Microflora of Feta cheese from four Greek manufacturers

UNIVERSITÀ DEGLI STUDI DI TORINO

Microflora of Feta Cheese from Four Greek Manufacturers

Kalliopi Rantsiou[1]*, Rosalinda Urso[2], Paola Dolci[1], Giuseppe Comi[2], Luca Cocolin[1]

[1]Dipartimento di Valorizzazzione e Protezione delle Risorse Agroforestali, Facoltà di Agraria, Università di Torino, Italy

[2]Dipartimento di Scienze degli Alimenti, Università di Udine, Italy

*Author for correspondence

Tel.: 0039-011-6708553, Fax: 0039-011-6708549, Email: kalliopi.rantsiou@unito.it

Abstract

The components of the microflora of four Feta cheeses, produced by different Greek manufacturers, were determined by culture-dependent and -independent techniques. Isolates from microbiological media were first grouped by Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis (PCR-DGGE) and then representatives of each DGGE group were sequenced for identification purposes. DNA and RNA, extracted directly from the cheese, were subjected to PCR-DGGE. Moreover, Feta cheeses were subjected to FISH analysis in order to identify viable bacterial populations. The microbial ecology, as represented by the Lactic Acid Bacteria (LAB) and yeast populations, was different for the four cheeses. The main LAB species isolated were *Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus coryniformis* and *Lactobacillus fermentum*. However, some inconsistencies were observed between the results obtained with the culture dependent and the culture independent approach. In the case of the yeasts, the results obtained by PCR-DGGE compared very well with those obtained by the conventional microbiological analysis and the main species found were *Kluyveromyces lactis*, *Pichia fermentans* and *C. zeylanoides*. FISH analysis highlighted viable but not culturable populations of *Streptococcus thermophilus* and *Lactococcus* spp. RAPD-PCR performed on the *Lb. plantarum* isolates revealed a cheese specific distribution and a temperature dependent clustering.

Keywords: feta; cheese microflora; PCR-DGGE

1. Introduction

Two microbial groups are important in cheese manufacture and ripening. The starter flora is responsible for acid development during cheese manufacture. The main species in starter flora, alone or in combination are *Lactococcus lactis, Streptococcus thermophilus, Lactobacillus helveticus* and *Lactobacillus delbrueckii*. Secondary flora are composed of complex mixtures of bacteria, yeasts and moulds, that are specific to particular cheese varieties (Beresford et al., 2001). The secondary microflora includes non-starter lactic acid bacteria (NSLAB) which grow internally in most cheese varieties, and other bacteria, yeasts and/or moulds, which grow internally or externally. NSLAB are mesophilic lactobacilli and pediococci, which form a significant portion of the microbial flora of most cheese varieties during ripening. They are not part of the normal starter flora, they generally do not grow well in milk, and they do not contribute to acid production (Beresford et al., 2001).

Feta is a Protected Denomination of Origin (PDO) cheese made out of sheep milk or a mix of sheep and goat milks. Acidification is aided by the addition of yoghurt starter cultures containing *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. The fresh cheese is dry-salted for 4-5 days and then put in brine with a salt content of 8 % for ripening. Feta cheese is commercialized after a period of ripering that lasts at least 60 days (Mauropoulos and Arvanitoyannis, 1999).

Previous studies, on the microflora of Feta cheese by conventional microbiological methods, have concluded that *Lactobacillus plantarum* is the most common NSLAB during ripening (Tzanetakis and Litopoulou-Tzanetaki, 1992; Manopoulou et al., 2003). In a survey of *Lb. plantarum* strains isolated from three batches of Feta, small phenotypic differences were identified and confirmed by Sodium Dodecyl Sulphate – PolyAcrylamide Gel Electrophoresis

(SDS-PAGE), and the strains showed marked variability in their proteolytic and acidifying activities (Xanthopoulos et al., 2000). The yeasts in Feta cheese or the brines used for Feta manufacture were found to be specific to individual processing plants (Westall and Filtenborg, 1998; Fadda et al., 2001; Psomas et al., 2001). The main species identified were *Saccharomyces cerevisiae*, *Debaryomyces hansenii*, *Candida famata*, *Pichia membranifaciens*, *Torulaspora delbrueckii*, *Kluyveromyces marxianus*, *Candida sake* and *Kluyveromyces lactis*.

Molecular methods for detection, identification and characterization of microorganisms in foods are currently considered indispensable tools for the description of food microflora. In addition, our understanding of microbial dynamics during food fermentations has improved significantly with the application of methods that analyze nucleic acids extracted directly from samples. Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis (PCR-DGGE) has been proven a very powerful method that allows a detailed and accurate description of fermented food microflora (Ercolini, 2004). Together with DGGE, fluorescence in situ hybridization (FISH) has been applied as a culture-independent method for identification of the locations of specific microbial populations in food matrices (Ercolini et al., 2003), and to detect viable populations (Amann et al., 1995).

In order to enrich our knowledge of the Feta cheese microflora and to compare the information obtained from the application of culture dependent and independent methods, we considered four different cheeses, on retail sale in Greece. We performed conventional microbiological analysis to identify the main microbial groups present in the cheeses and representative strains from the major groups were randomly selected and identified by molecular methods. In parallel, DNA and RNA were directly extracted from the cheeses and

PCR-DGGE was performed using universal primers for bacteria and yeasts. Regarding the viable populations in the cheeses, to confirm the results we obtained by PCR-DGGE on the RNA, we performed FISH analysis.

2. Materials and methods

2.1. Feta cheese collection and microbiological analysis

Samples of Feta cheese, that were produced by different, large manufacture plants were picked up at the retail level, from supermarkets in Athens, Greece. The cheeses were available for sale in a pre-packed format and had an approximate weight of 250 g each.

