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Variability of Bacterial Biofilms of the "Tina" Wood Vats Used in the Ragusano Cheese-Making Process▽

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Ragusano cheese is a "protected denomination of origin" cheese made in the Hyblean region of Sicily from raw milk using traditional wooden tools, without starter. To explore the Ragusano bacterial ecosystem, molecular fingerprinting was conducted at different times during the ripening and biofilms from the wooden vats called "tinas" were investigated. Raw milks collected at two farm sites, one on the mountain and one at sea level, were processed to produce Ragusano cheese. Raw milk, curd before and after cooking, curd at stretching time (cheese 0 time), and cheese samples (4 and 7 months) were analyzed by PCR-temporal temperature gel electrophoresis (PCR-TTGE) and by classical enumeration microbiology. With the use of universal primers, PCR-TTGE revealed many differences between the raw milk profiles, but also notable common bands identified as *Streptococcus thermophilus*, *Lactobacillus lactis*, *Lactobacillus delbrueckii*, and *Enterococcus faecium*. After the stretching, TTGE profiles revealed three to five dominant species only through the entire process of ripening. In the biofilms of the two tinas used, one to five species were detected, *S. thermophilus* being predominant in both. Biofilms from five other tinas were also analyzed by PCR-TTGE, PCR-denaturating gradient gel electrophoresis, specific PCR tests, and sequencing, confirming the predominance of lactic acid bacteria (*S. thermophilus*, *L. lactis*, and *L. delbrueckii* subsp. *lactis*) and the presence of a few high-GC-content species, like coryneform bacteria. The spontaneous acidification of raw milks before and after contact with the five tinas was followed in two independent experiments. The lag period before acidification can be up to 5 h, depending on the raw milk and the specific tina, highlighting the complexity of this natural inoculation system.

Ragusano cheese ("protected denomination of origin" since 1996) is a brine-salted pasta filata raw-milk cheese that is aged for 6 to 12 months and is produced on farms in eastern Sicily (16). No starters are added in the cheese-making process, and lactic acid is produced by natural milk flora and desirable flora from the surface of the traditional wooden cheese vats (tinas); the respective contributions of these two bacterial sources, the raw milk and the tina biofilm, are still not completely elucidated. Preliminary studies were performed on the Ragusano cheese bacterial ecosystem during the ripening by traditional microbiology after plating and by random amplified polymorphic DNA analysis of isolated clones (7). Forty different populations were identified, which can be predominant at different times of the ripening, among them *Lactococcus lactis*, *Enterococcus* sp., *Streptococcus thermophilus*, *Streptococcus macedonicus*, and various mesophilic lactobacilli. With both approaches, stretching was found to induce a strong modification of the bacterial profile. The presence of *L. lactis*, *S. thermophilus*, and *Streptococcus waius* was detected (6) in the wooden tina vat. However, considering the limits of traditional microbiological methods in exploring the microbial diversity of complex ecosystems (1), molecular fingerprinting can be a powerful complementary approach (11, 19). These methods are based on the extraction of DNA directly from the food matrix and the amplification of rRNA genes using universal primers. The amplicons are then separated, depending on their sequence, by several different methods: temporal temperature gel electrophoresis (TTGE), denaturating gradient gel electrophoresis (DDGE), and single-strand conformational polymorphism (2, 5, 9, 10, 12, 13, 20, 21, 25, 26). Randazzo et al. (24) used the DGGE method to study the dynamics and activities of bacterial communities during the production of some Ragusano cheese, but no information is given about the role and microbial composition of the tina vat biofilm.

The objectives of this study were (i) to further explore the dynamics of the Ragusano cheese bacterial ecosystem at different times of the cheese making by using direct molecular fingerprinting methods and (ii) to assess the potential role of the tina's biofilm in the inoculation of the milk.

MATERIALS AND METHODS

Experimental design. The first experiment was carried out by collecting raw milk from two different farms, one located at sea level and one on the mountain. The cheese making was performed in the *CoRFiLaC* experimental cheese plant using the traditional technology (see below). Samples for analysis were collected during the manufacture and ripening of the Ragusano cheese as follows: from the tina biofilm, milk before transfer to the tina vat, milk in the tina vat, curd after the first cooking, curd after the second cooking, curd after 24 h, curd after stretching (cheese 0 time), cheese at 4 months, and cheese at 7 months. The milk samples were withdrawn at 8°C before the milk was heated in the vat and at 35°C before the addition of rennet to the wooden vat.

Tinas from five farms in the Hyblean area (designated B, G, I, L, and S) were also tested for a separate biofilm exploration experiment. For each one, two biofilm samplings were carried out at a 2-week interval (week I and week III).

