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Diversity, Dynamics, and Activity of Bacterial Communities during Production of an Artisanal Sicilian Cheese as Evaluated by 16S rRNA Analysis†

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The diversity and dynamics of the microbial communities during the manufacturing of Ragusano cheese, an artisanal cheese produced in Sicily (Italy), were investigated by a combination of classical and culture-independent approaches. The latter included PCR, reverse transcriptase-PCR (RT-PCR), and denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes (rDNA). Bacterial and *Lactobacillus* group-specific primers were used to amplify the V6 to V8 and V1 to V3 regions of the 16S rRNA gene, respectively. DGGE profiles from samples taken during cheese production indicated dramatic shifts in the microbial community structure. Cloning and sequencing of rDNA amplicons revealed that mesophilic lactic acid bacteria (LAB), including species of *Leuconostoc*, *Lactococcus lactis*, and *Macrococcus caseolyticus* were dominant in the raw milk, while *Streptococcus thermophilus* prevailed during lactic fermentation. Other thermophilic LAB, especially *Lactobacillus delbrueckii* and *Lactobacillus fermentum*, also flourished during ripening. Comparison of the rRNA-derived patterns obtained by RT-PCR to the rDNA DGGE patterns indicated a substantially different degree of metabolic activity for the microbial groups detected. Identification of cultivated LAB isolates by phenotypic characterization and 16S rDNA analysis indicated a variety of species, reflecting to a large extent the results obtained from the 16S rDNA clone libraries, with the significant exception of the *Lactobacillus delbrueckii* species, which dominated in the ripening cheese but was not detected by cultivation. The present molecular approaches combined with culture can effectively describe the complex ecosystem of natural fermented dairy products, giving useful information for starter culture design and preservation of artisanal fermented food technology.

The practice of producing cheese from milk is largely based on fermentation by lactic acid bacteria (LAB), which may be added deliberately as starter cultures or are adventitious microbiota selected during the fermentation process (12). The final aim is to achieve an attractive and durable product. Manufacture of a typical cheese involves many different steps, including renneting of the milk, acid production, heating, salting, and ripening, that have marked effects on the microbial composition. The advantage of starter cultures, particularly with pasteurized milk, is the greater safety due to rapid acidification and greater uniformity of the final product (12).

Our present knowledge of microbial diversity in commercial cheeses made with raw or pasteurized milk produced with starter cultures is based mainly on cultivation studies followed by identification of some dominant microorganisms by taxonomic and/or phylogenetic methods (7, 9, 40). Raw milk microbiota and nonstarter LAB, especially those in Registered Denomination of Origin (RDO) cheeses, enhance the flavor of cheeses and therefore have received special attention (11, 24, 25, 26, 33). Only limited information is available concerning the microbiota involved in artisanal cheese varieties, i.e., no deliberately added starter, that are manufactured in farmhouses by traditional techniques (6, 22).

Ragusano cheese is an artisanal cheese manufactured throughout the year from raw milk produced by cows of the Modicana, Brown Swiss, or Holstein breed by traditional methods in the Hyblean area of Sicily (Italy) (20). The cheese has the specificity of RDO that implies that its unique organoleptic characteristics are derived from local environmental conditions, including the race and nutrition of the cows, as well as the cheese manufacturing practices (14). Briefly, the manufacturing process involves coagulation of raw milk by adding lamb or goat rennet paste; the curd is subsequently cooked twice and then incubated to allow lactic development by the adventitious microbiota. The curd is again heated to about 49°C, stretched into a block, and salted in brine, and may be ripened for up to a year (21). The resulting cheese, which belongs to the pasta filata or stretched curd group, is semihard, with a dense texture and a mild and pleasant flavor (3). The autochthonous microbiota also play a significant role in the organoleptic traits of the cheese, but no information is available on the evolution and

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the nature of the microbial groups during the manufacturing and ripening of this cheese.

Conventional cultivation methods, prior to characterization by physiological and biochemical tests, and molecular techniques such as ribotyping, amplified fragment polymorphism, and randomly amplified polymorphic DNA, can give significant insight into specific isolates and microbial populations during cheese manufacture (5, 32, 34, 38, 42). However, cultivation can over- or underestimate the microbial diversity, as media may not be sufficiently selective, and furthermore is laborious and time-consuming for monitoring population dynamics. Novel molecular approaches, especially those based on the use of rRNA and rRNA genes (rDNA), have provided the opportunity to analyze complex communities on the basis of sequence diversity (1).

Denaturing gradient gel electrophoresis (DGGE) and the related technique, temperature gradient gel electrophoresis (TGGE), have been applied in microbial ecology to resolve PCR-amplified regions of 16S rRNA genes or rRNA based solely on differences in nucleotide sequence (27). These techniques have proven to be valuable approaches to compare the structures of complex microbial communities and to monitor their dynamics in relation to environmental factors in several ecosystems (2, 45). More recently, methods for qualitative analysis of specific populations with a complex ecosystem based on genus-specific PCR and DGGE have been developed (15, 37).

The aims of this study were (i) to study the microbial communities in an artisanal fermented food product, Ragusano cheese, and (ii) to assess cultivation-independent molecular methods for analysis of population dynamics during cheese manufacture. The present study describes a method to isolate reverse transcription (RT)-PCR-quality DNA and rRNA from milk and cheese that, combined with DGGE, shows the changing biodiversity of the dominant as well as the specific *Lactobacillus* community during cheese manufacture. Comparison of the DNA- and RNA-derived DGGE profiles revealed the metabolically active members, and the value of the molecular versus cultivation approaches for analysis of food ecosystems is discussed.

MATERIALS AND METHODS

Cheese sampling. The cheese samples used in this study were obtained from three farmers who produce Ragusano-type cheeses by traditional methods located in different areas of the Hyblean region in Sicily, and the cheeses were chosen because of their high quality. Farmers I (best quality) and II used Modicana cow milk, and farmer III used Brown Swiss cow milk. Samples were taken aseptically from the raw milk and during manufacture of cheese and ripening and subjected to bacteriological analysis within 6 h or stored at −80°C.

Enumeration and isolation of microorganisms. Samples of milk containing added rennet, curd before cooking, curd after 24 h of fermentation, and fresh and ripened cheeses obtained from farmer I were homogenized in sterile physiological solution (0.9% NaCl) with a Stomacher Lab-Blender 400 (Seward Medical, London, United Kingdom) for approximately 5 min. Samples of raw milk and the homogenized samples were serially diluted in 0.9% NaCl. Aliquots of all samples were plated for microbial enumeration using the pour plate method, i.e., 1-ml portions of dilutions were inoculated directly into the molten media.

The media used for enumeration were as follows: PCA medium (Oxoid, Basingtoke, United Kingdom) for aerobic mesophilic microorganisms; MRS-glucose medium (Oxoid) acidified to pH 5.4 with HCl or Rogosa agar (Oxoid) for thermophilic lactobacilli; M17 medium (Oxoid) containing 1% lactose (LM17) for *Streptococcus*; LM17 medium containing cycloheximide (Fluka Chimica, Milan, Italy) (100 μg per liter added after sterilization) for *Lactococcus*; KAA (kanamycin-esculin-azide; Oxoid) agar base containing kanamycin selective supplement (Oxoid) for enterococci; MSE agar medium (Biolife, Milan, Italy) for *Leuconostoc*; and FH medium (16) for nonstarter mesophilic lactobacilli. Vancomycin antibiotic (50 ng μl^{-1}) (Sigma, Milan, Italy) was added after sterilization to FH medium. Plates containing PCA medium were incubated aerobically at 32°C for 48 h; MRS, M17, and KAA agar media were incubated under anoxic conditions using the Anaerocult A system (Merck, Darmstadt, Germany) at 37°C for 48 to 72 h. Plates containing MSE and FH were incubated at 30°C for 48 to 72 h, and M17 plates with cycloheximide were incubated at 22°C for 5 days.