Cheese pH was measured by means of a pin electrode of a pH meter (Radiometer Copenhagen pH M82, Cecchinato, Italy) inserted directly into the sample. Three independent measurements were made on each sample and means were calculated.

Samples were analyzed, in triplicate, by conventional microbiological methods. Ten g portions of Feta cheese were each added to 40 ml of saline-peptone water, containing NaCl, 8 g/l and bacteriological peptone, 1 g/l (Oxoid, Milan, Italy) in a sterile stomacher bag and the mixture was pummeled in a stomacher (PBI, Milan, Italy) for 1.5 min. Decimal dilutions of the stomacher fluid were made, using the same diluent, and the dilutions were used for enumeration of bacteria on duplicate agar plates: Total aerobic bacteria were spread on plates of gelatin peptone agar (GPA, Oxoid) which were incubated for 48 h at 30 ºC; mesophilic and thermophilic lactobacilli were enumerated on double layer deMan Rogosa Sharpe (MRS) agar (Oxoid) plates, after incubation at 22ºC and 42ºC respectively, for 48 h; mesophilic and thermophilic lactococci were enumerated on double layer M17 agar (Oxoid) plates, after incubation at 22ºC and 42ºC respectively, for 48 h; fecal enterococci were enumerated on

kanamycin aesculin azide agar (KAA) (Oxoid) plates, after incubation at 37ºC for 48 h; coagulase negative cocci (CNC) were enumerated on mannitol salt agar (MSA) (Oxoid) plates, after incubation at 30ºC for 48 h; yeasts and moulds were enumerated on malt extract agar (MEA) (Oxoid) plates, supplemented with tetracycline (1 μg/ml; Sigma, Milan, Italy) after incubation at 25ºC for 48 to 72 h; *Staphylococcus aureus* was enumerated on plates of Baird Parker medium (BP) (Oxoid) with added egg yolk tellurite emulsion (Oxoid), after incubation at 37ºC for 48 h; coliforms and *Escherichia coli* were enumerated on plates of Coli-ID medium (Bio-Merieux, Marcy d' Etoile, France), after incubation at 37ºC for 48 h. After counting, means and standard deviations were calculated. Wherever possible, 24 colonies were randomly selected from the following plates, containing approximately 30 to 300 colonies: MRS agar incubated at 22ºC and at 42ºC and malt extract agar. A total of 264 colonies that included 96 from MRS incubated at 22ºC, 96 from MRS incubated at 42ºC and 72 from MEA, were purified and stored at –20ºC with glycerol (30% final concentration). The isolates were then identified molecularly.

2.2. DNA extraction from pure cultures

One milliliter of an overnight culture of each isolate was centrifuged at 14,000 X *g* for 10 min at 4ºC to pellet the cells and the pellet was subjected to DNA extraction according to Andrigetto et al. (2002), with the addition of only lysozyme (50 mg/ml, Sigma, Milan, Italy) for cell lysis.

2.3. Direct extraction of nucleic acids from the Feta cheese samples

Two grams of each cheese were mixed with 20 ml of 2 % (wt/vol) sodium citrate and incubated for 30 min at 45ºC. The mix was vortexed for 5 min and centrifuged at 6,000 x g for 10 min and the pelleted material was re-suspended in 1 ml of 10 mM Tris - 5mM EDTA,

pH 8 (TE). After centrifugation at maximum speed (14,000 x g), the pellet was re-suspended in 300 µl of TE and the suspension was devided into two equal portions for the extraction of DNA and RNA. Each portion was centrifuged for 10 min at maximum speed. The pellet was resuspended in 120 µl of proteinase K buffer containing 50 mM Tris-HCl, 10 mM EDTA, 0.5 % (wt/vol) sodium dodecyl sulfate (SDS), pH 7.5, and the suspension was transferred to a 1.5 ml screw cap tube containing 0.3 g of glass beads with a diameter of 0.5 mm. Twenty microliters of solution of each of proteinase K (25 mg/ml; Sigma) and lysozyme (50 mg/ml; Sigma) were added and the mixture was incubated at 50ºC for 1 h. Then, 150 µl of 2X breaking buffer, composed of 4 % (vol/vol) Triton X-100, 2 % (wt/vol) SDS, 200 mM NaCl, 20 mM Tris, pH 8 and 2 mM EDTA, pH 8 were added. Three hundred microliters of phenol-chloroform-isoamyl alcohol, 25:24:1, pH 6.7 (Sigma) were added in the case of the extraction of DNA, while 300 µl of phenol-chloroform, 5:1, pH 4.7 (Sigma) were added for the extraction of RNA. Three 30-s bead beater (Fast Prep; Bio 101, Vista, CA, USA). treatments were performed, at maximum speed, with an interval of 10 s between each treatment. Three hundred microliters of TE were added and the tubes were centrifuged for 10 min at 12,000 x *g* at 4ºC. The aqueous phase was collected and nucleic acids were precipitated by addition of 1 ml of absolute ethanol. The DNA or RNA were pelleted by centrifugation at 14,000 x *g* for 10 min at 4ºC. Each pellet was air dried and 50 µl of sterile water was added. One microliter of DNase-free RNase (Roche Diagnostics, Milan, Italy) or 1 µl of RNase-free DNase (Roche Diagnostics) were added and the mixture was incubated at 37ºC for 1h to digest, respectively RNA or DNA. The RNA samples were checked for the presence of residual DNA by PCR amplification. When PCR products were obtained, the DNase treatment was repeated to eliminate the DNA.