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TABLE 1. Primers used in this study for the species-specific PCR assays

Target	Primer	Sequence (5′–3′)	Annealing temp (°C)	Source or reference
Arthrobacter spp.	Art1	Unpublished data	64	Furlan[a]
	Art2			
B. linens	Brevib	Unpublished data	64	Furlan[a]
	Blin			
E. faecalis	EFS1	CTGTAGAAGACCTAATTTCA	52	Firmesse[b]
	EFS2	CAGCTGAAGACCTAATTTCA		
E. faecium	EFM1	TKCAGCAATTGAGAAATAC	52	Firmesse[b]
	EFM2	CTTCTTTTATTTCTCCTGTA		
L. acidophilus	Laci01	GACCGCATGATCAGCTTATA	55	20
	Laci02	AGTCTCTCAACTCGGCTATG		
L. delbrueckii subsp. *lactis*	Ldel01	ACATGCATCGCATGATTCAAG	60	14
	Ldel02	AACTCGGCTACGCATCATTG		
Listeria monocytogenes	LIM 2	CTAAAGCGGGAATCTCCCTT	55	15
	LIMRE	CCATTGTCTTGCGCGTTAAT		
L. mesenteroides	Lnm1	TGTCGCATGACACAAAGTTA	58	4
	Lnm2	ATCATTTCCTATTCTAGCTG		
L. lactis	1RL	TTTGAGAGTTTGATCCTGG	45	20
	LacreR	GGGATCATCTTTGAGTGAT		
P. pentosaceus	Ppe	CGAACTTCCGTTAATTGATCAG	60	16
	Pu	ACCTTGCGGTCGTACTCC		
S. aureus	STAA-AuI	TCTTCAGAAGATGCGGAATA	55	13
	STAA-AuII	TAAGTCAAACGTTAACATACG		
S. xylosus	STAX-XyI	TCTTTAGAAGATGACAGAGG	55	13
	STAX-XyII	TGACTTTTAACACGACGAAG		
S. thermophilus	Sther03	TTATTTGAAAGGGGCAATTGCT	55	14
	Sther08	GTGAACTTTCCACTCTCACAC		

[a] S. Furlan et al., unpublished data.
[b] O. Firmesse et al., unpublished data.

Biofilm sampling. The tina biofilm samples were collected from the inner surface of the wooden vat (500 cm^2). Cells from the biofilm were withdrawn from five different areas of 100 cm^2, using sterile swabs suspended in 10 ml of peptone water, and then stored at −70°C. The sampling area of 100 cm^2 was delimited by a paper square previously sterilized under UV light in a laminate flow hood.

The tinas analyzed for the first experiment in the present work were used daily for milk manufacturing and cheese production on the mountain and sea-level farms. The five other farms whose tinas' biofilms were analyzed are designated B, I, G, L, and S.

Ragusano cheese-making process. The full process has been previously described (16). The following are the main critical points of interest of the present study. The native microflora present in the raw milk and in the wooden cheese-making equipment are the only sources of acid-producing bacteria during the cheese-making process. No starter culture is added. The amount of rennet paste (made from lamb and young goat) used is variable but must guarantee a firm coagulum in about 65 min. The curd is broken with the "rotula" (a wooden staff), using a circular stirring motion. Immediately following the cutting, hot water (80°C) is added (8 liters/hl of milk) to increase the temperature of the curd plus whey from 5.5°C to 39°C. The broken curd, approximately 4 mm in size, is allowed to settle to the bottom of the vat. The curd is removed from the whey after approximately 34 min. The drained whey is used to make ricotta, and then the whey from the ricotta ("scotta") is used, at about 84.5°C, for the second cooking of the Ragusano cheese curd. The mean pH of the curd immediately prior to the second cooking is 6.54, and the curd temperature just prior to the second cooking is 36.1°C. The curd remains in the scotta for 85 min during the second cooking. After the second cooking, the pH and the temperature of the curd are, respectively, 5.5 and 45°C. Next the curd is separated from the scotta and left to ripen for about 20 to 24 h at room temperature to reach the appropriate stretching pH (5 to 5.2). The stretching step starts with the curd being cut into slices (ca. 1 cm thick) in a copper or wooden container, the "staccio," and covered with water at a temperature of 85°C for about 8 min. Batches of about 14 to 15 kg of curd are stretched slowly with a flat wooden stick, a "manuvedda," until one spherical mass of cheese is formed that has a smooth outer surface. The curd pH at the end of stretching is 5.25, and the temperature of the curd is around 49°C. The stretched mass of curd is formed into the characteristic shape of a squared rectangle (50 cm by 16 cm by 16 cm) by turning it several times in a squared-off section of the open-topped table ("mastredda"). The cheese is kept on this table to cool for about 12 to18 h at room temperature. Next, the blocks of cheese are immersed in a small basin of saturated brine at room temperature. The conditions of the aging center are 14 to 16°C and 80 to 90% relative humidity during the 12 months of aging. In our experiment, samples were taken after 4 and 7 months of ripening.

DNA extraction. Cheese samples (10 g) were homogenized in 90 g of sterile 2% (wt/vol) trisodium citrate (Prolabo, Fontenay sous Bois, France) and homogenized by using a Waring blender (1 min on fast speed); 1 ml of the suspension was centrifuged at 10,000 rpm for 10 min at room temperature. The supernatants and the fat layers at the top were removed with pipette tips. The pellets were suspended in 180 μl or 400 μl (for pure culture or for dairy product, respectively) of buffer for enzymatic lysis (20 mM Tris HCl [pH 8], 2 mM EDTA, 1.2% Triton X-100, 20 mg/ml lysozyme, 10 μl/ml at a concentration of 5 U/ml of mutanolysine) and incubated at 37°C for 1 h. Protein digestion was performed by adding 25 μl of proteinase K (20 mg/ml; QIAGEN, Courtaboeuf, France), 200 μl of AL buffer (DNeasy tissue kit; QIAGEN, Courtaboeuf, France) and incubating at 70°C for 30 min. The suspensions were then transferred to a 2-ml microtube containing 0.3 g of zirconium beads (1-mm diameter), and the tubes were shaken twice for 90 s in a bead beater MM301 (30 Hz) (Retsch, Haan, Germany) and then centrifuged at 10,000 × *g* for 10 min at room temperature. Finally, the nucleic acids were precipitated from the supernatant with 200 μl of ice-cold absolute ethanol. DNA purification was performed as specified in QIAGEN's instructions.

