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Assessment of microbial diversity of the dominant microbiota in fresh and mature PDO Feta cheese made at three mountainous areas of Greece

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ABSTRACT

The aim of the present study was to assess the dynamics and the diversity of dominant microbiota in fresh and mature traditional Feta cheese produced in three mountainous areas and for that purpose a combined approach of culture-independent examination with conventional genotypic typing of the predominant NSLAB microbiota were applied. The microbial communities were monitored by PCR-DGGE on seven fresh and nine mature Feta cheese samples made by three artisanal producers. DGGE profiles suggested variability in the microbial composition of cheeses within the production area and distinctive differences in band profile of NSLAB in cheeses between the areas. PCR-DGGE analysis showed that *Lactococcus* spp. were the most widespread bacteria, *Streptococcus macedonicus* was detected often and, among *lactobacilli*, *Lactobacillus plantarum* prevailed. The diversity of NSLAB isolates from fresh and mature cheeses was assessed by RAPD-PCR and PFGE. Typing data indicated intraspecies genetic heterogeneity and specificity to the production area. Our results point to the conclusion that the manufacturing environment influence the dynamic and the diversity of microbial groups developed in the cheese and possibly the cheese flavour. In addition, the traditional Feta cheese may be a source of different NSLAB genotypes to make cheese with sensorial peculiarities appropriate for each area.

1. Introduction

The application of molecular methods, in particular the PCR-based fingerprinting techniques, is a powerful tool for determining the structure and monitoring changes in microbial communities of different environments (Randazzo, Caggia, & Neviani, 2009). Their microbial biodiversity at both genus and species level is regarded as a special feature of "*artisanal*" cheeses, such as Protected Designation of Origin (PDO) cheeses. It is generally believed that there is a link among the area of origin, the cheese-making procedure, and the microbial biodiversity as well as the specific characteristics of the final product (Ercolini, Mauriello, Blaiotta, Moschetti, & Coppola, 2004). Therefore, characterizing the cheese microbial population, especially the non-starter lactic acid bacteria (NSLAB), leads to understanding their ecosystem. In traditional Greek PDO cheeses, the NSLAB constitute the predominant cheese microbiota throughout cheese production and ripening (Litopoulou-Tzanetaki & Tzanetakis, 2011).

The diversity and the dynamics of cheese microbiota throughout cheese manufacture and ripening is currently studied by conventional culturing and non-culturing molecular methods. DNA fingerprinting techniques, such as pulsed-field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA (RAPD) are among the most powerful cultivation dependent molecular techniques applied for the intraspecific identification and for genotyping LAB isolated from several cheeses (Rossi, Gatto, Sabattini, & Torriani, 2012). Denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA gene is a culture-independent method, usually combined with culture-dependent methods, to study complex microbial communities and their dynamics during manufacture and ripening of artisanal cheeses (Randazzo, Pitino, Ribbera, & Caggia, 2010). This method was also successfully used to

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determine the geographic origin of cheeses, such as Fontina cheese made in alpine farms located in different altitudes (Giannino, Marzotto, Dellaglio, & Feligini, 2009), Grana Trentino from different factories (Rossi et al., 2012) and Pecorino Crotonese from two areas (Randazzo et al., 2010).

Feta is a semi-soft, white brined, PDO traditional Greek cheese, made from ovine or mixtures of it with caprine milk (up to 30%). The cheese is produced all over the country and Mytilini island. Traditionally, Feta cheese is made from raw milk in small family premises with elementary equipment. Sometimes, the producers apply a milder than HTST heat treatment of milk and traditional yoghurt as a starter is added (Moatsou, Massouras, Kandarakis, & Anifantakis, 2002). According to Greek standards (Codex Alimentarius, 1998), the cheese ripens for at least two months. Pasteurized milk and commercial starters are used for cheese production at large scale.

To our knowledge, no microbiological data are available on traditional Feta cheese, resulting from PCR-DGGE studies. Rantsiou, Urso, Dolci, Comi, and Cocolin (2008) studied in detail the components of the microbiota in four Feta cheese samples from pasteurized milk obtained from retail shops by PCR-DGGE and identified LAB and yeast species.

The aim of the present study was to assess the microbial diversity of raw milk Feta cheese made at three different mountainous areas of Greece, with emphasis on the NSLAB population. For this purpose, a combined approach of culture-independent examination by PCR-DGGE analyses with conventional culturing and genotypic typing of the predominant NSLAB microbiota were applied.

2. Materials and methods

2.1. Cheese manufacture and sampling

The milk was obtained from three different flocks of sheep owned by shepherds at farms, one in Northwest (NW) Greece at ~800 m altitude (area 1), one in Southwest (SW) at ~850 m (area 2) and one in SW Greek mountains at 1300–1500 m (area 3) above sea level. The cheeses were manufactured in a time period of three weeks as described previously (Bozoudi et al., 2015). Samples of fresh (24 h) and mature (2 months) Feta cheese were further analysed.