2.4. PCR and RT-PCR

Bacterial DNA was amplified with primers P1V1, 5'-GCG GCG TGC CTA ATA CAT GC-3' and P2, 5'-TTC CCC ACG CGT TAC TCA CC-3' (Klijn et al., 1991). The reaction mix was as follows: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1.25 U of *Taq* Polymerase (Applied Biosystems, Milan, Italy), 0.2 μM of each primer and 100 ng of DNA. In all amplifications, a negative control, containing all the reagents as described but no DNA, was included. Amplifications were carried out in a PTC-220 DNA Engine Dyad MJ Research thermalcycler (Celbio, Milan, Italy). The amplification cycle of denaturation at 95 ºC for 1 min, annealing at 42 ºC for 1 min and extension at 72 ºC for 1 min was repeated 35 times. The cycle was preceded by an initial denaturation at 95 ºC for 5 min and followed by a final extension at 72 ºC for 7 min. For yeast DNA amplification, primers NL1, 5′-GCC ATA TCA ATA AGC GGA GGA AAA G-3′ and LS2, 5′-ATT CCC AAA CAA CTC GAC TC-3′ were employed (Cocolin et al., 2000). The reaction mix was 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each of dNTP, 1.25 U of *Taq* Polymerase and 0.2 μM of each primer and 100 ng of DNA. The amplification cycle of denaturation at 95 ºC for 1 min, annealing at 42 ºC for 1 min and extension at 72 ºC for 1 min was repeated 35 times. The cycle was preceded by an initial denaturation at 95 ºC for 5 min and followed by a final extension at 72 ºC for 7 min.

PCR products were analyzed by electrophoresis in 0.5 X Tris-Borate-EDTA (TBE) agarose gel. Primers P1V1 and NL1 were carrying a GC-clamp (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G -3′) when used for DGGE analysis (Sheffield et al., 1989). The RT-PCR reactions were performed using the M-MLV reverse transcriptase (Promega, Milan, Italy). One microgram of RNA was mixed with 10 μM of primer P2 or

LS2, for bacterial or yeast RNA, respectively, and sterile water for a final volume of 10 µl. The mix was denatured at 75 ºC for 5 min and then it was placed on ice and the reverse transcription reaction mix was added. The final concentrations in the 25 µl RT reaction mix were 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 2 mM of each dNTP, 4 µM primer, 200 units M-MLV and 0.48 to 0.96 units of RNasin ribonuclease inhibitor. The reverse transcription was carried out at 42 ºC for 1 h. Appropriate PCR reactions were performed, as described above, with addition of 1 µl of completed RT reaction mix to 25 µl of PCR reaction mix.

2.5. DGGE analysis

The Dcode universal mutation detection system (Bio-Rad, Hercules, CA, USA) was used, with a 0.8 mm thick gel of 8 % (wt/vol) acrylamide-bisacrylamide, 37.5:1, for DGGE analysis. For PCR products obtained with primer set P1V1/P2V1, electrophoresis was performed with a denaturant gradient from 40 % to 60 %, with 100 % corresponding to 7 M urea and 40 % (wt/vol) formamide, increasing in the direction of the electrophoretic run; at 130 V and 60 ºC, for 4 h. Amplicons obtained with primer set NL1/LS2 were analyzed in a denaturant gradient from 30 % to 60 %; at 120 V and 60 ºC, for 4 h. Gels were stained for 20 min in 1.25 X Tris-acetate-EDTA containing 1 X SYBR Green (Molecular Probes, Eugene, OR, USA). They were visualized under UV, digitally captured, and analyzed using the GeneGenius Bio Imaging System (SynGene, Cambridge, United Kingdom).

2.6. FISH analysis

The 4 feta samples were analysed by FISH, using probes specific for eubacteria, *S. thermophilus*, *Lactococcus* spp. and *Lb. plantarum*, according to Cocolin et al. (2007), by a commercial facility (Vermicon AG, Munich, Germany).

2.7. Sequencing of DGGE bands and sequence analysis

Blocks of polyacrylamide gels containing selected DGGE bands were collected using sterile pipette tips. Each block was placed in 50 µl of sterile water and it was left overnight at 4 ºC. Three microliters of the water were then used in a 25 µl PCR reaction mix, performed with primers containing the GC clamp. The products were run on a DGGE gel together with an appropriate DNA or RNA control amplified with bacterial or yeast primers. When the product migrated as a single band that corresponded to the control band, the excised band was re-amplified, using primers without the GC clamp, and the product was cloned into the pGEM-T easy vector (Promega, Milan, Italy) following the instructions of the manufacturer. Clones were checked by co-migration with control, as before, and the insert of appropriate clones was sequenced by a commercial facility (MWG Biotech, Edersberg, Germany). Sequence comparisons were performed using the BLAST program (Altschul et al., 1997).

2.8. Identification of the isolates by PCR-DGGE

Both bacterial and yeast isolates were identified by groupings based on their PCR-DGGE profiles and sequencing of representative isolates of each group. DNA extracted from bacterial isolates was amplified with primer set P1V1/P2V1 and the PCR products were analyzed by DGGE as described by Cocolin et al. (2001). The DGGE profiles were grouped and representative isolates of each group were amplified with primers P1V1 and P4V3, as described by Klijn et al. (1991), targeting 700 bp of the V1-V3 region of the 16S rRNA gene. The PCR products were sequenced by a commercial facility (MWG Biotech) and the resultant sequences were aligned with those in GeneBank using the Blast program, to determine the closest known relatives, based on the partial 16S rRNA gene homology. Similarly, for yeast isolates, the DNA was first amplified with primers NL1/LS2 and the

products run on DGGE, according to Cocolin et al. (2000). Representatives of the different DGGE profile groups were identified by sequencing the partial 26S rRNA gene that was amplified with primers NL1/NL4, as previously described (Kurtzman and Robnett, 1997).

