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Raw Cow Milk Bacterial Population Shifts Attributable to Refrigeration

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We monitored the dynamic changes in the bacterial population in milk associated with refrigeration. Direct analyses of DNA by using temporal temperature gel electrophoresis (TTGE) and denaturing gradient gel electrophoresis (DGGE) allowed us to make accurate species assignments for bacteria with low-GC-content (low-GC%) (<55%) and medium- or high-GC% (>55%) genomes, respectively. We examined raw milk samples before and after 24-h conservation at 4°C. Bacterial identification was facilitated by comparison with an extensive bacterial reference database (~150 species) that we established with DNA fragments of pure bacterial strains. Cloning and sequencing of fragments missing from the database were used to achieve complete species identification. Considerable evolution of bacterial populations occurred during conservation at 4°C. TTGE and DGGE are shown to be a powerful tool for identifying the main bacterial species of the raw milk samples and for monitoring changes in bacterial populations during conservation at 4°C. The emergence of psychrotrophic bacteria such as *Listeria* spp. or *Aeromonas hydrophila* is demonstrated.

The diversity in the microbial flora of raw milks contributes to the great differences in organoleptic characteristics among raw milk cheeses. Indeed, although many of the characteristics desired by consumers are not present in pasteurized cheeses (4, 6, 11, 20, 28), few studies address the characterization of microbial flora of raw milks. To date, identification has been limited to the enumeration of the most represented microbiological groups, with partial identification. In brief (2, 10, 12, 13, 14, 25), the dominant microflora of raw milk generally include (i) species of lactic acid bacteria (LAB; *Lactococcus* and/or *Lactobacillus* spp.), (ii) *Pseudomonas* spp., (iii) the group *Micrococcaceae* (*Micrococcus* and *Staphylococcus* spp.), and (iv) yeasts. Other microbial groups present in raw milks belong to the LAB (including *Leuconostoc*, *Enterococcus*, and *Streptococcus* spp.), *Bacillus*, *Clostridium*, and *Listeria* spp. and *Enterobacteriaceae*; there are also many gram-negative (*Acinetobacter*, *Alcaligenes*, *Flavobacterium*, and *Aeromonas*) and gram-positive (*Arthrobacter*, *Corynebacterium*, *Brevibacterium*, and *Propionibacterium*) species.

Many factors influence milk composition and hence the nature and abundance of the microbial load. The conditions of raw milk production, in particular the hygienic practices of farmers (e.g., washing of milking equipment and pre- and post-milking udder preparation), determine the contents in useful cheese-making and spoilage microorganisms (25). Intensive washing of milking equipment and udder preparation (individual washings) result in raw milks containing a majority of spoilage microorganisms (such as coliforms and *Pseudomonas* spp.) (29). In contrast, minimal hygiene around the udder preserves microorganisms, including salt-tolerant flora (such as *Micrococcus*, *Arthrobacter*, *Microbacterium*, *Brevibacterium*, and *Staphylococcus* spp.) and also the LAB (15), yielding raw milks in which useful cheese-making microorganisms are dominant. The health status of animals, the nature of their feed (forage, ensilage, etc.), and the storage conditions of raw milk are also important factors that determine the composition of their microbial flora. Intensive washing of milking equipment associated with storage of the raw milk at low temperatures gives higher levels of contamination by *Pseudomonas* spp. Fifty percent of the psychrotrophs in refrigerated raw milk (the first day) belong to the genus *Pseudomonas*, with *Pseudomonas fluorescens* being the predominant species (32). Other psychrotrophs present in refrigerated raw milk belong to the genera *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, *Aeromonas*, *Bacillus*, *Listeria*, and *Arthrobacter*; *Enterobacteriaceae* such as *Hafnia alvei*, *Citrobacter freundii*, or *Serratia liquefaciens* are also found (12).

Until recently, the bacterial community of raw milk was described by classical microbiological methods, which are generally long and tedious, and allow only a partial inventory of the bacterial microflora. New molecular approaches based on direct analyses of DNA (or RNA) in its environment without microbial enrichment have allowed more precise descriptions of microbial dynamics in complex ecosystems. The most-developed methods are single-strand conformational polymorphism (17, 19, 30), denaturing gradient gel electrophoresis (DGGE) (18, 27, 33), temperature gradient gel electrophoresis (22, 34), and temporal temperature gel electrophoresis (TTGE) (26, 35). In all of these methods, the total DNA (or RNA) is extracted from environmental samples, and a zone corresponding to the 16S or 28S rRNA gene is PCR amplified. Nucleotide variation in these conserved sequences is the basis for separa-