Physiological and biochemical characterization of isolates. To analyze the total LAB population, 32 colonies were randomly picked from different agar plates with different samples, and each colony was purified by streaking three times. All isolates were characterized by determining their Gram reaction, catalase activity, spore formation, and ability to grow in MRS broth at 10 and 45°C in stationary tubes. Cell morphology was observed with a phase contrast microscope. Carbohydrate fermentation patterns were determined using API 50 CH test strips, and the isolates were identified using the bioMérieux SA software (bioMérieux, Nurtingen, Germany). All isolates were stored in liquid cultures with 20% glycerol at −80°C.

Preparation of total DNA from pure cultures. Cell cultures (1.5 ml) in the late exponential growth phase were centrifuged at 8,000 rpm for 10 min, and the cell pellets were washed and resuspended in 0.5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The suspension was homogenized in a 2-ml screw-cap tube containing 0.3 g of sterile zirconium beads (diameter, 0.1 mm) in a bead beater (Biospec Products, Bartlesville, Okla.) at 5,000 rpm for 180 s and cooled on ice. The homogenate was centrifuged at 13,000 rpm for 5 min, and the supernatant fluid was used as the template for PCR or stored at −20°C until use.

Nucleic acid isolation from milk and cheese samples. Dairy samples (2 g) were incubated at 45°C for 30 min with 20 ml of sodium citrate solution (2% [wt/vol] trisodium citrate dihydrate) and glass pearls (diameter, 3 mm; Tamson, Zoetermeer, The Netherlands). The suspensions were mixed with a Vortex mixer for approximately 5 min, the large material was left to settle, and the supernatants were transferred to clean tubes. After centrifugation for 10 min at 8,000 rpm, the fat layer at the top was removed with a cotton tip. The cell pellets were resuspended in 1 ml of TE buffer and centrifuged at 8,000 rpm for 10 min. The supernatant fluid (approximately 900 μl) was removed, and the remaining 100 μl and pellet were mixed and transferred to a 2-ml screw-cap tube containing 0.3 g of zirconium beads and 150 μl of phenol-TE (phenol equilibrated with TE; Life Technologies, Gaithersburg, Md.). The samples were treated at 5,000 rpm for 5 min in a bead beater. After the addition of 150 μl of CI solution, consisting of chloroform and isoamyl alcohol at a 24:1 (vol/vol) ratio, the tubes were vortexed briefly and centrifuged at 13,000 rpm for 5 min. The aqueous phase was divided into two aliquots of 0.5 ml, one each for DNA and RNA isolation.

For the DNA isolation, phenol-chloroform extractions were performed on the 0.5-ml aliquots with 150 μl of phenol-TE and 150 μl of CI solution until a clear interface was obtained. After a final CI extraction, the DNA was precipitated by addition of 2 volumes of ethanol (−20°C) to the aqueous phase. After incubation at −20°C for 30 min, the DNA was collected at 13,000 rpm for 20 min, washed briefly with 70% ethanol, and resuspended in 500 μl of TE. Five units of DNase-free RNase (Promega) was added, and the sample was incubated at 37°C for 15 min. Several CI extractions were performed until a clear interface was obtained. The DNA was precipitated with 3 M sodium acetate (pH 5.2) and 96% ethanol (−20°C) and stored at −20°C for 30 min. DNA was collected by centrifugation and resuspended in 50 μl of TE buffer. The amount and integrity of the nucleic acids were determined visually after electrophoresis on a 1.2% agarose gel containing ethidium bromide and comparison to standard concentrations of DNA markers.

Total RNA was extracted from the second 0.5-ml lysate as previously described (45) except the DNase (Roche) treatment was repeated once to remove all traces of DNA.

PCR amplification. PCR amplification was performed with the *Taq* DNA polymerase kit from Life Technologies. Reaction mixtures consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 50 mM each of the four deoxynucleoside triphosphates (dNTP), 1.25 U of *Taq* polymerase, 5 pmol of each primer, and 1 μl of appropriately diluted template DNA in a final volume of 50 μl.

To investigate the dominant bacterial communities by DGGE analysis, PCR products were generated with PCR primers U968-GC and L1401-r (Table 1) to amplify the V6 to V8 region of eubacterial 16S rDNA (31). The 40-nucleotide GC-rich sequence at the 5′ end of primer U968-GC improves the detection of sequence variations of amplified DNA fragments by subsequent TGGE or DGGE (30). The samples were amplified in a Perkin-Elmer Applied Biosystems

TABLE 1. Primers used in this study

Primer[a]	Sequence (5′ to 3′)	Use	Specificity or target	Reference or source
7-f	AGA GTT TGA TC/TA/C TGG CTCAG	PCR	Eubacterial 16S	19
124-f	CAC GGA TCC GGA CGG GTG AGT AAC ACG	PCR	Eubacterial 16S	19
342-r	CTG CTG CCT CCC GTA G	Sequencing	Eubacterial 16S	19
515-r	ATC GTA TTA CCG CGG CTG CTG CTG GCA	PCR	Universal 16S	19
533-f	GTG CCA GC (AC) GCC GCG GTA A	Sequencing	Universal 16S	19
968-f	AAC GCG AAG AAC CTT A	Sequencing	Eubacterial 16S	19
1100-r	GGG TTG CGC TCG TTG	Sequencing	Eubacterial 16S	19
1510-r	ACG G (C/T) T ACC TTG TTA CGA CTT	PCR	Universal 16S	19
S-G-Lab-0677-r	CAC CGC TAC ACA TGG AG	PCR	*Lactobacillus* 16S	15
L1401-r	GCG TGT GTA CAA GAC CC	PCR/RT-PCR	Eubacterial 16S	31
U 968-f	AAC GCG AAG AAC CTT AC	PCR/RT-PCR	Eubacterial 16S	31
T7	AAT ACG ACT CACT ATA GGG	Sequencing	pGEM^T	Promega Corp.
Sp6	ATT TAG GTG ACA CTA TAG	Sequencing	pGEM^T	Promega Corp.
GC clamp	CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G	PCR/DGGE		30

[a] f, forward; r, reverse.

(Foster City, Calif.) GenAmp PCR System 9700 programmed as follows: initial denaturation of DNA for 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 56°C, and 40 s at 68°C; and extension of incomplete products for 7 min at 68°C.

PCR was performed with primer pairs 7-f and 1510-r plus T7 and Sp6 (Table 1) to amplify the bacterial 16S rDNA prior to cloning and sequence analysis by using the cell lysates as the template to confirm an insert of the correct size. DNA amplification was carried out with the reaction mixtures as described above under the following conditions: 94°C for 3 min; 30 cycles of 94°C for 30 s, 52°C for 30 s, and 68°C for 1.5 min; and finally 68°C for 7 min.

