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Biodiversity of Bacterial Ecosystems in Traditional Egyptian Domiati Cheese[▽]

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Bacterial biodiversity occurring in traditional Egyptian soft Domiati cheese was studied by PCR-temporal temperature gel electrophoresis (TTGE) and PCR-denaturing gradient gel electrophoresis (DGGE). Bands were identified using a reference species database (J.-C. Ogier et al., Appl. Environ. Microbiol. 70:5628–5643, 2004); de novo bands having nonidentified migration patterns were identified by DNA sequencing. Results reveal a novel bacterial profile and extensive bacterial biodiversity in Domiati cheeses, as reflected by the numerous bands present in TTGE and DGGE patterns. The dominant lactic acid bacteria (LAB) identified were as follows: *Leuconostoc mesenteroides*, *Lactococcus garvieae*, *Aerococcus viridans*, *Lactobacillus versmoldensis*, *Pediococcus inopinatus*, and *Lactococcus lactis*. Frequent non-LAB species included numerous coagulase-negative staphylococci, *Vibrio* spp., *Kocuria rhizophila*, *Kocuria kristinae*, *Kocuria halotolerans*, *Arthrobacter* spp./*Brachybacterium tyrofermentans*. This is the first time that the majority of these species has been identified in Domiati cheese. Nearly all the dominant and frequent bacterial species are salt tolerant, and several correspond to known marine bacteria. As Domiati cheese contains 5.4 to 9.5% NaCl, we suggest that these bacteria are likely to have an important role in the ripening process. This first systematic study of the microbial composition of Domiati cheeses reveals great biodiversity and evokes a role for marine bacteria in determining cheese type.

Domiati cheese (Gbnah Beeda) is the most popular soft white pickled cheese in Egypt and makes up about 75% of the cheese produced and consumed in that country (52). It differs chiefly from other pickled cheese varieties, such as feta, Brinza, or Telema cheese, in that the milk is salted at the first step of its manufacture. The proportion of salt (5 to 14%) depends on the season of manufacture and on the temperature of cheese ripening (1). The cheese is made from either cow or buffalo whole milk or a mixture of the two. The salted milk can be curdled fresh or sometimes after pasteurization, without the addition of any starter cultures. The cheese can be consumed either fresh or, more often, after pickling in salted whey or a brine solution for up to 2 to 4 months.

During the last decades, several investigators have isolated and identified different lactic acid bacterial species from Domiati cheese, such as *Lactococcus lactis* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. casei* (21), *L. farciminis*, *L. alimentarius*, *Enterococcus faecalis*, *E. faecium* (16), *Lactobacillus plantarum*, and *L. paracasei* (15). Other bacterial species were also isolated, including coliforms (2), *Micrococcus* spp. (20), *Arthrobacter* spp. (14), *Propionibacterium jensenii*, *Microbacterium lacticum*, *Brevibacterium linens* (16), *Staphylococcus aureus* (9), and *Aeromonas* spp. (8). In all the above-mentioned studies, results were obtained using culture methods.

Recently, molecular methods such as temporal temperature gel electrophoresis (TTGE) and/or denaturing gradient gel electrophoresis (DGGE) (5, 17, 38) were successfully used to identify the bacterial biodiversity of different types of cheese such as artisanal Sicilian (43), Stilton (18), mozzarella (6, 19, 34), Beaufort, Saint Nectaire, Morbier, Epoisse (41), Mish (10), Karish (11), and hard Ras cheeses (13). Large-scale analyses of dairy samples in INRA, France, have led to the establishment of a reference database, allowing comparative identification of some 170 bacterial species, including some food pathogens (41). The aim of the present work was to use these molecular methods to characterize the bacterial biodiversity of the popular Egyptian Domiati cheese.

MATERIALS AND METHODS

Sampling. Eleven samples of Domiati cheese made by traditional methods were collected aseptically from different cheese producers from Alexandria, Behira, and Domiatta governorates. Gross analysis of Domiati cheese samples showed that moisture, salt content, and pH values ranged between 59.83 and 63.15%, 5.46 and 9.50%, and 5.30 and 6.15, respectively.

Genomic DNA extraction. Total genomic DNA was extracted from each Domiati cheese sample (7 g) as previously described (41). After undergoing a protein digestion step by the pronase (Boerhinger, Mannheim, Germany), bacterial cells were mechanically lysed. DNA purification was performed as previously described (7). Pellets of DNA were then dissolved in 100 μl Tris-EDTA buffer plus RNase (Sigma, Saint Quentin Fallavier, France) and then analyzed by 0.8% agarose gel electrophoresis.