The tina biofilm suspensions were centrifuged (10,000 × *g* for 10 min), and the pellet was submitted to the same lysis and DNA purification protocol.

PCR amplification and TTGE migration parameters. The V3 region of the 16S rRNA gene was amplified by using the primers V3P3-GC-Clamp (5′-GCC CGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTCGGGA GGCAGCAG-3′) and V3P2 (5′-ATTACCGCGGCTGCTGG-3′), giving a PCR product of about 233 bp. The PCR mixture (50 μl) contained *Taq* polymerase buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$), 200 μM of desoxynucleoside triphosphate mix, 1 μM each primer, 2.5 U *Taq* polymerase (Q-BIOgene, Illkirch, France), and 1 μl of template DNA. PCR amplification was performed on an iCycler Therman cycler (Bio-Rad Laboratories, Hercules, CA). The amplification program was 94°C for 2 min; 35 cycles of 95°C for 30 s, 63°C for 30 s, and 72°C for 1 min; and finally, 72°C for 5 min. The purity and length of the PCR products were checked on a 1.5% (wt/vol) agarose gel (Eu-

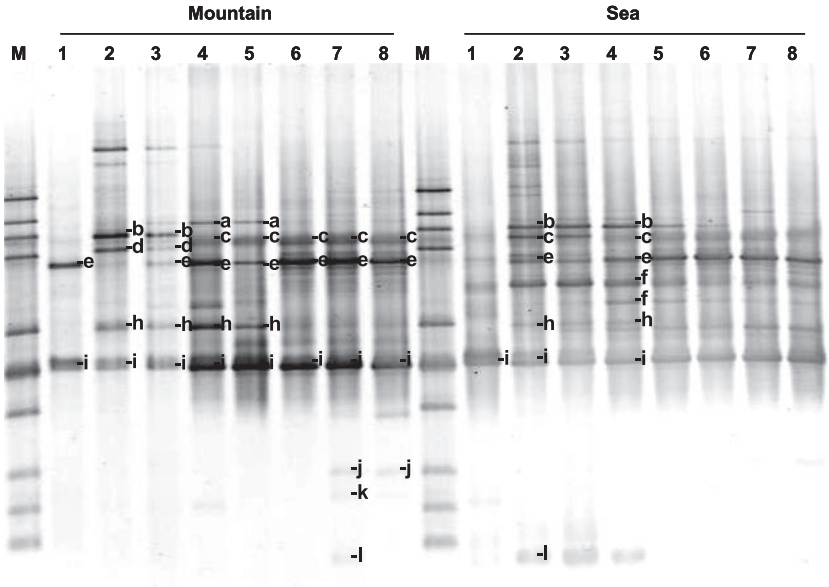

FIG. 1. PCR-TTGE analysis of V3 16S rRNA gene fragments from samples collected during Ragusano cheese manufacture and ripening in farms located, respectively, on the mountain and at sea level. Lanes: M, genomic DNA marker (*Lactobacillus plantarum*, *Lactobacillus fermentum*, *Enterococcus faecium*, *Lactobacillus helveticus*, *Lactococcus lactis*, *Streptococcus thermophilus*, *Corynebacterium moorparkense*, *Lactobacillus paracasei*, *Arthrobacterium nicotianae*, and *Brevibacterium casei*); 1, tina wood vat biofilm; 2, raw milk before heating; 3, milk after heating; 4, curd after first cooking; 5, curd after second cooking; 6, curd after stretching; 7, cheese at 4 months; 8, cheese at 7 months. Assignment of species to PCR-TTGE bands obtained from tina biofilm and cheese samples: a, *Staphylococcus equorum*/*Leuconostoc cremoris* or *mesenteroides*; b, *Enterococcus faecium*/*Staphylococcus equorum*; c, *Lactobacillus delbrueckii* subsp. *lactis*/*Lactobacillus brevis*; d, *Lactobacillus helveticus*/*Lactobacillus acidophilus*/*Lactobacillus crispatus*; e, *Lactobacillus delbrueckii* subsp. *lactis*; f, *Enterococcus faecalis*/*Staphylococcus succinus*; g, *Streptococcus macedonicus*; h, *Lactococcus lactis*; i, *Streptococcus thermophilus*; j, *Lactobacillus casei* or *paracasei* or *zeae* or *rhamnosus*; k, *Brachybacterium tyrofermentans*/*Arthrobacterium globiformis* or *nicotianae*; l, *Propionibacterium freudenreichii*/*Brachybacterium nesterenkovii*.

robio, Les Ullis, France) in comparison with a 100-bp DNA ladder (Fermentas Life Sciences, Vilnius, Lithuania).

