



HAL
open science

Assessment of the bacterial diversity of treated and untreated milk during cold storage by T-RFLP and PCR-DGGE methods

Eric Rasolofo, Lapointe, Roy

► **To cite this version:**

Eric Rasolofo, Lapointe, Roy. Assessment of the bacterial diversity of treated and untreated milk during cold storage by T-RFLP and PCR-DGGE methods. Dairy Science & Technology, 2011, 91 (5), pp.573-597. 10.1007/s13594-011-0027-4 . hal-00930586

HAL Id: hal-00930586

<https://hal.science/hal-00930586>

Submitted on 11 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Assessment of the bacterial diversity of treated and untreated milk during cold storage by T-RFLP and PCR-DGGE methods

Eric Andriamahery Rasolofo · Gisele LaPointe · Denis Roy

Received: 5 January 2011 / Revised: 12 April 2011 / Accepted: 28 April 2011 /
Published online: 26 May 2011
© INRA and Springer Science+Business Media B.V. 2011

Abstract The evaluation of the effect of treatments on the dynamics of milk microbiota by appropriate and reliable methods is critical for a better control of the microbiological quality of milk. The aim of this study was to compare the diversity and the alterations of the microbiota in treated and untreated milk samples during cold storage at 4 °C or 8 °C up to 7 days. Three treatments were applied to raw milk, carbon dioxide addition (CO₂), thermization (TH), and microfiltration (MF), and untreated raw milk (UT) was used as a control. Two molecular fingerprinting methods, terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) were used. According to the T-RFLP and polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) profiles, the dominant bacteria in milk were presumably affiliated with the psychrotolerant genera *Streptococcus*, *Staphylococcus*, *Pseudomonas*, and *Aerococcus*. At day 7, phylotypes related to *Pseudomonas* spp., *Trichococcus flocculiformis* and *Prevotella* spp. were dominant in UT and CO₂ milk samples but not in TH milk samples while *Staphylococcus* spp. and *Stenotrophomonas* spp. were specifically dominant in TH and MF milk samples, respectively. Principal component analysis of T-RFLP profiles revealed the strong effect of treatment on the dynamics of specific bacteria. PCR-DGGE profiles revealed the dominant bacteria associated with the effect of treatments (TH, CO₂, and MF) on milk microbiota, which can then be used as markers for the quality of treated milk. The T-RFLP method had a higher resolution than PCR-DGGE in this study and can thus be used as an accurate tool for analysis of the bacterial community in milk.

E. A. Rasolofo · G. LaPointe · D. Roy (✉)

Institut des Nutraceutiques et des Aliments Fonctionnels (INAF), Université Laval,
2440 Hochelaga Blvd, Québec, QC, Canada G1V 0A6
e-mail: denis.roy@inaf.ulaval.ca

采用 T-RFLP 和 PCR-DGGE 方法评价处理乳和非处理乳在冷藏过程中细菌多样性

摘要 采用恰当、可靠的方法评价处理过程对乳微生物动力学的影响,对更好地控制乳微生物质量是非常重要的。本研究比较了处理乳与非处理乳在冷藏条件下(4 °C 或 8 °C 下 7 天)微生物的多样性及其变化。分别采用充二氧化碳、热杀菌 (TH) 和微过滤 (MF) 方法对原料乳进行处理。两种分子指纹图谱方法,即末端限制片段长度多态性 (Terminal restriction fragment length polymorphism, T-RFLP) 和变性梯度凝胶电泳 (Denaturing gradient gel electrophoresis, DGGE) 用于本研究中。根据 T-RFLP 和 PCR-DGGE 的图谱,推测乳中主要微生物为耐寒性微生物 *Streptococcus*, *Staphylococcus*, *Pseudomonas* 和 *Aerococcus* 属。冷藏 7 天后,在 UT 和 CO₂ 处理的乳样中,以 *Pseudomonas* spp., *Trichococcus flocciformis*, *Prevotella* spp. 为主导菌群,TH 处理乳中无上述菌群,但以 *Staphylococcus* spp. 为主, MF 处理乳样中则以 *Stenotrophomonas* spp. 为主导菌群。T-RFLP 的主成分分析揭示了处理方法对特定微生物有强烈的影响。PCR-DGGE 图谱揭示了主导菌群与处理方法 (TH、CO₂ 和 MF) 对乳样微生物菌群的作用是密切联系,这种方法可以作为评价处理乳质量的标记。T-RFLP 方法与 PCR-DGGE 方法相比,就有更高的分辨率,因此可以作为乳中微生物群落分析的精确工具。

Keywords Raw milk · Microfiltration · Thermization · Carbon dioxide · PCR-DGGE · T-RFLP · Microbial dynamics · Cloning · Ribosomal 16S RNA

关键词 鲜乳 · 微滤 · 热杀菌 · 二氧化碳 · PCR-DGGE · T-RFLP · 微生物动力学 · 克隆 · 核糖体 16S RNA

1 Introduction

The microbiological quality of milk may vary considerably due to seasonal variations, farm environment, sanitary conditions of milk processing, and the health of dairy cattle (Frank et al. 2002). Cold storage on farms and processing plants has been used to maintain the microbiological quality of milk, but this creates selective conditions for the growth of psychrotrophic bacteria (Champagne et al. 1994). Moreover, treatments such as thermization (Champagne et al. 1994), carbon dioxide (Rowe 1988), and microfiltration (Elwell and Barbano 2006) have been used to control and to improve the microbiological quality of milk. Rasolofo et al. (Rasolofo et al. 2010) studied the main spoilage microbiota in untreated milk and milk treated with carbon dioxide, thermization, and microfiltration during cold storage. They found that dominant bacterial species were affiliated with *Staphylococcus*, *Streptococcus*, *Clostridia*, *Aerococcus*, *Facklamia*, *Corynebacterium*, *Acinetobacter*, and *Trichococcus*.

