Food Microbiology 28 (2011) 465–471

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Molecular analysis of bacterial communities in raw cow milk and the impact of refrigeration on its structure and dynamics

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ARTICLE INFO

Article history:
Received 20 July 2010
Received in revised form
29 September 2010
Accepted 19 October 2010
Available online 27 October 2010

Keywords:
Raw milk
Microbial dynamics
Psychrotolerant
DGGE
16S rRNA gene
Actinobacteria
Pseudomonadales

ABSTRACT

The impact of refrigeration on raw cow milk bacterial communities in three farm bulk tanks and three dairy plant silo tanks was studied using two methods: DGGE and cloning. Both methods demonstrated that bacterial taxonomic diversity decreased during refrigeration. *Gammaproteobacteria*, especially *Pseudomonadales*, dominated the milk after refrigeration. Farm samples and dairy plant samples differed in their microbial community composition, the former showing prevalence of Gram-positive bacteria affiliated with the classes *Bacilli*, *Clostridia* and *Actinobacteria*, the latter showing prevalence of Gram-negative species belonging to the *Gammaproteobacteria* class. *Actinobacteria* prevalence in the farm milk samples immediately after collection stood at about 25% of the clones. A previous study had found that psychrotolerant *Actinobacteria* identified in raw cow milk demonstrated both lipolytic and proteolytic enzymatic activity. Thus, we conclude that although *Pseudomonadales* play an important role in milk spoilage after long periods of cold incubation, *Actinobacteria* occurrence may play an important role when assessing the quality of milk arriving at the dairy plant from different farms. As new cooling technologies reduce the initial bacterial counts of milk to very low levels, more sensitive and efficient methods to evaluate the bacterial quality of raw milk are required. The present findings are an important step towards achieving this goal.

1. Introduction

Psychrotolerant bacteria play a leading role in spoilage of refrigerated milk and milk products. They are defined as bacteria that grow at 7 °C, although their optimal growth temperature is higher (Cousin et al., 2001). Most psychrotolerants are easily destroyed at high temperatures of the pasteurization and sterilization processes; however, their extracellular enzymes resist heat processing and cause deterioration and spoilage of dairy products during cold storage (Cousin, 1982; Koka and Weimer, 2001; Hantsis-Zacharov and Halpern, 2007). The presence of psychrotolerants and their enzymes in refrigerated raw milk is of great concern to dairy manufacturers and processors. In Israel for instance, it is estimated that psychrotolerants can cause about 10% loss in milk fats and proteins (Hantsis-Zacharov and Halpern, 2007).

Although bacterial communities in raw milk have been described, most studies used culture-dependent methods for bacterial identification (Sorhaug and Stepaniak, 1997; Hilton et al., 2002; Sanjuan et al., 2003; Holm et al., 2004; Hantsis-Zacharov and Halpern, 2007). Raw milk is generally believed to harbor a large fraction of culturable species. However, culture-dependent methods, which are usually time consuming, will not recover all the microorganisms in this environment. Only few studies have monitored bacterial communities from raw milks by culture-independent methods (Lafarge et al., 2004; Ogier et al., 2004; Delbes et al., 2007; Giannino et al., 2009; Kuang et al. 2009; Rasolofo et al. 2010). The effect of cold incubation on raw milk bacterial communities has been even less researched (Lafarge et al., 2004; Rasolofo et al., 2010).

The current study is a follow up research of our recently published study on the diversity, dynamics and enzymatic traits of culturable psychrotolerant bacteria in raw cow milk (Hantsis-Zacharov and Halpern, 2007). In the present study, the effect of refrigeration on the diversity and dynamics of raw cow milk bacterial communities from dairy farms and silo tanks was investigated by two molecular approaches based on 16S rRNA gene analysis: DGGE, and cloning.

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doi:10.1016/j.fm.2010.10.009

2. Materials and methods

2.1. Milk sampling

Raw milk samples were collected in October 2006 from bulk tanks at three dairy farms in northern Israel and from three silo tanks at the main industrial dairy processing plant of that region. All sampled farms are equipped with modern automated milking facilities and are considered farms with very good milk quality ($<10^4$ mesophilic CFU/ml). There is no grazing area in any of the sampled farms, and the herd's nutrition trough is a feed mixture produced at a regional center. Samples were taken under sterile conditions from a mix of several milkings. The milk was stored at 4 °C from milking until it was analyzed in the laboratory. Bacterial DNA was extracted 2 h after sample collection, that is, approximately 22 h and 54 h after milking, for the farm and dairy tank samples respectively. All samples were further incubated in the laboratory at 4 °C, for 24 and 48 h respectively, and bacterial DNA was extracted from the samples again. In sum, 18 DNA samples were obtained. Raw milk samples from the farms were designated F1, F2 and F3, those from the dairy plant silo tanks T1, T2 and T3. The samples' ID corresponding to source and the incubation time are listed in Table 1.