The *Lactobacillus* group-specific primer S-G-Lab-0677-r and primer 7-f (Table 1) were used to amplify the V1 to V3 region of 16S rDNA, and the resulting amplicons were used as templates in nested PCRs and resolved by DGGE as previously described (15). Primer S-G-Lab-0677-r is specific for members of the *Lactobacillus* group and was used to obtain specific fingerprints for the *Lactobacillus* community in Ragusano cheese samples during manufacture or from pure cultures.

PCR products were quantified by electrophoresis on a 1.2% (wt/vol) agarose gel containing ethidium bromide and, where necessary, were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. To examine the biodiversity and to select unique clones prior to sequence analysis, restriction fragment length polymorphism (RFLP) analysis of 16S rDNA PCR products was performed by restriction enzyme digestion with *Hae*III and *Msp*I (Gibco-BRL, Paisley, United Kingdom), followed by electrophoresis of the products on a 2% (wt/vol) agarose gel in 1× TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA [pH 8.0]) containing ethidium bromide.

RT-PCR amplification. Segments of 16S rRNA were reverse transcribed and subsequently amplified by applying the *Tfl* DNA polymerase (Promega Access RT-PCR System) and the primers described for PCR (Table 1). RT-PCR mixtures (final volume, 50 μl) consisted of 10 μl of 5× avian myeloblastosis virus (AMV)-*Tfl* reaction buffer, 1 μl of 10 mM each dNTP, 6 μl of 25 mM $MgSO_4$, 1 μl of 5U AMV Reverse Transcriptase, 1 μl of 5 U *Tfl* DNA polymerase, 1 μl each of primers U968-GC and L1401-r, and 1 μl of appropriately diluted RNA. Reverse transcription and PCR amplification were performed as follows: 45 min at 48°C to encourage full-length cDNA synthesis; incubation for 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 56°C, and 40 s at 68°C; and extension of incomplete products for 7 min at 68°C. The reactions were subsequently cooled to 4°C. PCR without a reverse transcription step was performed to verify the absence of DNA.

DGGE analysis of PCR amplicons. DGGE analysis of PCR amplicons was performed on the Dcode or Dgene System apparatus (Bio-Rad, Hercules, Calif.) essentially as described previously (30, 37). Samples were applied to an 8% (wt/vol) polyacrylamide gel (acrylamide-bisacrylamide, 37.5:1) in 0.5× TAE buffer (2 M Tris base, 1 M glacial acetic acid, 50 M EDTA [pH 8.0]) (35). Optimal separation of the PCR products for the species within these cheese samples was achieved with a 30 to 60% urea-formamide denaturant gradient, increasing in the direction of electrophoresis. A 100% denaturant corresponds to 7 M urea and 40% (vol/vol) formamide. Electrophoresis was performed at a constant voltage of 85 V and a temperature of 60°C for 16 h. The DNA bands were visualized by silver staining and developed as previously described (36).

Cloning and sequence analysis of 16S rDNA in plasmid inserts and from pure strains. Clone libraries of the 16S rDNA amplicons from curd and the 15-day-old cheese of farmer I were constructed. Amplicons derived from PCR of 16S rDNA using primer pairs 7-f and 1510-r or 7-f and S-G-Lab-0677-r were purified and cloned in *Escherichia coli* JM109 using the pGEM-^{T} plasmid vector system (Promega, Madison, Wis.) in accordance with the manufacturer's instructions. Colonies containing 16S rDNA inserts were screened by RFLP analysis, which confirmed an insert of the correct size and the biodiversity within the microbial community (data not shown). The appropriate regions of the 16S rDNA in the cell lysates of transformants were amplified, and their mobilities were compared to the rDNA-derived patterns of curd and cheese samples by DGGE (data not shown). The clones that produced a single DGGE amplicon with a melting position identical to that of one of the dominant bands in the curd and cheese DNA patterns were selected for sequence analysis.

The plasmid inserts from the corresponding clones were sequenced (see Table 1 for primers) using the Sequenase sequencing kit (Amersham, Slough, United Kingdom) to obtain the complete double-stranded sequence of the 16S rRNA genes (see Table 1 for primers). For pure strains, 10 μl of PCR product of the 16S rDNA was directly sequenced. Products of sequencing reactions were analyzed with an automatic Li-Cor (Lincoln, Neb.) DNA sequencer 4000L and corrected manually. Similarity searches of 16S rDNA sequences derived from cheese clones were performed using the Baylor College of Medicine nucleic acid sequence search service available on the Internet (http://www. hgsc.bcm. tmc.edu/SearchLauncher). The complete 16S rDNA sequences were checked for chimerical constructs using the CHECK-CHIMERA program of the Ribosomal Database Project (23) and the ARB software package (41).

Nucleotide sequence accession numbers. The sequences of the 16S rDNA clones were deposited in the GenBank database. The accession numbers of the 12 cheese clones are as follows (clone code in parentheses): AF349917 (20CR), AF349918 (32CR), AF349920 (39CR), AF349924 (50CR), AF349922 (51CR), AF349923 (45CR), AF349919 (37CR), AF349921 (41CR), AF349925 (54CR), AF349926 (60CR), AF349928 (G3-CR), and AF349927 (R3-CR). The accession numbers of the 16S rDNAs for the 10 LAB isolates are (strain code in parentheses): AF349929 (CR300S), AF349930 (CR303S), AF349931 (CR310S), AF349932 (CR314S), AF349933 (CR316S), AF349934 (CR317S), AF349935 (CR6AS), AF349936 (CR7AS), AF349937 (CR609S), and AF349938 (CR6S).

RESULTS

Application of a PCR-DGGE method to monitor population dynamics in cheeses. To investigate the diversity and the dynamics of the dominating microbial communities during Ragusano cheese production, samples from milk through to the 30-day ripened cheese and from a 15-day-old cheese produced after a period of 6 months, all provided by farmer I, were analyzed by PCR-DGGE (Fig. 1). The appearance and disappearance of amplicons in the DGGE pattern indicate impor-