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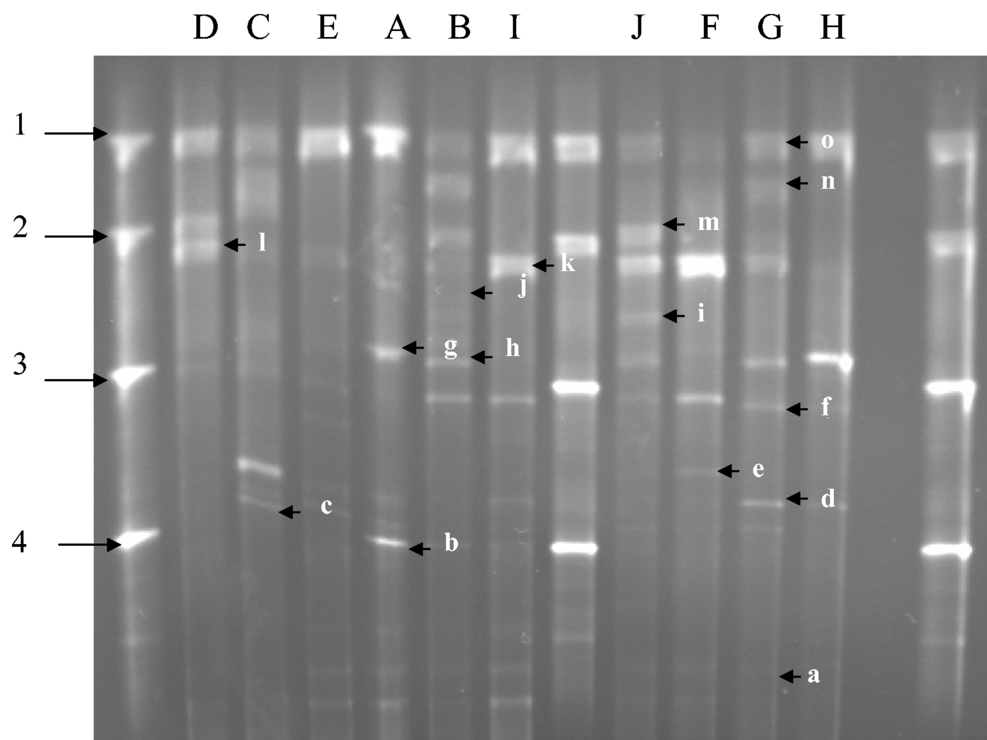

FIG. 1. TTGE profiles of 16S rRNA gene V3 regions obtained from different raw milk samples (as indicated above each lane). Lanes 1, 8, and 13, marker. Bands: a, unnidentified; b, *Lactococcus garvieae*; c, *Lactobacillus plantarum*/*Lactobacillus pentosus*; d, *Listeria innocua*/*Listeria monocytogenes*/*Lactobacillus fermentum*; e, *Staphylococcus epidermidis*; f, *Pseudomonas fluorescens*/*Enterococcus faecium*/*Enterococcus durans*/*Enterococcus hirae*/*Leuconostoc carnosum*; g, *Leuconostoc lactis*/*Staphylococcus xylosus*/*Acinetobacter johnsonii*; h, *Chryseobacterium*; i, *Lactobacillus acidophilus* group; j, *Lactobacillus delbrueckii* subsp. *bulgaricus*; k, *Streptococcus uberis*/*Bacillus circulans*; l, *Staphylococcus warneri*; m, *Pseudomonas stutzeri*; n, *Streptococcus dysgalactiae*/*Hafnia alvei*/*Pseudomonas alcaligenes*; o, *Lactococcus lactis*. Markers (arrowed): 1, *Lactococcus lactis* subsp. *lactis* bv. diacetylactis CNRZ260; 2, *Enterococcus faecalis* CE17; 3, *Lactococcus raffinolactis* CNRZ1214; 4, *Lactococcus garvieae* CNRZ1323.

tion during electrophoresis. For the low-GC-content (low-GC%) bacterial species (T_m of V3 sequence of <75°C), optimal resolution was achieved by TTGE; for bacteria with medium- or high-GC% DNA (T_m of V3 sequence of >75°C), the best separation was obtained by DGGE.

We used TTGE and DGGE here to study the evolution of the bacterial community in some raw milks upon conservation at 4°C.

MATERIALS AND METHODS

Raw milk samples. Ten raw milk samples (A to J) were collected in the same area (Ile-de-France, France), except for sample H (Normandy, France), and were analyzed by TTGE and DGGE. Samples A, B, C, D, E, F, G, and H were sampled in farms; samples A, B, C, D, and E were nonrefrigerated, and samples F, G, and H were refrigerated at 4°C to 8°C for 12 h. Milk samples I and J, refrigerated at 4 to 8°C for 24 to 48 h, were collected from tanks in two industrial dairies. All raw milk samples (~250 ml each) were collected in sterile conditions and carried at 4°C to the laboratory. Two DNA extractions were performed. The first was performed at the most 3 h after sampling (for all samples). The second was performed on seven raw milk samples (i.e., samples A, B, C, D, E, F, and G) after conservation at 4°C for 24 h.