Few studies focus on the investigation of whole microbial communities in milk, by using culture-independent methods based on conserved genomic regions coding for the 16S rRNA gene such as gene clone library analysis (Rasolofo et al. 2010) or direct sequencing (Fricker et al. 2010). Additional molecular methods such as terminal restriction fragment length polymorphism (T-RFLP; Kitts 2001; Marsh 2000) and polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE; Muyzer et al. 1993) are generally used in environmental microbiology, and they have been applied in food microbiology as well (Giannino et al. 2009). The T-RFLP method was reported to generate a reliable fingerprint of complex bacterial communities and provided information on the number of different fragments and their relative abundance (Osborn et al. 2000). The major advantages of T-RFLP are

its suitability for routine analysis of large sample numbers, its high reproducibility, and its high throughput. T-RFLP analysis is a powerful method in microbial ecology for comparing the spatial and temporal changes in bacterial community structure (Schutte et al. 2008). Principal component analysis (PCA) has been applied to the data from T-RFLP analysis to facilitate the comparison of microbial communities with large data sets (Park et al. 2006; Talbot et al. 2009). In the PCR-DGGE method, DNA fragments amplified with universal primers are separated on denaturing gels based on nucleotide content. The dominant bands can be further excised and sequenced, and phylogenetic relationships can be determined among dominant bacteria (Giraffa and Neviani 2001). The T-RFLP does not require several gel conditions to separate sequences, whereas different gradients are necessary to separate sequences of low or high GC% in DGGE analysis (Ogier et al. 2004). The PCR-DGGE and T-RFLP analyses have both been used in the study of microbial communities, and their potential was compared to emphasize bacterial diversity (Enwall and Hallin 2009). Moreover, the PCR-DGGE method has been used to describe the bacterial community of milk (Lafarge et al. 2004; Ogier et al. 2004). However, to our knowledge, there is no report about the application of the T-RFLP method to elucidate the bacterial structure and composition of milk microbiota during cold storage.

The aim of this study was to analyze the diversity and the dynamics of the dominant microbial community in treated (thermized, microfiltered, and acidified) and untreated milk samples stored at 4 °C and 8 °C up to 7 days by a molecular approach based on T-RFLP and PCR-DGGE. Particularly, the purpose of this work was to evaluate the effectiveness of the T-RFLP method based on 16S rDNA as a tool to monitor microbial community shifts in treated milk. Knowledge of the spoilage organisms and their dynamics in treated milk during cold storage are necessary to correctly apply treatments to maintain high milk quality for cheese processing.

2 Materials and methods

2.1 Milk treatments

Three treatments, carbon dioxide addition (CO₂), thermization (TH), and microfiltration (MF), were applied the same day of raw milk reception as described previously (Rasolofot et al. 2010), and untreated raw milk (UT) was used as a control (Rasolofot et al. 2010). For each treatment, milk (between 60 L to 75 L) was stored at 4 °C and 8 °C up to 7 days. Milk samples (150 mL) from each milk treatment were taken aseptically after 1, 3, and 7 days of storage and stored at -20 °C.

2.2 Microbial analysis

The population of different microorganisms was determined in milk samples during 7 days of storage at 4 °C. The psychrotrophic bacteria were evaluated on Plate Count Agar (Difco, Detroit, MI, USA) after incubation at 7 °C for 10 days. Total and coagulase-positive staphylococci were determined on Baird Parker Agar supplemented with Bacto YE tellurite (Difco, Detroit, MI, USA), incubated at 37 °C for 24 h. Proteolytic microorganisms were determined on standard method caseinate

agar after incubation at 30 °C for 3 days. Lipolytic microorganisms were obtained on Tributyrin Agar (Oxoid, Basingstoke, Hants, UK) incubated at 30 °C for 3 days. Coliforms were determined on Violet Red Bile Agar (Difco, Detroit, MI, USA) incubated at 37 °C for 24 h. Yeasts were counted on potato dextrose agar acidified with tartaric acid (Difco, Detroit, MI, USA) after incubation at 22 °C for 5 days. For spore-forming bacteria, a milk sample of 10 mL was heated at 80 °C for 15 min. After heating, aerobic spore-forming bacteria were determined on plate count agar supplemented with a 0.1% starch solution (Difco, Detroit, MI, USA) and incubated at 30 °C during 3 days. Anaerobic spore-forming bacteria were determined on trypticase peptone glucose yeast extract agar after incubation at 37 °C in an anaerobic room for 7 days.

2.3 Terminal restriction fragment length polymorphism (T-RFLP) analysis

DNA extraction was performed according to the method described by Rasolofo et al. (Rasolofo et al. 2010). The concentration of purified DNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and DNA was stored at -20 °C. The bacterial 16S small subunit rRNA genes were amplified from purified DNA using primer set F27 (5'-AGAGTTT-GATCMTGGCTCAG-3') labeled at the 5'-end with 6-carboxyfluorescein (6-FAM) and R788 (5'-GGACTACCAGGGTATCTAA-3') to generate an amplicon of ca. 800 bp (Hartmann and Widmer 2006; Therese et al. 1998). The PCR mixture (50 µL per reaction) contained 1× PCR buffer (New England Biolabs (NEB), Mississauga, ON, Canada), 0.2 mmol.L⁻¹ concentration of each deoxynucleoside triphosphate (Invitrogen, Burlington, ON, Canada), 300 nmol.L⁻¹ of each primer (Integrated DNA Technologies (IDT), Coralville, IA), 2.5 U of *Taq* polymerase (NEB), and 5 µL of genomic DNA (200 ng.µL⁻¹). A negative control (PCR mixture without DNA) was included in all PCR experiments. The PCR amplification was carried out with a TGradient thermocycler (Biometra, Göttingen, Germany), and the cycle parameters were 1 min at 94 °C for initial denaturation followed by 33 cycles with denaturation for 30 s at 94 °C, annealing for 30 s at 53 °C, and extension for 1 min 72 °C; and with a final elongation at 72 °C for 5 min. The amplified products were visualized on a 2% (w/v) agarose gel containing 0.5 µg.mL⁻¹ ethidium bromide along with a 100 bp DNA mass ladder (Invitrogen). PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Mississauga, ON, Canada), and the concentration of purified PCR products was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Duplicate 6-FAM-labeled PCR products for each sample were pooled and split in two tubes. A quantity of 300 ng of PCR product was digested in separate reactions with 20 U of the restriction enzymes BstUI, HhaI, HaeIII, MspI, RsaI, and HpyCh4IV in a final volume of 20 µL for 12 h according to the instructions provided by the manufacturer (NEB). The efficiency of restriction digestion was further confirmed by agarose gel electrophoresis. Aliquots of the digested amplicons were desalted by dilution with sterile water and concentrated by using Microcon YM-10 spin columns (Millipore Corporation, Billerica, MA, USA). Concentrations of digested and desalted amplicons were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Approximately 40 ng of digested amplicons were further dehydrated by vacufuge (Eppendorf, Westbury, NY, USA) and were suspended with 10 µL of Hi-Di formamide (Applied