For TTGE analysis, a DCode universal mutation detection system (Bio-Rad Laboratories, Hercules, CA) was used to separate the V3 region PCR products. Ten microliters of PCR product was added to 5 μl of loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol, 70% glycerol). Gels were prepared with 10% (wt/vol) bisacrylamide (37.5:1), 7 M urea and run with 1.25× TAE buffer diluted from 50× TAE buffer (2 M Tris base, 1 M glacial acetic acid, 50 mM EDTA, pH 8). Migration was performed at 41 V for 16 h with a temperature gradient of 63°C to 70°C (rate of 0.4°C · h^{-1}) for bacteria of low GC content. The gels were stained with ethidium bromide (0.6 μg of ethidium bromide per ml of 1.25× TAE buffer) for 15 min and then rinsed for 15 min in distilled water and photographed on a UV transillumination table. The bands were analyzed by using the database developed by Parayre et al. (22) for presumptive species identification.

PCR-DGGE analyses. DGGE analyses of V3 amplicons were performed for high-GC-content bacteria as previously described (20). The denaturing gradient gel contained a 40 to 70% gradient of urea and formamide (a 100% denaturing solution consisted of 7 M urea and 40% [vol/vol] deionized formamide). Electrophoresis was conducted with 1.25× TAE buffer (92 V at 60°C for 16 h for one gel). After the gels were run, they were stained for 15 min with an ethidium bromide solution (0.5 μg/ml of 1× TAE buffer), rinsed for 20 min in 1× TAE buffer, and photographed on a UV transillumination table.

Gel analysis and band identification using species database. The TTGE and DGGE gels were analyzed using Gel Compar software (Applied-Maths, Belgium) as previously described (21). The software standardizes TTGE and DGGE profiles to minimize migration differences between gels by alignment of the identification ladder with a standard gel. Band identifications are performed by comparison to a TTGE species database (22) and DGGE database (20) which both include fingerprints of pure strains isolated from dairy ecosystems.

Species-specific PCR assay. Specific PCR tests were carried out using different species-specific primers (Table 1) on 1 μl of DNA obtained from tina biofilm samples. The primers (MWG Biotech AG, Ebersberg, Germany) were prepared at a final concentration of 60 μM in deionized autoclaved water. Amplification was performed with a model 2400 GenAmp system (Perkin Elmer, France), and all reactions were carried out following conditions previously prescribed by the authors (Table 1). For primers EFS1/EFS2 and EFM1/EFM2, the PCR conditions were 94°C for 10 min for denaturation and then 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s. For primers Art1/Art2, the PCR conditions were 94°C for 5 min for denaturation and then 30 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s. The sizes of the PCR products were determined by using 1.5% agarose gel electrophoresis (Seakem CTG agarose; TEBU, France).

Sequencing of bands. Some bands obtained from the TTGE and DGGE gels were excised, purified, and sequenced as described previously (21). The sequences were compared to those present in the Ribosomal Database (17) to determine the closest known species.

Acidification assay. Raw milk samples taken before and after 5 min of contact with the tinas (from farms B, I, G, S, and L) and kept at −20°C were thawed at room temperature, enumerated (total counts), and submitted in parallel to spontaneous acidification in a water bath at 37°C. A CINAC device (multiple-electrode pH-tracking device) was used to continuously follow the pH decrease (8). The lag phase, maximal speed, and time to reach pH 5.0 were determined to characterize each acidification curve and to quantify the impact of the contact with the tina.

RESULTS

PCR-TTGE profiles and enumeration of Ragusano cheese bacterial ecosytems during the ripening. Raw milk, curd before and after cooking and stretching, and cheese (aged 4 and 7 months) were analyzed by PCR-TTGE and enumeration microbiology for cheese made on the mountain and at sea level. The total counts for raw milks from the mountain and sea-level farms were, respectively, 6×10^4 and 2.3×10^5 CFU/ml. With the use of universal primers, PCR-TTGE revealed many differences between the raw-milk profiles, except for a few common bands identified as *S. thermophilus*, *L. lactis*, *Lactobacillus delbrueckii*, and *Enterococcus faecium* (Fig. 1).