DNA extraction. To 35 ml of raw milk sample was added 50 mg of pronase (Roche Diagnostics, Meylan, France) and 100 μl of β-mercaptoethanol (Serva, Heidelberg, Germany). After 1.5 h of incubation of suspensions in a 37°C water bath, bacterial pellets, obtained by centrifugation at 12,000 × *g* for 15 min at 4°C, were washed once with sterile water and once with 10 ml of TES buffer (25 mM Tris-HCl, 0.1 M EDTA, 25% [wt/vol] saccharose; pH 8). Supernatants were discarded, and bacterial pellets were stored at −20°C.

Bacterial pellets were resuspended in 500 μl of TES. Bacteria were mechanically lysed with zirconium beads (diameter, 150 to 200 μm; Sigma, St. Louis, Mo.) by six cycles of 30 s of vortexing, with 1 min of storage in ice between each cycle.

DNA purification was performed as described previously (9).

PCR amplification. The V3 region of the 16S rRNA gene is the substrate for PCR amplification. The extracted DNA (1 μl) was amplified by two successive PCR amplifications. A 700-bp fragment, including the 16S rRNA gene V3 region, was first PCR amplified as described previously (26) by using the primers W01 [5′-AGA GTT TGA TC(AC) TGG CTC-3′] and W012 [5′-TAC GCA TTT CAC C(GT)C TAC A-3′] (MWG-Biotech AG, Ebersberg, Germany). The PCR fragment containing the V3 region was then used as substrate to amplify an ~200-bp fragment, as described previously (26). Two primers from MWG-Biotech AG were used: HDA1_GC-clamp (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3′; GC-clamp is underlined) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA-3′). PCRs were performed by using the Gene Amp system (model 2400; Perkin-Elmer, Courtaboeuf, France). The purity and lengths of PCR products were verified on 2% agarose gels (FMC Bioproducts, Rockland, Maine) in comparison with a standard containing DNA fragments of defined lengths (λ/BstE II; Q-BIOgene, Illkirch, France). The latter fragments were used to perform TTGE and DGGE species identifications.

TTGE analysis for identification of bacteria with AT-rich genomes. For TTGE analysis, the Dcode universal mutation detection system (Bio-Rad, Marnes la Coquette, France) was used to separate the V3 region PCR products. PCR products (5 μl) were added to 10 μl of loading buffer (100 mM EDTA, bromophenol blue [1.5 mg/ml], 40% saccharose). Samples were electrophoresed on 8% (wt/vol) polyacrylamide gels containing 6 M urea in 1.25× TAE running buffer (2 M Tris base, 1 M glacial acetic acid, 50 mM EDTA). A marker containing four reference species (*Lactococcus garvieae* CNRZ1323, *Lactococcus raffinolactis* CNRZ1214, *Enterococcus faecalis* CE17, and *Lactococcus lactis* subsp. *lactis* bv. diacetylactis CNRZ260) was loaded onto every gel. Migration was performed at