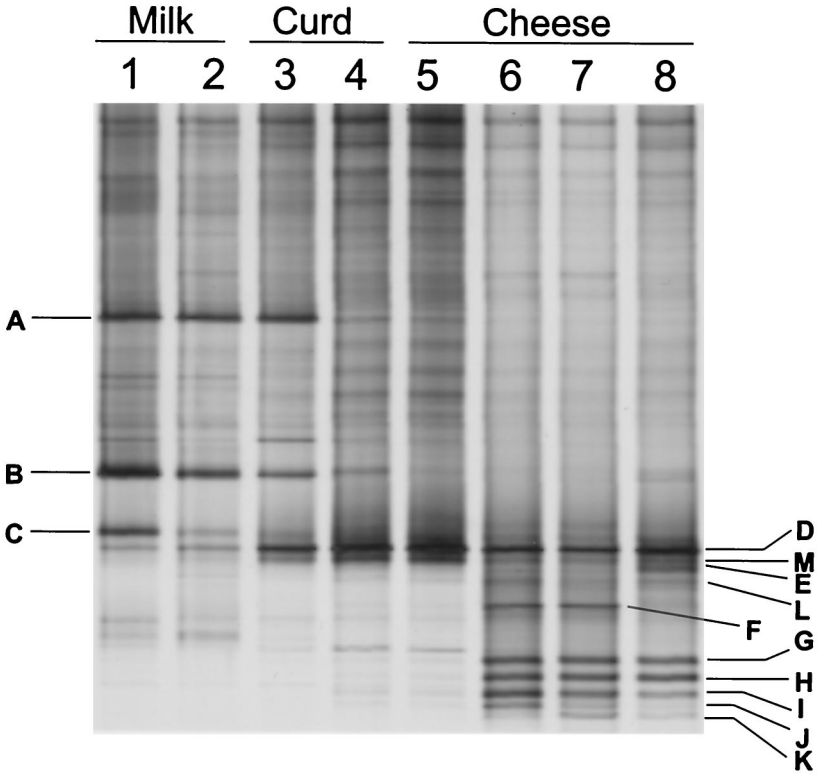

FIG. 1. DGGE of PCR products of the V6 to V8 regions of 16S rDNA that represent the biodiversity in samples taken during Ragusano cheese manufacture provided by farmer I. Lanes: 1, milk; 2, milk with added rennet; 3, curd; 4, curd after fermentation; 5, fresh cheese; 6 and 7, 15- and 30-day ripened cheese, respectively; and 8, a 15-day ripened cheese provided by the same farmer after a period of 6 months. The positions of bands discussed in the text are indicated by letters that correspond to species of bacteria: A, *Macrococcus caseolyticus*; B, *Lactococcus lactis*; C, *Leuconostoc mesenteroides*; D, *Streptococcus thermophilus*; E: *Streptococcus bovis*; F, G, H, I, and J, *Lactobacillus delbrueckii* subsp. *bulgaricus*; K, *Lactobacillus fermentum*; L, *Lactobacillus casei*; and M, *Enterococcus hirae*. The identification of the bands is discussed in the text.

tant shifts in the microbial community structure. The intensity of an individual band is a semiquantitative measure for the relative abundance of this sequence in the population (30).

Analysis of samples taken during manufacture, ranging from raw milk to ripened cheeses, showed quite dramatic changes in the profiles, with an increase in the diversity during the ripening period. Most of the amplicons (A, B, and C) that dominated in the milk, milk with added rennet, and curd (Fig. 1, lanes 1 to 3) disappeared during fermentation (lane 4) and did not reappear during the ripening process. Other bands (E to M) appeared only during the ripening period (lane 6, 7, and 8). Only one band, D, was present in all samples; it became more intense in the curd after the 24-h fermentation and also dominated the profile in the fresh cheese. Similar profiles were present in the 15- and 30-day ripened cheese (lanes 6 and 7) and in a 15-day ripened cheese of another fermentation provided by the same farmer after a period of 6 months (lane 8), and only amplicon F was not present in the latter. Weaker bands were also present (for example, K, L, and M), but other weak bands have not been labeled.

To determine if the diversity and population changes were similar between different Ragusano cheeses made on different farms, samples taken during cheese manufacture provided by three farmers, I, II, and III, were analyzed by PCR-DGGE (Fig. 2). Comparison of the three cheeses showed similar trends, with higher bands in the curd (lanes 1 and 2) disappearing during fermentation (lanes 2 and 3) and lower bands becoming more apparent in the ripened cheeses (lanes 4 and 5). There were many amplicons at the same position in all three cheeses (a to e in lanes 1, 2, and 3 and i and j in lanes 3 to 5), strongly suggesting similarity in the microbial composition. The profiles of cheeses from farmers I and II were clearly very similar; for example, amplicons g, h, and k (lanes 4 and 5) were observed only in these DGGE patterns. The composition of the third cheese appeared the most different, and the profiles contained new distinct dominant amplicons (bands s, t, and u, lanes 1 and 2). Amplicon d appeared to be the most dominant at some stage in samples from all three farmers.

Culture-independent identification of the dominant species. Clone libraries of the complete 16S rDNA amplicons from the curd and the 15-day-old cheese samples from farmer I were constructed in order to identify some of the dominant bands in the rDNA-derived patterns (Table 2 and Fig. 1). Band A in the curd sample (Fig. 1, lane 2) originated from *Macrococcus caseolyticus.* The closest relative corresponding to the strong band D in the 15-day-old cheese originated from a *Streptococcus thermophilus*-like sequence. Most of the other clones from the 15-day cheese resulted in sequences derived from species belonging to the *Lactobacillus* genus and corresponded to amplicons F to K (Fig. 1, lane 6, and Table 2). No clones were detected corresponding to dominant bands B and C (Fig. 1, lane 2) in the clone library for the curd.