2.2. Enumeration of microorganisms

Milk samples were diluted and plated on sterile standard plate count (SPC) agar (Oxoid, Hampshire, UK) and incubated at 32 °C for 48 h.

2.3. DNA extraction

Bacterial pellets were obtained from 35 ml of raw milk sample by centrifugation at 10,000*g* for 20 min at 4 °C. Bacterial pellets were then washed with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). This procedure was repeated four times. Bacterial pellets were further washed with lysis buffer [Tris–HCl 20 mM, EDTA 2 mM, Triton 1.2% (wt/vol), pH 8.0], and then resuspended in 600 μl lysozyme buffer containing lysozyme (5 mg/ml), and incubated at 65 °C for 30 min. DNA was purified with Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany) according to the manufacturer's instructions.

2.4. PCR amplification

The extracted DNA was amplified with puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Freiburg, Germany) in two successive PCR amplifications. All oligonucleotides used in this study were specific for bacterial 16s rRNA gene. The numbers in the primer names indicate the position of the 5′ nucleotide in the 16S rRNA gene of *Escherichia coli*. An 896-bp fragment, including the 16S rRNA gene V3 region, was first PCR amplified by means of the primers 11F (GGA TCC AGA CTT TGA TYM TGG CTC AG) (modified from Felske et al., 2007) and 907R (CCG TCA ATT CMT TTG AGT TT) (Lane et al., 1985). The thermal cycling conditions were 94 °C for 4 min; 30 cycles at 94 °C for 30 s, at 54 °C for 40 s, and at 72 °C for 70 s; then at 72 °C for 20 min. The PCR products' lengths were verified on 1.5% agarose gels, and the 896-bp fragments were sliced and purified by Invisorb Spin DNA Extraction kit. The purified fragments were then used as substrate to amplify the V3 region using 341F GC-clamp and 534R primers (ATT ACC GCG GCT GCT GG) (Muyzer et al., 1993). The V3 region of the 16S rRNA gene corresponds to a 193-bp fragment, and was the substrate for PCR–DGGE electrophoresis. PCRs were performed on MyCycler Personal Thermal Cycler (Bio-Rad Hercules, CA, USA).

Table 1
Samples analyzed in the current study. Each sample name indicates place of sampling [farm (F) or dairy plant tank (T)] and approximate refrigeration (4 °C) duration (numbers after the hyphen) from time of milking. At time of sampling, samples from the farm and the dairy plant tank had already been stored at 4 °C for 22 h and 54 h respectively. All samples were analyzed by DGGE.

	22 h	46 h	70 h		54 h	78 h	102 h
Farm 1	F1_22[a]	F1_46	F1_70	Tank 1	T1_54[a]	T1_78[a]	T1_102[a]
Farm 2	F2_22[a]	F2_46	F2_70	Tank 2	T2_54	T2_78	T2_102[a]
Farm 3	F3_22[a]	F3_46	F3_70[a]	Tank 3	T3_54[a]	T3_78	T3_102

[a] Samples also analyzed by cloning.

2.5. DGGE analysis

For DGGE analysis, the Dcode universal mutation detection system (Bio-Rad, Hercules, CA, USA) was used to separate the V3 region PCR products. PCR products were electrophoresed on 8% (wt/vol) polyacrylamide gels containing a denaturating gradient from 40 to 60% urea and formamide [a 100% denaturant corresponds to 7 M urea and 40% (vol/vol) formamide] in 1× TAE running buffer (2 M Tris base, 1 M glacial acetic acid, 50 mM EDTA). Migration was performed at 90 V for 16 h, and the running buffer temperature was kept constant at 60 °C. Gels were stained with GelStar Nucleic Acid Stain (0.1 μl/ml) (Cambrex Bio Science, Rockland, USA), photographed by UV GelDoc (Bio-Rad Hercules, CA, USA) and analyzed with GelCompar software (Applied Maths, Kortrijk, Belgium). DGGE bands were excised from the gel, eluted by incubation in 50 μl sterile distilled water at 4 °C overnight, and amplified. PCR products were sequenced at the Technion Medical School, Haifa, Israel. A dendrogram of the DGGE profiles was constructed based on the Ward method using the negative value of the cosine coefficient as distance (Ward, 1963).

2.6. Cloning and clone library analysis

Analysis of bacterial communities by cloning was performed on nine of the 18 milk samples. The samples that were analyzed using cloning are marked by an "a" in Table 1. For cloning, an 896-bp fragment of the 16S rRNA gene was obtained as described above. The purified PCR products were cloned into *E. coli* DH5α using the CR®II-TOPO TA cloning vector (Invitrogen, Paisley, UK). About 100 recombinant clones were randomly selected for analysis. The plasmids were extracted using Wizard Plus SV Minipreps kit (Promega, Madison, USA). Clones were analyzed by comparison of V3 region migration patterns of each clone on a DGGE gel. Clones that migrated the same distance in the DGGE gel were temporarily grouped into the same operational taxonomic unit (OTU). To ensure that the migration patterns of the clones grouped in the same OTU were identical, clones affiliated to the same OTUs were simultaneously examined on the same DGGE gel. This procedure was performed twice. A number of representative clones for each OTU were selected, sequenced and compared, to verify the OTU's identity. In sum, 356 clones (representing 896 clones) were selected and sequenced.