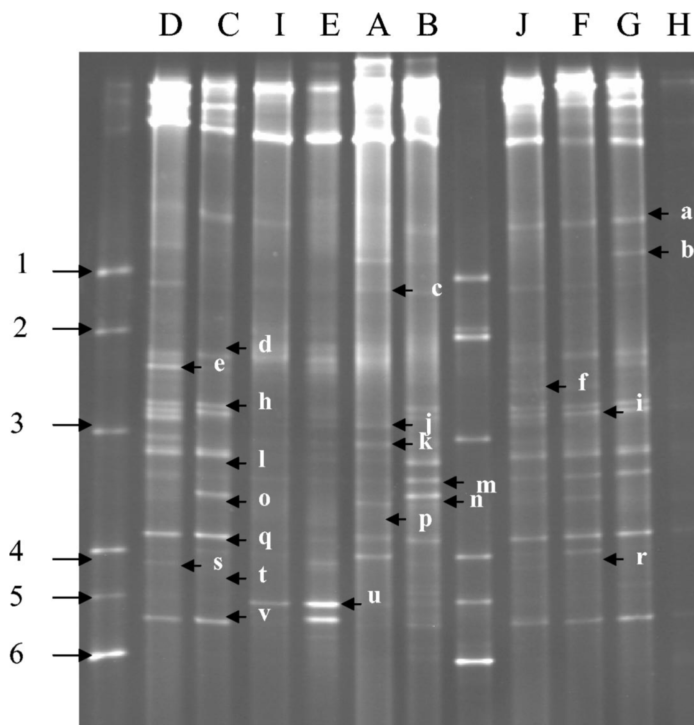

FIG. 2. DGGE profiles of 16S rRNA gene V3 regions obtained from different raw milk samples as indicated above each lane. Lanes 1, 8, and 13, marker. Bands: a, *Escherichia coli*/*Klebsiella pneumoniae*; b, *Citrobacter freundii*; c, unidentified; d, *Enterobacter sakazakii*/*Aeromonas hydrophila*/*Lactobacillus reuteri*; e, unidentified; f, *Serratia marcescens*; h, *Klebsiella pneumoniae*; i, *Clostridium sporogenes*; j, *Pantoea* sp.; k, unidentified; l, *Kocuria rosea*/*Klebsiella pneumoniae*; m, *Brevibacterium linens*; n, *Arthrobacter* species/*Klebsiella pneumoniae*/*Brachybacterium tyrofermentans*/*Corynebacterium ammoniagenes*; o, unidentified; q, *Brevibacterium linens*/*Klebsiella pneumoniae*; r, unidentified; s, unidentified; t, *Brevibacterium linens*; u, *Kocuria kristinae*/*Brevibacterium linens*; v, *Propionibacterium acidipropionici*/*Kocuria* sp. Markers (arrowed): 1, *Bacillus pumilus* ATCC 7725; 2, *Klebsiella oxytoca* ATCC 103434^T; 3, *Kytococcus sedentarius* CNRZ880; 4, *Arthrobacter citreus* CNRZ928^T; 5, *Kocuria kristinae* CNRZ872; 6, *Propionibacterium jensenii* Z87.

41 V for 16 h with a running buffer temperature of 63°C at the beginning and 70°C at the end. Gels were stained in an ethidium bromide solution (0.5 μg of 1× TAE buffer/ml) for 20 min, rinsed in 1× TAE buffer for 20 min, and photographed on a UV transillumination table. Gel photographs were converted into a file image (Photo Capt Imager Software) and analyzed by using GelCompar software (Applied Maths, Kortrijk, Belgium).

DGGE analysis for identification of bacteria with GC-rich genomes. For DGGE analysis, the Dcode universal mutation detection system (Bio-Rad) was used to separate the V3 region PCR products. PCR products were prepared as for TTGE. Samples were electrophoresed on 8% (wt/vol) polyacrylamide gels containing a denaturating gradient from 40 to 70% urea and formamide (a 100% denaturant corresponds to 7 M urea and 40% [vol/vol] formamide) in 1.25× TAE running buffer. A marker containing six reference species (*Kytococcus sedentarius* CNRZ880, *Arthrobacter citreus* CNRZ928^T, *Kocuria kristinae* CNRZ872, *Bacillus pumilus* ATCC 7725, *Propionibacterium jensenii* Z87, and *Klebsiella oxytoca* ATCC 103434^T) was loaded onto every gel. Migration was performed at 92 V for 16 h, and the running buffer temperature was kept constant at 60°C. Gels were stained, photographed, and analyzed as described above.

Analysis of TTGE and DDGE gels. GelCompar software used to analyze TTGE and DGGE gels adjusted for migration differences between gels by aligning the standardization markers included in all gels with a standard gel (31). Bacterial species isolated from dairy products were then identified by comparison with our recently reported reference dairy bacteria database (26, 26a).

Cloning and sequencing of TTGE and DGGE fragments. In cases where new bands appeared or where assignments were ambiguous, DNA bands on TTGE and DGGE gels were excised and purified as described previously (26). The excised band (corresponding to the V3 region) was then amplified with the HDA1 primer without the GC clamp and with HDA2. PCR products were quantified on 2% agarose gels, purified by using Concert Rapid PCR purification system (Life Technologies, Gaithersburg, Md.), and cloned into the pTOPOI plasmid vector (using the TOPO TA cloning kit; Invitrogen, Carlsbad, Calif.). To verify the insert, an amplification of the V3 region searched was sequenced (ABI Prism 310; Applied Biosystems, Courtaboeuf, France) after an amplification of a region of 500 bp containing the insert with the primers M13 Reverse and M13 Forward from the TOPO TA cloning kit (Invitrogen). Sequences were compared to the Ribosomal Database Project sequences (24) for species assignment.

RESULTS

Bacterial biodiversity in raw milk samples. TTGE and DGGE profiles varied in complexity. In TTGE (for identification of bacteria with low-GC% genomes), some raw milk samples displayed simple profiles, with three or four bands (Fig. 1, samples A, D, E, I, and H). Other milk samples displayed were complex profiles, with as many as 10 bands (Fig. 1, samples B, C, F, G, and J). In DGGE (for identification of bacteria with medium- or high-GC% genomes), only one sample displayed simple profiles with three bands (Fig. 2, sample I). The other sample profiles displayed six or more bands. All milk samples showed the same major band (band o) in TTGE except sample F. Many common bands were present in the different milk samples, as revealed by both TTGE and DGGE.