PCR amplification. Amplicons for TTGE and DGGE analyses were prepared by performing two successive PCRs using a Gene Amp system model 2400 (PerkinElmer, France) and appropriate primers. First, a 700-bp fragment of the 16S rRNA gene including the V3 region was amplified with primers W01 and W012. Second, the V3 region of 200 bp was amplified using primers HDA1-GC and HDA2. The PCR mixtures and amplification programs were as previously described (40). Sizes and quantities of PCR products were determined by 2%

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

Target	Primers	Sequence (5′–3′)	Annealing temp (°C)	Source or reference[a]
Brevibacterium casei	Brevib, Bcas	Unpublished data	64	Furlan
Brevibacterium linens	Brevib, Blin	Unpublished data	64	Furlan
Corynebacterium variabile	Cvar, Corb	Unpublished data	60	Furlan
Escherichia coli	Eco 223	ATCAACCGAGATTCCCCCAGT		
	Eco 455	TCACTATCGGTCAGTCAGGAG	64	46
Enterococcus casseliflavus	EC1, EC2	Unpublished data	52	Firmesse
Enterococcus durans	ED1, ED2	Unpublished data	52	Firmesse
Enterococcus faecalis	EFS1, EFS2	Unpublished data	52	Firmesse
Enterococcus faecium	EFM1, EFM2	Unpublished data	52	Firmesse
Enterococcus hirae	EH1, EH2	Unpublished data	52	Firmesse
Lactococcus garvieae	1RL	TTTGAGAGTTTGATCCTGG	45	42
	LgR	AAGTAATTTTCCACTCTACTT		
Lactococcus lactis	1RL	TTTGAGAGTTTGATCCTGG	45	42
	LacreR	GGGATCATCTTTGAGTGAT		
Lactococcus lactis subsp. *cremoris*	CreF	GTGCTTGCACCGATTTGAA	58	42
	LacreR	GGGATCATCTTTGAGTGAT		
Lactococcus lactis subsp. *lactis*	LacF	GTACTTGTACCGACTGGAT	58	42
	LacreR	GGGATCATCTTTGAGTGAT		
Lactococcus raffinolactis	1RL	TTTGAGAGTTTGATCCTGG	45	42
	PipLraR	CGTCACTGAGGGCTGGAT		
Lactobacillus acidophilus	Laci01	GACCGCATGATCAGCTTATA	55	25
	Laci02	AGTCTCTCAACTCGGCTATG		
Lactobacillus brevis	LbBreF	CTTGCACTGATTTTAACA	40	26
	LbBreR	GGGCGGTGTGTACAAGGC		
Lactobacillus casei	PrI 16S-23S	CAGACTGAAAGTCTGACGG	55	50
	CasII	GCGATGCGAATTTCTTTTTC		
Lactobacillus gasseri	GasI	GAGTGCGAGAGCACTAAAG	55	50
	GasII	CTATTTCAAGTTGAGTTTCTCT		
Lactobacillus johnsonii	Joh 16SI	GAGCTTGCCTAGATGATTTTA	57	50
	16SII	ACTACCAGGGTATCTAATCC		
Lactobacillus plantarum	Lfpr16S-23S	GCCGCCTAAGGTGGGACAGAT	55	50
	PlanII	TTACCTAACGGTAAATGCGA		
Leuconostoc citreum	Lcit-f	AAAACTTAGTATCGCATGATATC	60	30
	Lcit-r	CTTAGACGACTCCCTCCCG		
Leuconostoc mesenteroides	Lnm1	TGTCGCATGACACAAAGTTA	58	4
	Lnm2	ATCATTTCCTATTCTAGCTG		
Pseudomonas aeruginosa	Paer16SH	AGGGCAGTAAGTTAATACCTTGCTG	65	51
	Paer16SIR	CCACCTCTACCGTACTCTAGCTCAG		
Serratia marcescens	Smar16SV	GGGAGCTTGCTCACTGGGTG		
	Smar16SWR	GCGAGTAACGTCAGTTGATGAGCGTATTA	66	51
Staphylococcus aureus	STAA-AuI	TCTTCAGAAGATGCGGAATA		
	STAA-AuII	TAAGTCAAACGTTAACATACG	55	24
Staphylococcus chromogenes	STAC-ChrI	ACGGAATATCGCTTTTAAGC		
	STAC-ChrII	CGTTTACATTCGGCTTTCG	52	24
Staphylococcus epidermidis	STAE-EpI	TCTACGAAGATGAGGGATA		
	STAE-EpII	TTTCCACCATATTTTGAATTGT	52	24
Staphylococcus saprophyticus	fStSap	TCAAAAAGTTTTCTAAAAAATTTAC		
	rStSap	ACGGGCGTCCACAAAATCAATAGGA	55	33
Staphylococcus simulans	STAS-SiI	ATTCGGAACAGTTTCGCAG		
	STAS-SiII	ATTGTGAGTAATCGTTTGCC	55	24
Staphylococcus xylosus	STAX-XyI	TCTTTAGAAGATGACAGAGG		
	STAX-XyII	TGACTTTTAACACGACGAAG	55	24
Streptococcus thermophilus	Sther03	TTATTTGAAAGGGGCAATTGCT		
	Sther08	GTGAACTTTCCACTCTCACAC	55	25

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

agarose gel electrophoresis (Seakem CTG agarose; TEBU, France) against a standard containing DNA fragments of defined lengths (Smart Ladder, France).