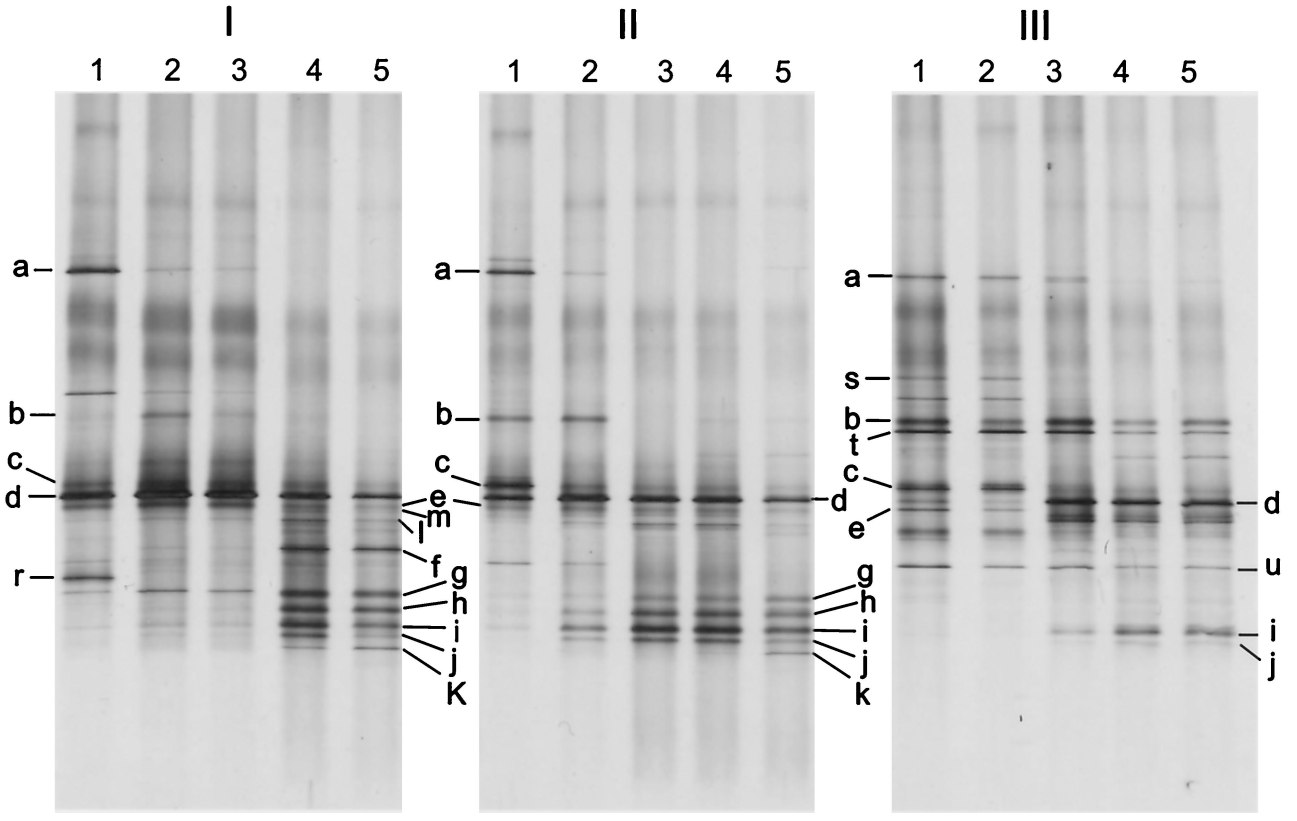

FIG. 2. DGGE of PCR amplicons of the V6 to V8 regions of 16S rDNA that represent the biodiversity in samples taken during Ragusano cheese manufacture provided by three different farmers, I, II, and III. Lanes: 1, curd; 2, curd after fermentation; 3, fresh cheese; 4 and 5, 15- and 30-day ripened cheese, respectively. The positions of bands discussed in the text are indicated by the letters. Bands a to m correspond to the same bands in Fig. 1.

Classical enumeration and phenotypic characterization of isolates. The microbiota present in Ragusano cheese during the manufacturing and ripening process were enumerated by cultivating on media with the intention of selecting different groups of bacteria (Table 3). The initial total aerobic counts of microorganisms (PCA medium) in raw milk was 1.6×10^8 CFU g^{-1}, and these reached their highest levels after acid development in the curd. The presumptive streptococcal (LM17 medium) and lactococcal (LM17 medium with cycloheximide) counts were reasonably high in the raw milk and increased in the curd during lactic acid development. The low counts of enterococci (KAA medium) and leuconostocs (MSE medium) in the milk increased slowly during manufacture but remained low in the final ripened cheese. The initial high

TABLE 2. Identification of dominant fragments in DGGE patterns of the total microbial community and the *Lactobacillus* group population

Community	Sample	Clone	Fragment	Length (bp)	Closest sequence relative (species)	% Identity	Genbank accession no.
Total[a]	Curd	20CR	A	1,193	*Macrococcus caseolyticus*	98	AF349917
		32CR	D	1,479	*Streptococcus thermophilus*	97	AF349918
	Cheese, 15-day	39CR	D	1,471	*Streptococcus thermophilus*	98	AF349920
		50CR	E	1,251	*Streptococcus bovis*	96	AF349924
		51CR	F	1,487	*Lactobacillus delbrueckii* subsp. *bulgaricus*	97	AF349922
		45CR	G	1,189	*L. delbrueckii* subsp. *bulgaricus*	97	AF349923
		37CR	H	1,484	*L. delbrueckii* subsp. *bulgaricus*	99	AF349919
		41CR	I	1,183	*L. delbrueckii* subsp. *bulgaricus*	97	AF349921
		54CR	J	1,486	*L. delbrueckii* subsp. *bulgaricus*	99	AF349925
		60CR	K	1,498	*Lactobacillus fermentum*	99	AF349926
Lactobacillus[b]	Curd	G3-CR	B	673	*Leuconostoc lactis*/*Leuconostoc argentinum*	99	AF349928
		R3-CR	C	823	*L. delbrueckii* subsp. *lactis*	98	AF349927

[a] Letters correspond to fragments in Fig. 1.
[b] Letters correspond to fragments in Fig. 4.

TABLE 3. Microbial enumeration of samples taken during Ragusano cheese production on different media[a]

Sample	Bacterial count (log_{10} CFU/g of sample)						
	PCA (aerobic mesophiles)	M17 (*Streptococcus* spp.)	M17$^+$ (*Lactococcus* spp.)	FH (mesophilic *Lactobacillus* spp.)	MRS (pH 5.2) (thermophilic *Lactobacillus* spp.)	MSE (*Leuconostoc* spp.)	KAA (*Enterococcus* spp.)
Milk	8.2	7.9	8.2	8.0	5.3	4.3	5.3
Milk & rennet	8.5	8.4	8.5	8.5	6.4	4.5	6.4
Cooked curd	8.8	8.9	8.8	7.9	7.5	5.6	5.7
Curd after 24 h	8.8	9.0	9.1	7.8	8.4	6.8	5.8
Fresh cheese	8.5	8.8	8.3	8.3	8.3	6.1	6.1
Cheese, 15-day	8.5	9.2	7.8	8.7	8.6	7.1	6.8
Cheese, 30-day	8.4	8.5	7.6	8.7	8.6	7.7	7.4

[a] See the text for an explanation of the media. The targeted microorganisms are shown for each medium. M17$^+$ is M17 containing cycloheximide.

mesophilic lactobacillus (FH medium) counts decreased during the cooking process but increased again during ripening, while the initial low thermophilic lactobacillus (MRS medium) counts increased throughout manufacture to reach high numbers. It is noteworthy that the media were not always very selective for the groups (see below), but overall the plate counts suggested the predominance of lactobacilli and streptococci in the final cheese.

A total of 32 isolates were randomly selected from agar plates of a range of selective media for phenotypic and biochemical characterization as described in Materials and Methods. The sources of the isolates and the media and incubation conditions by which they were isolated are presented in Table 4. All 32 isolates were considered LAB based on their positive Gram reactions, nonmotility, absence of catalase activity and spore formation (data not shown), and rod or coccal shape. Fifteen of the isolates produced gas from glucose, indicating a heterofermentative metabolism (Table 4). Ten of the isolates grew at both 10 and 45°C after incubation for 5 days and 48 h, respectively. Six of the isolates were mesophilic and grew at 10°C but not at 45°C. Sixteen of the isolates grew well only at 45°C.

The bioMérieux SA software, based on carbohydrate fermentation patterns, assigned 11 homofermentative coccal strains to the *Lactococcus lactis* subsp. *lactis* species with a probability of identification ranging from 48 to 99%. The homofermentative strain CR6, showing coccal cells in tetrads, was identified as *Pediococcus acidilactici.* The two heterofermentative coccoid isolates were classified as *Leuconostoc mesenteroides*. Five homofermentative strains were identified as *Lactobacillus casei* (strains CR600, CR601, CR606, and CR609), and *Lactobacillus plantarum* (CR7A). The 13 remaining homo- and heterofermentative lactobacilli were classified as *Lactobacillus fermentum* species.

