Molecular evaluation of microbial diversity occurring in different types of Mozzarella cheese

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Aims: The microbial community of different types of unripened Pasta Filata cheese was investigated by culture-independent methods with the aim of rapidly achieving knowledge about cheese microbiota and discriminating traditional and industrial cheeses.

Methods and Results: The microbial DNA extracted directly from the samples was used as a template in PCR experiments to amplify the 16S–23S rDNA spacer region and the V3 region of the 16S rDNA. Conventional electrophoresis of the amplified spacers allowed known classes of these DNA fragments belonging to genera and species of lactic acid bacteria to be distinguished. Denaturing gradient gel electrophoresis analysis of V3 amplicons was supported by reference cultures of LAB used as markers.

Conclusions: Both molecular approaches furnished the expected information about microbial diversity and were quite valid for discriminating industrial, semi-artisanal or traditional cheeses, characterized by increasingly complex DNA profiles.

Significance and Impact of the Study: Both methods could be used for legal purposes when products obtained through prescribed manufacturing regulations are to be analysed.

INTRODUCTION

Mozzarella is the most popular member of the ‘Pasta Filata’ cheeses. At present, in Europe, the following are produced: water-buffalo Mozzarella cheese from Campania (EC Rule no. 1107/96), Mozzarella from cow’s milk, and another very common cow’s milk product in Italy called ‘Fior di latte’. Apart from the different origins of the milk used, these unripened dairy products are manufactured either according to traditional procedures (raw milk inoculated with natural whey or milk cultures, raw milk ripened under special conditions, without starter addition) or by using pasteurized milk and commercial starter cultures of lactic acid bacteria (LAB). Moreover, Mozzarella cheese is also known to be produced by direct acidification with lactic acid, citric acid or glucono-δ- lactone (Parente and Moschetti 1997).

Within the complex bacterial community of traditional raw milk cheeses, lactic acid bacteria are considered to be the dominant microflora, but many genera and species of these micro-organisms are necessarily involved in the curd ripening process in order to assure the typical taste and aroma of the final cheese through appropriate acidifying, proteolytic and flavouring activities (Coppola et al. 1988, 1990). The variety of such organisms in cheeses depends on the starter used and could therefore represent a good marker to discriminate traditional from industrial products.

Research on the microflora of dairy products relies on the isolation, on suitable substrates, of the cultivable organisms that are not always representative of the complex ecosystem. It has been reported (Ward et al. 1990; Engelen et al. 1998) that only a small fraction of micro-organisms is analysed by conventional methods and often, the isolated strains do not seem to represent the real spectrum of micro-organisms and their genes active in the habitat of choice. By contrast, culture-independent methods can provide a more realistic view of microbial diversity in the ecosystem, as recently demonstrated in lactic acid-fermented maize dough by Ampe et al. (1999).

To analyse microbial communities without their cultivation, denaturing gradient gel electrophoresis (DGGE) (Fischer and Lerman 1979) by sequence-specific separation...
of 16S rDNA amplified fragments has been developed (Fleske et al. 1998). This technique has recently been applied to evaluate microbial diversity in various environments (Ampe et al. 1999; Gelsomino et al. 1999), to ‘profile’ complex microbial communities (Muyzer et al. 1993; Heuer et al. 1997) and to infer the phylogenetic affiliation of the community members (Muyzer et al. 1995).

In addition, the study of the LAB community is generally performed by way of culture-dependent methods associated with a variety of molecular techniques, amongst which PCR 16S–23S rDNA spacer polymorphism has been successfully performed for the genetic differentiation of several species of LAB such as Lactococcus lactis and Streptococcus thermophilus (Nour et al. 1995; Moschetti et al. 1998), Enterococcus faecalis (Hall 1994), Ent. faecium (Moschetti et al. 1998), Lactobacillus delbrueckii subsp. lactis (Moschetti et al. 1997) and Lactobacillus spp. (Berthier and Ehrlich 1998). This technique has also been applied to describe the microbial diversity in situ of different soils and sea waters (Garcia-Martinez et al. 1999). As far as is known, no other studies have exploited PCR 16S–23S rDNA spacer polymorphism analysis to evaluate the microbial diversity of dairy products using DNA extracted directly from food materials.