TTGE and DGGE analyses. TTGE and DGGE analyses of V3 amplicons were applied on 16-cm by 16-cm by 1-mm gels (Bio-Rad DCode universal mutation detection system; Marnes La Coquette, France) and performed as previously described (41). After runs, gels were stained for 15 min with an ethidium bromide solution (0.5 μg/ml of 1× Tris-acetate-EDTA [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. TTGE and DGGE gels were analyzed by GelCompar software (Applied-Maths, Belgium) as previously described (41). The software standardizes TTGE and DGGE profiles to minimize migration differences between gels by alignment of the identification ladder with a standard gel (40). Band identifications are performed by comparison to a species database which includes TTGE and DGGE fingerprints of about 170 bacterial species isolated from dairy ecosystems (41). In some cases, specific PCR tests and/or cloning and sequencing were undertaken to confirm species assignments or to distinguish between comigrating species.

Species-specific PCR tests. Specific PCR tests were carried out using different species-specific primers (Table 1) with DNA obtained from the cheese samples. Primers (MWG Biotech AG, Ebersberg, Germany) were prepared at a final

TABLE 2. Range of viable counts of different bacterial groups in Domiati cheese samples

Medium	Group targeted	Incubation temp (°C), time, anaerobic or aerobic conditions[a]	Log CFU/g[b]	
			Lower	Higher
MSA	*Staphylococcus* spp.	37, 48 h, A	7.09 ± 0.25	7.57 ± 0.37
M17	Mesophilic streptococci	30, 48 h, A	5.21 ± 0.23	7.40 ± 0.26
BHI + 5% NaCl	Salt-tolerant flora	25, 48 h, A	6.36 ± 0.28	7.12 ± 0.32
M17	Thermophilic streptococci	42, 48 h, A	5.50 ± 0.19	6.86 ± 0.31
MSE	*Leuconostoc* spp.	30, 48 h, A	5.55 ± 0.21	6.63 ± 0.22
BEA	*Enterococcus* spp.	37, 48 h, An	6.16 ± 0.18	6.62 ± 0.28
YEL	*Propionibacterium* spp.	25, 7 days, An	5.92 ± 0.25	6.12 ± 0.21
CFC	*Pseudomonas* spp.	30, 48 h, A	4.02 ± 0.12	5.94 ± 0.26
MRS	Mesophilic *Lactobacillus*	30, 48 h, An	4.75 ± 0.16	5.57 ± 0.24
MRS	Thermophilic *Lactobacillus*	42, 72 h, An	3.03 ± 0.09	5.45 ± 0.14
VRBA	Total coliform	30, 48 h, A	2.10 ± 0.15	3.78 ± 0.19

[a] A, aerobic; An, anaerobic.
[b] The values shown are means ± standard deviations.

concentration of 60 μM in deionized, autoclaved water. PCR was performed in a GenAmp system model 2400 (PerkinElmer, France), and all reactions were carried out following conditions previously provided by the authors (Table 1). Sizes of PCR products were determined using 1.5% agarose gel electrophoresis (Seakem CTG agarose; TEBU, France).

Sequencing of bands. Some bands obtained from TTGE and DGGE analyses of Domiati cheese samples were excised, purified, cloned, and sequenced, as described previously (40). Sequences of the clones of the V3 16S rRNA genes were compared to those present in the Ribosomal Database Project (31) to determine the closest known relative species.

Enumeration of different bacterial groups. Domiati cheese samples (11 g) were emulsified in 99 ml of sterile 2% (wt/vol) tri-sodium citrate solution (Merck) and homogenized using an Ultra-Turrax mechanical blender (19,000 rpm for 45 s). Serial dilutions were prepared in sterile 1% (wt/vol) peptone water, plated on selective agar medium using a spiral platter (Spiral system; Cincinnati, OH), and incubated at the appropriate temperatures (Table 2). Bacterial enumerations were done on M17 (Difco, Elancourt, France) for lactococci and streptococci, on MRS (Difco) (pH adjusted to 5.2) for mesophilic and thermophilic lactobacilli, on MSE (35) for *Leuconostoc*, on BEA (Difco) for enterococci, on MSA (Difco) for staphylococci, on BHI (Difco)