Identification and diversity of isolates by 16S rRNA analysis. The 16S rDNA genes of the 32 pure isolates were screened by RFLP and PCR-DGGE (based on migration differences of amplicons generated with primers U968GC-f and 1401-rev), which distinguished 10 unique types (data not shown). The identity of the 10 isolates was determined by sequence analysis

TABLE 4. Physiological characterization of isolates from Ragusano cheese

Isolate(s)	Source	Medium	Growth at:		CO_2 from glucose	Morphology	% Probability of identification (species)[a]
			10°C	45°C			
CR300	Milk	KAA	+	+	−	Cocci in pairs	53 (*L. lactis*)
CR303	Milk	LM17$^+$	+	+	−	Cocci in pairs	48 (*L. lactis*)
CR304	Milk	LM17$^+$	+	−	−	Cocci in pairs	51 (*L. lactis*)
CR314	Milk	LM17	−	+	−	Cocci in chains	88 (*L. lactis*)
CR301	Milk + rennet	KAA	+	+	−	Cocci in pairs	48 (*L. lactis*)
CR305	Milk + rennet	LM17$^+$	+	−	−	Cocci in chains	89 (*L. lactis*)
CR315, CR316	Milk + rennet	LM17	−	+	−	Cocci in pairs	89 (*L. lactis*)
CR317, CR318	Curd	LM17	+	−	−	Cocci in pairs	89 (*L. lactis*)
CR310	Curd 24 h	LM17$^+$	+	−	+	Cocci in chains	99 (*L. mesenteroides*)
CR319	Curd 24 h	LM17	+	−	+	Cocci in pairs	99 (*L. mesenteroides*)
CR322	Fresh cheese	LM17	+	+	−	Cocci in chains	87 (*L. lactis*)
CR2	Fresh cheese	MRS (pH 5.4)	−	+	+	Rods	98 (*L. fermentum*)
CR10, CR11	15-day cheese	Rogosa	+	+	+	Rods	98 (*L. fermentum*)
CR1, CR3, CR4, CR5	15-day cheese	MRS (pH 5.4)	−	+	+	Rods	98 (*L. fermentum*)
CR6	15-day cheese	MRS (pH 5.4)	−	+	−	Cocci in tetrads	99 (*P. acidilactici*)
CR7, CR8, CR9, CR4A CR5A	15-day cheese	Rogosa	−	+	+	Rods	99 (*L. fermentum*)
CR600, CR609	15-day cheese	MRS (pH 5.4)	+	+	−	Rods	99 (*L. casei*)
CR6A	30-day cheese	Rogosa	−	+	+	Rods	99 (*L. fermentum*)
CR7A	30-day cheese	Rogosa	−	+	−	Rods	99 (*L. plantarum*)
CR601, CR606	30-day cheese	MRS (pH 5.4)	+	+	−	Rods	99 (*L. casei*)

[a] Probability of correct identification by the bioMérieux software.

TABLE 5. Identities of pure isolates

Strain	16S rDNA sequence	Closest relative	Identity (%)	Fragment[a]	GenBank accession no.	Related isolates[b]
CR300	1,503	*Enterococcus faecalis*	99	E–L[c]	AF349929	
CR303	1,400	*Enterococcus hirae*	98	M	AF349930	CR301, CR305, CR304, CR318
CR310	1,399	*Leuconostoc mesenteroides*	99	C	AF349931	CR319
CR314	1,445	*Streptococcus thermophilus*	98	D	AF349932	CR315, CR322
CR316	1,456	*Enterococcus sulfurans*	97		AF349933	
CR317	1,228	*Lactococcus lactis*	97	B	AF349934	
CR6A	1,513	*Lactobacillus fermentum*	99	K	AF349935	CR4A, CR5A, CR1, CR2, CR3, CR4, CR5, CR7, CR8, CR9, CR10
CR7A	1,250	*Lactobacillus plantarum*	98		AF349936	CR11
CR609	1,220	*Lactobacillus casei*	99	L	AF349937	CR600, CR601
CR6	1,530	*Pediococcus acidilactici*	98		AF349938	CR606

[a] Letters correspond to fragments in Fig. 1.
[b] Isolates that had the same RFLP and DGGE patterns.
[c] Fragment was positioned between these bands in Fig. 1.

of most (1,220 to 1,530 bp) of the 16S rDNA. The rDNA sequences demonstrated the highest identity (96 to 99%) with 16S rDNA sequences of members of the *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Pediococcus*, and *Lactococcus* genera in GenBank (Table 5).

The positions of the PCR products from the 10 isolates were compared with the microbial community DGGE patterns of samples from Ragusano cheese manufacture obtained from farmer I (Fig. 1) in order to further identify the origins of the amplicons. Seven of the 10 PCR products corresponded precisely to one of the bands identified in the DGGE patterns (Table 5). Intense bands B and C (lane 1, Fig. 1) corresponded to isolates CR317 and CR310, whose closest relatives were *Lactococcus lactis* and *Leuconostoc mesenteroides*, respectively. *Streptococcus thermophilus* isolate CR314 and *Lactobacillus fermentum* isolate CR6A (Table 5) corresponded to bands D and K (Fig. 1), respectively, as expected from the clone library results (Table 2). *Lactobacillus casei* (CR609) showed the same position as the weak amplicon L (lane 6, Fig. 1). Isolate CR303, identified as *Enterococcus hirae*, corresponded to amplicon M (lanes 3 to 8, Fig. 1). A weak band between bands E and L appeared to correspond to *Enterococcus faecalis* CR300. *Enterococcus sulfurans* CR316, *Pediococcus acidilactici* CR6, and *Lactobacillus plantarum* CR7A (Table 5) did not appear to correspond to any bands in the profiles.

Comparison of DNA- and RNA-derived DGGE profiles. DGGE profiles derived from PCR and RT-PCR of DNA and rRNA, respectively, of milk and cheese samples were compared in order to determine the expression level of the 16S rRNA genes of the most predominant bacteria, which may reflect their contribution to total activity (10). The DGGE patterns derived from DNA and RNA isolated from milk, curd, and 15-day ripened cheese samples provided by farmer I showed high similarity (Fig. 3). Amplicons that corresponded to *Lactococcus lactis* (B) and *Leuconostoc mesenteroides* (C) in the milk and curd samples appeared more intense in the DNA-derived profiles compared to the RNA-derived profiles, indicating that these bacteria might not be metabolically active. Similarly, in the cheese sample, *Lactobacillus acidophilus* (I) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (J) appeared to be metabolically active. In contrast, amplicons of *Macrococcus caseolyticus* (A), *Streptococcus thermophilus* (D) in the milk and curd, as well as other amplicons in the 15-day cheese showed similar intensities between DNA- and RNA-derived profiles.

Population dynamics and diversity of the *Lactobacillus* community. The diversity and population dynamics of *Lactobacillus* spp. in samples taken during cheese production obtained from farmer I were studied by *Lactobacillus*-specific PCR and DGGE. The *Lactobacillus*-specific primer targets all members of the *Lactobacillus* group, which includes *Leuconostoc* and *Pediococcus* spp. (15). The evolution of the *Lactobacillus* community during the manufacturing and ripening process was reflected in the unstable DGGE profiles (Fig. 4). Changes in both the positions of specific amplicons and numbers of the amplicons during manufacture were observed. Only the 15-day and 30-day ripened cheese profiles (lanes 6 and 7) showed almost identical DGGE patterns.

To identify the origin of the bands in the *Lactobacillus* DGGE patterns (Fig. 4), the migration of the V1 to V3 regions of the 16S rDNA amplicons of all the pure *Lactobacillus* genus isolates (Table 5) and the *Lactobacillus*-related clones (Table 2) isolated from the 15-day-old cheese were compared with the *Lactobacillus* DGGE profiles. Most of the remaining lactobacillus bands were identified by using clone libraries of approximately 700-bp 16S rRNA amplicons and primers 7-f and S-G-Lab-0677-r DNA for the milk and curd samples. *Leuconostoc mesenteroides* strain CR310S corresponded to amplicon A, which was present in milk and became more intense in the curd after fermentation (Fig. 4, lane 4). Amplicon D of the 15- and 30-day-old cheese samples (Fig. 4, lanes 6 and 7) originated from *Lactobacillus fermentum* strain CR6AS (clone 60CR). Intense amplicon B and amplicon C (Fig. 4, lanes 1 to 3) were identified from clones G3-CR and R3-CR and showed 99% identity with *Leuconostoc lactis* or *Leuconostoc argentinum* and 99% identity with *Lactobacillus delbrueckii* subsp. *lactis*, respectively (Table 2). Amplicons E and F (Fig. 4, lanes 4 to 7), which became more intense during and after acid development, corresponded to *Lactobacillus delbrueckii* subsp. *bulgaricus* clone 41CR, and clones 37CR, 45CR, 51CR, and 54CR, respectively.