2.2. DNA extraction from cheese samples

Feta cheese samples (5 g) were homogenized with 45 mL sterile warm (45 °C) TE buffer pH 8.0 for 2 min in a Colworth Stomacher 400 (Seward Ltd., UK). Ten mL of the mixture was centrifuged (1000 rpm, 1 min, 4 °C) and the supernatant was then again centrifuged (8000 rpm, 5 min, 4 °C) to collect bacterial cells. The pellet was re-suspended in 500 μL TE buffer containing 10 mg/mL lysozyme (Sigma), incubated (37 °C, 30 min) and 10% SDS (w/v) was added to a final concentration 0.5%, followed by RNA and protein digestion. The DNA was finally purified with chloroform:isoamyl alcohol extractions and precipitated in 70% vol/vol ethanol. It was then suspended in 50 μl TE buffer and stored in −80 °C until the analysis.

2.3. PCR amplification for denaturing gradient gel electrophoresis (DGGE) analysis

To investigate the dominant bacterial species, the variable V2–V3 region of 16S rRNA gene was amplified using the universal primers HDA1-GC and HDA2, and following the procedures described by Tannock et al. (2000). The primers Lac1 and Lac2-GC, that amplified a ~340 bp fragment of the V3 region of the 16S rRNA gene, were used to specifically detect NSLAB species (Walter et al., 2001). The reaction mixture and the amplification program was determined according to Walter et al., (2001).

2.4. DGGE analysis

Amplicons were separated using a DCode Universal Mutation Detection System (Bio-Rad, USA). PCR samples (35 μL) were directly loaded into an 7.7% (w/v) polyacrylamide gel using a solution of acrylamide:N,N′-methylenebisacrylamide 37.5:1 (Fluka, USA) with 1× TAE buffer. The gels contained a 40–55% gradient of urea and formamide for universal primers HDA1-GC/HDA2 and a 30–50% gradient for primers Lac1/Lac2-GC that increased in the direction of electrophoresis (Tannock et al., 2000). A 100% denaturing solution contained 40% vol/vol formamide and 7 M urea. The electrophoresis was conducted with a constant voltage of 50 V at 60 °C for 16 h. Bands were visualized using a UV transilluminator after staining with ethidium bromide (5 μg/mL).

2.5. Sequencing analysis

Selected DGGE bands were excised from the acrylamide gels and stored in 100 μl of TE buffer. DNA from picked bands was purified in TE buffer just by spontaneous diffusion and it was used as template for subsequent re-amplification using the primers HDA1 without GC clamp and HDA2, or Lac1 and Lac2 without GC clamp, depending on the origin of the DGGE band. Single-band products were purified by a commercial PCR Clean-Up System kit (Macherey-Nagel, Germany) and Sanger sequenced (BMR Genomics, Italy). The sequences identities were determined using the nucleotide BLAST database of GenBank (http://www.ncbi.nlm.nih.gov/BLAST/).

2.6. Isolation of the predominant cheese microbiota

Totally, 58 and 38 isolates were obtained from each, MRS and M17 agar plates of the fresh and mature cheese, respectively. The isolation conditions are described in our previous study (Bozoudi et al., 2015), in which those isolates were initially grouped at species level by SDS-PAGE and finally were identified by sequencing the 16S rRNA gene.

2.7. RAPD-PCR analysis of the NSLAB isolates

Isolates from fresh and mature Feta cheese were sub-cultured twice in MRS broth (30 °C for 24 h) and 1 mL from the final culture was used for DNA extraction using a commercial kit (Invitrogen, USA) following the manufacturer's instructions. DNA was amplified (PTC-100TM, MJ Research, USA) according to Andrighetto et al., (2001) for the isolates from fresh cheese and Torriani et al. (2001) for those from mature, using the pair of primers M13 (5′-GAGGGTGGCGGTTCT-3′) and D8635 (5′-GAGCGGCCAAAGGGAG-CAGAC-3′), as well as M14 (5′-GAGGGTGGGGCCGTT-3′) and COC (5′-AGCAGCGTGG-3′), respectively. PCR products were separated in 1.5% agarose gels in 0.5× TBE buffer and photographed under UV-light (Minibis, DNR Bio-Imaging Systems, Israel) after staining with ethidium bromide (0.5 μg/mL). RAPD-PCR patterns of the captured images were analysed using the GelCompar software v. 4.6 (Applied Maths, Belgium). Calculation of the similarity of the band profile and grouping of the RAPD patterns was based on the Pearson product moment correlation coefficient (r) and the Unweighted Pair Group Method using Averages Linkages (UPGMA) cluster analysis. RAPDs were performed at least twice for each isolate.