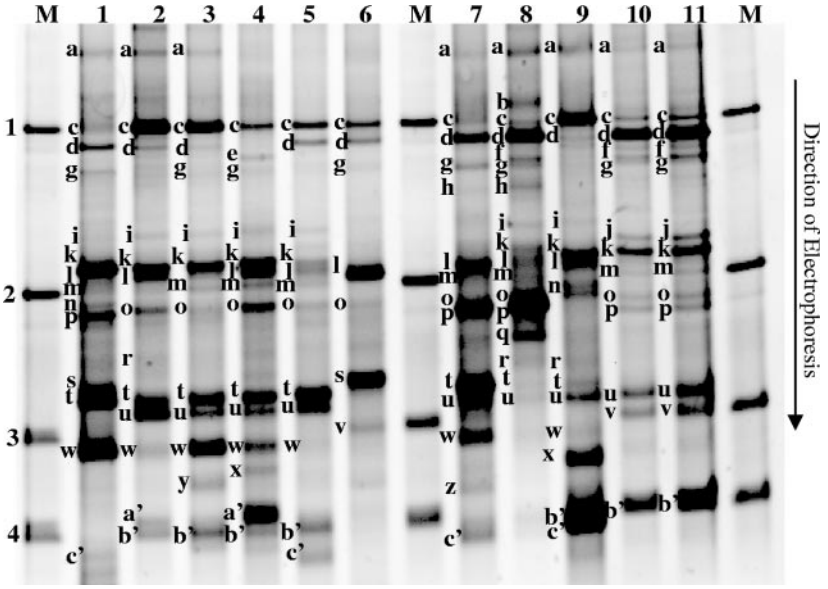

FIG. 1. TTGE electrophoresis of V3 16S rDNA fragments from different samples of Domiati cheese. Samples 1 to 11 correspond to V3 16S rDNA regions PCR amplified from genomic DNA extracted from cheese samples (see Materials and Methods). After standardization of the gel by GelCompar software, bands are identified by comparison with the species database. Band a, PCR artifact; b, unidentified band; c, *Lactococcus garvieae*; d, *Aerococcus viridans*; e, unidentified band; f, *Lactobacillus johnsonii*, *Lactobacillus gasseri*; g, *Lactobacillus plantarum*, *Lactobacillus pentosus*; h, *Acinetobacter lwoffii*; i, *Leuconostoc citreum*; j, *Enterococcus casseliflavus*, *Staphylococcus lentus*; k, *Leuconostoc mesenteroides*; l, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Staphylococcus simulans*; m, *Lactococcus raffinolactis*, *Staphylococcus equorum*, *Staphylococcus capitis*; n, unidentified band; o, *Enterococcus faecium* group, *Pseudomonas fluorescens*, *Leuconostoc pseudomesenteroides*; p, *Leuconostoc lactis*, *Staphylococcus xylosus*, *Lactobacillus brevis*; q, *Pseudomonas putida*; r, *Lactobacillus acidophilus* group; s, *Staphylococcus saprophyticus*, *Pseudomonas aeruginosa*; t, *Pediococcus pentosaceus*, *Macrococcus caseolyticus*; u, *Streptococcus uberis*, *Moraxella bovis*; v, *Enterococcus faecalis*, *Staphylococcus warneri*; w, unidentified band; x, unidentified band; y, *Staphylococcus chromogenes*, *Hafnia alvei*, *Pseudomonas alcaligenes*; z, unidentified band; *a′*, *Acinetobacter* spp.; b′, *Lactococcus lactis*; c′, *Streptococcus thermophilus*. Markers: 1, *Lactococcus garvieae* CNRZ1323; 2, *Lactococcus raffinolactis* CNRZ1214; 3, *Enterococcus faecalis* CE17; 4, *Lactococcus lactis* subsp. *lactis* bv. *diacetilactis* CNRZ260. Lane M, TTGE standardization ladder; lane 1 to lane 11, cheese samples from 1 to 11.