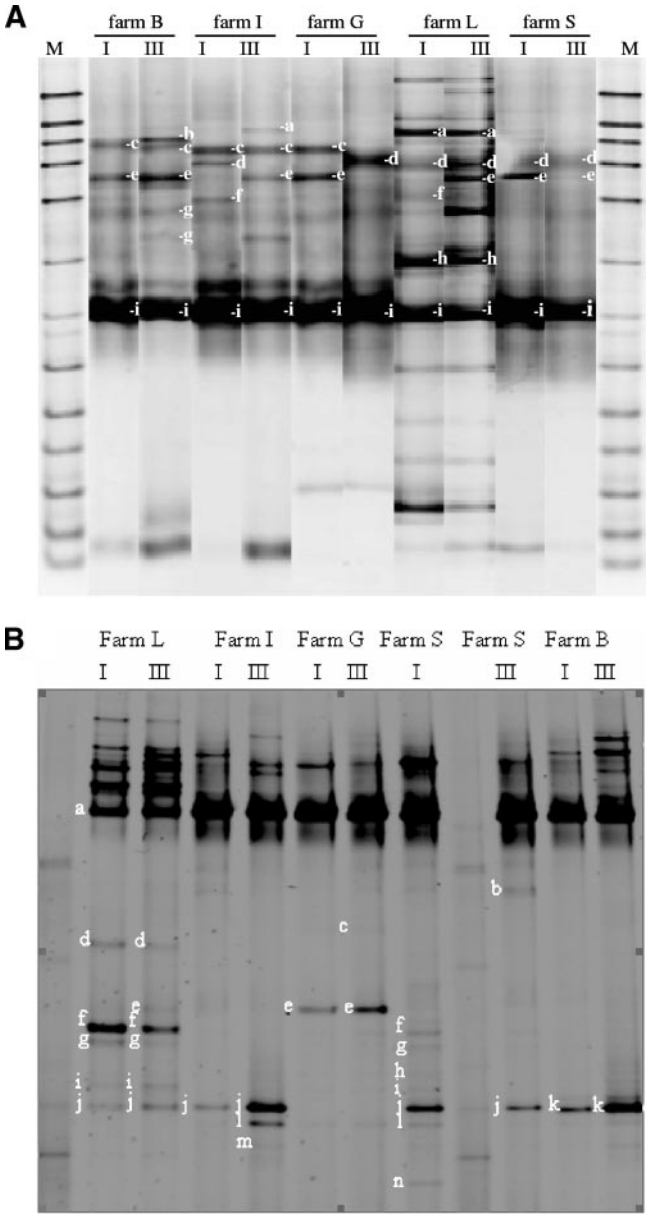

FIG. 2. (A) PCR-TTGE of tina biofilms collected on five different farms designated B, I, G, L, and S. Lanes: M, genomic DNA marker (*Lactobacillus plantarum*, *Lactobacillus fermentum*, *Enterococcus faecium*, *Lactobacillus helveticus*, *Enterococcus faecalis*, *Lactococcus lactis*, *Streptococcus thermophilus*, *Corynebacterium moorparkense*, *Lactobacillus reuteri*, *Lactobacillus paracasei*, *Arthrobacterium nicotianae*, *Brevibacterium casei*, and *Propionibacterium cyclohexanicum*); I, week I; III, week III. Assignment of species to PCR-TTGE bands obtained from tina biofilm samples: a, *Staphylococcus equorum*/*Leuconostoc cremoris or mesenteroides*; b, *Enterococcus faecium*/*Staphylococcus equorum*; c, *Lactobacillus delbrueckii* subsp. *lactis*/*Lactobacillus brevis*; d, *Lactobacillus helveticus*/*Lactobacillus acidophilus*/*Lactobacillus crispatus*; e, *Lactobacillus delbrueckii* subsp. *lactis*; f, *Enterococcus faecalis*/*Staphylococcus succinus*; g, *Streptococcus macedonicus*; h, *Lactococcus lactis*; i, *Streptococcus thermophilus*. (B) PCR-DDGE analysis of tina biofilms collected on five different farms with a 2-week interval (lanes: I, week I; III, week III); a, *S. thermophilus*; b, *Enterobacter sakazakii*/*L. reuteri*; c, *Lactobacillus casei*/*Microbacterium gubbeenensee*; d, *Corynebacterium vitaeruminis*; e, *Arthrobacter* sp.; f, *Microbacterium* sp.; g, unknown band; h, *Brevibacterium* sp.; i, *Brevibacterium linens*; j, *Kocuria kristinae*/*Brevibacterium linens*; k, *Kocuria kristinae*; l, *Brevibacterium linens*; m, unknown band; n, unknown band.

The stretching step induced a simplification of the milk profiles, and only three of five dominant species were detected by TTGE through the entire process of ripening (Fig. 1). No changes were observed in the predominant species, i.e., bands attributed to *S. thermophilus*, *L. delbrueckii* subsp. *lactis*, *L. lactis*, and *S. macedonicus*, after the stretching step. Classical enumeration confirmed the presence of viable, predominantly thermophilic lactic acid bacteria growing until the stretching; e.g., thermophilic lactobacilli (as enumerated on MRS [pH 5.4] at 42°C) reached 9.6×10^8 CFU after stretching and were reduced to 4×10^5 CFU after 7 months of ripening. In the same way, enumeration on M17 medium at 42°C, correlated to *S. thermophilus*, reached 1.2×10^9 CFU after stretching and only 3×10^4 CFU after 7 months (data obtained for cheeses from the mountain farm; similar results were obtained for the cheeses from the sea-level farm [data not shown]).

The two tinas exhibited different biofilms: the tina from the farm on the mountain was colonized by two clearly predominant species identified on the TTGE database as *S. thermophilus* and *L. delbrueckii* subsp. *lactis*, whereas the one used on the farm at sea level exhibited a predominant band corresponding to *S. thermophilus* and four weak bands (Fig. 1, mountain and sea, respectively, lanes 1).