The aim of this work was to develop culture-independent methods, such as PCR 16S–23S rDNA spacer polymorphism and PCR-DGGE analyses, to study the microbial diversity of unripened Pasta Filata cheeses, in order to discriminate traditional and industrial products on the basis of their DNA profiles of microbial origin.

**MATERIALS AND METHODS**

**Reference strains and growth conditions**

The reference strains used in this study are listed in Table 1. Lactococci, enterococci and Streptococcus thermophilus were grown in M17 broth (Oxoid) supplemented with 10 g l⁻¹ lactose (LM17) at 30, 37 and 44°C, respectively. Leuconostocs and lactobacilli were cultured in MRS broth (Oxoid) at 30 and 44°C, respectively. They were streaked on appropriate agar plates before DNA extraction.

**Samples**

**Unripened ‘Pasta Filata’ cheese samples.** Mozzarella and Fior di Latte cheeses were purchased at local supermarkets and dairy factories in Campania (southern Italy). Samples analysed were: (i) industrial Mozzarella cheeses produced with pasteurized cow’s milk and commercial starter cultures (samples a and b); (ii) water-buffalo Mozzarella cheeses of Campania produced with raw milk and natural whey cultures (samples c to f); (iii) Fior di latte cheeses produced with raw milk and natural thermophilic milk cultures (samples g and h); (iv) traditional Fior di latte cheeses produced with ripened raw milk without any addition of starter cultures (samples i and j).

Immediately after collection, all samples (20 ml or 20 g) were stored at 4°C for not more than 6 h before analysis.

**DNA extraction of reference strains**

Preparation of crude cell extracts was carried out as previously reported (Moschetti et al. 1998). A 1 μl aliquot of the mixture was used directly as template for PCR amplification.

**Isolation of bacterial DNA from dairy samples**

Bacterial DNA extraction from dairy samples was carried out by modification of the method of Lick et al. (1996). In brief, 1 ml or 1 g of sample was frozen at −30°C for 2 h. Then, 600 μl of freshly prepared 400 mmol l⁻¹ NaOH plus 300 μl of 40% (w/v) trisodium citrate dihydrate (Wash Buffer A) were added to the de-frozen sample. After shaking, the sample was incubated for 15 min at room temperature and then centrifuged for 4 min at 10 000 g. The supernatant fluid containing the fat layer was discarded. This step (adding Wash Buffer A) was repeated twice. The pellet was resuspended twice in 1 ml 5x SSC and 150 μl 40% (w/v) trisodium citrate dihydrate (Wash Buffer B), and then incubated for 10 min at room temperature. After centrifugation (4 min at 10 000 g), the pellet was purified with a synthetic resin (Instagene Bio-Rad Matrix, 732–6211 Bio-Rad Laboratories, Richmond, CA, USA) according to the supplier’s instructions. The resulting purified DNA was stored at −30°C until use.

<table>
<thead>
<tr>
<th>Table 1 Reference strains used in this study</th>
<th>Origin*</th>
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<tbody>
<tr>
<td>Enterooccus casseliflavus ATCC 25788T</td>
<td>ATCC</td>
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<tr>
<td>Ent. durans ATCC 11576</td>
<td>ATCC</td>
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<tr>
<td>Ent. faecalis ATCC 19433T</td>
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<tr>
<td>Ent. faecium ATCC 19434T</td>
<td>ATCC</td>
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<tr>
<td>Lactococcus garvieae DSM 20684T</td>
<td>DSM</td>
</tr>
<tr>
<td>L. lactis subsp. lactis ATCC 19435T</td>
<td>ATCC</td>
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<tr>
<td>Streptococcus thermophilus NCDO 822</td>
<td>NCMB</td>
</tr>
<tr>
<td>Lactobacillus delbrueckii subsp. bulgaricus DSM 20081T</td>
<td>DSM</td>
</tr>
<tr>
<td>Lact. delbrueckii subsp. lactis DSM 20072T</td>
<td>DSM</td>
</tr>
<tr>
<td>Leuconostoc lactis DSM 20202T</td>
<td>DSM</td>
</tr>
<tr>
<td>Leuc. mesenteroides subsp. mesenteroides DSM 20343T</td>
<td>DSM</td>
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</tbody>
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* DSM: Deutsche Sammlung von Mikroorganismen und Zelkulturen, Braunschweig, Germany; NCIMB: National Collection of Industrial and Marine Bacteria, Abberdeen, Scotland, UK; ATCC: American Type Culture Collection, Rockville, Maryland, USA. 