2.7. Sequence analysis

Sequences obtained in our study were aligned with the sequences in the Ribosomal Database Project (BLASTN; www.ncbi.nlm.nih.gov) to identify their closest relatives. Phylogenetic analysis of the clone sequences together with their closest relatives was performed using ARB software (http://www.arb-home.de). The phylogenetic tree was constructed by the neighbor-joining method. Sequences with less than 99% similarities in the 16S rRNA gene were considered dissimilar OTUs.

2.7.1. Nucleotide sequence accession numbers

The sequences in this study were deposited in the GenBank database under accession numbers EU029233–EU029588.

2.8. Statistical analysis

Coverage of microbial communities represented by clone libraries was calculated according to Good (1953). The diversity of microbial communities was analyzed by UniFrac, a web application available at http://bmf.colorado.edu/unifrac (Lozupone et al., 2006). UniFrac facilitates comparison of microbial communities simultaneously by means of hierarchical clustering with the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm. The Jackknife Environment Clusters option (Lozupone and Knight, 2005) was used to evaluate clustering robustness and evenness.

Shannon–Weiner and Dominance indexes were calculated with Past software (http://folk.uio.no/ohammer/past/Estimates). Non-parametric species richness, Chao 1, was calculated with Estimates software version 8.0 (http://purl.oclc.org/estimates). Pearson's correlation between the diversity of the bacterial communities and the time of milk storage at 4 °C, and linear regression, were calculated with SPSS software version 12.0.

3. Results

Diversity of raw milk bacterial communities and the effect of refrigeration on their dynamics were studied on milk samples from three dairy farms and three silo tanks. Bacterial mesophilic culturable counts of the milk samples were $<10^4$ CFU/ml, attesting that the milk was of good quality.

3.1. Analysis of DGGE profiles

Milk samples exhibited a complex DGGE genetic profile, composed of many bands corresponding to a great variety of bacterial populations (Fig. 1). The dendrogram obtained by Ward's analysis for the farm milk samples showed two distinct groups (Fig. 1a). The first contained DGGE profiles of the milk samples from farms F1 and F2 immediately after sampling (F1_22, F2_22) and after 24 h incubation at 4 °C (F1_46, F2_46). The second group contained DGGE profiles from farms F1 and F2 after 48 h of cold incubation (F1_70, F2_70) together with all the samples from farm F3 (Fig. 1a). Dominant bands in samples F1 and F2 immediately after sampling and after 24 h cold incubation were affiliated to the phylum *Firmicutes* (e.g., bands 4, 9 and 12 corresponding to *Staphylococcus*, *Streptococcus* and *Bacillus* respectively, Fig. 1a and Table 2). Significant changes in the bacterial community's composition were observed after 48 h cold incubation (samples F1_70, F2_70), when other bands, affiliated to the phylum *Proteobacteria*, became visible (e.g., bands 8 and 11 corresponding to *Pseudomonas* and *Acinetobacter* respectively, Fig. 1a and Table 2). Different bacterial population dynamics was observed in the samples from farm F3 where identical bands could be found in all the F3 samples [e.g., bands 2 and 5 corresponding to *Staphylococcus* and *Macrococcus* (*Firmicutes*), Fig. 2a, Table 2].

Dairy plant DGGE profiles differed from the farm DGGE profiles and were divided into two groups (Fig. 1b). The group that contained DGGE profiles of samples T1_54, T2_54 and T3_78 was characterized by a great number of bands distributed all over the gel gradient. Major changes in the DGGE band's composition after 48 h of cold incubation could be observed in samples T1_102 and T2_102. Reduction in the band's numbers in these samples was