Most of the bands were assigned to a species or a group of species of our reference database (26, 26a). However, in some cases, e.g., when migration of V3 fragments was the same for different species (comigration as for the same V3 sequences or the same melting temperature) or to confirm an electrophore-

TABLE 1. Identification of 16S rRNA gene (V3 region) cloned sequences

Band	Milk[a]	TTGE or DGGE	Species assigned by reference database[b]	Sequence analysis		
				Species	GenBank accession no.	% Identity
k	D_{NR}	TTGE	*S. uberis/B. circulans*	*S. uberis*	AB023573	99
d	C_{NR}	TTGE	*L. innocua/L. monocytogenes/E. casseliflavus/L. fermentum*	*L. monocytogenes*	AJ549929	91
				L. innocua	AJ549928	91
k	F_{NR}	TTGE	*S. uberis/B. circulans*	*S. uberis*	AB002527	100
d	G_{NR}	TTGE	*L. innocua/L. monocytogenes/E. casseliflavus/L. fermentum*	*L. monocytogenes*	AJ549929	99
				L. innocua	AJ549928	99
f	G_{NR}	TTGE	*P. fluorescens/E. faecium/E. durans/E. hirae/L. carnosum*	*E. faecium*	AJ420800	100
k	I_{NR}	TTGE	*S. uberis/B. circulans*	*S. uberis*	AB023576	99
n	G_{NR}	TTGE	*S. dysgalactiae/H. alvei/P. alcaligenes*	*S. dysgalactiae*	AY121362	99
o	D_{NR}	TTGE	*L. lactis*	*L. lactis*	AF515226	99
d	D_R	TTGE	*L. innocua/L. monocytogenes/L. fermentum*	*L. monocytogenes*	AJ508749	91
				L. innocua	AL596172	91
n	G_R	TTGE	*S. dysgalactiae/H. alvei/P. alcaligenes*	*S. dysgalactiae*	AY121362	99
o	F_R	TTGE	*L. lactis*	*L. lactis*	AF515226	100
m	G_{NR}	DGGE	*B. linens*	*K. pneumoniae*	AF130982	97
u	E_{NR}	DGGE	*K. kristinae/B. linens*	*C. bifermentans*	AF320283	85
m	B_{NR}	DGGE	*B. linens*	*K. pneumoniae*	AF453251	97
l	G_{NR}	DGGE	*K. rosea/K. pneumoniae*	*K. pneumoniae*	AY291290	100
v	D_R	DGGE	*P. acidipropionici/Kocuria* sp.	*Propionibacterium* sp.	AY096033	99
u	E_R	DGGE	*K. kristinae/B. linens*	*Kocuria* sp.	AY043546	99
q	D_R	DGGE	*B. linens/K. pneumoniae*	*K. pneumoniae*	AY291290	98
n	B_R	DGGE	*Arthrobacter* sp./*K. pneumoniae/B. tyrofermentans/C. ammoniagenes*	*C. bifermentans*	AF320283	100
h	D_R	DGGE	*K. pneumoniae*	*K. pneumoniae*	AF390084	100
b	D_R	DGGE	*C. freundii*	*C. freundii*	AF458082	97

[a] Subscripts: NR, nonrefrigerated milk at 4°C for 24 h; R, refrigerated milk at 4°C for 24 h.
[b] See legends to Fig. 1 to 4 for full genes and species names.