† Type strain.
PCR conditions

Amplification of 16S–23S rDNA spacer region. Oligonucleotide primers used for amplifying the 16S–23S rRNA intergenic spacer region were selected from the conserved regions at the 3′ end of the 16S rRNA and the 5′ end of the 23S rRNA genes (Jensen et al. 1993). The sequences of the primers were: G1, 5′ GAAGTCGTAACAAGG 3′ and L1, 5′ CAAGGCATCCACCCTG 3′. Polymerase chain reaction conditions consisted of 25 cycles (1 min at 94°C, 2 min at 55°C, 3 min at 72°C) plus one additional cycle with a final 7 min chain elongation. The presence of specific PCR products was controlled by agarose gel electrophoresis at 7 V cm⁻¹ for 2 h.

Amplification of V3 region of 16S rDNA and DGGE analysis. The primers V3f (5′ CCTACGGGAGGCAGCAG 3′) and V3r (5′ ATTACCGCGGCTGCTGG 3′) spanning the V3 region of the 16S ribosomal DNA of Escherichia coli (Muyzer et al. 1993) were used. A GC-clamp was added to the forward primer, according to Muyzer et al. (1993). Amplification was performed in a programmable heating incubator (MJ Research Inc., Madison, USA). Each mixture (final volume, 25 µl) contained 1 µl of template DNA (about 25 ng), each primer at a concentration of 0·2 µmol l⁻¹, each deoxynucleoside triphosphate at a concentration of 0·25 mmol l⁻¹ MgCl₂, 2·5 µl of 10× PCR buffer and 2·5 U of Taq polymerase (Gibco BRL). Template DNA was denatured for 5 min at 94°C. To increase the specificity of amplification and to reduce the formation of spurious by-products, a ‘touchdown’ PCR was performed (Muyzer et al. 1993). The initial annealing temperature used was 10°C above the expected annealing temperature (65°C), and the temperature was decreased by 1°C every second cycle until the touchdown temperature (55°C) was reached; then, an additional 10 cycles was carried out at 55°C. Primer extension was carried out at 72°C for 3 min. Finally, the samples were incubated for 10 min at 72°C (final extension). PCR products were analysed by denaturing gradient gel electrophoresis (DGGE) using a Dcode apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Samples were applied to 8% urea–formamide denaturing gradient (100% corresponded to 7 mol l⁻¹ urea and 40% (w/v) formamide) in the direction of electrophoresis. The gels were electrophoresed for 10 min at 50 V and for 3·5 h at 200 V, stained with ethidium bromide for 5 min and rinsed for 20 min in distilled water.

Estimation of DNA similarity

The scoring of similarities between individual lanes was performed by computer-aided recognition using the programme Phoretix 1D version 3·01 (Phoretix International Limited, Newcastle upon Tyne, UK). The correlation matrix of the PCR 16S–23S spacer patterns of each sample was performed using the formula described by Upholt (1977) and Nei and Li (1979):

\[ F_{xy} = \frac{2n_{xy}}{n_x + n_y} \]

where \( F_{xy} \) is the proportion of the bands that are common to the samples compared, \( n_{xy} \) is the number of the bands shared by both samples \( x \) and \( y \), and \( n_x \) and \( n_y \) are the total number of bands observed in samples \( x \) and \( y \), respectively. \( F_{xy} \) values were estimated normalizing the spacer fingerprints with molecular weight markers. \( F_{xy} \) values were included in a similarity matrix that was used in the average linkage method by the Cluster procedure of Systat 5.2.1 (Systat 1992) in order to estimate the percentage of similarity (S) in the PCR pattern between samples.