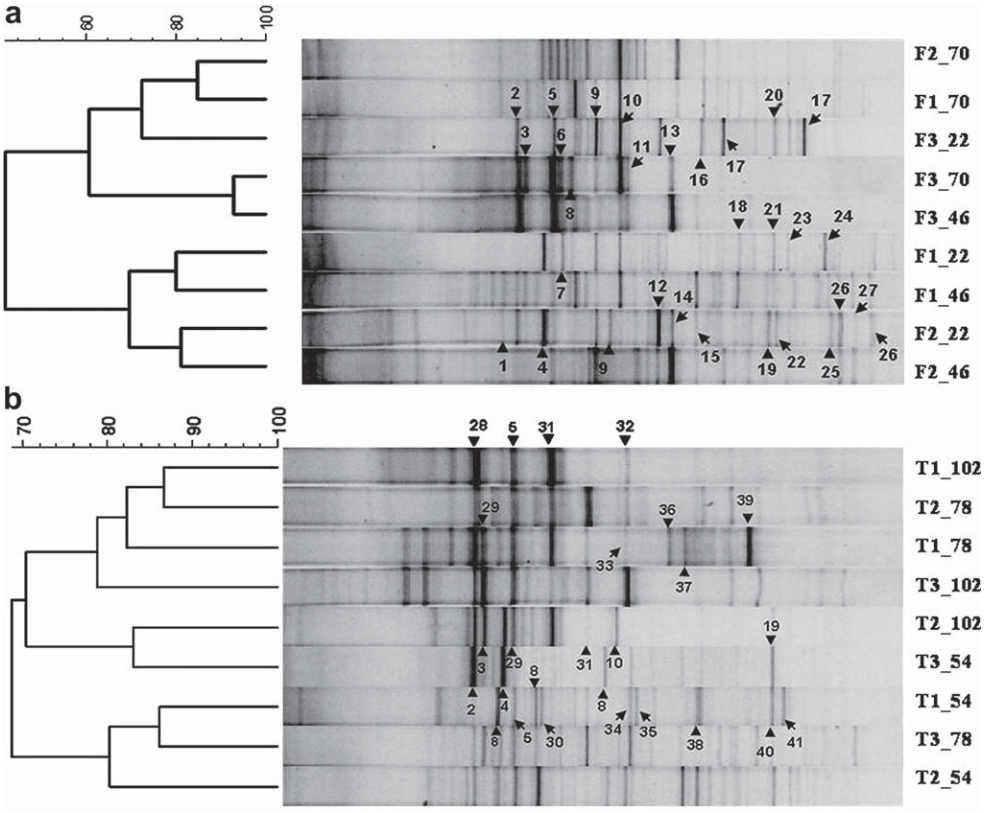

Fig. 1. Effect of refrigeration on bacterial community composition. Bacterial community compositions of raw milk from (a) farms F1–3 and (b) dairy plant tanks T1–3 were compared based on PCR–DGGE patterns of V3 region of 16S rRNA gene. Dendrogram of the PCR–DGGE profiles was constructed by Ward's hierarchical clustering method. Numbers after the hyphen in the sample name indicate approximate refrigeration (4 °C) duration. Numbered bands were identified from the 16S rRNA gene clone library described in Table 2.

Table 2
Identification of bands in DGGE patterns of raw milk bacterial communities. (a) Dominant bands, (b) other bands. Closest relative as determined by ARB phylogenetic analysis, GenBank accession numbers and percentage of similarity are shown.

DGGE-band no.	Closest species by ARB phylogenetic analysis	GenBank accession no. ^{T} (type strain)	%
a			
2[a]	*Staphylococcus sciuri*	S83569	98.0–99.9
3[a]	*Pseudomonas brenneri*	AF268968^{T}	99.7–100
4[a]	*Staphylococcus saprophyticus*	AP008934	99.9
5[a]	*Macrococcus caseolyticus*	EF204307	99.6–100
6	*Streptococcus parauberis*	AF284579	99.6–99.9
8[a]	*Acinetobacter johnsonii*	EF204263	99.9
9	*Streptococcus dysgalactiae*	AY584478	100
10[a]	*Lactococcus lactis*	AB100803^{T}	100
12	*Bacillus thuringiensis*	AF160221	100
17	*Corynebacterium variabile*	AJ222815^{T}	99.4–99.9
19[a]	*Paenibacillus apiarius*	AB073201	99.3–99.5
28	*Pseudomonas synxantha*	EF204250	99.6–100
29	*Pseudomonas* sp.	EF111224	97.7–100
31	*Acinetobacter* sp.	AJ551155	99.1–100
39	Uncultured *Actinomycetaceae*	EF419390	93.5
b			
1	*Enterococcus aquimarinus*	AJ877015^{T}	99.3
7	*Pseudomonas* sp.	AY689078	99.9
11	*Pseudomonas putida*	EF204247	99.4–99.7
15	*B. thuringiensis*	AF160221	99.3
16	*Thauera* sp.	AM084104	97.1–100
18	*Stenotrophomonas koreensis*	AB166885^{T}	99.9
20	*Corynebacterium variabile*	AJ222815^{T}	96.6
21	*Phyllobacterium myrsinacearum*	AJ011330	94.8
22	*Microbacterium xinjiangensis*	DQ923131^{T}	96.5
23	*Corynebacterium macginleyi*	AJ439345^{T}	95.4
24	*Yania halotolerans*	AY228479^{T}	96.7
25	*Clostridium lituseburense*	M59107	96.8
26	*Janibacter anophelis*	AY837752^{T}	99.3–99.4
27	*Ornithinicoccus* sp.	AB188224	97.0
30	*Janthinobacterium lividum*	AY247410	99.9
32	*Pseudomonas pertucinogena*	AB021380^{T}	97.7
33	*Caryophanon latum*	X70319	97.5–98.3
34	*Streptococcus thermophilus*	AY687383	99.7–99.9
35	*Adhaeribacter aquaticus*	AJ626894^{T}	91.3
36	Uncultured bacterium (*Clostridiales*)	AF317376	96.2
37	Uncultured bacterium (*Clostridiales*)	AF371782	95.6
38	*Bosea thiooxidans*	AF508803	98.9
40	*Corynebacterium macginleyi*	AJ439345^{T}	99.8–100
41	*Brevibacterium samyangensis*	DQ344485^{T}	95.6