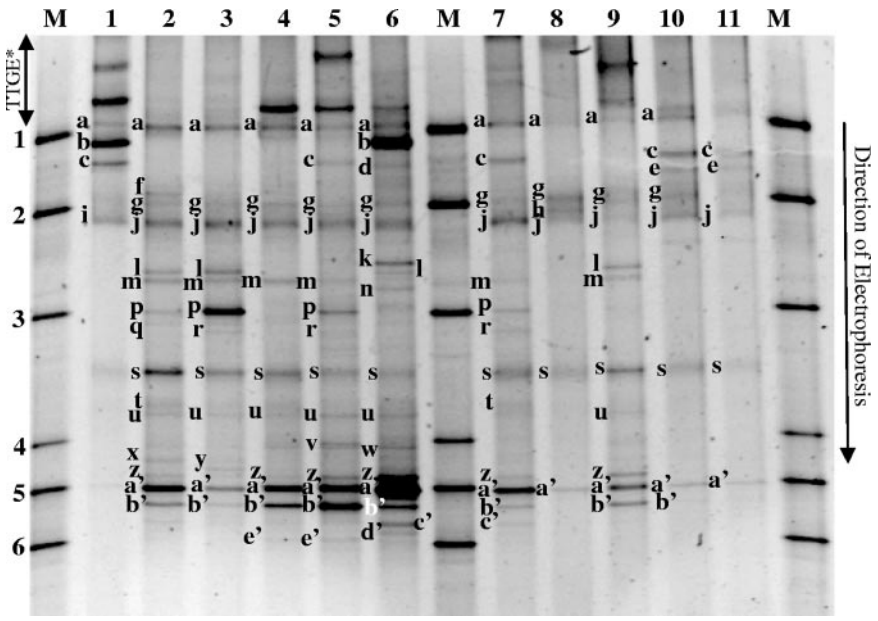

FIG. 2. DGGE electrophoresis of V3 16S rRNA gene fragments from different samples of Domiati cheese. Samples 1 to 11 correspond to V3 16S rRNA gene regions PCR-amplified from genomic DNA extracted from cheese samples (see Materials and Methods). After standardization of the gel by GelCompar software, bands are identified by comparison with the species database. Band a, *Klebsiella pneumoniae*; b, *Enterobacter amnigenus*, *E. coli*; c, *Citrobacter freundii*; d, unidentified band; e, unidentified band; f, *Serratia marcescens*, *Raoultella planticola*; g, *Corynebacterium variabile*; h, *Enterobacter cloacae*, *Klebsiella oxytoca*; i, *Serratia liquefaciens*; j, unidentified band; k, *Corynebacterium flavescens*; l, *Microbacterium gubbeenense*; m, *Lactobacillus casei*; n, *Corynebacterium vitaeruminis*, *Kluyvera ascorbata*; o, *Clostridium sporogenes*; p, *Micrococcus* spp., *Microbacterium* spp.; q, unidentified band; r, *Corynebacterium ammoniagenes*; s, *Arthrobacter* spp., *Brachybacterium tyrofermentans*; t, unidentified band; u, *Brevibacterium* spp.; v, unidentified band; w, *Brevibacterium linens* (50%); x, unidentified band; y, *Brevibacterium casei*, *Corynebacterium bovis*; z, *Propionibacterium freudenreichii*; a′, *Kocuria kristinae*, *Brevibacterium linens* (50%); b′, *Propionibacterium acidipropionici*, *Kocuria* spp.; c′, unidentified band; d′, unidentified band, e′, unidentified band. Markers, 1, *Bacillus pumilus* ATCC 7725; 2, *Klebsiella oxytoca* ATCC 103434T; 3, *Kytococcus sedentarius* CNRZ880; 4, *Arthrobacter citreus* CNRZ928T; 5, *Kocuria kristinae* CNRZ872; 6, *Propionibacterium jensenii* Z87. Lane M, DGGE standardization ladder; lane 1 to lane 11, cheese samples from 1 to 11. TTGE*, bands separated by TTGE method.

supplemented with 5% (wt/vol) NaCl for salt-tolerant flora, on YEL (yeast extract-sodium lactate medium) for *Propionibacterium* spp., on VRBA (Difco) for total coliform, and on cetrimide, fucidin, and cephalosporin (CFC) agar (Oxoid) for *Pseudomonas* spp.

RESULTS

Considerable bacterial biodiversity existed within the 11 samples of Domiati cheese that were analyzed by TTGE and DGGE (Fig. 1 and 2). In TTGE profiles, all cheese samples showed complex profiles ranging from 7 bands (Fig. 1, sample 6) to 18 bands (Fig. 1, sample 8). In DGGE profiles, some Domiati cheese samples showed relatively simple profiles with 5 bands (Fig. 2, sample 1), whereas other samples showed 17 bands (Fig. 2, sample 6).

Biodiversity among low-G+C-percentage species present in Domiati cheeses. TTGE profiles of the Domiati cheese samples revealed 29 different bands (Fig. 1). Among them, 22 could be potentially identified using the species database, while 7 others (Fig. 1, bands a, b, e, n, w, x, and z) were novel. Results showed different major bands corresponding to *Leuconostoc mesenteroides* (band k), *Lactococcus garvieae* (band c), and *Aerococcus viridans* (band d) (Fig. 1). High band intensities and frequencies for these species may reflect their strong dominance in Domiati cheese samples. The presence of these three species was further confirmed using species-specific PCR tests (Table 3) and/or a cloning and sequencing strategy (Table 4). Other major bands (Fig. 1, bands p, t, u, and w) gave ambiguous assignments using the reference database and were then sequenced for some Domiati samples. They were identified as close relatives of *Staphylococcus sciuri* (band p), *Pediococcus inopinatus* and/or *Macrococcus caseolyticus* (band t), *Lactobacillus versmoldensis* (band u), and *Vibrio* spp. (band w) (Table 4). We note that some bands may correspond to different bacterial species (Table 4).

Lactococcus lactis (band b′) appeared as a relatively major band, as observed from the TTGE profile (Fig. 1). The presence of *L. lactis* was confirmed in all tested samples using a species-specific PCR assay (Table 3). In most cases, subspecies belonging to the same species (e.g., *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) could not be differentiated by the TTGE approach (40). Specific PCR tests were then applied to determine which one was more dominant in the Domiati cheese samples. *L. lactis* subsp. *lactis* was detected in all 11 samples, whereas *L. lactis* subsp. *cremoris* was detected in only three samples (Table 3). The presence of *L. lactis* subsp. *lactis* was also confirmed by sequencing of the corresponding bands (Table 4).

Many other bacterial species, belonging to different genera (e.g., *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Streptococcus*, *Pseudomonas*, and *Acinetobacter*), were identified as subdominant or minor species in Domiati cheese (Fig. 1). Most of the band assignments using the species database

TABLE 3. Presence of bacterial species in individual Domiati cheese samples using specific primer tests