Other, weaker bands detected after fermentation corresponded to *Pediococcus acidilactici* CR6 (G), *Lactobacillus ca-*

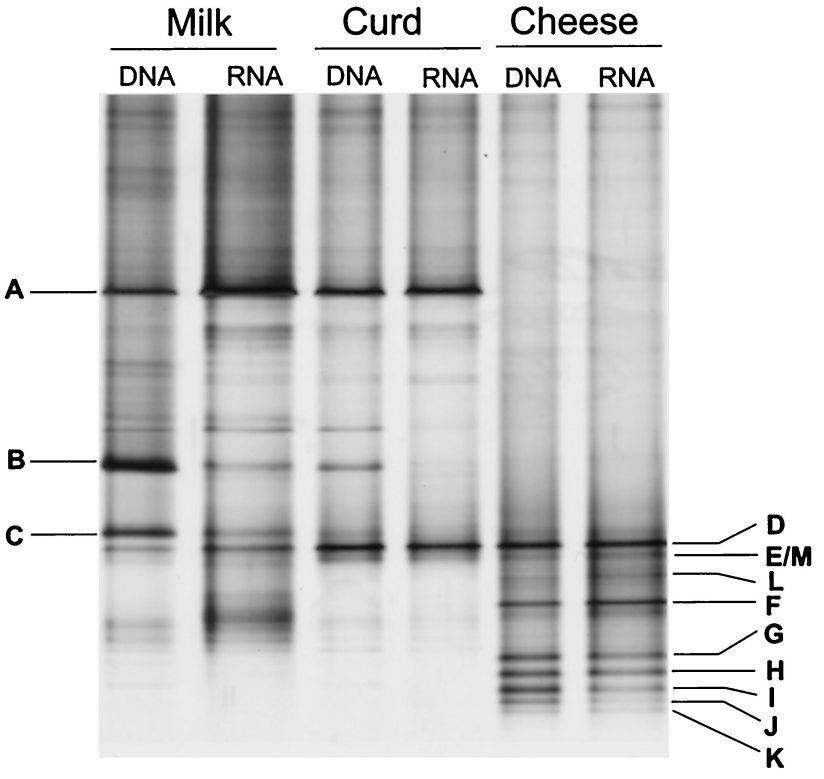

FIG. 3. Comparison between DGGE patters of PCR and RT-PCR products of the V6 to V8 regions from simultaneous DNA and rRNA isolations of milk, curd, and 15-day ripened cheese samples from farmer I. The bands are discussed in the text, and the positions of the bands are indicated by letters that correspond to those in Fig. 1.

sei CR609 (H), and *Lactobacillus plantarum* CR7A (I). It is noteworthy that both the total (Fig. 1) and the *Lactobacillus* (Fig. 4) DGGE profiles indicated that *L. delbrueckii* strains in particular were dominant during the cheese-ripening period, although this species was not isolated by culturing on the selective media.

DISCUSSION

In the present study, the dramatic change in diversity of the microbial communities during the manufacturing process of an artisanal Ragusano cheese was revealed by using a combination of classical and culture-independent PCR and DGGE. The initial microbiota in the raw milk was remarkably different from that of the final ripened cheese. The mesophilic LAB, including species of *Leuconostoc* and *Lactococcus lactis* as well as *Macrococcus caseolyticus*, that dominated in the raw milk disappeared during the cooking and fermentation of the curd. The thermophilic *S. thermophilus*, which not only survived the cooking of the curd but whose growth was concomitantly enhanced, effectively performed the lactic fermentation and remained dominant in the cooked and salted fresh cheese. Thermophilic lactobacilli, including *Lactobacillus delbrueckii* and *Lactobacillus fermentum*, flourished during the ripening of the cheese, but enterococci also appeared in reasonable numbers, and the bacterial composition stabilized in the 15- and 30-day-old cheeses.

While the microbial ecology during manufacture of one excellent-quality Ragusano cheese was followed in detail, a similar microbiology for other Ragusano cheeses was suggested by the DGGE profiles of good-quality cheeses provided by two other farms. Bands corresponding to *M. caseolyticus*, *Leuconostoc mesenteroides*, and *Lactococcus lactis* were present in fresh or fermented curd of all cheeses. *S. thermophilus* was dominant at some stage during manufacture in either the fermented or the fresh cheese, and finally, microbes with a high GC content, which include members of the *Lactobacillus delbrueckii* subgroup of lactobacilli, emerged during ripening. Presumably, similar environments and cheese manufacturing conditions are selecting similar species. The similar DGGE profile for the 15-day cheese made by farmer I 6 months later supports similar conditions for manufacture and indicates the reproducibility of the method. The conservation in DGGE patterns due to similar microbiota suggests that the method has potential for monitoring the quality of these cheeses.

The mesophilic *Lactococcus* and *Leuconostoc* species present in the raw milk were apparently not very metabolically active, as suggested by the weak bands generated by RT-PCR of their rRNA compared to the DNA-derived profiles. These strains may not be well adapted to the milk environment and may not possess the highly efficient lactose metabolism or proteolytic traits of typical dairy starter LAB. Presumably, unfavorable conditions such as the high temperature (approximately 40°C) suppressed the growth and inactivated the mesophilic LAB in the curd. In contrast, *M. caseolyticus* appears active both in the milk and in the cooked curd.