[a] Band was found in DGGE patterns of both farm and dairy plant tanks.

followed by increased intensity of some of the bands. For example, bands 34, 40, 41, corresponding to *Streptococcus*, *Corynebacterium* and *Brevibacterium* (*Firmicutes* and *Actinobacteria*) respectively, in sample T1_54, disappeared in sample T1_102, while bands 28, 31 and 32, corresponding to *Pseudomonas*, *Acinetobacter* and another *Pseudomonas* species (*Proteobacteria*) respectively, became visible (Fig. 2b). Band 5, identified as *Macrococcus caseolyticus* (*Firmicutes*), showed high intensity in all the dairy plant samples that were incubated at 4 °C for 24 or 48 h and was also observed in all the samples from farm 3 (F3).

3.2. Bacterial diversity analysis using cloning

Clone libraries were constructed for nine of the raw milk samples. Four were from farms (F1_22, F2_22, F3_22 and F3_70) and five from dairy plant tanks (T1_54, T1_78, T1_102, T2_102 and T3_54). Clone analysis revealed 191 OTUs (<99% similarities in the 16S rRNA gene). Most (59%) could not be classified as culture-defined bacterial species. Farm samples and plant silo tank samples differed in their microbial community composition. While Gram-positive bacteria affiliated with classes *Bacilli*, *Clostridia* and

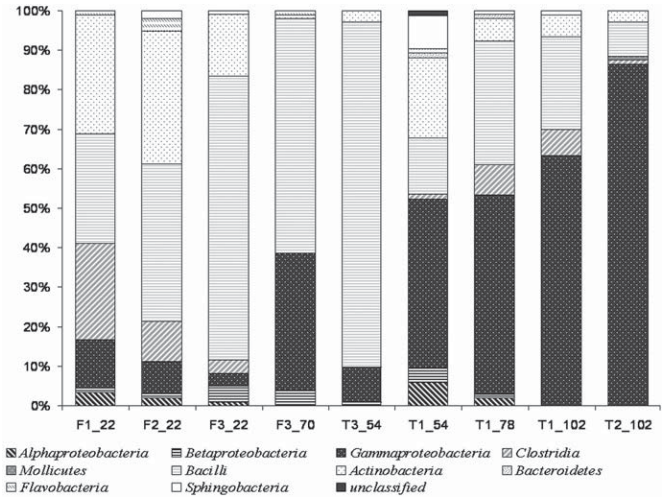

Fig. 2. Composition of raw milk bacterial communities at class phylogenetic level. Relative abundance of partial 16S rRNA gene clone sequences affiliated to the class level in raw milk clone libraries from farms (F1_22, F2_22, F3_22 and F3_70) and dairy plant tanks (T1_54, T1_78, T1_102, T2_102 and T3_54). Numbers after the hyphen in the sample name indicate approximate refrigeration (4 °C) duration.

Actinobacteria prevailed in farm bulk tanks after milking (Fig. 2, samples F1_22, F2_22, F3_22), milk samples from plant silo tanks were dominated by Gram-negative species belonging to *Gammaproteobacteria* class (except for dairy plant T3_54 where species belonging to *Bacilli* were dominant) (Fig. 2, Table 3).

Actinobacteria prevalence was 25% on average in farm samples F1_22, F2_22, F3_22 and in one dairy plant tank sample T1_54. All these samples were analyzed immediately after sampling and without incubation at 4 °C in the laboratory (Fig. 2, Fig. S1, Table 3, Table S1).

The raw milk clones were affiliated to 22 orders and 108 different genera. The most abundant genera found in all the farm samples immediately after milking were *Streptococcus* and *Lactococcus*. *Bacillus* and *Corynebacterium* were found in two out of three samples (Table 3). The most abundant genera in all the dairy plant tanks samples after cold incubation were *Pseudomonas* and *Acinetobacter*. *Staphylococcus*, *Macrococcus* and *Clostridium* were found in some of the various farms and dairy plant tank clone's samples (Table 3 and Table S1)

3.3. UniFrac metric analysis, Shannon–Weiner and Dominance indexes

Comparison of the microbial diversity of the clone libraries by UniFrac software revealed that the origin of a sample exerted major influence on the bacterial community structure (Fig. 3). The libraries were divided into two major groups. One group contained samples from all the farms and the dairy plant tank sample T3_54. All the other plant dairy tanks samples were found together in the second group. Sample T3_54 from the dairy plant was grouped with the farm samples probably due to prevalence of class *Bacilli* (87.4%).