The correlation matrix of the PCR-DGGE patterns of each sample and the linkage method used was performed using the formula and procedure described above.

RESULTS

PCR 16S–23S rDNA spacer of unripened Pasta Filata cheeses

In this work, DNA extracted directly from unripened Pasta Filata cheese samples was used as template in PCR assays. By amplifying the 16S–23S rDNA spacer of the microbial community of different cheeses, the results shown in Fig. 1 were obtained.

PCR profiles of industrial Mozzarella (Fig. 1, lanes a, b) displayed a single band located at 350 bp that could be referred to the spacer region of Streptococcus thermophilus (Fig. 1, lane 1).

Traditional water-buffalo Mozzarella cheese samples (Fig. 1, lanes c to f) are characterized by complex PCR profiles with nine to 10 bands between 700 and 320 bp. In all these samples, in addition to the band of 350 bp referable to the spacer regions of Strept. thermophilus, only one 380 bp band concerning the spacer region of Lactococcus lactis (Fig. 1, lane k) could be recognized. The 520 and 320 bp fragments, representing the two classes of 16S–23S rRNA spacer region of Lactobacillus spp. (Fig. 1, lane m), were only shown by three traditional water-buffalo Mozzarella cheeses (Fig. 1, lane d to f).

Samples g and h of Fior di latte cheese were characterized by DNA profiles with only one major and intense PCR band located at 350 bp (referable to the spacer region of...
Strep. thermophilus), plus four to six minor and less intense PCR bands between 650 and 380 bp.

Finally, samples i and j of Fior di latte cheeses (Fig. 1, lane i and j) were characterized by complex DNA profiles with 10 PCR bands located in the range of 650–320 bp. In particular, the 350 bp band of Strep. thermophilus, which was less intense in sample i, the 380 bp band of L. lactis and the 520 and 320 bp bands of Lactobacillus spp., could be distinguished in these samples.

The PCR 16S–23S rDNA spacer profiles obtained underwent cluster analysis to estimate the percentage of similarity (S) in the spacer profile between microbial communities of the cheeses. As shown in Panel A of Fig. 2, three main clusters were defined at a similarity level of 51%: (i) cluster 1 (S of 100%) comprised the two industrial Mozzarella cheeses; (ii) cluster 2 (S of 80%) included the two Fior di Latte cheeses produced with raw milk and natural thermophilic milk starter; and (iii) cluster 3 (S of 51%) gathered water-buffalo Mozzarella and samples i and j of Fior di latte cheese.

With a second approach, microbial diversity of the same Pasta Filata cheeses was studied by application of denaturing gradient gel electrophoresis to PCR products obtained amplifying the V3 region of 16S rDNA from reference strains and from the microbial community occurring in the cheeses.

The V3 16S regions of 11 reference strains listed in Table 1 were submitted to denaturing gradient gel electrophoresis in order to use the migrating bands as markers (results not shown). Then, marker bands were loaded
together as shown in Fig. 3: Marker 1 (M1), *Lact. delbrueckii* subsp. *lactis* (fragment 4) and *Enterococcus casseliflavus* (fragment 9); M2, *Ent. faecalis* (fragment 3), *Ent. durans* (fragment 8) and *L. garvieae* (fragment 11); M3, *L. lactis* subsp. *lactis* (fragment 2), *Ent. faecium* (fragment 7) and *Leuconostoc mesenteroides* subsp. *mesenteroides* (fragment 10); M4, *Strep. thermophilus* (fragment 1), *Lact. delbrueckii* subsp. *bulgaricus* (fragment 5) and *Leuc. lactis* (fragment 6).