2.9. RAPD analysis

For RAPD analysis, primer M13, 5′GAG GGT GGC GGT TCT-3′, was used as described previously (Andrighetto et al., 2001). Reactions were carried out in a final volume of 25 μl, containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μM primer, 1.25 U *Taq* polymerase and 100 ng of DNA. The amplification was 35 cycles of 94 °C for 1 min, 38 °C for 1 min, ramp to 72 °C at 0.6 °C/s, and 72 °C for 2 min. Initial denaturation was at 94 °C for 5 min and final extension at 72 °C for 5 min. RAPD-PCR products were analyzed by electrophoresis on 1.5 % (wt/vol) agarose gels containing 6.25 μg/ml ethidium bromide (Sigma) in 0.5 X TBE, at 120 V for 2 h. Pictures of the gels were digitally captured by the GeneGenius Bio Imaging System (SynGene), and Gel Compare version 4.1 (Applied Maths, Kortrijk, Belgium) was used for pattern analysis. The calculation of similarities in the profiles of bands was based on the Pearson product moment correlation coefficient. Dendrograms were obtained by the unweighted pair group method with arithmetic averages clustering algorithm (Vauterin and Vauterin, 1992). The isolates were subjected to RAPD-PCR analysis at least twice.

3. Results

3.1. Conventional microbiological analysis of the cheeses

The pH values for cheeses A, B, C and D were 4.32, 4.72, 5.05 and 4.42, respectively. The counts on GPA, MSA and MRS, at both 22°C and 42°C, were similar for all four cheeses (Table 1). No colonies were recovered on BP. Three of the cheeses gave no colonies on ColiID and sample B gave no colonies on KAA. Presumptive yeasts on MEA were recovered at levels of 10^3 cfu/g from cheeses A, C and D but were not recovered from cheese B. Colonies on M17 at 22 °C and 42 °C were obtained from cheese A only and from cheeses A, C and D, respectively.

3.2.Identification of isolates by PCR-DGGE

The results of the identification by PCR-DGGE are presented in Table 2. *Lb. plantarum* was the predominant organism recovered from cheese A at 22ºC and the only organism recovered at 42ºC. In cheese B, 3 species of lactobacilli were identified: *Lb. plantarum*, that represented the 58.3 % of the isolates at 22ºC and the 100 % at 42°C, *Lb. brevis* and *Lactobacillus paraplantarum*. Four species were identified in cheese C. *Lb. paraplantarum* was isolated only from plates incubated at 22ºC, at a percentage of 12.5 %, while *Lb. plantarum*, *Lb. brevis* and *Lactobacillus coryniformis* were isolated at both temperatures but with different percentages. In cheese D we isolated *Lb. plantarum* and *Lb. brevis* at 22ºC (with percentages of 91.7 and 8.3 % respectively) and only *Lb. fermentum* at 42ºC.

Regarding the yeasts, we identified *K. lactis* in cheeses A and C (79.2 % and 83.3 % of the total yeast populations respectively), *P. membranifaciens* in cheese A (20.8 %) and *Candida krisii*/*zeylanoides* in cheese C (16.7 %). In cheese D, all 20 isolates that were subjected to molecular identification resulted to be about 90 % homologous to *P. fermentans*.

3.3. DGGE analysis of the cheeses

The DNA and RNA DGGE gels are shown in Figure 1 while the results of the identification of bands are presented in Table 3. At the DNA level, with the universal primers for bacteria, a band that resulted to correspond to *S. thermophilus* (Figure 1A, band 1), was present for all four cheeses. The same band was present at the RNA level for cheeses A, B and C. For cheese A, in addition to the *S. thermophilus* band, at the RNA level we sequenced a band corresponding to *Lb. plantarum* (Figure 1A, band 6). For cheese C, at both DNA and RNA level, we detected *Lb. delbrueckii* subsp. *bulgaricus* (Figure 1A, bands 2 and 8) and *L. lactis* that gave double bands (Figure 1A, bands 3 and 4 at the DNA level and bands 9 and 10 at the RNA level). In cheese D, we detected a species that it was not possible to identify (Figure 1A, bands 5, 11, 12 and 13). The cut bands gave a similarity of 97 % to *Lactobacillus suntoryeus/helveticus/gallinarum*. In the DGGE gels for the yeasts, *K. lactis* (Figure 1B, bands 14 and 19) and *P. membranifaciens* (Figure 1B, bands 15 and 20) were identified, at both DNA and RNA level, for cheese A. In cheese C, at the DNA level *C. zeylanoides* (Figure 1B, band 17), was identified while in cheese D the band 18 (that was visible also for the RNA sample) was sequenced and gave a low homology (90 %) to *P. fermentans.* No DGGE profile was obtained for cheese B with primers NL1GC/LS2. Bands 16 and 21 did not produce any PCR products after re-amplification and for this reason we believe they represent the excess of the primers that did not get amplified.

3.4. FISH analysis

When subjected to FISH analysis all Feta samples showed a total living population of about $5x10^8$ cells/g. *Lb. plantarum* was found only in cheese A, while viable populations of *S. thermophilus* and *Lactococcus* spp. were detected mainly in cheeses A, B and C (data not

shown).