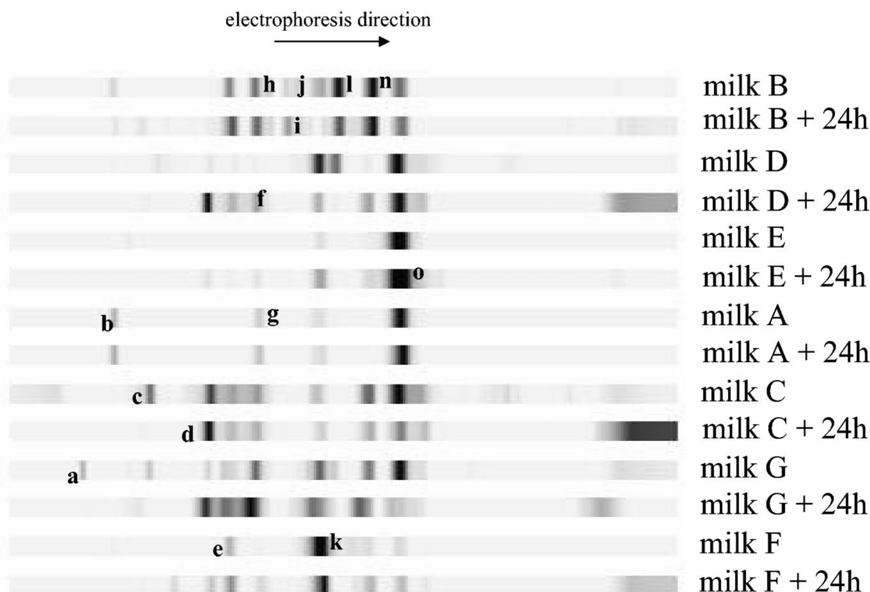

FIG. 3. TTGE profiles of rRNA gene V3 regions obtained from different raw milk samples before and after conservation at 4°C for 24 h. After standardization of band migration with the GelCompar software (Applied Maths), species were identified by comparison with known species in the reference database. Bands: a, unidentified; b, *Lactococcus garvieae*; c, *Lactobacillus plantarum/Lactobacillus pentosus*; d, *Listeria innocua/Listeria monocytogenes/Lactobacillus fermentum*; e, *Staphylococcus epidermidis*; f, *Pseudomonas fluorescens/Enterococcus faecium/Enterococcus durans/Enterococcus hirae/Leuconostoc carnosum*; g, *Leuconostoc lactis/Staphylococcus xylosus/Acinetobacter johnsonii*; h, *Chryseobacterium*; i, *Lactobacillus acidophilus* group; j, *Lactobacillus delbrueckii* subsp. *bulgaricus*; k, *Streptococcus uberis/Bacillus circulans*; l, *Staphylococcus warneri*; n, *Streptococcus dysgalactiae/Hafnia alvei/Pseudomonas alcaligenes*; o, *Lactococcus lactis*.

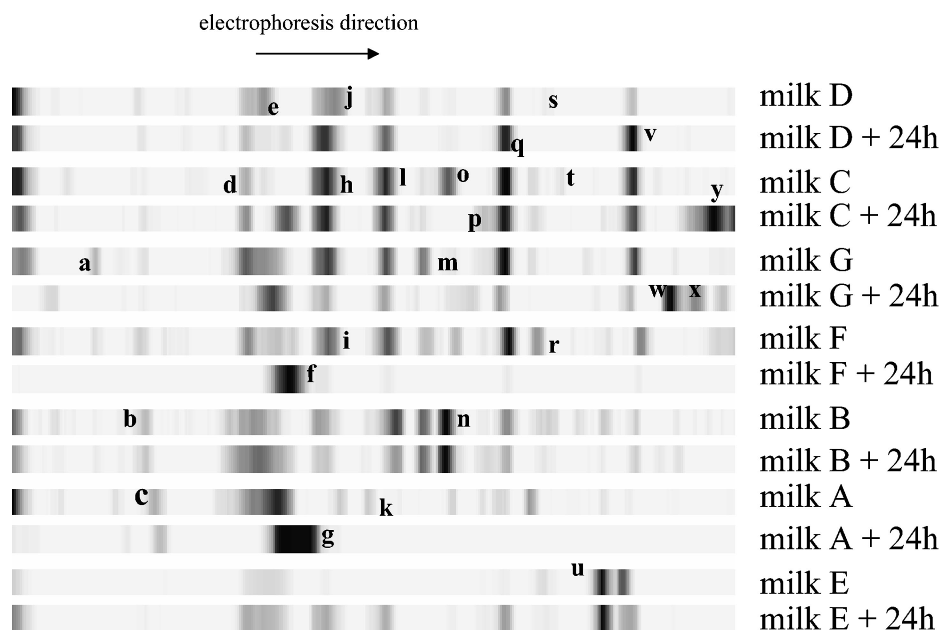

FIG. 4. DGGE profiles of rRNA gene V3 regions obtained from different raw milk samples before and after conservation at 4°C for 24 h. After standardization of band migration with the GelCompar software (Applied Maths), species were identified by comparison with known species in the reference database. Bands: a, *Escherichia coli*/*Klebsiella pneumoniae*; b, *Citrobacter freundii*; c, unidentified; d, *Enterobacter sakazakii*/*Aeromonas hydrophila*/*Lactobacillus reuteri*; e, unidentified; f, *Serratia marcescens*; g, *Aeromonas hydrophila*; h, *Klebsiella pneumoniae*; i, *Clostridium sporogenes*; j, *Pantoea* sp.; k, unidentified; l, *Kocuria rosea*/*Klebsiella pneumoniae*; m, *Brevibacterium linens*; n, *Arthrobacter* species/*Klebsiella pneumoniae*/*Brachybacterium tyrofermentans*/*Corynebacterium ammoniagenes*; o, unidentified; p, unidentified; q, *Brevibacterium linens*/*Klebsiella pneumoniae*; r, unidentified; s, unidentified; t, *Brevibacterium linens*; u, *Kocuria kristinae*/*Brevibacterium linens*; v, *Propionibacterium acidipropionici*/*Kocuria* sp.; w, *Propionibacterium thoenii*/*Propionibacterium jensenii*; x, unidentified; y, nonidentified.

sis band, sequencing was performed to confirm the identification.