The results of DGGE analysis of the microbial community of unripened Pasta Filata cheeses are shown in Fig. 3. DGGE profiles of industrial Mozzarella cheeses (Fig. 3, lanes a, b) displayed three bands, one of which migrated the same distance in the gel of the 16S V3 fragment of *Strep. thermophilus* (Fig. 3, lane M4, fragment 1). Traditional water-buffalo Mozzarella cheeses (Fig. 3, lanes c to f) were characterized by complex DGGE profiles with 8–13 bands. In all the four samples analysed, DNA bands referred to the 16S V3 region of *Strep. thermophilus* (Fig. 3, lane M4, fragment 1) and *L. lactis* (Fig. 3, lane M3, fragment 2) could be recognized. The 16S V3 region of *Ent. faecalis* (Fig. 3, lane M2, fragment 3) was detected in sample e; its occurrence in sample c is supposed, since a double band is located in the corresponding part of the gel. The 16S V3 region of *Leuc. lactis* (Fig. 3, lane M4, fragment 6) was detected only in sample c. By contrast, the 16S V3 region of *Lact. delbrueckii* subsp. *lactis* (Fig. 3, lane M1, fragment 4) and *Lact. delbrueckii* subsp. *bulgaricus* (Fig. 3, lane M4, fragment 5) were well detected in samples d, e and f.

Samples g and h of Fior di latte cheese (Fig. 3, lanes g and h) were characterized by different DNA profiles with only three DNA fragments, one of which was referable to the 16S V3 region of *Strep. thermophilus* (Fig. 3, Lane M4, fragment 1).

Finally, Fior di latte cheeses i and j (Fig. 3, lane i and j) were characterized by DNA profiles with nine and five DGGE bands, respectively, some of which can be referred to the 16S V3 region of *Strep. thermophilus*, *L. lactis* (Fig. 3, lanes i and j), *Ent. faecalis* (Fig. 3, lanes i and j), *Leuc. lactis* (Fig. 3, lane j) and *Lact. delbrueckii* subsp. *lactis* (Fig. 3, lane i).

To estimate the percentage of similarity (S) in the DGGE profile between microbial communities of Pasta Filata cheeses, a simple dendrogram was performed using a cluster analysis procedure. As shown in panel (b) of Fig. 2, three main clusters were defined at a similarity level of 52%: (i) cluster 1 (S of 67%) comprising the two industrial Mozzarella cheeses; (ii) cluster 2 (S of 80%) including the two Fior di Latte cheeses produced with natural milk cultures as starter; and (iii) cluster 3 (S of 52%) including water-buffalo Mozzarella and Fior di latte cheeses produced with ripened raw milk, without starter addition.

**Fig. 3** DGGE of PCR products obtained with primers V3f and V3r targeting the 16S rDNA V3 region. DNA of microbial origin was extracted directly from the dairy samples and used as templates in PCR amplification. Lanes a, b: industrial Mozzarella cheeses; lanes c-f: water-buffalo Mozzarella cheeses of Campania; lanes g, h: Fior di latte cheeses; lanes i, j: traditional Fior di latte cheeses; M1: *Lactobacillus delbrueckii* subsp. *lactis* DSM 20072T (fragment 4) and *Enterococcus casseliflavus* ATCC 25788T (fragment 9); M2: *Ent. faecalis* ATCC 19433T (fragment 3), *Ent. durans* ATCC 11576 (fragment 8) and *Lactococcus garvieae* DSM 20684T (fragment 11); M3: *L. lactis* subsp. *lactis* DSM 20481T (fragment 2), *Ent. faecium* ATCC 19434T (fragment 7) and *Leuconostoc mesenteroides* subsp. *mesenteroides* DSM 20343T (fragment 10); M4: *Streptococcus thermophilus* CNZR 302 (fragment 1), *Lact. delbrueckii* subsp. *bulgaricus* DSM 20081T (fragment 5) and *Leuc. lactis* DSM 20202T (fragment 6).
DISCUSSION

In this work, a culture-independent DNA extraction combined with PCR 16S–23S rDNA spacer polymorphism and PCR-DGGE analyses was developed to study the microbial diversity of unripened Pasta Filata cheeses manufactured differently. The results obtained with these approaches showed that it is possible to discriminate traditional products from those produced with industrial criteria without any culture-dependent methods.