Biosystems, Forest City, CA, USA) containing 0.15 μL of GeneScan 500 LIZ size standard (Applied Biosystems), denatured for 5 min at 94 $^{\circ}\text{C}$, cooled on ice, and resolved by an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) using the Genemapper software ver. 3.7 (Applied Biosystems). The ABI PRISM 3100 sequencer was equipped with 50 cm capillaries filled with POP-6 polymer. The injection time was 22 s, and the injection voltage was 1 kV. Samples were electrophoresed at 15 kV for 100 min at 60 $^{\circ}\text{C}$. To avoid detecting primers and uncertainties in size determination, terminal restriction fragments (T-RFs) smaller than 50 bp and bigger than 500 bp were excluded from the analysis. The baseline threshold for signal detection was set to 35 fluorescence intensity units. Relative peak areas of each T-RF were determined by dividing the area of the peak of interest by the total area of peaks. A threshold for relative abundance was applied at 0.5%, and only T-RFs with higher relative abundances were included in the remaining analyses. Putative identities for the dominant peaks were predicted by *in silico* digestion with selected enzymes in the Ribosomal Database Project II using the T-RFLP analysis program TAP (<http://rdp8.cme.msu.edu/html/TAP-trflp.html>). Reproducibility of patterns was confirmed by duplicate T-RFLP analysis using the same DNA sample. Each individual T-RF was designated as a putative phylotype, and the mean T-RF number in each sample was determined. Sequences from identified clones (Rasolofu et al. 2010) were used as references for the identification of the T-RF peaks (Table 1).

T-RF data were transformed with the Hellinger transformation prior to PCA. The Hellinger transformation $y'_{ij} = \sqrt{\frac{y_{ij}}{y_{i+}}}$, can be used to make community composition data containing many zeros suitable for analysis by linear methods such as PCA where y_{ij} is the original species value for site i and species j , y_{i+} represents the sum of all species values for site i . This transformation is particularly recommended when rare species are not truly rare because sampling was performed blindly (Legendre and Gallagher 2001).

2.4 Denaturing gradient gel electrophoresis (DGGE) analysis

Gene fragments of 16S rDNA were amplified with primers GC_F27 (5'-CGCCCCGGGCGCGCCCCGGGCGGGGCGGGGCACGGGGG-AGAGTTT-GATCMTGGCTCAG-3') and R534 (5'-ATTACCGCGGCTGCTGG-3'), which are specific for universally conserved bacterial 16S rRNA gene sequences to generate an amplicon of ca. 500 bp (Muyzer et al. 1993; Ogier et al. 2004). A 40-bp GC-rich sequence (GC-clamp) was added to the 5' end of primer GC_F27 to allow suitable migration and separation of PCR products in the DGGE gel. PCR amplification was carried out in a TGradient thermocycler (Biometra). Reaction mixtures of 50 μL contained 2 μL of genomic DNA (200 $\text{ng}\cdot\mu\text{L}^{-1}$), 2.5 U of *Taq* polymerase (NEB), 1 \times PCR buffer (NEB), 0.2 mM concentration of each deoxynucleoside triphosphate (Invitrogen), and 300 nM of each primer (IDT). A negative control (PCR mixture without DNA) was included in all PCR-DGGE experiments. Samples were amplified using a touchdown thermal cycling program with the following steps: initial denaturing at 94 $^{\circ}\text{C}$ for 1 min; 20 cycles of 94 $^{\circ}\text{C}$ for 30 s, 30 s at 67 $^{\circ}\text{C}$ initially with the temperature sequentially decreasing by 0.5 $^{\circ}\text{C}$ each cycle, and 72 $^{\circ}\text{C}$ for 30 s; 15 cycles of 94 $^{\circ}\text{C}$ for 30 s, 57 $^{\circ}\text{C}$ for 30 s, and 72 $^{\circ}\text{C}$ for 30 s; and final extension at 72 $^{\circ}\text{C}$ for 5 min. The PCR products were first visualized on a 2% (w/v)

Table 1 Identification of peaks obtained from T-RFLP analysis and major bands from PCR-DGGE analysis of the bacterial community of untreated (UT) and treated (TH, CO₂, MF) milk samples

T-RFLP		PCR-DGGE		Closest match obtained from RDP II					
Peaks	Actual T-RF size (bp) ^a	Theoretical T-RF size (bp)	Ladder Bands ^b	Corresponding clone ^c	Accession no.	Species	Accession no.	% Identity	Class
p58	58	61	b10	A3UT4_A8	GQ466855	<i>Jeotgaliococcus psychrophilus</i>	GQ015357	97%	Bacilli
p90	90	95		A3MF4_A1	GQ467065	<i>Ochrobactrum anthropi</i>	AY730720	100%	Alphaproteobacteria
p93	93	95		C3MF4_B5	GQ467799	Unclassified Rhizobiales	DQ088793	99%	Alphaproteobacteria
p109	109	113	b13	A3UT4_F6	GQ466847	<i>S. agalactiae</i>	AE009948	98%	Bacilli
p109	109	113	b15	A3UT4_A7	GQ466849	<i>Streptococcus uberis</i>	AM946015	98%	Bacilli
p110	110	113	L7	A3UT4_C12	GQ466886	<i>Lactococcus lactis</i>	AB008215	98%	Bacilli
p113	113	117	b17	A7MF4_G9	GQ467482	<i>Sphingobacterium multivorum</i>	AB100738	97%	Sphingobacteria
p117	117	119		A3TH4_C8	GQ467029	<i>Prevotella</i> sp.	EU728757	95%	Bacteroidetes
p124	124	127		A3UT4_B11	GQ466879	<i>Lachnospiraceae</i>	DQ824605	88%	Clostridia
p194	194	197		A3UT4_C4	GQ466831	<i>Brevundimonas nasdae</i>	AB071954	100%	Alphaproteobacteria
p196	196	199		A3MF4_C9	GQ467124	<i>Methylobacterium populi</i>	CP001029	99%	Alphaproteobacteria
p201 / 202	201 / 202	203 / 205	b7	A3MF4_B3	GQ467081	<i>D. acidovorans</i>	AB074256	99%	Betaproteobacteria
p212	212	213	b11	A3MF4_C2	GQ467075	<i>S. maltophilia</i>	EU573409	99%	Gammaproteobacteria
p213	213	215		A3MF4_C5	GQ467097	<i>S. acidaminiphila</i>	AF273079	98%	Gammaproteobacteria
p214	214	215		A3MF4_D11	GQ467138	<i>Aeromonas punctata</i>	X74674	98%	Gammaproteobacteria
p221	221	222	L14	A3UT4_E1	GQ466812	<i>Corynebacterium</i> sp.	AF276640	97%	Actinobacteria
p222	222	223		A3CO4_F5	GQ466927	<i>Chryseobacterium bovis</i>	EF204446	95%	Flavobacteria
p224	224	226		C7MF4_C1	GQ468131	<i>Microbacterium</i>	EF143430	98%	Actinobacteria