Primer-specific species	Presence of bacteria in indicated Domiati cheese sample[a]											Frequency (samples/total)[b]
	1	2	3	4	5	6	7	8	9	10	11	
Brevibacterium casei	−	+	+	−	−	−	+	−	−	−	−	3/11
Brevibacterium linens	−	−	−	−	−	−	−	−	−	−	−	0/11
Corynebacterium variabile	+	+	+	+	+	+	+	−	+	+	+	10/11
Escherichia coli	+	+	+	+	+	+	+	+	−	+		9/11
Enterococcus casseliflavus	−	−	−	−	−	−	−	−	−	−	−	0/11
Enterococcus durans	−	−	−	−	−	−	−	−	−	−	−	0/11
Enterococcus faecalis	+	+	+	−	−	−	−	+	−	−	+	5/11
Enterococcus faecium	+	−	−	−	−	−	−	+	−	−	−	2/11
Enterococcus hirae	−	−	−	−	−	−	−	−	−	−	−	0/11
Lactococcus garvieae	+	+	+	+	+	+	+	+	+	+	+	11/11
Lactococcus lactis	+	+	+	+	+	+	+	+	+	+	+	11/11
Lactococcus lactis subsp. *cremoris*	+	+	−	+	−	−	−	−	−	−	−	3/11
Lactococcus lactis subsp. *lactis*	+	+	+	+	+	+	+	+	+	+	+	11/11
Lactococcus raffinolactis	−	−	−	−	−	+	−	−	−	−	−	1/11
Lactobacillus acidophilus	−	+	+	+	−	−	−	+	+	−	−	5/11
Lactobacillus brevis	−	−	−	−	−	−	−	−	−	−	−	0/11
Lactobacillus casei	+	+	+	−	+	+	+	+	+	+	+	10/11
Lactobacillus gasseri	−	−	−	−	−	−	−	−	−	−	−	0/11
Lactobacillus johnsonii	+	+	+	−	+	−	+	−	+	+	+	8/11
Lactobacillus plantarum	−	−	−	−	−	−	−	−	−	−	−	0/11
Leuconostoc citreum	+	+	+	+	+	+	+	−	+	+	+	10/11
Leuconostoc mesenteroides	+	+	+	+	+	+	+	+	+	+	+	11/11
Pseudomonas aeruginosa	−	−	−	−	−	−	−	−	−	−	−	0/11
Serratia marcescens	+	+	−	−	−	+	−	−	−	−	−	3/11
Staphylococcus aureus	−	+	−	−	−	−	−	−	−	−	−	1/11
Staphylococcus chromogenes	−	+	−	−	−	−	−	−	−	−	−	1/11
Staphylococcus epidermidis	−	+	+	−	−	−	−	−	−	−	−	2/11
Staphylococcus saprophyticus	+	+	−	−	+	+	+	+	−	+	+	8/11
Staphylococcus simulans	−	−	+	+	−	−	−	+	−	−	−	3/11
Staphylococcus xylosus	−	−	−	−	−	−	−	−	−	−	−	0/11
Streptococcus thermophilus	+	−	+	+	−	−	+	−	+	−	−	5/11

[a] +, positive; −, negative.
[b] Frequency, ratio of numbers of positive samples to numbers of tested cheese samples.

were confirmed by specific PCR tests and/or by cloning and sequencing strategies (Tables 3 and 4). These complementary tests were very useful for identifying precisely the bacteria at the species level. For example, band g was identified as *Lactobacillus plantarum* and *L. pentosus* by the reference database and also by band sequencing (Fig. 1 and Table 4), whereas, using the specific PCR tests, *L. plantarum* was absent (Table 3). Consequently, it supposed that this band might correspond to *Lactobacillus pentosus*.

Biodiversity among high-G+C-percentage species present in Domiati cheese. The DGGE profile for Domiati cheese samples (Fig. 2) included 31 different bands. The species database allowed the identification of only 21 bands, whereas the other 10 (Fig. 2, bands d, e, j, q, t, v, x, c′, d′, and e′) could not be identified. The major bands (Fig. 2) putatively identified were those of *Kocuria kristinae* or *Brevibacterium linens* (band a′) and *Propionibacterium acidipropionici* or *Kocuria* spp. (band b′). A species-specific PCR assay failed to identify *B. linens* (Table 3), thus making it likely that band a′ was amplified from *K. kristinae*. Sequencing of the V3 region fragment of band b′ identified *K. rhizophila* as the species (Table 4). Some other frequent bands of moderate intensity identified *Klebsiella pneumoniae* (band a), *Arthrobacter* spp./*Brachybacterium tyrofermentans* (band s), *Corynebacterium variabile* (band g), and *Propionibacterium freudenreichii* (band z) as the corresponding species. Other bacterial species were identified by the DDGE approach (Fig. 2) and confirmed, in some cases, *Escherichia coli* (band b), *Citrobacter freundii* (band c), *L. casei* (band m), and *B. casei* (band y) (Table 3 and 4).

Enumeration of different bacterial groups. Several selective media were used to enumerate the different bacterial groups present in Domiati cheese samples (Table 2). The highest bacterial counts (ranging between 7.1 and 7.6 $\log_{10}$ CFU/g) were obtained with MSA medium, which is generally selective for the staphylococcal populations. Total coliforms were recorded as the lowest number of cells (2.1 to 3.8 $\log_{10}$ CFU/g). The counts of salt-tolerant flora ranged between 6.4 and 7.1 $\log_{10}$ CFU/g. In general, the counts of coccal lactic acid bacteria (LAB) (*Lactococcus*, *Streptococcus*, *Leuconostoc*, and *Enterococcus*), as presented in Table 2, were higher than those of rod members of LAB (mesophilic and thermophilic lactobacilli).