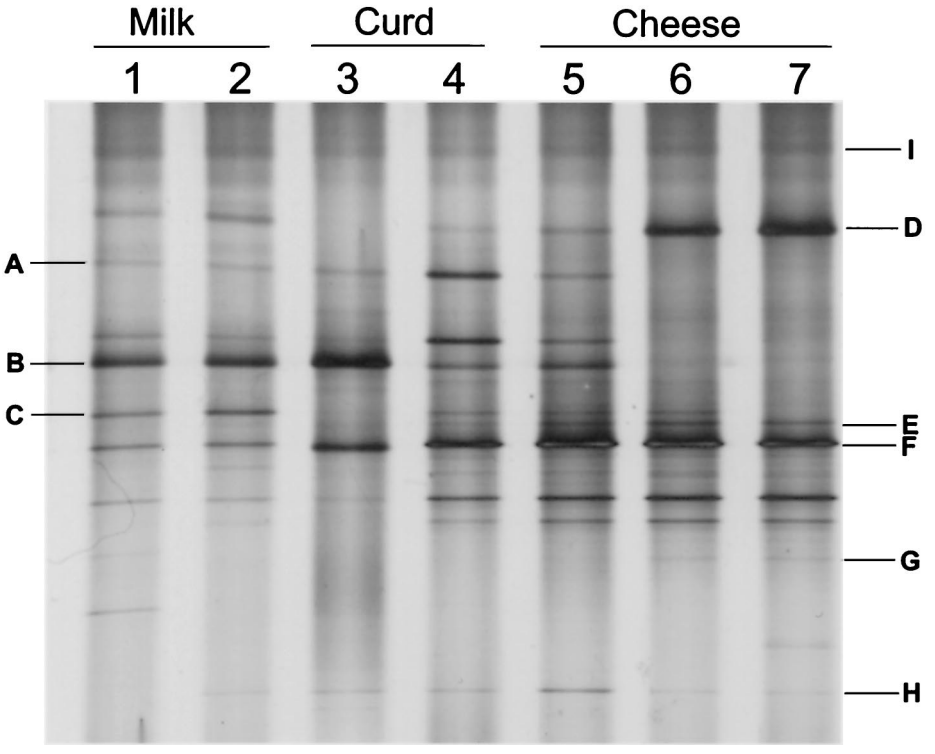

FIG. 4. DGGE profiles of amplicons generated by PCR with the *Lactobacillus*-specific primer that represent the *Lactobacillus* genus biodiversity in samples taken during Ragusano cheese manufacture provided by farmer I. Lanes: 1, milk; 2, milk with added rennet; 3, curd; 4, curd after fermentation; 5, fresh cheese; 6 and 7, 15- and 30-day ripened cheese samples, respectively. The positions of bands discussed in the text are indicated by letters that correspond to species of bacteria: A, *Leuconostoc mesenteroides*; B, *Leuconostoc lactis*/*Leuconostoc argentinum*; C, *Lactobacillus delbrueckii* subsp. *lactis*; D, *Lactobacillus fermentum*; E and F, *Lactobacillus delbrueckii* subsp. *bulgaricus*; G, *Pediococcus acidilactici*; H, *Lactobacillus casei*; and I, *Lactobacillus plantarum*. The identification of the bands is discussed in the text.

M. caseolyticus forms part of the normal microbiota of cattle and other animals and can hydrolyze casein, which explains its presence in the milk (18, 39). Since the optimum growth temperature is 35°C, the organisms would survive the cooking of the curd, but their respiratory metabolism would rapidly become a disadvantage in the curd as lactic fermentation commenced.

It is possible that some of the numerous mesophilic bacteria in the milk form part of the nutrition for the emerging bacteria in the ripening cheese. During the ripening process, sequences derived from both *Streptococcus thermophilus* and *Lactobacillus delbrueckii* showed high-intensity bands in DNA- and rRNA-derived patterns. Therefore, these species can be considered as both the most dominant and metabolically most important bacteria in this artisanal cheese. Furthermore, *Lactobacillus delbrueckii* as well as the other LAB that appeared during the ripening process are likely to be involved in the flavor and aroma development of the cheese and are well adapted to the particular environmental conditions of cheese ripening. The *Lactobacillus*-targeted cloning and PCR-DGGE indicated that a species closely related to *Leuconostoc lactis* or *Leuconostoc argentinum* was dominant in milk and curd, while cultivation and PCR-DGGE for the dominant microbiota pointed to *L. mesenteroides*. Since the two results were obtained by different approaches, the former by direct cloning and the latter through cultivation, the true dominant *Leuconostoc* species cannot be discerned at this stage.

The abundance of *Lactobacillus* species was further investigated by using group-specific PCR and DGGE, which also substantiated the role of *Lactobacillus delbrueckii* and *Lactobacillus fermentum* as predominant species in the ripened cheese. As least five different amplicons corresponding to *Lactobacillus delbrueckii* subsp. *bulgaricus* were detected in the dominant microbiota and two in the *Lactobacillus*-specific community DGGE profiles, generated by PCR of the V6 to V8 and V1 to V3 16S rRNA regions, respectively. It is noteworthy that several bands for a species in a DGGE profile does not necessarily mean that different strains of that species are present. This is especially true in the case of *Lactobacillus delbrueckii*, which harbors at least six copies of the rRNA operon that are heterogeneous in their sequence (28).

When investigating whole communities, a reliable method for extraction of DNA and RNA from the sample is one of the most critical steps, since all further analyses assume the complete and representative presence of accessible nucleic acids (44). During the course of this study, an effective and reproducible method for isolation of DNA and RNA from milk and cheese samples was developed (see Material and Methods) based on a combination of previously reported methods (17, 45), but several phenol and CI extractions were included and a cotton tip was used to remove the fat from the samples. In addition, a mechanical bead-beating procedure was used to lyse the bacterial cells to ensure efficient extraction of nucleic acids. The method yielded DNA or RNA of sufficient quality

for PCR or RT-PCR amplification, as demonstrated in this study.

The fresh curd of pasta filata cheeses is kneaded in hot water, which imparts a characteristic structure as well as melting and stretching properties. Well-known members of traditionally produced pasta filata cheeses include mozzarella and provolone in Italy and kashkavel in the Balkans (4). The microbiota of all these cheeses appear to be unique due to different manufacturing processes, e.g., the dominant acidifying species in provolone and mozzarella are *Lactobacillus helveticus* and *S. thermophilus* plus *Lactococcus lactis*, respectively (13, 27). Recently, PCR-DGGE was used to compare the microbial diversity of unripened Italian pasta filata cheeses by comparing the amplified rRNA V3 regions and could discriminate between industrial and traditional cheeses (8).

Microbial enumeration on different media for LAB reflected to a large extent the results obtained by PCR-DGGE and 16S rDNA sequence analyses. However, it is noteworthy that some of the media were not very selective. For example, in addition to streptococci and lactococci, enterococci and *Leuconostoc* were also isolated on LM17 and LM17$^+$, so presumably the collective counts of these species were increasing during the initial production of the cheese. Counts of streptococci and mesophilic and thermophilic lactobacilli were high in the ripened cheese samples obtained from farmer I. Although the presumptive *Leuconostoc* number increased on MSE medium, especially during cheese ripening, the latter has also been reported to be poorly selective (43), which may explain why no *Leuconostoc* bands were detected by DGGE or from the clone libraries of the ripened cheese.

Following cultivation, a variety of isolates were identified by both classical phenotypic methods and 16S rDNA sequence analysis. Some of the 16S rDNA sequences of the cultured isolates demonstrated less than 98% similarity with described LAB. Also, some of the carbohydrate fermentation patterns were atypical in comparison to their closest relative (data not shown) and these isolates will be the subject of further study. While one would not expect to obtain all isolates from the dairy samples due to the relatively small number of strains identified (32 total), it is remarkable that *Lactobacillus delbrueckii,* which was quite dominant in the ripened cheese as indicated by PCR-DGGE and the clone library, was never isolated on the selective media. Presumably, the media or cultivation conditions must be adjusted to favor the growth of these strains, which without prior knowledge of their presence would be overlooked. Research on the microbiota of dairy products has relied so far mainly on cultivation, which, as demonstrated in this study, may not always be representative of the complex ecosystem.

Throughout Europe and other parts of the world, artisanal cheeses with unique characteristics such as Ragusano cheese are produced that are part of the culture of various peoples. Younger generations of farmers are not always willing to continue these arduous traditions, and centralization to co-ops will be necessary to maintain these practices. The value of a molecular approach in combination with classical methods for establishing the microbiology of the product is clearly illustrated here. This provides a more informed choice for starter culture design and can play an important role in the preservation of artisanal cheese.

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