Variability of the tina biofilms and identification of the species. As the biofilm samples from the two tinas from the mountain farm and the farm at sea level were different, the variability of the biofilm, which can be a source of variability in the final cheese quality, was further explored. The PCR-TTGE profiles of the biofilms from five other tinas were established, with an independent duplicate analysis after 2 weeks. The results (Fig. 2A) revealed from 2 (tina from farm S) to 10 major bands per profile (tina from farm L). The species assigned from the database were *S. thermophilus*, *L. delbrueckii* subsp. *lactis*, *L. lactis*, and other thermophilic lactobacilli species. *S. thermophilus* was present and largely predominant in the five tinas in the two independent assays, confirming the result obtained in the first two tinas (Fig. 1). By using PCR-DGGE, some high-GC-content bacterial species belonging to the coryneform bacterial group were detected (Fig. 2B), highlighting the fact that the biofilm is not exclusively composed of lactic acid bacteria. The species identifications were further confirmed either by sequencing (Table 2) or by specific PCR tests (using primers described in Table 1). With the specific PCR tests, the presence of *S. thermophilus* was confirmed in the five tinas, *L. lactis* was found in tina L, *Lactobacillus acidophilus* group was found in tinas I and L, *Leuconostoc mesenteroides* was detected only in tina S, and *Arthrobacter* spp. were detected in tinas I, S, and G, whereas *Pediococcus pentosaceus*, *Staphylococcus xylosus*, *Brevibacterium linens*, *Enterococcus faecium*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Listeria monocytogenes* were not detected in any of the tinas. The sequencing of some DGGE bands confirmed the presence of coryneform bacteria in the tinas from farms L, G, and I. Interestingly, the independent replication done two weeks later (resulting in more than 10 different cheese-making assays with different raw milks) confirmed the stability of *S. thermophilus* as the predominant species but highlighted obvious variations in the minor bands in some cases (tinas I and S). This result raised the question of the complex exchange of

TABLE 2. Band identification by sequencing

Band	Type of assay	Species[a]	Farm (wk) of tina biofilm sample collection	Closest relative (species) by sequencing	% Identity	GenBank accession no.
i	TTGE	*Streptococcus thermophilus*	G (I)	*Streptococcus thermophilus*	100	CP000419
			I (I)	*Streptococcus thermophilus*	100	CP000419
			S (III)	*Streptococcus thermophilus*	100	CP000419
			B (III)	*Streptococcus thermophilus*	100	CP000419
			L (III)	*Streptococcus thermophilus*	100	CP000419
h	TTGE	*Lactococcus lactis*	L (I)	*Lactococcus lactis* subsp. *lactis*	98	AM411114
			L (III)	*Lactococcus lactis* subsp. *lactis*	100	EF114309
e	TTGE	*Lactobacillus delbruecki* subsp. *lactis*	S (I)	*Lactobacillus species*	97	DQ975786
e	DGGE	Unassigned band	L (I)	*Microbacterium* spp.	100	DQ985071
			L (III)	*Microbacterium* spp.	100	DQ985071
e	DGGE	*Arthrobacter* sp.	G (III)	*Microbacterium* spp.	100	AB266564
k	DGGE	Unassigned band	B (III)	*Arthrobacter globiformis*	98	EF177369

[a] Assigned using the species database developed by Parayre et al. (22).

populations which might occur between the raw milk and the tina biofilm during each cheese-making process.

Contribution of the tina to raw-milk inoculation and improvement of the acidification process. To explore the role of the tina in inoculating the milk with bacteria, the total bacterial count of the milk before and after 5 min of contact with the tinas was determined (Table 3). Either no increase or an increase of the total bacterial count of from 0.3 to 1 log was shown. The absence of an increase in total bacterial count after contact does not prove that no bacteria were inoculated. The level of inoculation, if any, may be far below the initial level of milk contamination and therefore unquantifiable (for example, an inoculation of 10^4 CFU/ml in a milk already at 10^6 cannot be detected in the total count).

In order to detect if lactic acid bacteria were inoculated by the tina biofilm, the milks before and after the 5-min contact were incubated at 37°C to assess the spontaneous acidification (Fig. 3). Depending on the raw milk and on the tina, a 4-h reduction of the lag period before acidification can be observed, as well as a reduction of 6 h in the time to reach pH 5 (farm I, week 1). An increase in V_{max} also occurred in the milks from some farms, providing evidence for the rapid inoculation (in just a few minutes) of acidifying species by the tina. In other cases, no acceleration was observed, the curves being rigorously identical before and after contact, indicating either that the raw milk was already very rich in lactic acid bacteria or that the tinas did not inoculate any lactic acid bacteria. However, two observations support the first hypothesis: (i) the raw milks exhibiting the lowest counts (Table 3, counts below 5×10^4) were ones for which the improvement of acidification was systematic, and (ii) in the repeated assays with the same tina (2-week interval), an acceleration or no acceleration at all can be observed (farms G and S), suggesting that the tina inoculates lactic acid bacteria but the acidification impact of this inoculation also depends on the raw milk.