Table 1 (continued)

T-RFLP		PCR-DGGE		Corresponding clone ^c		Accession no.		Closest match obtained from RDP II	
Peaks	Actual T-RF size (bp) ^a	Theoretical T-RF size (bp)	Ladder Bands ^b			Accession no.	Species	Accession no.	Class
p226	226		L12	A3UT4_B6		GQ466843	<i>B. crudilactis</i>	AY952450	Actinobacteria
p226	226		L13	A3UT4_B12		GQ466885	<i>Clostridium dispersicum</i>	Y18176	Clostridia
p235	235		L3	A3UT4_B2		GQ466815	<i>Staphylococcus aureus</i>	F1463828	Bacilli
p235	235		L4	A3UT4_C6		GQ466844	<i>F. tabacinusalis</i>	Y17820	Bacilli
p236	236		L9	C3UT4_D1		GQ467506	<i>Bacillus cereus</i>	AY224384	Bacilli
p239	239		L11	A3UT4_A6		GQ466842	<i>Aerospaera taetra</i>	AJ279038	Bacilli
p245	245		L5	A7CO4_C1		GQ467333	<i>T. flocculiformis</i>	FJ660575	Bacilli
p245	245		L11	A3UT4_A12		GQ466884	<i>Nostocoida limicola I</i>	AF255736	Bacilli
p247	247		L5	A3UT4_C9		GQ466864	<i>E. faecium</i>	AY172570	Bacilli
p250	250		L8	A3UT4_B4		GQ466830	<i>L. kefiranoformis</i>	AM113781	Bacilli
p261	261		L1	A3UT4_A9		GQ466862	<i>W. paramesenteroides</i>	EU855224	Bacilli
p381	381		L1	A3UT4_E12		GQ466888	<i>Succinivibrio dextrinosolvens</i>	EU794288	Gammaproteobacteria
p382	382		L6	A3MF4_A2		GQ467073	<i>Pseudomonas pseudocataligenes</i>	AI628163	Gammaproteobacteria
p384	384		L6	A3MF4_A4		GQ467088	<i>Pseudomonas</i> sp.	EU037276	Gammaproteobacteria
p384	384		L4	A7MF4_D6		GQ467457	<i>Pseudomonas putida</i>	AF094741	Gammaproteobacteria
p386	386		L10	A3MF4_B11		GQ467136	<i>Comamonas testosteroni</i>	EF522133	Betaproteobacteria
p387	387		L10	A3UT4_A4		GQ466829	<i>Acinetobacter</i> sp.	FN395271	Gammaproteobacteria
p389	389		L12	C3MF4_D3		GQ467786	<i>Serratia proteamaculans</i>	DQ088739	Gammaproteobacteria
p390	390		L12	A3UT4_G12		GQ466890	<i>Rothia</i> sp.	EU873349	Actinobacteria

Table 1 (continued)

T-RFLP	PCR-DGGE		Corresponding clone ^c	Accession no.	Closest match obtained from RDP II			
Peaks	Actual T-RF size (bp) ^a	Theoretical T-RF size (bp)	Ladder Bands ^b		Species	Accession no. % Identity	Class	
p396	396	401	L2	A3UT4_A1	GQ466808	<i>Aerococcus</i> sp.	F1405326 99%	<i>Bacilli</i>
p397	397	397		A7MF4_C3	GQ467433	<i>Arcanobacterium pyogenes</i>	EU268193 99%	<i>Actinobacteria</i>

Labels refer to A=batch A, C=batch C, 3 or 7=sampling day (3 or 7), UT=untreated milk, CO=CO₂ milk, TH=thermized milk, MF=microfiltered milk

^a T-RF length (bp) after digestion with BstUI

^b All bands are named in Fig. 4

agarose gel containing $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ ethidium bromide along with a 100 bp DNA mass ladder (Invitrogen). PCR products were then analyzed by DGGE based on the method cited by Muyzer et al. (Muyzer et al. 1993), using a DCode universal mutation detection system (Bio-Rad, Hercules, CA, USA). Polyacrylamide (6%) gels (16 by 16 cm; 1 mm thickness) were run with $1\times$ TAE (Tris-acetate-EDTA) buffer diluted from $50\times$ TAE buffer ($40 \text{ mmol}\cdot\text{L}^{-1}$ Tris base, $20 \text{ mmol}\cdot\text{L}^{-1}$ glacial acetic acid, and $1 \text{ mmol}\cdot\text{L}^{-1}$ EDTA). The polyacrylamide gels (acrylamide/bisacrylamide, 37.5:1 ratio) were made with denaturing gradients ranging from 30% to 60%. Denaturant (100%) contained $7 \text{ mol}\cdot\text{L}^{-1}$ urea and 40% (v/v) formamide (Sigma, Oakville, ON, Canada). Gels were polymerized by adding $100 \mu\text{L}$ of a 10% ammonium persulfate solution and $10 \mu\text{L}$ TEMED into each of the denaturing solutions immediately prior to pouring the gradient gel. The electrophoresis was run at 60°C for 8 h at 75 V. After electrophoresis, the gels were stained for 15 min in aqueous ethidium bromide ($0.5 \mu\text{g}\cdot\text{mL}^{-1}$) solution followed by 5 min destaining in water and visualized by UV transillumination on a Syngene Chemigenius Bio Imaging System using GeneSnap v.6.00.19 software (Synoptics Ltd., Cambridge, UK). Amplicons identified from clone libraries (Rasolofu et al. 2010) were added as references during PCR-DGGE analysis (Table 1). Cluster analysis of PCR-DGGE profiles was done in the dendrogram type of the unweighted pair group method using arithmetic averages, with the Dice coefficient using InfoQuest FP software, version 4.5 (Bio-Rad). A densitometric curve was obtained for each gel lane. PCR-DGGE profiles of milk samples were compared in standardized electrophoretic conditions which were set up to optimize the migration of 14 reference clones selected from clone libraries created from the same milk samples (Rasolofu et al. 2010). Reference clones were chosen as an identification ladder and used to normalize gel profiles allowing the comparison between gels. Other reference clones were also used to identify bands in PCR-DGGE analysis (Table 1). All of the sequences from clone libraries used for the choice of reference clones on PCR-DGGE profiles corresponded to portions of the 16S rRNA gene, and none of the sequences was found to be chimeric. The 16S rRNA gene partial sequences from clone libraries used in this study are available in the National Center for Biotechnology Information (NCBI) database under the accession numbers shown in Table 1.