Family level with coverage of 4.0–7.5 was chosen for calculation of Shannon–Weiner index of diversity, Dominance and Chao 1 richness estimator. In contrast to the results obtained by UniFrac analysis, showing that the source of the sample had a major influence on the bacterial community structure, Shannon–Weiner and Dominance indexes showed significantly negative and positive correlations respectively between taxonomic diversity and increase of incubation time at 4 °C (SW: $r = -0.58$, $P = 0.05$; D: $r = 0.74$, $P < 0.05$). Chao 1 richness decreased with longer duration of cold incubation.

Table 3
Composition of raw milk bacterial communities. Relative abundance of partial 16S rRNA gene clone sequences affiliated to the different taxonomic groups detected within clone libraries. Only the ten most abundant genera are specified. The partial 16S rRNA gene clone libraries were constructed for F1_22, F2_22, F3_22 and F3_70 raw milk samples and for the dairy plant tanks T1_54, T3_54, T1_78, T1_102 and T2_102. Detailed description of all the 896 clones including species abundances in the different milk samples is given in Table S1.

Phylum/class	Order/genus	Farms (% clones)				Dairy plant tanks (% clones)					Average (%)
		F1_22	F2_22	F3_22	F3_70	T3_54	T1_54	T1_78	T1_102	T2_102	
Proteobacteria		16.7	11.2	8.3	38.6	9.7	52.4	53.3	63.3	86.5	**37.1**
Alphaproteobacteria		3.3	2	0.8	–	–	6	1.9	–	–	1.5
	Sphingomonadales	–	–	–	–	–	1.2	–	–	–	0.1
	Rhodobacterales	1.1	–	0.8	–	–	–	–	–	–	0.2
	Rhizobiales	2.2	2	–	–	–	4.8	1.9	–	–	1.1
Betaproteobacteria		1.1	1	4.1	4	1	3.6	1	–	–	1.8
	Burkholderiales	–	1	1.7	4	1	3.6	1	–	–	1.3
	Rhodocyclales	1.1	–	2.5	–	–	–	–	–	–	0.4
Gammaproteobacteria		12.2	8.2	3.3	34.7	8.7	42.9	50.5	63.3	86.5	**33.8**
	Xanthomonadales	1.1	–	–	–	–	1.2	–	–	1.9	0.4
	Pseudomonadales	11.1	2	2.5	30.7	7.8	36.9	48.6	63.3	84.6	**31.4**
	(3, 28, 29) *Pseudomonas*	–	–	–	8.9	–	2.4	17.1	37.8	57.7	13.7
	(8, 31) *Acinetobacter*	–	–	–	8.9	1.9	20.3	24.8	20	16.3	10
	Alteromonadales	–	–	–	–	–	–	1	–	–	0.1
	Enterobacteriales	–	6.1	0.8	4	1	4.8	1	–	–	1.9
Firmicutes		52.2	50	75.2	59.4	87.4	15.5	39	30	10.6	**47.9**
Clostridia	*Clostridiales*	24.4	10.2	3.3	–	–	1.2	7.6	6.7	1	5.8
Mollicutes	*Anaeroplasmatales*	–	–	–	–	–	–	–	–	1	0.1
Bacilli		27.8	39.8	71.9	59.4	87.4	14.3	31.4	23.3	8.7	**42**
	Bacillales	20	29.6	33.9	37.6	83.5	6	22.9	16.7	7.7	**29.5**
	(12) *Bacillus*	–	6.1	8.3	–	–	–	–	–	–	1.8
	(2, 4) *Staphylococcus*	8.9	13.2	9.9	11.9	72.8	–	–	5.6	6.7	**14.7**
	(5) *Macrococcus*	–	–	13.2	25.7	–	3.6	16.2	7.8	–	7.7
	(19) *Paenibacillus*	–	3.1	–	–	8.7	–	–	–	1	1.5
	Lactobacillales	6.7	10.2	38	21.8	3.9	7.1	8.6	6.7	1	**12.3**
	(9) *Streptococcus*	3.3	6.1	14.9	4	–	–	–	–	–	3.5
	(10) *Lactococcus*	1.1	1	17.4	8.9	–	1.2	1	1.1	1	4
	Unclassified Bacilli	1.1	–	–	–	–	1.2	–	–	–	0.2
Actinobacteria		30	33.7	15.7	1	2.9	20.2	5.7	5.6	2.9	**12.7**
	Acidimicrobiales	5.6	1	–	–	–	–	–	–	–	0.7
	Rubrobacterales	–	–	–	–	–	1.2	–	–	–	0.1
	Actinomycetales	24.4	32.7	15.7	1	2.9	19	5.7	3.3	2.9	**11.7**
	(26[a]) *Janibacter*	–	13.3	–	–	–	–	–	–	–	1.5
	(40[a] 17) *Corynebacterium*	–	1	9.1	–	–	11.9		–	–	2.4
	Bifidobacteriales	–	–	–	–	–	–	–	2.2	–	0.2
Bacteroidetes		1.1	5.1	0.8	1	–	10.7	1.9	1.1	–	**2.2**
	Bacteroidales	–	–	–	–	–	1.2	1	–	–	0.2
	Flavobacteriales	1.1	3.1	–	1	–	1.2	–	–	–	0.7
	Sphingobacteriales	–	2	0.8	–	–	8.3	1	1.1	–	1.3
Unclassified		–	–	–	–	–	1.2	–	–	–	0.1

Numbers in parentheses to the left of genera names correspond to the DGGE bands (Fig. 1 and Table 2).
[a] Minor band in DGGE pattern.