3.5. Cluster analysis of L. plantarum strains isolated from the four cheeses

In order to understand the ecology of the most numerous group of bacterial isolates from the four samples, represented by *Lb. plantarum*, we performed cluster analysis of the RAPD profiles obtained with primer M13. Strains isolated from the same cheese were grouped in order to observe the effect of the temperature of incubation (22 and 42ºC), on the selection of strains, while strains isolated at 22ºC from all four samples were grouped to understand the effect of sample-specific environment on the strain selection. For cheese D we did not isolate *Lb. plantarum* at 42°C, thereby we did not perform the intra-cheese cluster analysis. Figures 2A, 2B and 2C show the clusters of the strains from cheeses A, B and C respectively while figure 2D shows the clusters of all the *Lb. plantarum* strains isolated at 22ºC.

Using a coefficient of similarity of 70 %, the strains of *Lb. plantarum* isolated from cheese A, formed 3 clusters. One of them (A*III*) contained all the strains isolated at 22ºC, while the strains isolated at 42ºC were split between the other two clusters. For cheese B we identified 2 big clusters. One was homogeneous and contained strains isolated only at 42ºC (cluster B*I*). The second cluster (B*II*) contained strains isolated at both 22 and 42ºC. Similarly, for cheese C, we identified two clusters, one contained strains isolated at 42ºC (C*II*) and a second one that was mixed, with strains isolated at both temperatures. When strains isolated at 22ºC from all four cheeses were compared, 3 clusters were obtained. Two of them, *II* and *III*, were homogeneous and composed of strains from cheeses D and A respectively. The third cluster was the most numerous, consisting of 54 strains, originating from all 4 cheeses.

4. Discussion

The conventional microbiological data obtained for three out of the four Feta cheeses were similar. As expected, we found high numbers of lactobacilli and moderate numbers for yeasts and enterococci, all of which are involved in the ripening of Feta and other cheeses, while members of thermophilic and mesophilic cocci were lower. The thermophilic and mesophilic cocci are mainly the starters used in cheese manufacture, which die off at the end of ripening, because of the low pH and the high salt concentration (Bintsis and Papademas, 2002). In one cheese, we did not detect lactococci, and surprisingly we could not count yeasts or enterococci. Possibly the enterococci had been outgrown by the lactobacilli (Beresford et al., 2001). The pH values of the cheeses ranged from 4.30 to 5.05. The large difference in pH values seem not to interfere or influence the microbiological counts.

The main species numerically was *Lb. plantarum*. This result confirms previous studies on Feta as well as on other cheeses that have highlighted *Lb. plantarum* as an important agent for the ripening process (Xanthopoulos et al., 2000; Beresford et al., 2001). In addition, we identified *Lb. brevis*, *Lb. coryniformis*, *Lb. paraplantarum* and *Lb. fermentum*. Sample C resulted to be the most heterogeneous, harboring 4 out of the 5 *Lactobacillus* species. *Lb. fermentum*, a species that according to Bergey's manual shows generally good growth at 45ºC (Kandler and Weiss, 1986), was only identified in cheese D and was isolated only from the plates incubated at 42ºC.

From the yeasts molecular identification we conclude that there was cheese specificity of the species isolated: *K. lactis*, that was present in cheeses A and C, *P. membranifaciens* was specific to cheese A, while *C. krisii/zeylanoides* to cheese C. Cheese D was characterized by

a species that has not been previously described or for which sequence information of the D1-D2 loop of the 26S rRNA gene is not available.

The picture of the bacterial ecology of the four cheeses, obtained from the direct analysis, was different from that obtained by conventional plating and molecular identification of the isolates. In particular, from the direct analysis we detected *S. thermophilus*, *L. lactis* and *Lb. delbrueckii* subsp. *bulgaricus* that we did not isolate from the plates. On the M17, medium commonly used to isolate cocci, the isolates were identified as *Lactobacillus* spp. by 16S rRNA gene sequencing (data not shown). Since we were not able to isolate the species detected by DGGE on any of the media employed for the conventional analysis, we hypothesize that these media favored the growth of other LAB populations. Such limitation, due to the non-selective medium used, has been previously reported (Lynch et al., 1996). The species identified by DGGE, *S. thermophilus*, *L. lactis*, *Lb. delbrueckii* subsp. *bulgaricus*, are commonly responsible for the fermentation and it is unusual that they are present and active during the ripening and marketing of cheeses. It is also surprising that, with the exception of *Lb. plantarum* in the RNA sample A, by direct analysis we were not able to detect the main *Lactobacillus* populations that we isolated on the plates. It is possible that, the nucleic acids of the dead but intact cells of thermophilic LAB populations, which are added as starter for the Feta manufacture and reach high numbers during the fermentation, may persist during ripening and the marketing of the product, creating a masking effect that does not allow us to detect the *Lactobacillus* populations. However, since we obtained similar results when the RNA was analyzed, and knowing that RNA is less resistant and generally RNA presence is considered as indication of vitality, we considered the possibility that these thermophilic LAB populations are present in viable non-culturable (VNC) state. The VNC state has been

described for *L. lactis* as a physiological response to carbohydrate starvation, a situation that characterizes a cheese after fermentation (Stuart et al., 1999). In order to verify the VNC state of *S. thermophilus* and *L. lactis*, FISH analysis was carried out. FISH is widely used for the cultivation-independent detection and analysis of alive complex microbial populations, for example in soil and sediments and activated sludge (Amann et al., 1995). Applying FISH to the feta samples we confirmed the presence of alive populations of *S. thermophilus* and *Lactococcus* spp.

The direct analysis of the yeasts populations compared very well with the conventional microbiological analysis. The species detected have been previously isolated from Feta or Feta-type cheeses (Westall and Filtenborg, 1998; Fadda et al., 2001; Bintsis and Papademas, 2002), highlighting their adjustment to the specific ecosystem and possibly their importance and involvement in the ripening process. The unidentified species of yeast isolated by plating in cheese D, was also detected, by direct analysis, at both DNA and RNA level.