2.5 Shannon–Weaver diversity index (H')

The Shannon–Weaver diversity index (H') was calculated using the equation $H' = -\sum P_i \ln P_i$, where P_i is the importance probability of the bands in a lane (Shannon and Weaver 1963). H' was calculated on the basis of the bands on the gel lanes by taking the intensities of the bands as judged by peak heights in the densitometric curves. The importance probability, P_i , was calculated as $P_i = n_i/N$ where n_i is the height of a peak and N is the sum of all peak heights in the densitometric curve. For the calculation of bacterial diversity in T-RFLP profiles, n_i is the area of a peak, and N is the sum of all peak areas in each electropherogram.

2.6 Statistical analysis

One-way analyses of variance and Tukey–Kramer HSD tests were performed using JMP Version 7.0 (SAS Institute Inc, Cary, NC, USA), with a level of significance of 0.05. The

PCA of T-RFLP profile was done in the statistical analysis package PC-ORD (McCune and Mefford MJ 2006). A Mantel test was performed in order to test the significance of the correlation between PCR-DGGE-based community structure and T-RFLP-based community structure (PC-ORD). The Mantel test evaluates the null hypothesis of no correlation between two distance matrices that contain the same set of sample units. Mantel statistics can be interpreted as a correlation coefficient, with identical ordinations yielding a value of 1. The Mantel test was performed by using Euclidian distance measures and the randomization (Monte Carlo) procedure with 1,000 randomized runs. Two-way analysis of similarity (ANOSIM) based on Euclidian distance was performed with PAST software (<http://folk.uio.no/ohammer/past/>). Values were considered significantly different if $R > 0.5$ and $P < 0.05$.

3 Results

3.1 Microbial analysis in untreated and treated milks by culture-dependent methods

The evolution of microorganisms in UT, CO₂, TH, and MF milks was determined during 7 days of storage at 4 °C. The population of psychrotrophic bacteria seemed to be stable during 3 days (3.9 Log₁₀ CFU.mL⁻¹) of storage at 4 °C but increased rapidly and reached 6.9 Log₁₀ CFU.mL⁻¹ at day 7 in untreated milk. However, the number of psychrotrophic bacteria (4.5 Log₁₀ CFU.mL⁻¹) was lower in CO₂ milk compared with that in untreated milk at day 7. Moreover, populations of psychrotrophs were lower in TH and MF milks. The initial reduction of psychrotrophic bacteria was 2 Log₁₀ CFU.mL⁻¹ in TH milk and 3 Log₁₀ CFU.mL⁻¹ in MF milk. The populations of psychrotrophic bacteria in TH and MF milks were stable during storage (1.7 and 0.9 Log₁₀ CFU.mL⁻¹, respectively).

The population of staphylococci (total, 3.4 Log₁₀ CFU.mL⁻¹ and coagulase-positive, 3.0 Log₁₀ CFU.mL⁻¹) was stable during storage in UT and CO₂ milk samples but was affected by thermization and MF treatments. Particularly, the total staphylococci population was reduced by 0.5 Log₁₀ CFU.mL⁻¹ in TH milk while it was reduced by 3 Log₁₀ CFU.mL⁻¹ in MF milk. Coagulase-positive staphylococci were not detected in TH and MF milk samples at day 1. The population of spore-forming bacteria (aerobic and anaerobic) was not affected by CO₂ and TH treatments (1.5 Log₁₀ CFU.mL⁻¹ for UT, CO₂, and TH milk samples). The populations of total staphylococci, coagulase-positive staphylococci, aerobic spore-forming, and anaerobic spore-forming bacteria were generally stable during cold storage. MF treatment was the only treatment which reduced the initial population of spore-forming bacteria to 0.3 Log₁₀ CFU.mL⁻¹. While the population of aerobic spore-forming bacteria was stable during storage, the population of anaerobic spore-forming bacteria increased to 0.8 Log₁₀ CFU.mL⁻¹ in MF milk.

The addition of CO₂ did not affect the initial population of proteolytic and lipolytic bacteria as compared with UT milk samples (4.0 Log₁₀ CFU.mL⁻¹). However, at day 7, the number of proteolytic and lipolytic bacteria in CO₂ milk samples was 2 Log₁₀ CFU.mL⁻¹ and 1 Log₁₀ CFU.mL⁻¹ lower, respectively, compared with that of UT milk samples (6.9 Log₁₀ CFU.mL⁻¹). The populations of proteolytic and lipolytic bacteria were initially reduced by MF and TH treatments

(2.7 and 1.3 Log₁₀ CFU.mL⁻¹, respectively). Coliforms and yeasts were eliminated in TH and MF milk samples but were not affected by CO₂ treatment (2.0 Log₁₀ CFU.mL⁻¹ for UT and CO₂ milk samples).

3.2 T-RFLP analysis of milk microbiota

3.2.1 T-RFLP profiles of untreated and treated milk during cold storage

The T-RFLP is a fingerprinting method which gives a visual representation of the diversity and evenness of each community. The choice of restriction enzyme greatly affected the number of peaks in T-RFLP profiles. In this study, the enzymes BstUI and MspI gave the highest number of peaks. However, peak number obtained with the BstUI enzyme was the most correlated with the number of OTUs calculated from clone libraries (correlation coefficient (R^2) of 0.77; Rasolofo et al. 2010). A correlation between the Shannon diversity indexes calculated from clone libraries and from T-RFLP profiles derived from the BstUI enzyme was also observed with the R^2 of 0.76. Therefore, T-RFLP profiles from the BstUI enzyme were used subsequently for ordination and multivariate analysis.