Lactococcus lactis (band o) was the major raw milk species identified by TTGE and confirmed by sequencing (see below) (Table 1). Some *Staphylococcus* species were also present. *Staphylococcus warneri* (band l) was detected as a major species in the samples B, D, and J; *Staphylococcus epidermidis* (band e) was also identified in four samples (samples B, C, F, and G). Several bands were assigned to a group of species of our database reference (26, 26a) (bands c, d, f, g, i, k, and n). In that case, Table 1 shows the results of sequencing, the GenBank accession number, and the percentage of identity with V3 of a known species. Band k was identified as "*Streptococcus uberis*" at 99% certainty for milk sample D, 100% for milk sample F and 99% for milk sample I. Band d (present in samples C and G) was ambiguously identified as being either *Listeria innocua*, *Listeria monocytogenes*, or *Lactobacillus fermentum*. After sequencing, it was identified as *Listeria* sp. at 91 and 99% certainties in samples C and G, respectively. Band f (in milk sample G) was confirmed as corresponding to *Enterococcus faecium* at a 100% certainty, and band n was confirmed as corresponding to *Streptococcus dysgalactiae* at a 99% certainty.

Some major bands were detected by DGGE. *Klebsiella pneumoniae* (band h) was detected as a major species in the milk sample C. Band l (in milk sample C) was identified as *Kocuria rosea* or *Klebsiella pneumoniae*. Band n (in milk sample B) was identified as *Arthrobacter* species, *Klebsiella pneumoniae*, *Brachybacterium tyrofermentans*, or *Corynebacterium ammoniagenes*. Band q was identified as *Brevibacterium linens* or *Klebsiella pneumoniae* in samples C, F, and G, and band u (in milk sample E) was identified as *Kocuria kristinae* or *Brevibacterium linens* (Fig. 2). Many of the bands gave ambiguous assignments. For example, band q was assigned to the species *Brevibacterium linens* or *Klebsiella pneumoniae*, and band n was assigned to *Arthrobacter* species, *Klebsiella pneumoniae*, *Brachybacterium tyrofermentans*, or *Corynebacterium ammoniagenes*. Sequencing clarified some of these uncertainties: band m (samples B and G) and band l (milk G) were identified as *Klebsiella pneumoniae*. Band u (milk E) was identified as *Clostridium bifermentans*.

Bacterial biodiversity of raw milk samples after conservation at 4°C for 24 h. To determine whether bacterial dynamics in milk is affected by refrigeration, TTGE and DGGE profiles were determined for samples A, B, C, D, E, F, and G after 24 h of incubation at 4°C and then compared to profiles of nonrefrigerated samples (Fig. 3 and 4 and Tables 2 and 3). Many of the species identified after refrigeration were present in the initial sample. However, the relative proportions of bacteria were clearly altered by refrigeration. The intensities of some bands increased (e.g., in samples B, C, E, F, and G in TTGE [Table 2] and in samples A, B, D, E, F, and G in DGGE [Table 3]). Other populations decreased (for samples A, F, and G in DGGE, the band intensity showed an overall decrease). In several cases, new bands appeared after refrigeration (e.g., samples D and F in TTGE and samples A, C, E, and G in DGGE). The more pertinent changes due to refrigeration are presented below.

We detected increases in (i) *Listeria innocua*, *Listeria monocytogenes*, or *Lactobacillus fermentum*; (ii) *Staphylococcus epi-*

TABLE 2. Band intensity after storage of milk samples at 4°C for 24 h

Milk sample	Intensity[a] of band:													
	a	b	c	d	e	f	g	h	i	j	k	l	n	o
B		−			+	=		=	+	=	D	−	=	=
D				A	A	A					−	D	A	=
E											+		+	=
A		=					=				=			=
C			D	+	=	=					=		−	−
G	D		D	+	+	+					+		+	−
F				A	=	A					−		+	+

[a] Key: A, appearance; D, disappearance; +, increase; −, decrease; =, no change.

dermidis; (iii) *Pseudomonas fluorescens*, *Enterococcus faecium*, *Enterococcus durans*, *Enterococcus hirae*, and/or *Leuconostoc carnosum* (iv) *Streptococcus dysgalactiae*, *Hafnia alvei*, and/or *Pseudomonas alcaligenes* (TTGE bands d, e, f, and n); (v) *Serratia marcescens*; (vi) *Klebsiella pneumoniae*; (vii) *Kocuria rosea* and/or *Klebsiella pneumoniae*; (viii) *Brevibacterium linens* and/or *Klebsiella pneumoniae*; and (ix) *Propionibacterium acidipropionici* and/or *Kocuria* sp. (DGGE bands f, h, l, q, and v). For milk sample D, bands d, e, f, and n in TTGE appeared, and the bands h, l, q, and v in DGGE intensified.