Lb. plantarum is one of the main species of non-starter LAB isolated during the ripening of Feta cheese (Tzanetakis and Litopoulou-Tzanetaki, 1992; Xanthopoulos et al., 2000; Manopoulou et al., 2003). This was confirmed in our study. Furthermore, the results of the RAPD PCR molecular characterization indicate genetic heterogeneity within the *Lb. plantarum* populations from each cheese that may potentially be translated into physiological and biochemical heterogeneity of the isolates. As a matter of fact, such heterogeneity has been previously observed (Xanthopoulos et al., 2000).

In this study we determined the microbial ecology of 4 Feta cheeses at the retail level. By conventional methods we counted the main microbial groups while by direct PCR-DGGE analysis we defined the main species of LAB and yeasts that are potentially involved in the

ripening process and remain viable during the marketing of the product. *Lb. plantarum* resulted to be the most frequently isolated LAB, confirming its status as an important component of the NSLAB microflora of Feta cheese. An unidentified species of yeast was detected in one of the four samples by plating as well as directly. Comparision of the DNA and RNA analysis indicated the presence of VNC populations of *S. thermophilus* and *Lactococcus* spp.

Acknowledgements

We are grateful to Ingrid Bergmaier and Claudia Beimfohr from Vermicon AG (Munich, Germany) for the analysis of the Feta samples by FISH.

REFERENCES

Altschul, S. F., Madden, T. L., Shaffer, A. A., Zhang, J., Zhang, Z., Miller, W., Lipman, D. J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389-3402.

Amann, R. I., Ludwig, W., Schleifer, K.-H., 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiological Reviews 59, 143-169.

Andrighetto, C., Zampese, L., Lombardi, A., 2001. RAPD-PCR characterization of lactobacilli isolated from artisanal meat plants and traditional fermented sausages of Veneto region. Letters in Applied Microbiology 33, 26-30.

Beresford, T. P., Fitzsimons, N. A., Brennan, N. L., Cogan, T. M., 2001. Recent advances in cheese microbiology. International Dairy Journal 11, 259-274.

Bintsis, T., Papademas, P., 2002. Microbiological quality of white-brined cheeses: a review. International Journal of Dairy Technology 55, 113-120.

Cocolin, L., Bisson, L. F., Mills, D. A., 2000. Direct profiling of the yeast dynamics in wine fermentations. FEMS Microbiology Letters 189, 81-87.

Cocolin, L., Diez, A., Urso, R., Rantsiou, K., Comi, G., Bergmaier, I., Beimfohr, C., 2007. Optimization of conditions for profiling bacterial populations in food by culture-independent methods. International Journal of Food Microbiology 120, 100-109.

Cocolin, L., Manzano, M., Cantoni, C., Comi, G., 2001. Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. Applied and Environmental Microbiology 67, 5113-5121.

Ercolini, D., Hill, P. J., Dodd, C. E. R., 2003. Bacterial community structure and location in Stilton cheese. Applied and Environmental Microbiology 69, 3540-3548.

Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. Journal of Microbiological Methods 56, 297-314.

27 Fadda, M. E., Cosentino, S., Deplano, M., Palmas, F., 2001. Yeast populations in Sardinian feta
28 cheese. International Journal of Food Microbiology 69, 153-156.
29 Kandler, O., Weiss, N., 1986. Regular, nonsporing gram-positive rods, p. 1208-1234. *In* P. H. A.
30 Sneath (ed.), Bergey's manual of Systematic Bacteriology, vol. 2. Williams & Wilkins,
31 Baltimore.
32 Klijn, N., Weerkamp, A. H., de Vos, W. M., 1991. Identification of mesophilic lactic acid bacteria
33 by using polymerase chain reaction-amplified variable regions of 16S rRNA and specific
34 DNA probes. Applied and Environmental Microbiology 57, 3390-3393.
35 Kurtzman, C. P., Robnett, C. J., 1997. Identification of clinically important ascomycetous yeasts
36 based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA
37 gene. Journal of Clinical Microbiology 35, 1216-1223.
38 Tzanetakis, N., Litopoulou-Tzanetaki, E., 1992. Changes in numbers and kinds of lactic acid
39 bacteria in Feta and Teleme, two Greek cheeses from Ewes' milk. Journal of Dairy Science
40 75, 1389-1393.
41 Lynch, C. M., McSweeney, P. L. H., Fox, P. F., Cogan, T. M., Drinan, F. D., 1996. Manufacture of
42 cheddar cheese with and without adjunct Lactobacilli under controlled microbiological
43 conditions. International Dairy Journal 6, 851-867.
44 Manopoulou E., Sarantinopoulos, P., Zoidou, E., Aktypis, A., Moschopoulou, E., Kandarakis, I.G.,
45 Anifantakis, E. M., 2003. Evolution of microbial populations during traditional Feta cheese
46 manufacture and ripening. International Journal of Food Microbiology 82, 153-161.
47 Mauropoulos, A. A., Arvanitoyannis, I. S., 1999. Implementation of hazard analysis critical control
48 point to Feta and Manouri cheese production lines. Food Control 10, 213-219.
49 Psomas, E., C. Andrighetto, E. Litopoulou-Tzanetaki, A. Lombardi, N. Tzanetakis. 2001. Some
50 probiotic properties of yeast isolates from infant feaces and Feta cheese. International
51 Journal of Food Microbiology 69, 125-133.