A total of 119 T-RF peaks potentially representing different phylotypes were found in T-RFLP profiles of 72 samples of treated (TH, CO₂, MF) and untreated (UT) milk. The mean peak number in T-RFLP profiles of all milk samples varied between 8.7 and 40.7 peaks. One-way analysis of variance on peak number in T-RFLP profiles showed the significant effect of treatments (CO₂, TH, and MF), temperature (4 °C and 8 °C), and time of storage (1, 3, and 7 days) on the microbial community of milk (results not shown).

The peak number in UT and CO₂ milk samples significantly decreased ($P < 0.05$) during cold storage. The decrease of peaks in UT milk was more pronounced compared with that in CO₂ milk. Peak number remained stable in MF and TH milk samples with a mean number of peaks of 13.4 and 29, respectively (Table 2). Microfiltered milk was characterized by the lowest number of peaks. No decrease of peak number was observed in MF milk samples because the dominant population was successfully removed by microfiltration, thus eliminating its effect on reducing

Table 2 Number of T-RF peaks in T-RFLP profiles from BstUI digestion of treated (TH, CO₂, and MF) and untreated (UT) milk samples during cold storage

Day	UT	CO ₂	TH	MF
1	33.8±5.8 a	33.5±2.8 a	31.8±4.2 ns	11.3±2.6 ns
3	27.5±4.5 ab	28.0±4.1 ab	26.3±5.7 ns	15.3±2.8 ns
7	14.5±1.9 b	19.8±2.5 b	29.0±6.0 ns	13.7±4.2 ns

Values are mean±standard error SE ($n=6$). Different letters indicate that values of peak number at different time of sampling were significantly different ($P < 0.05$), as determined by one-way analysis of variance and Tukey–Kramer HSD (Honestly Significant Differences) test

ns not significant

microbial diversity. Moreover, the dynamics of the remaining population in MF milk samples were different compared with that of UT and CO₂ milk samples as seen in our previous study (Rasolofo et al. 2010).

3.2.2 Principal component analysis of the bacterial community of untreated and treated milk samples during cold storage

Untreated milk was first analyzed separately (Fig. 1). The first and second principal components in the PCA plot of T-RFLP profiles of three batches of untreated milk samples accounted for 25.4% and 18.3% of the variation, respectively (Fig. 1). When loadings for the components were examined, the first component was representative of the time of storage (positive loading for days 1 and 3), and the second component was representative of the batch of milk (positive loading for batch A and C). As all batches were treated similarly, conditions affecting bacterial growth were comparable, leading to convergence of batches toward a composition

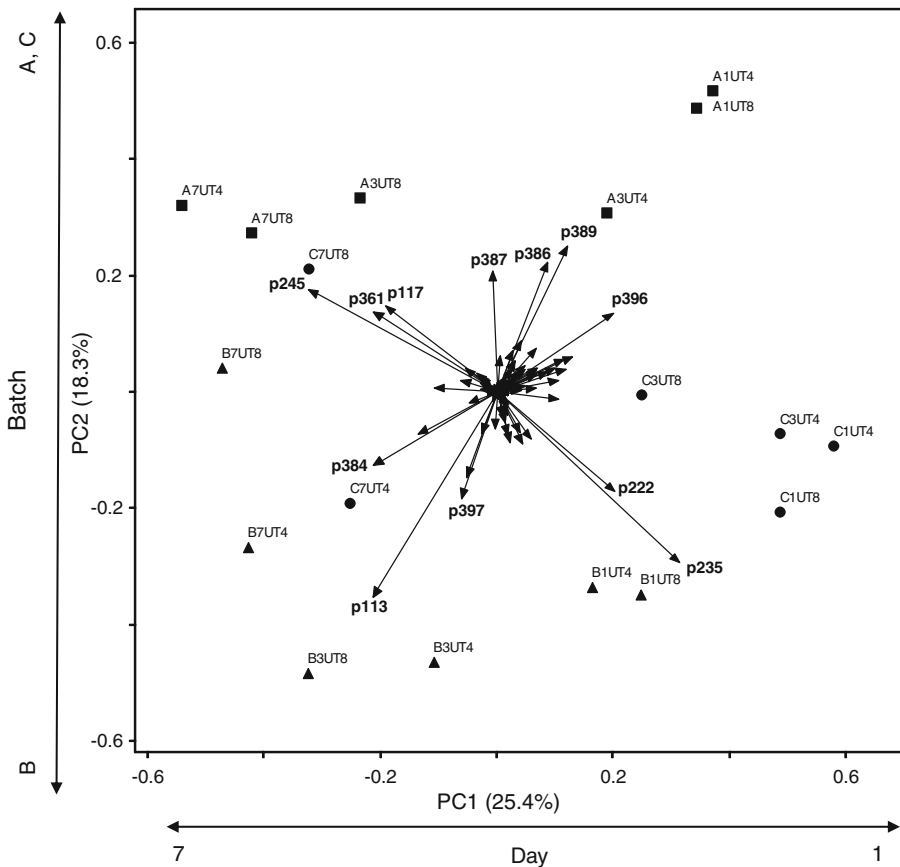


Fig. 1 Principal component analysis of the T-RFLP dataset derived from BstUI digestion of three independent batches **a** (filled square), **b** (filled triangle), and **c** (filled circle) of untreated milk samples (UT) stored at 4 °C and 8 °C for 1, 3, and 7 days. Labels refer to A=batch A, B=batch B, C=batch C, PC =Principal component. Specific T-RF peaks are indicated in Table 1

representing the condition applied. Peaks p235, p222, p396, p245, p384, p113, and p117 had the strongest loading on the first principal component (PC1) while peaks p113, p235, p397, p389, p386, and p387 produced the most important loading on the second principal component (PC2). The first principal component (PC1) separates UT milk at different days of sampling based on the presence of peaks presumably corresponding to the genera *Staphylococcus* or *Facklamia* (p235), *Chryseobacterium* (p222), and *Aerococcus* (p396) at day 1 and *Trichococcus* (p245), *Sphingobacterium* (p113), *Prevotella* (p117) and *Pseudomonas* (p384) at day 7 (Table 1). Meanwhile, the second principal component (PC2) separates the batch of milk samples by the presence of peaks presumably corresponding to the genera *Serratia* (p389), *Acinetobacter* (p387), and *Comamonas* (p386) in batch A, *Staphylococcus* or *Facklamia* (p235) and *Sphingobacterium* (p113) in batch B, *Arcanobacterium* (p397) and *Chryseobacterium* (p222) in batch C. In a previous study using clone libraries, the relative abundance of *Staphylococcus* was higher than that of *Facklamia* so peak p235 could presumably be related to *Staphylococcus* sp. even though peaks corresponding to both species are coeluted (Rasolofa et al. 2010). Moreover, untreated milk samples stored for 1 day at 4 °C or 8 °C and those stored for 3 days at 4 °C were clustered together while those stored for 3 days at 8 °C were clustered with milk samples stored for 7 days.

