the amplification of the whole microbial community. This is due to the possible competition for primers by the DNA of dominant species, which would result in the amplification of the 16S rDNA of the dominant species only [5, 8], therefore not reflecting the minor LAB representatives. Taking into account this drawback of culture-independent PCR approach, identical ARDRA profiles could be explained although initial diverse profiles were expected due to the use of raw milk in the cheese production. It has been reported by other authors [3, 4, 11, 14, 15] that population structure of LAB community present in the traditional raw milk cheeses is diverse and complex.

The dominance of only one species was also confirmed by PCR-DGGE. The V3-16S rDNA pattern of the Karakačanski skakutanac consisted of one dominant band in all samples (Fig. 2). Multiple copies

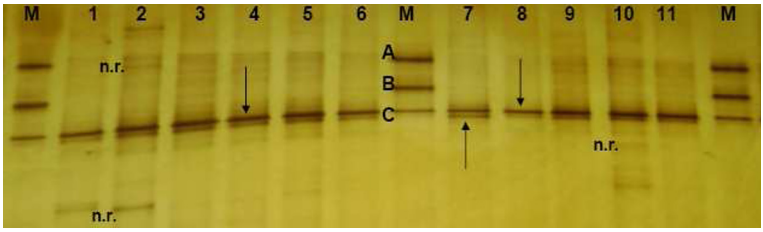

Figure 2. DGGE profiles of V3-16S rDNA amplicons of total DNA extracts from 11 Karakačanski skakutanac cheeses. The samples were collected from a dairy farm at 14-day intervals from April (1) to September (11). M – marker composed of A-*Lb. delbrueckii* subsp. *lactis*, B-*Ec. faecalis*, and C-*Lc. lactis* subsp. *lactis*. Sequences of selected bands (indicated by arrows) were 100% homologous to *Lc. lactis* subsp. *cremoris* (Accession No. AM944595). n.r. – not reamplifed. The gel shown is not normalized.

of the dominant band were observed on the DGGE gel, similar to the results reported by other authors [1, 5]. Sequencing of the dominant bands revealed 100% identity with *Lc. lactis* subsp. *cremoris*. The single species detected in Karakačanski skakutanac cheese indicated that most probably only the dominant species was amplified. It has been reported that the detection limit of culture-independent PCR-DGGE, with the use of universal bacterial primer, is ~ 10^4 cfu·g^{-1} [2, 8]. Similar results have also been observed in PCR-DGGE analysis of an artisanal Robiola di Roccaverano cheese. In that study, in the area named B, during summer and winter sampling of the Robiola cheese, the only detected species by PCR-DGGE was *Lc. lactis* subsp. *lactis* [1]. The discrepancy of the results between PCR-ARDRA and PCR-DGGE in our study might be due to close genetic relationship between subspecies *lactis* and *cremoris*, different primers used and discrimination pitfalls of the 16S rDNA in the identification of closely related species. It has been reported previously that these two subspecies co-migrated in DGGE by the use of primers targeting V3-16S rDNA [7]. Furthermore, no single region of the 16S rDNA can differentiate among all LAB and the use of different sets of primer targeting different regions might improve the analysis [5, 6]. Molecular analysis of the Stilton cheese demonstrated that the use of different targets of the same DNA template revealed remarkably different results [6]. Moreover, partial 16S rDNA sequences do not always allow discrimination between closely related species and one band may represent two species with identical partial sequence [12, 14–16].

The drawbacks of the methods are to be taken into consideration at the interpretation of the results obtained by analysing the 16S rDNA of mixed bacterial population. However, it should be also kept in mind that microbiota of each traditional cheese is unique, since specific environmental and processing factors influence the microbiota structure.

4. CONCLUSION

This first study of the microbiota of Karakačanski skakutanac cheese indicated the predominance of *Lc. lactis* population. Probably due to highly competitive DNA of predominant species, minor populations were not detected by culture-independent approaches. A polyphasic culture-dependent and -independent approach, including

molecular quantification, could allow to elucidate better the microbiota of Karakačanski skakutanac.

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