DISCUSSION

Traditional cheeses like Domiati are widely consumed regionally and also contribute to the cultural heritage. To date, little was known about the bacterial communities responsible for fermentation and ripening of Domiati cheese. This is due in part to the sole use of culture-dependent methods for bacterial identification, which provide relatively poor discrimination of species present in a product. In particular, media used for

TABLE 4. Bacterial species identified in Domiati cheese samples using cloning and sequencing strategy

Band	TTGE or DGGE	Bacterial species or groups assigned by species database	Domiati sample no.	Closest sequence relative species	Identity (%)	GenBank accession no.
c	TTGE	*Lactococcus garvieae*	2, 3, 6,	*Lactococcus garvieae*	100	AY699289.1
d	TTGE	*Aerococcus viridans*	3, 7	*Aerococcus viridans*	100	DQ402378.1
g	TTGE	*Lactobacillus plantarum*, *Lactobacillus pentosus*	5	*Lactobacillus plantarum*, *Lactobacillus pentosus*	100, 100	DQ860149, AY362458
k	TTGE	*Leuconostoc mesenteroides*	1, 3	*Leuconostoc mesenteroides*	99	AB120036.1
n	TTGE	Unidentified	9	Marine sediment bacterium	99	AY669172.1
p	TTGE	*Staphylococcus xylosus*, *Latobacillus brevis*, *Lactobacillus lactis*	7, 8	*Staphylococcus sciuri*	98	AB233332
s	TTGE	*Staphylococcus saprophyticus*, *Pseudomonas aeruginosa*	6	*Staphylococcus saprophyticus*	99	AY375294.1
t	TTGE	*Pediococcus pentosaceus*, *Macrococcus caseolyticus*	4	*Pediococcus inopinatus*	100	AF404741.1
			7	*Macrococcus caseolyticus*	98	AY15711
u	TTGE	*Streptococcus uberis*, *Moraxella bovis*	4, 10	*Lactobacillus versmoldensis*	100	AJ496791.1
v	TTGE	*Enterococcus faecalis*, *Staphylococcus warneri*	11	*Enterococcus faecalis*	99	AY850358.1
w	TTGE	Unidentified	7	*Vibrio* spp.	98	AY836815.1
			1, 3	*Staphylococcus* spp., *Staphylococcus arlettae*	100, 100	DQ888572, DQ872460.1
x	TTGE	Unidentified	9	*Streptococcus parauberis*	100	AY584477.1
b′	TTGE	*Lactococcus lactis*	10	*Lactococcus lactis* subsp. *lactis*	100	AF515224.1
c′	TTGE	*Streptococcus thermophilus*	7	*Streptococcus thermophilus*	100	CP000024.1
c	DGGE	*Citrobacter freundii*	1	*Citrobacter freundii*	93	AF458082.1
j	DGGE	Unidentified	6	*Kocuria halotolerans*, *Rothia* spp.	100, 100	DQ979377, DQ822568
b′	DGGE	*Propionibacterium acidipropionici*, *Kocuria* spp.	4	*Kocuria rhizophila*	100	AF542072.1

isolation might fail to monitor bacteria that cannot multiply outside the cheese environment (40).

The combination of molecular tools (i.e., PCR-TTGE, PCR-DGGE, species-specific PCR assays, and cloning/sequencing analyses) allowed us to identify 46 different bacterial species that are present in Domiati cheese. Most of the bacteria were first presumably identified by the assignation of the TTGE/DGGE bands to a complex species database (40, 41). But the limitation of this method concerns the species comigrations. Despite sequence differences, melting temperatures of comigrated V3 fragments are similar, and thus, they migrate at the same position in denaturing gels (37). To confirm species assignments or to distinguish between comigrating species, species-specific PCR tests and/or cloning and sequencing were performed. This strategy proved to be very useful to more precisely identify the bacterial species. According to the frequencies and intensities of the bands on TTGE and DGGE gels (TTGE/DGGE methods are considered semiquantitative techniques [53] and generally, band intensities reflect the relative proportion of each species in the total bacterial population [39]), we could differentiate the bacterial population of Domiati cheeses into three groups. (i) The first is composed of dominant bacterial species (they are almost identified in each sample), e.g., *Leuconostoc mesenteroides*, *Lactococcus garvieae*, *Aerococcus viridans*, *Lactobacillus versmoldensis*, *Pediococcus inopinatus*/*Macrococcus caseolyticus*, and *Lactococcus lactis* subsp. *lactis*. The bacteria of this group may play the main role in the fermentation and organoleptic properties of Domiati cheeses because of their common presence in the tested samples. (ii) The second group is composed of frequently encountered bacterial species. These bacteria belonged to both LAB species, e.g., *Leuconostoc citreum*, *Lactobacillus casei*, *Lactobacillus johnsonii*, *Staphylococcus thermophilus*, *E. faecalis*, *E. faecium*/*Leuconostoc pseudomesenteroides*, and to non-LAB species, e.g., *Vibrio* spp., *K. kristinae*, *C. variabile*, *K. rhizophila*, *Arthrobacter* spp./*B. tyrofermentans*, plus numerous species of coagulase-negative staphylococci. (iii) The third group consists of occasionally encountered bacterial species, e.g., *Lactococcus raffinolactis*, *Acinetobacter lwoffii*, *Staphylococcus lentus*, *S. chromogenes*, *Enterobacter cloacae*, and *Klebsiella oxytoca.* The bacteria of the second and third groups may have a secondary activity in the fermentation process of Domiati cheeses, but the origins and potential roles of these as well as the bacteria of the first group would need further investigation.