With respect to all milk treatments combined, the first and second principal components in the PCA plot of T-RFLP profiles accounted for 21.6% and 14.4% of the variation, respectively (Fig. 2). The first principal component (PC1) was representative of milk treatment (positive loading for UT, CO₂ and TH) and the second principal component (PC2) was representative of storage time (positive loading for day 1 and 3). T-RFLP profiles of MF milk samples were principally placed at negative values of PC1 whereas those of UT and CO₂ milk samples sampled at day 7 were scattered at negative values of PC2. Moreover, T-RFLP profiles of UT, CO₂, and TH milk samples at days 1 and 3 formed a distinct cluster at positive values of PC2. The contribution of each peak (putative species) to the first two principal components is summarized in the corresponding plot of variable loadings (Fig. 2). Peaks p235, p109, p112, and p213 had the strongest loading on the first principal component (PC1) while peaks p384, p245, p212, p213, and p389 produced the most important loading on the second principal component (PC2). T-RFLP profiles of MF milk samples were characterized by higher ratios for peaks p212 and p213 presumably corresponding to *Stenotrophomonas maltophilia* and *Stenotrophomonas acidaminiphila* and a low ratio of peak p245, presumably corresponding to *Staphylococcus* or *Facklamia* (Table 1). Our previous study confirmed that *Staphylococcus* was removed by microfiltration (Rasolofa et al. 2010). At day 7, UT, CO₂, and MF milk samples were characterized by higher ratios for peaks p384 and p245 presumably corresponding to *Pseudomonas* sp. and *Trichococcus flocculiformis*, respectively. The second principal component (PC2) separates milk samples at different sampling times derived from the presence of *Pseudomonas* sp. (p384) in UT and CO₂ milk samples at day 7 and the presence of the genera *Staphylococcus* (p235), *Streptococcus* (p109), *Aerococcus* (p396), and *Serratia* (p389) in UT, CO₂, and TH milk samples at days 1 and 3.

Finally, all T-RFLP data were analyzed by cold storage duration (Fig. 3). At day 1, MF milk samples were separated from the other treated milks, forming a distinct

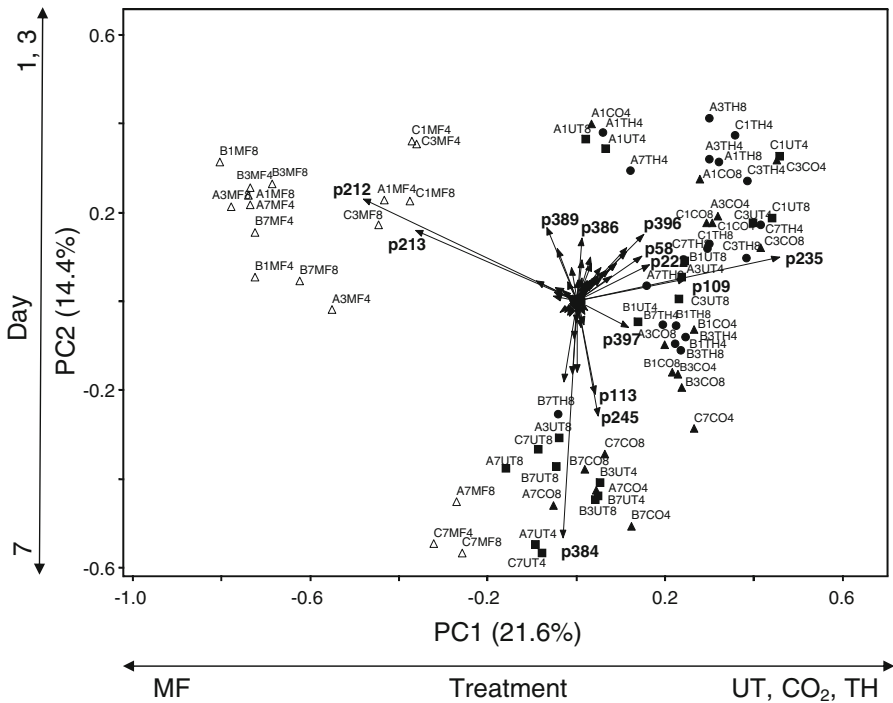
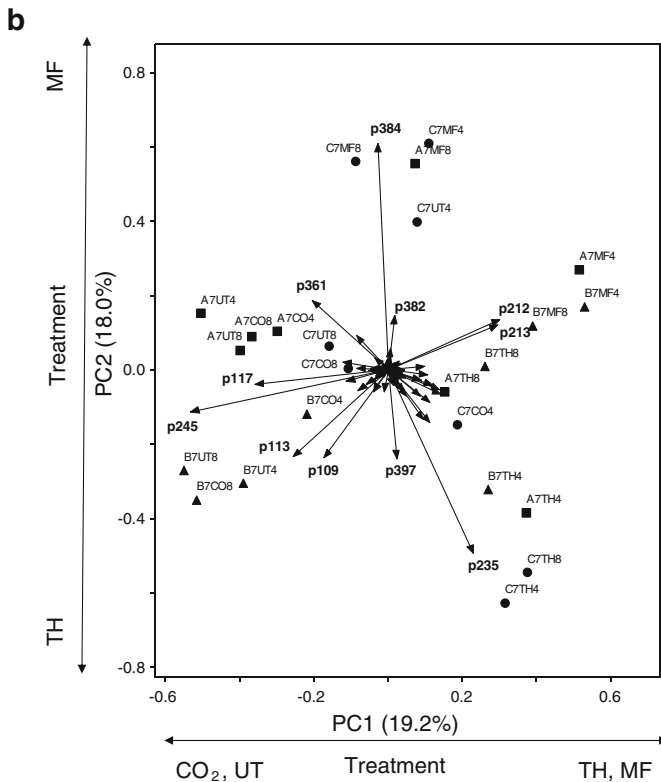
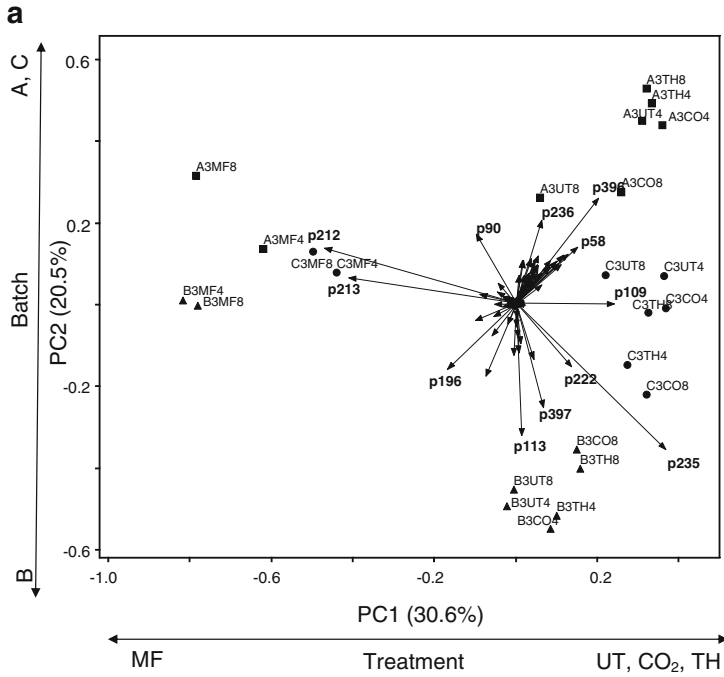