Refrigeration resulted in decreased representation of *Lactococcus lactis*, the major raw milk bacterial component (TTGE band o in samples C and G). Decreases in *Streptococcus uberis* (TTGE band k in milk sample D) and in *Brevibacterium linens*/*Klebsiella pneumoniae* (DGGE band q in milk samples F and G) were also observed. *Lactobacillus plantarum*/*Lactobacillus pentosus*, a minority species, disappeared after incubation of the raw samples at 4°C for 24 h (TTGE band c in samples C and G). In DGGE, some bands disappeared as band o (milk sample C); band m (milk sample G); bands i, l, m, o, q, r, and v (milk sample F); and bands j, k, n, q, and r (milk sample A). Bands f (milk sample F) and g (milk sample A) appeared in the majority of samples after incubation of the raw samples at 4°C for 24 h. The results of the present study reveal that refrigeration has a clear impact on the bacterial community of raw milk.

DISCUSSION

Bacterial communities and dominant populations in food products may evolve during different food fermentation processes or during storage. TTGE and/or DGGE, specifically, have been used previously for analysis of the microflora of other food systems such as artisanal cheeses (27), malt whisky (33), Mexican maize dough (3), Italian sausages (8), dairy products (26, 26a), and traditional sour cassava starch (1). In the present study, we used TTGE and DGGE to characterize the bacterial population in raw samples and to examine changes within the bacterial community due to milk refrigeration. To our knowledge, the dynamics of the bacterial population during a simple process such as raw milk conservation at 4°C for 24 h has not been studied previously.

The combined TTGE and DGGE approaches allow us to generate a global picture of the main bacterial species of raw milk samples, generally within 3 days. Strains were identified by using the reference database established by Ogier et al. (26, 26a); the database was enriched with new species in the course of this work. Overall, the results of our analyses of raw milk bacterial composition were in agreement with previous studies (12, 13, 25). The reference database we established was exhaustive and representative of the bacterial species present in raw samples. *Lactococcus lactis* was confirmed to be a major raw milk species.

The present study provides important information on the sanitary state of animals and the conditions of raw milk production in dairies. Bacteria known to cause mastitis, such as *Streptococcus uberis*, *Streptococcus dysgalactiae*, or *Serratia marcescens* were detected in many samples as majority species. Using this molecular method, detection of *Listeria*, a serious problem in the dairy industry particularly in raw milk cheeses, was achieved within 3 days. Some rapid classical microbiological methods (e.g., ALOA medium), immunological methods (e.g., Vidas *Listeria*), or molecular methods (e.g., Probelia *Listeria*) can also detect *Listeria* within 3 days, but the advantage with TTGE and DGGE is that all pathogenic bacteria can be analyzed simultaneously. To date, however, since all *Listeria* species have the same 16S rRNA gene, assignments go as far as genus identification. The use of more specific primers (21) will allow us to distinguish *Listeria monocytogenes* from other *Listeria* species.

TTGE and DGGE profiles of raw milk samples evolved after conservation at 4°C for 24 h. An emergence of psychrotrophic bacteria such as *Listeria* (samples C, D, and G) and *Aeromonas hydrophila* (milk sample A) was observed. The increase of psychrotroph flora in raw samples stored at 4°C was reported as requiring 48 h when tested by classical microbiological methods (5, 7, 16). Our results reveal that the time for psychrotrophic populations to increase is markedly shorter

TABLE 3. Band intensity after storage of milk samples at 4°C for 24 h

Milk sample	Intensity[a] of band:																								
	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u	v	w	x	y
D				=	−			+	=	−		+					+		D			+			
C				=	D	A		=				=			D	A	=			D		=		A	A
G	D			−	+	=		−				−	D				−	D				−	A	A	A
F		D		D		+			−			D	D		D		−	D				D			
B		=		=	=	+			=			−	=	=		+	=	−			−	=			
A			=	D	D	+	A		D		D			D		D	D	D			D	D			
E				=	=	=			+			A					+	=			=	−			

[a] Key: A, appearance; D, disappearance; +, increase; −, decrease; =, no change.

than previously reported, and such populations are present within 24 h. Furthermore, we noted that bacterial dynamics showed considerable variation between samples. This may indicate that the presence of a single different strain may have a significant effect on the microbial balance in dairy products such as milk. In some cases (e.g., samples A and F), low temperatures amplified some bacterial species that were barely detectable in the initial sample and also eliminated initially major species (see Fig. 3 and 4 and Tables 2 and 3). These results should have an impact on the storage protocols used in the future for raw milk samples.

The results obtained are of interest not only for their contribution to the knowledge on the bacterial flora of raw milk samples but also essentially for elucidating the power of these molecular approaches to rapidly and precisely describe the consequences of a simple process, milk refrigeration, on the quality of dairy products and its impact on health. Recently, a link was hypothesized between Crohn's disease and the emergence of psychrotroph bacteria during the cold chain used for foods, leading to chronic infestation of the digestive tract (23). This potential association could be detailed and confirmed by using the approach described here.

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