DISCUSSION

The bacterial ecosystems occurring in two entire Ragusano cheese processes were explored in this work. One analysis was done with samples from a farm located at sea level, and the second with samples from a farm located on the mountain. The results showed that the cheese-making process for Ragusano cheese (pH and temperature, mainly during stretching) has a major role in selecting the predominant natural microflora from raw milk. Whatever the initial raw-milk species, thermo-

TABLE 3. Total bacterial counts and acidification parameters of the milks before and after contact with the indicated tina[a]

Farm (wk) of tina biofilm sample collection	CFU in:		Increase in PCA medium count (log)	Acidification parameters					
	MBT	MAT		Lag phase (h)		V_{max} (10^{-3} pH units/min)		Time (h) to reach pH 5	
				MBT	MAT	MBT	MAT	MBT	MAT
B (I)	1.4×10^3	1.5×10^4	1	7.5	4.4	−10.9	−8.8	13.8	11.3
B (III)	1.6×10^4	2.6×10^5	1	6.6	2.8	−9.9	−8.4	11.2	8
I (I)	8.2×10^3	2.2×10^4	0.5	6.0	5.3	−4.5	−8.8	16.8	10.3
I (III)	8.4×10^3	1.7×10^4	0.3	6.6	6.6	−7.3	−11.1	13.9	11.5
G (I)	3×10^5	8.9×10^5	0.5	3.2	4.8	−4.9	−5.3	10.8	11.3
G (III)	1.3×10^5	1.5×10^5		5.8	3	−7.4	−5.9	12	10.6
S (I)	1.5×10^5	6.4×10^5	0.6	3	1.7	−8.6	−9.2	8.8	6.8
S (III)	9.6×10^5	9.3×10^5		1.1	1.2	−4.4	−5.3	8.7	8.3
L (I)	4.9×10^5	4.9×10^5		1.5	0.8	−5.0	−7.8	10	7.8
L (I)	1.2×10^6	2.5×10^6	0.3	2.4	1.8	−7.7	−9.6	7.8	5.8

[a] The results are from before and after 5 min of contact with the tina; the spontaneous acidification was performed at 37°C in a water bath. The experiment was repeated twice (week I and week III). MBT, milk before contact with tina; MAT, milk after contact with tina; PCA, plate count agar.

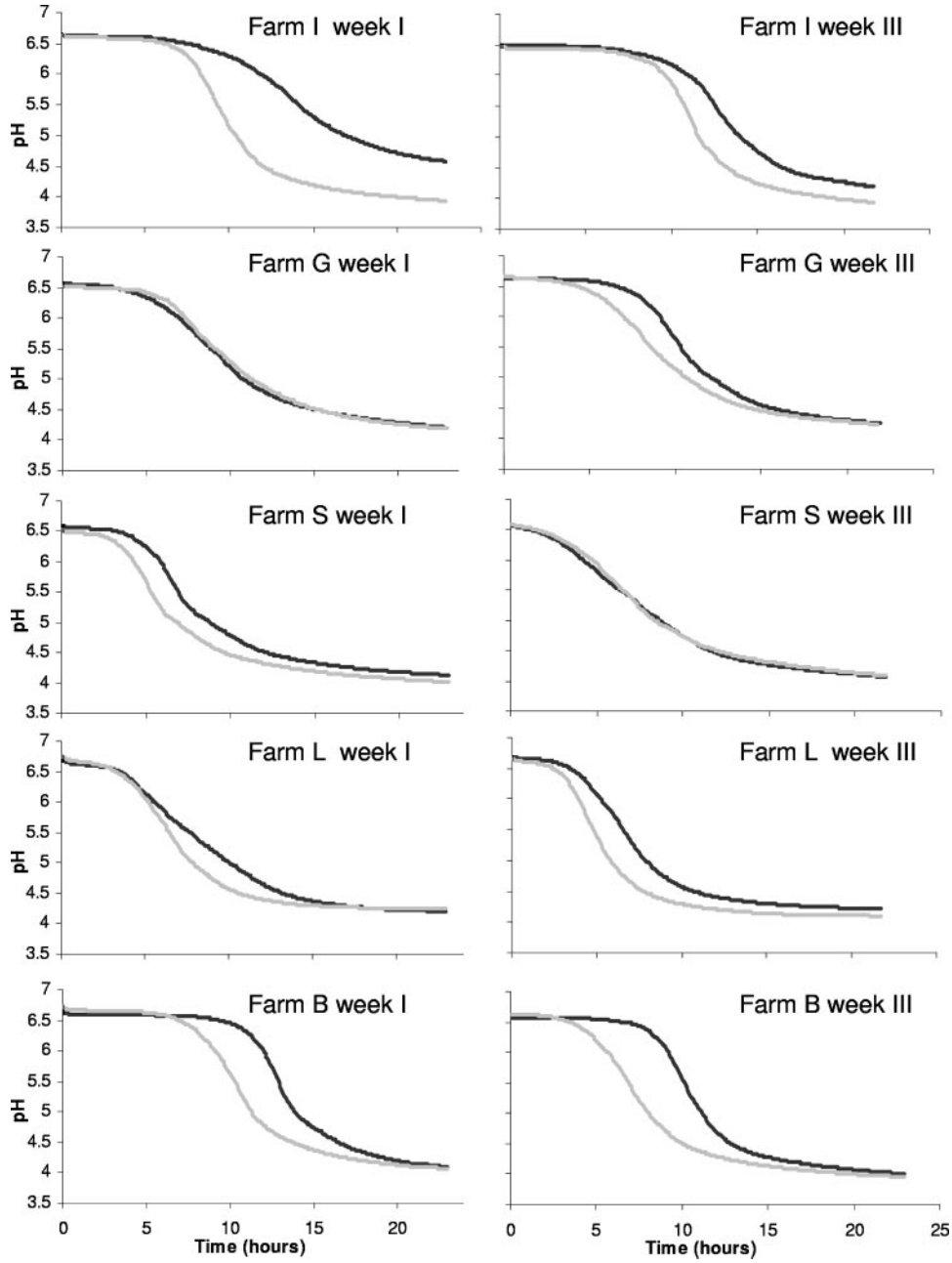

FIG. 3. Spontaneous acidification assay at 37°C. Black line, milk before contact with tina; gray line, milk after 5 min of contact with tina.