Domiati cheese manufacture involves several technological steps, including natural fermentation of milk, salting, rennetting, and ripening (pickling) in brine or salted whey solutions. The salt added to cheese milk plays an important role during manufacturing by favoring or inhibiting bacterial growth (the salt content in our cheese samples ranged between 5.46 and 9.50% of wet weight). Many of the identified bacterial species could be recognized as partially or totally salt-tolerant bacteria, e.g., *Leuconostoc mesenteroides*, *Lactobacillus versmoldensis* (up to 14% salt tolerance) (28), *Lactococcus lactis* subsp. *lactis*, *Staphylococcus saprophyticus*, *K. rhizophila*, *K. halotolerans*, *Brachybacterium tyrofermentans* (up to 16% salt tolerance) (48), *K. kristinae*, and *Microbacterium gubbeenense*. In some cases, the identified species were directly related to a marine environment, e.g., *Lactococcus garvieae* (which often occurs in dairy environments [3, 12, 23] and is considered one of the major pathogens responsible for fish mortality [44]), *Aerococcus viridans*, *Vibrio* spp., and marine sediment bacterium. As

previously described (47), our results indicate that salt-tolerant and marine bacteria may play a role in the ripening process of Domiati cheese. Other studies of red-smear soft cheeses have demonstrated that a fraction of cheese flora was composed of microorganisms related to a marine environment (22, 32).

This study allows us to clarify reports of the occurrence of staphylococcal species in Domiati cheese, as numerous species have been identified by molecular tools. These results reflected the high bacterial counts using MSA medium (7.1 to 7.6 $\log_{10}$ CFU/g). Previous studies focused on the occurrence of *Staphylococcus aureus* because of its important role in food poisoning. Our results indicate a relative predominance of *S. sciuri* and *S. saprophyticus* (both coagulase-negative staphylococci) in Domiati cheese. Fortunately, *S. aureus* was detected in only one cheese sample, indicating either that good sanitation procedures were applied during Domiati cheese manufacturing or that *S. aureus* is not a good competitor with other bacterial species (36). This study also revealed the presence of other coagulase-negative staphylococci: *S. simulans* and *S. chromogenes* were identified for the first time in Domiati cheese. These species are common causes of subclinical mastitis (27, 29). However, it is notable that *S. simulans* produces lysostaphin, a cell wall-degrading enzyme that lyses practically all known staphylococcal species (45), and was recently developed for its bactericidal antistaphylococcal effects (49). Its presence might actually control development of populations of susceptible staphylococci in Domiati cheese. Other bacterial species known to cause mastitis were also identified (e.g., *Streptococcus parauberis*, *Staphylococcus epidermidis*, *Citrobacter freundii*, and *Serratia marcescens*) but were detected as minor species.

In the present study, several bacterial species (never previously isolated by culturing from Domiati cheese) were detected for the first time in Domiati cheese samples, e.g., LAB (*Lactococcus garvieae*, *A. viridans*, *Pediococcus inopinatus*, *Lactobacillus versmoldensis*, *L. johnsonii*, *Leuconostoc citreum*, and *S. parauberis*), and non-LAB (*Pseudomonas putida*, *Staphylococcus sciuri*, *S. chromogenes*, *S. simulans*, *Macrococcus caseolyticus*, *Citrobacter freundii*, *Corynebacterium variabile*, *Microbacterium. gubbeenense*, *Propionibacterium freudenreichii*, *K. rhizophila*, *K. halotolerans*/*Rothia* spp., and marine sediment bacterium). These results confirm interest in using the molecular methods for exhaustive and precise identification of complex microbial biodiversity occurring in artisanal cheeses such as Domiati.

The considerable bacterial biodiversity found in Domiati cheeses may explain the heterogeneous production of cheeses available in Egyptian markets. It is expected that each bacterial species present in Domiati cheese may contribute in some way to the ripening process but would vary according to its tolerance toward the probably highly variable cheese production conditions (e.g., salt concentrations, pH, ripening temperatures, and type of pickling solutions [whey or brine]).

Although traditional Domiati cheese is made from raw milk without the addition of any starters, most of the bacteria identified as dominant belonged to LAB. Nevertheless, improvements in Domiati cheese manufacture may be necessary to obtain a safe and homogenous product. This would require the systematic use of clean raw materials, controlled manufacturing steps, and selection of the appropriate LAB for the ripening process. The origin and role of the salt-tolerant and marine bacteria present in Domiati cheese should be further investigated. Finally, the application of culture methods will be valuable for the isolation of "positive" bacteria that characterize Domiati cheese for future use as cheese starter cultures.

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