52 Sheffield, V. C., Cox, D. R., Lerman, L. S., Myers, R. M., 1989. Attachment of a 40-base pairs
53 G+C rich sequence (GC clamp) to genomic DNA fragments by the polymerase chain
54 reaction results in improved detection of single-base changes. Protocols of the National
55 Academy of Sciences, 86 297-303.
56 Stuart, M. R., Szu Chou, L., Weimer, B. C., 1999. Influence of carbohydrate starvation and arginine
57 on culturability and amino acid utilization of *Lactococcus lactis* subsp. *lactis*. Applied and
58 Environmental Microbiology 65, 665-673.
59 Vauterin, L., Vauterin, P., 1992. Computer-aided objective comparison of electrophoretic patterns
60 for grouping and identification of microorganisms. European Microbiology 1, 37-41.
61 Westall, S., Filtenborg, O., 1998. Yeast occurence in Danish feta cheese. Food Microbiology 15,
62 215-222.
63 Xanthopoulos, V., Hatzikamari, M., Adamidis, T., Tsakalidou, E., Tzanetakis, N., Litopoulou-
64 Tzanetaki, E., 2000. Heterogeneity of *Lactobacillus plantarum* isolates from Feta cheese
65 throughout ripening. Journal of Applied Microbiology 88, 1056-1064.

Table 1. Results of the microbiological analysis of the 4 Feta cheeses. Mean values and standard deviations of the log (cfu/g) for each sample, analyzed in triplicate, are reported. Refer to materials and methods for media specifications and incubation conditions.

Medium and incubation temperature	Cheese A		Cheese B		Cheese C		Cheese D	
	Mean log (cfu/g)	Standard deviation	Mean log (cfu/g)	Standard deviation	Mean log (cfu/g)	Standard deviation	Mean log (cfu/g)	Standard deviation
MRS at 22°C	7,19	0,17	6,84	0,02	6,47	0,11	5,95	0,05
MRS at 42°C	6,26	0,17	5,90	0,12	6,39	0,11	5,92	0,10
M17 at 22°C	4,18	0,18	n.a.[a]		n.a.[a]		n.a.[a]	
M17 at 42°C	3,30	0,00	n.a.[a]		3,63	0,21	5,32	0,14
MSA	0,95	0,00	1,11	0,20	1,08	0,05	0,39	0,12
MEA	3,54	0,21	n.a.[b]		3,27	0,07	3,07	0,05
GPA	3,39	0,12	3,65	0,07	3,15	0,21	3,20	0,54
Coli ID	1,60	0,00	n.a.[a]		n.a.[a]		n.a.[a]	
KAA	1,39	0,12	n.a.[b]		3,33	0,23	1,87	0,23

Abbreviation: n.a.: not applicable. n.a.[a]: counts < 5 cfu/g, n.a.[b]: counts < 50 cfu/g.

Table 2. Molecular identification of isolates on MRS incubated at 22 and 42°C and on MEA. The isolates were selected randomly, grouped based on co-migration in PCR-DGGE and representatives of each group were sequenced to obtain an identification for the whole group.

Isolates on MRS at 22°C					
	Cheese A (24 isolates)	Cheese B (24 isolates)	Cheese C (24 isolates)	Cheese D (24 isolates)	Total (96 isolates)
Lactobacillus plantarum	95.8 %	58.3 %	75 %	91.7 %	80.2 %
Lactobacillus brevis	4.2 %	25 %	4.2 %	8.3 %	10.4 %
Lactobacillus coryniformis	-	-	8.3 %	-	2.1 %
Lactobacillus paraplantarum	-	16.7 %	12.5 %	-	7.3 %
Isolates on MRS at 42°C					
	Cheese A (24 isolates)	Cheese B (24 isolates)	Cheese C (24 isolates)	Cheese D (24 isolates)	Total (96 isolates)
Lactobacillus plantarum	100 %	100 %	50 %	-	62.5 %
Lactobacillus brevis	-	-	8.3 %	-	2.1 %
Lactobacillus coryniformis	-	-	41.7 %	-	10.4 %
Lactobacillus fermentum	-	-	-	100 %	25 %
Isolates on MEA					
	Cheese A (24 isolates)	Cheese B	Cheese C (24 isolates)	Cheese D (24 isolates)	Total (72 isolates)
Kluyveromyces lactis	79.2 %	-	83.3 %	-	54.2 %
Pichia membranifaciens	20.8 %	-	-	-	6.9 %
Candida krisii/zeylanoides	-	-	16.7 %	-	5.6 %
Pichia fermentans	-	-	-	100 %	33.3 %

Table 3. Identification, based on BLAST comparison in GenBank, of the bands obtained by PCR-DGGE using universal primers for bacteria and yeasts. Based on the sequence obtained from bacterial bands 5, 11, 12 and 13, no final identifiation was possible. [(a)]Abbreviations: ns, no sequence was obtained.