philic lactic acid bacteria were predominant after stretching and then decreased drastically during aging. Molecular fingerprinting established by PCR-TTGE confirmed the simplification of the initial ecosystem through stretching and the predominance of bands attributed to *S. thermophilus* and *L. delbrueckii.* Using PCR-DDGE and by sequencing a clonal library, Randazzo et al. (24) found similar results. By reverse transcriptase PCR-DGGE, they also showed that *S. thermophilus* and *L. delbrueckii* were the only two species metabolically active after stretching. Unfortunately, profiles were established only until 1 month of ripening. In our study, the viable count of thermophilic lactic acid species decreased drastically after stretching, and a 4- to 5-log reduction was evident after 7 months of ripening, indicating that this lactic flora is probably not metabolically active anymore. As lactose was shown to be rapidly exhausted in Ragusano cheese (G. Licitra, personal communication) lactic acid bacteria likely underwent lysis after few weeks of ripening, releasing their intracellular enzymes into the Ragusano curd. This was observed in many other cheeses (3), where lysis was shown to significantly contribute to the aging process and the development of flavor. During this drastic loss of viability of thermophilic lactic acid bacteria, the PCR-TTGE profiles did not exhibit any change (from stretching to 7 months of ripening), underlining the limit of this

approach based on DNA and universal primers (11). Indeed, the DNA of dead cells can still be predominant, masking by competition during the PCR step the DNA corresponding to the growth of minor floras during the ripening period. Therefore, to further understand the viable ecosystem of Ragusano cheese, reverse transcriptase PCR-TTGE should be applied until the end of the ripening. As the RNA of dead cells is rapidly degraded, the competition problem indicated above would not occur. This could allow the determination of, first, what lactic acid flora are intact and metabolically active during different stages of the ripening and, second, which other species are growing in parallel.

For Ragusano cheese making, each farm uses its own tina wood vat, whose surface is covered by a natural biofilm. In addition to the two tinas from sea level and the mountain, biofilm profiles were established for tinas from five other farms, with an independent duplicate analysis 2 weeks later. Previous assays to identify the main species of the tina biofilms by classical enumeration and some specific PCR did not allow a clear view of the ecosystem (6). As the composition and variability of this biofilm can be a source of variability in the final cheese quality, it was explored here by PCR-TTGE for the first time. Moreover, band sequencing from TTGE gels and exhaustive specific PCR tests were also performed to confirm the identities of most of the bacterial species in the tina biofilm samples. The main conclusion was that *S. thermophilus* was the predominant and stable species in all the tinas investigated. The richness of the tina biofilm, its variability from one tina to another (2 to 10 codominant species), and the dominance of lactic acid bacteria (*S. thermophilus*, *L. lactis*, *L. delbrueckii lactis*, and *L. acidophilus*) were determined, as was the presence of high-GC-content species (coryneform bacteria). An overall stability of the profile over a two-week period was observed, although with some variations in the minor bands. This last observation raised the question of the complex exchange of populations which might occur between raw milk and tina biofilm during each cheese-making process.

To further characterize this rich biofilm, further work should explore by reverse transcriptase PCR-TTGE which of all these species are metabolically active. Is one strain of *S. thermophilus* present or are there many coexisting strains? Are the *S. thermophilus* strains colonizing the tinas physiologically similar? The stability of the tina biofilm profiles for a longer period of time should be determined as well. Since only bacterial species were explored in this work, the presence or absence of yeasts or molds should also be considered.

As no starter is used in the Ragusano cheese-making process, one of the main interesting questions is the exact role played by the tina in inoculating raw milk and the impact of the species variability observed in the biofilm on the rate of acidification and the overall cheese quality (due to proteolysis, lipolysis, and aroma compounds). In the tinas from each of the same five farms and in duplicate (at a 2-week interval), the raw milks were compared for their total bacterial count and spontaneous acidification before and after a very short contact with the tina. In 80% of the cases, a highly significant improvement of acidification was observed (reduction of lag phase and increase of the rate of acid production), definitively proving the role and efficiency of the tina in inoculating the raw milk with lactic acid bacteria. In the most extreme case, 6 h were gained in reaching pH 5.0, which is crucial from a technological and practical point of view. In the case where no improvement was noted, milk acidification curves being rigorously identical before and after contact with the tina, the most likely explanation is that the raw milk was already very rich in lactic acid bacteria. This hypothesis is supported by the fact the raw milks exhibiting the lowest counts (below 10^5) were the ones for which a systematic improvement of acidification was noted. Above 10^5 CFU/ml of total count, the result was apparently tina and raw milk dependent, highlighting the complexity of this natural inoculation system. However, it can already be concluded from our data that the tina biofilms quickly and efficiently release lactic acid bacteria into the raw milk, making the acidification process faster, and that the tina's use is especially crucial for raw milks with low initial counts of lactic acid bacteria.

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