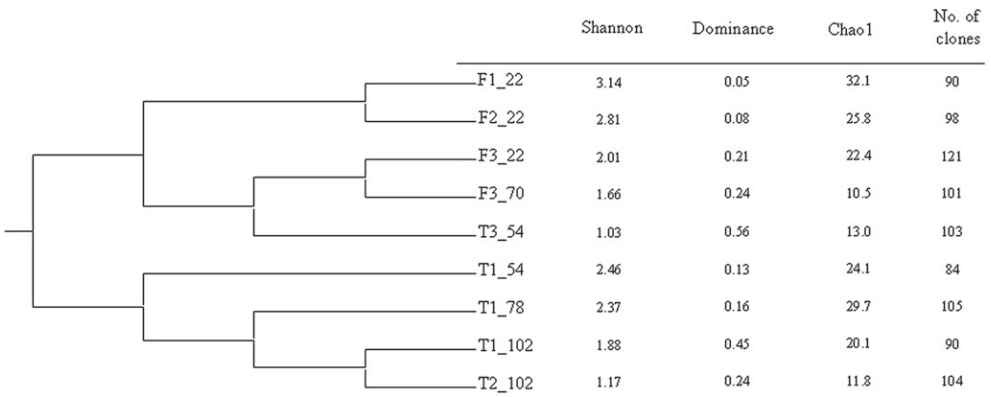

Fig. 3. UniFrac metric analysis of raw milk bacterial communities. UPGMA tree based on pairwise UniFrac metric distances between the bacterial communities as revealed by partial 16S rRNA gene clone sequence libraries from farms (F1_22, F2_22, F3_22 and F3_70) and dairy plant tanks (T1_54, T1_78, T1_102, T2_102 and T3_54) (based on 356 sequences and their frequency in each clone library). Numbers after the hyphen in the sample name indicate approximate refrigeration (4 °C) duration. Shannon–Weiner index of diversity, Dominance and Chao 1 richness estimator were calculated for the different communities based on family affiliation of the clone sequences.

4. Discussion

The current study demonstrated that considerable evolution of bacterial communities occurred during cold storage of raw milk. Taxonomic diversity decreased in consequence of some microbial populations' dominance during refrigeration (Figs. 1 and 3). Milk samples from farm bulk tanks and from the dairy plant silo tanks differed in the composition of their microbial communities (Figs. 1–3, Table 3 and Table S1). While Gram-positive bacteria affiliated to *Bacilli*, *Clostridia* and *Actinobacteria* prevailed in the farm tanks during the incubation, milk samples from the dairy plant tanks were dominated by Gram-negative species belonging to *Gammaproteobacteria* (Fig. 2, Table 3). An exception was the dairy plant sample T3_54, where 73% of the species belonged to the genus *Staphylococcus* (Fig. 2, Table 3). Cold incubation changed the bacterial community of sample T3 and Gram-negative bacteria became dominant (T3_78 and T3_102, Figs. 1 and 3).

Gammaproteobacteria, in particular *Pseudomonadales*, were present in milk after refrigeration (Table 3, Table S1). Using culturable methods, we found in our previous study that psychrotolerant isolates of *Pseudomonas* and *Acinetobacter* species showed mainly lipolytic activity (Hantsis-Zacharov and Halpern, 2007). Some members of *Pseudomonadales* are considered important psychrotolerants that cause spoilage of milk and dairy products (Cousin, 1982; Ercolini et al., 2009; Sorhaug and Stepaniak, 1997). Marchand et al. (2009) demonstrated that *Pseudomonas lundensis* and *Pseudomonas fragi* were predominant milk spoilers in Belgian raw milk samples. *Pseudomonas* species dominated the bacterial population of raw milk and carbon dioxide-treated milk that were cold incubated for 7 d, while *Acinetobacter* was dominant in TH milk (thermized milk – heated at 65 °C for 16 s) (Rasolofo et al., 2010). However, in this study we showed that 37–54% of the psychrotolerant bacteria—Gram-negative as well as Gram-positive—that inhabit cold-stored milk samples belong to taxa other than *Pseudomonas* (Fig. 2, Table 3 and Table S1). For example, psychrotolerant species found in the raw milk farm samples belonged to the genera *Staphylococcus*, *Streptococcus*, *Bacillus*, *Lactobacillus*, *Enterococcus*, *Clostridium*, *Corynebacterium*, *Micrococcus* and *Microbacterium* (Table 3. For species abundance in each genera, see Table S1).