The amplification of the 16S–23S rDNA spacer region, using DNA extracted directly from cheese samples as template, allowed us to obtain a variety of PCR profiles in which the number of bands increased from industrial to artisanal or traditional dairy products. DNA profiles of this latter type of cheese were grouped in a main cluster with a similarity level of 51%; by contrast, they exhibited a similarity level of 40% with respect to semi-artisanal cheeses and a level of 22% with industrial products.

As far as could be determined, the results presented here mark the first study of the microbial community of dairy products using PCR 16S–23S rDNA spacer polymorphisms combined with a culture-independent DNA extraction.

The drawback of the technique was the difficulty of recognizing a lot of species or genera of LAB when the PCR profiles were very complex. In fact, only the spacer region of Strep. thermophilus, L. lactis and Lactobacillus spp. could be distinguished. The spacer region of Strep. thermophilus was present in all the dairy samples analysed, confirming the importance of this species in the processing of Pasta Filata cheeses (Parente and Moschetti 1997). By contrast, L. lactis was detected only in traditional cheese samples, demonstrating the occurrence of this species in dairy products from unselected microflora and non-pasteurized milk (Coppola et al. 1988; Parente et al. 1997). Finally, Lactobacillus spp. was also found only in traditional cheeses, with the exception of sample c in which no class of spacer region referred to this genus was detected.

The results of the amplification of the 16S V3 region combined with DGGE analysis allowed discrimination of all the typologies of dairy products analysed as well, even though the industrial Mozzarella cheeses showed lower similarity levels compared with that furnished by spacer analysis.

However, using the two different approaches, the level of similarity between samples was quite different. In fact, it must be considered that in 16S–23S spacer analysis, one species or genus can provide one or more bands in the spacer profile, while in PCR–DGGE analysis of the V3 region, each species gives only one band in the pattern.

It was possible to recognize clearly at least six species, as their amplified V3 regions migrated the same distance in the denaturing gel as the reference strain amplicons used as markers. However, due to the complexity of the profiles, some results can be only supposed, requiring the sequencing of the V3 region involved (i.e. Ent. faecalis).

Consistent with the results of spacer analysis, Strep. thermophilus was found in all the samples and L. lactis only in the traditional cheeses, while Lactobacillus spp. were still undetectable in sample c. Only two traditional cheeses showed the amplicon referred to Leuc. lactis, a species previously recognized as responsible for the typical aroma of the finished cheese (Coppola et al. 1990; Moio et al. 1993).

Due to the larger number of bands detected and their resolution in the gel, DGGE analysis is considered to be more discriminating than 16S–23S rDNA spacer analysis and is suitable for analysing the complex microbial community as natural ecosystems. With reference to food products, this technique was used by Ampe et al. (1999) to evaluate the typical microflora of Mexican Pozol using culture-independent DNA extraction.

The other V3 regions found but not identified demonstrated the complexity of the microbial community of raw milk dairy products. Therefore, the microbial diversity of these cheeses requires further investigation. First, this approach requires the use of a variety of other V3 regions from reference strains of important species of lactic acid bacteria as migration markers. Alternatively, different denaturing conditions (i.e. temperature gradient gel electrophoresis, TGGE) could be tested with reference to their resolution capability. Moreover, cloning and sequencing of the V3 region amplicons common in only traditional cheese samples could reveal some technologically interesting species responsible for particular features of the final product.

However, in addition to the potential of the two molecular approaches exploited in the present study for extending the knowledge of fermented food microbiota, the above methods may also be considered as valid for discriminating traditional and industrial cheeses, for legal purposes, when products obtained through prescribed manufacturing regulations are analysed.

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