Band #	Identification	Similarity
	Bacteria	
1	*Streptococcus thermophilus*	100 %
2	*Lactobacillus delbrueckii* subsp. *bulgaricus*	99 %
3	*Lactococcus lactis*	100 %
4	*Lactococcus lactis*	100 %
5	*Lactobacillus suntoryeus* or *Lactobacillus helveticus* or *Lactobacillus gallinarum*	97 %
6	*Lactobacillus plantarum*	99 %
7	*Streptococcus thermophilus*	100 %
8	*Lactobacillus delbrueckii* subsp. *bulgaricus*	99 %
9	*Lactococcus lactis*	100 %
10	*Lactococcus lactis*	100 %
11	*Lactobacillus suntoryeus* or *Lactobacillus helveticus* or *Lactobacillus gallinarum*	97 %
12	*Lactobacillus suntoryeus* or *Lactobacillus helveticus* or *Lactobacillus gallinarum*	97 %
13	*Lactobacillus suntoryeus* or *Lactobacillus helveticus* or *Lactobacillus gallinarum*	97 %
	Yeasts	
14	*Kluyveromyces lactis*	99 %
15	*Pichia membranifaciens*	99 %
16	ns[(a)]	
17	*Candida zeylanoides*	100 %
18	*Pichia fermentans*	90 %
19	*Kluyveromyces lactis*	100 %
20	*Pichia membranifaciens*	99 %
21	ns[(a)]	

92 **FIGURE LEGENDS**

93 Figure 1. DGGE profiles of the nucleic acids extracted directly from the Feta cheeses. Panel A,
94 bacterial DNA and RNA. Panel B, yeast DNA and RNA. Bands indicated by numbers were excised
95 and after reamplification (as described in materials and methods), subjected to sequencing. The
96 identification of the bands is reported in Table 3.

97 Figure 2. RAPD-PCR cluster analysis of profiles obtained by PCR with primer M13 from
98 *Lactobacillus plantarum* strains isolated from the Feta cheeses. Panel A. *Lb. plantarum* isolated
99 from sample cheese A. Panel B. *Lb. plantarum* isolated from cheese B. Panel C. *Lb. plantarum*
100 isolated from cheese C. Panel D. *Lb. plantarum* isolated from all 4 cheese on plates incubated at 22
101 °C. For strains in figures 4A, 4B and 4C, apart from the code, the temperature of incubation of the
102 plates from where they were isolated is also indicated. A line was drawn at 70 % of similarity and
103 clusters are indicated with roman numerals.

104

Figure 1

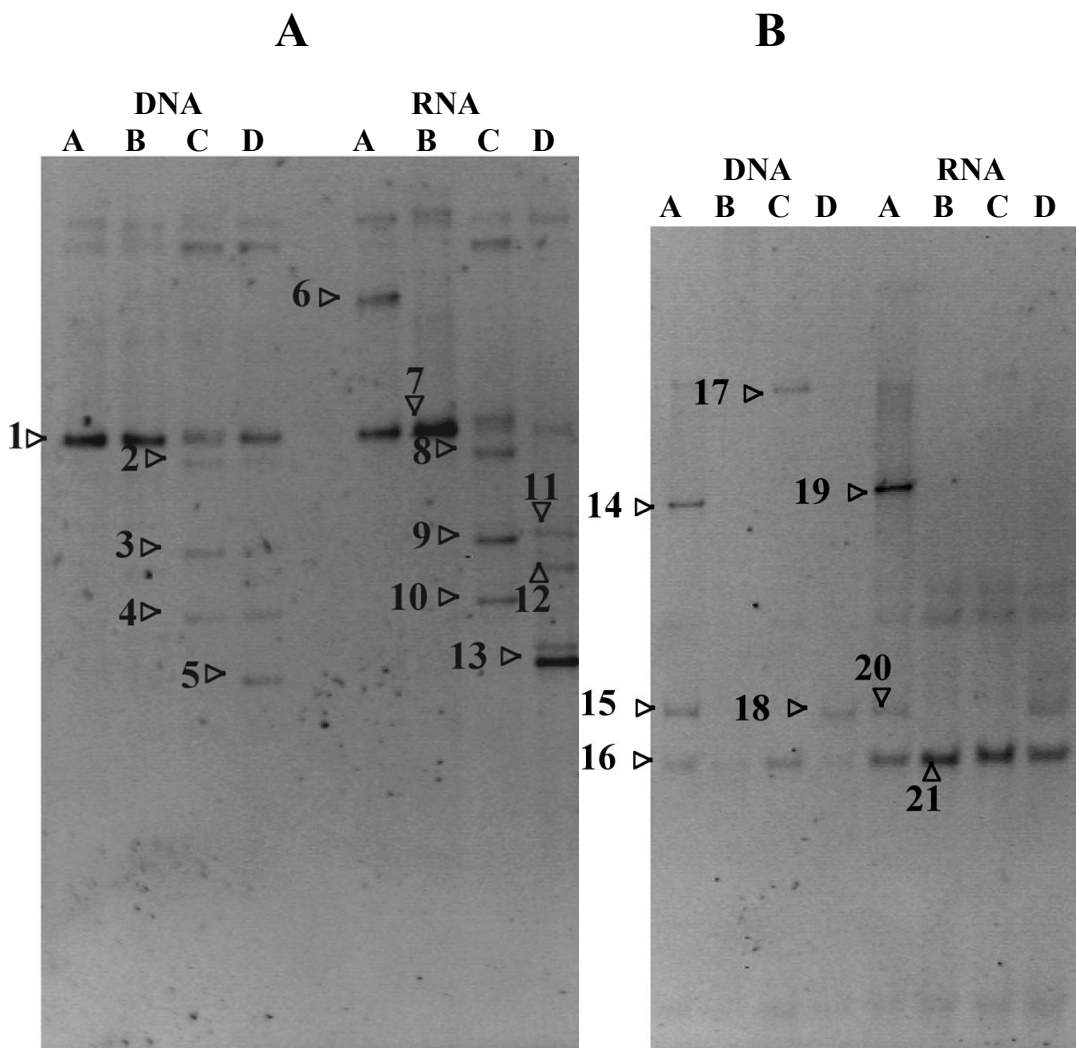
A
DNA
A B C D
RNA
A B C D
1
2
3
4
5
6
7
8
9
10
11
12
13
B
DNA
A B C D
RNA
A B C D
14
15
16
17
18
19
20
21

Figure 2