Lactic acid bacteria (LAB) comprised 11.6% of the clones (Table S1); *Lactococcus lactis* was the most abundant species identified, albeit at low frequencies in most samples (Table S1). Lafarge et al. (2004) found *L. lactis* to be a dominant species in ten raw milk samples from farms in France, and that its abundance decreased after refrigeration. LAB species show reduced proteolytic activity and very low lipolytic activity (Hantsis-Zacharov and Halpern, 2007).

Streptococcus thermophilus was detected in all plant silo tank samples immediately after sampling (samples T1_54, T2_54 and T3_54), both as minor bands in the DGGE gel (Table 2b, band 34) and at low frequencies in the clones (Table S1 taxon no. 129). The fact that this species is thermophilic explains why it disappeared after cold incubation (except for sample T3_78). Using the DGGE method, Giannino et al. (2009) found that *S. thermophilus* was present in five out of 13 raw milk samples that were collected from farms at different altitudes of alpine pastures. The prevalence of *S. thermophilus* increased to eight out of ten (80%) in curd milk samples (that were mildly heated) (Giannino et al., 2009).

Obligate anaerobes belonging to *Clostridiales* (with an average of 5.8% of the clones) were identified in the current study in all samples except F3_70 and T3_54 (Table 3, Fig. 1 band 25, Table S1). *Clostridiales* were also recovered from raw milk and from TH milk (Delbes et al., 2007; Rasolofo et al., 2010). The reduction in abundance of *Clostridia* over storage time was most noticeable in the current study (Table 3) and in that of Rasolofo et al. (2010). A study on the occurrence of *Clostridium* in four dairy farms found that the *Clostridium* originated from environments other than the milk itself (Julien et al., 2008). Most (83%) of the taxa belonging to anaerobes detected in raw milk were also found in grass or maize silage (Julien et al., 2008).

In our study no pathogenic bacteria were detected in the raw milk samples. This finding is in contrast to findings of Jayarao and Henning (2001), Lafarge et al. (2004), Salo et al. (2006) and Jayarao et al. (2006), who identified pathogenic species such as *Listeria*, *Bacillus cereus* and *Aeromonas*.

UniFrac analysis (Fig. 3) demonstrated that the farm samples differed significantly from the diary plant samples and were dominated by Gram-positive bacteria. The differences in the microbial communities' composition might be due to the re-inoculation of the raw milk with biofilm microflora. In the industrial environment, microbial biofilms are a major problem (Maukonen et al., 2003). They are liable to cause contamination of dairy products due to their development on the surfaces and interfaces of milk-processing equipment (Bremer et al., 2006). Additional study is required to evaluate the contribution of the biofilm to the bacterial load of raw milk.

In the samples F1_22, F2_22, F3_22 and in one dairy plant tank sample T1_54, *Actinobacteria* prevalence stood at 25% on average (Fig. 2, Fig. S1, Table 3). Using culturable methods, we previously found that when raw milk samples taken from farms were screened for psychrotolerant bacteria, species belonging to *Actinobacteria* were highly lipolytic and proteolytic (Hantsis-Zacharov and Halpern, 2007). These results demonstrate that raw milk reaching the dairy plant is already populated by psychrotolerant species, which potentially possess the ability to secrete heat-stable proteolytic and lipolytic enzymes. Since many variables may affect the composition of raw milk bacterial communities (e.g., the cows' race or food, the climate), our results may provide information only on bacterial population in raw milk in Israel. Rasolofo et al. (2010) detected a low prevalence of *Actinobacteria* in cold incubated milk samples in Canada. However, in the Canadian study, milk samples were not screened before cold storage immediately after milking. When micro-filtered milk was studied, it was found that the abundance of *Actinobacteria* increased the longer the milk was kept in cold storage (Rasolofo et al., 2010). In France, Lafarge et al. (2004) identified *Corynebacterium ammoniagenes* (*Actinobacteria*) as one of the major bands in a DGGE analysis of raw cow milk before refrigeration. *Actinobacteria* species were also present in raw milk collected immediately after milking from cows in alpine farms in Italy (Giannino et al., 2009).

Indigenous raw milk bacterial communities are already present when the milk arrives at the dairy plant, and among them are potentially lipolytic and proteolytic psychrotolerant *Actinobacteria*. Even if pasteurization is performed at once, the heat-stable enzymes secreted by those psychrotolerants may spoil the milk and later, its products. If the pasteurization procedure is delayed, other psychrotolerants, mainly *Pseudomonadales*, will proliferate. Rapid and reliable identification of raw milk microbiota based on rRNA gene sequence analysis is important for the study of the microbiological quality of raw milk. We suggest exploring the function of persistent populations of *Actinobacteria* especially in farm milk arriving at dairy plants.

Acknowledgments

This study was supported by a grant from Tnuva Research Institute (Israel).

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fm.2010.10.009.

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