Fig. 2 Principal component analysis of the T-RFLP dataset derived from BstUI digestion from three independent batches (A, B, and C) of untreated (filled square), CO₂ (filled triangle), thermized (filled circle), and microfiltered (open triangle) milk samples stored at 4 °C and 8 °C for 1, 3, and 7 days. Labels refer to A=batch A, B=batch B, C=batch C, UT=untreated milk, CO=CO₂ milk, TH=thermized milk, MF=microfiltered milk, PC=principal component. Specific T-RF peaks are indicated in Table 1

cluster (results not shown). At day 3, the first and the second principal components explained 51.1% of the bacterial community variation (Fig. 3a). The plot of variable loadings showed major contributions of p235, p109, p212, and p213 to PC1 and p235, p113, p397, p396, and p236 to PC2. As was seen on day 1, the MF milk groups at day 3 were still separated from the UT, CO₂, and TH milk sample groups on the PC1 axis. In contrast to day 1, the three batches of milk were clearly separated on the PC2 axis at day 3. Milk profiles from batch A were characterized by higher ratios for peaks presumably corresponding to the genera *Aerococcus* (p396), *Ochrobactrum* (p90), *Jeotgalicoccus* (p58), and *Bacillus* (p236). Milk profiles from batch B were characterized by higher ratios for peaks presumably corresponding to *Staphylococcus* (p235), *Sphingobacterium* (p113), *Arcanobacterium* (p397), and *Methylobacterium* (p196) while milk profiles from batch C were characterized by higher ratios for peaks p235 and p109 presumably corresponding to *Staphylococcus* and *Streptococcus*, respectively. The first principal component (PC1) at days 1 and 3

Fig. 3 Principal component analysis of T-RFLP profiles from three independent batches **a** (filled square), **b** (filled triangle), and **c** (filled circle) of untreated (UT), CO₂ (CO), thermized (TH), and microfiltered (MF) milk samples stored at 4 °C and 8 °C for 3 (Fig. 3a) and 7 (Fig. 3b) days. Labels refer to A=batch A, B=batch B, C=batch C, UT=untreated milk, CO=CO₂ milk, TH=thermized milk, MF=microfiltered milk, PC=Principal component. Specific T-RF peaks are indicated in Table 1



separates treated milk while the second principal component (PC2) separates the three batches of milk samples based on the presence of dominant bacteria in each batch of milk. The PCA score plot of T-RF data at day 7 revealed that the untreated (UT) and treated (TH, CO₂, and MF) milk samples were distinctly separated along the first and the second principal components, which described 19.2% and 18% of the total variance, respectively (Fig. 3b). The plot of variable loadings showed major contributions of p245, p117, p113, p212, and p213 to PC1 and p384, p382, p235, and p397 to PC2. Milk profiles at day 7 were clustered into three groups. The first group was composed of MF milk samples which were characterized by higher ratios for peaks presumably corresponding to the genera *Pseudomonas* (p384 and p382) and *Stenotrophomonas* (p212 and p213). The second group was composed of TH milk which was characterized by higher ratios of peaks presumably corresponding to *Staphylococcus aureus* or *Fackliama tabacinensis* (p235), and *Arcanobacterium* (p397). The last group consisted of UT and CO₂ milk samples and was characterized by higher ratios for peaks p245, p117, p113, and p109 related to the genera *Trichococcus*, *Prevotella*, *Sphingobacterium*, and *Streptococcus*, respectively. No clusters with regard to storage temperature were observed in T-RFLP profiles (Fig. 3b).

3.3 PCR-DGGE profiles of untreated and treated milk during cold storage

PCR-DGGE profiles contained a variable number of bands ranging from four to 16 dominant bands, which differed for each batch and each treatment, although some common bands were observed (Fig. 4). Batch A contained the highest number of bands, followed by batch B with mean numbers of bands of 10.9 and 8.0, respectively. Batch C had the lowest mean number of 5.7 bands. At day 7, PCR-DGGE of untreated, microfiltered, and CO₂ milk samples showed similar profiles which were composed of 7.6 bands. In contrast, higher biodiversity was observed in TH milk as expressed by the greater average number of bands (9.8 bands), but the difference in the number of bands was not significant compared with other milk treatments.

Major bands that showed the highest intensity were identified by comparison with bands from reference clones listed in Table 1 and confirmed by species-specific PCR (Rasolofo et al. 2010). The number of bands in UT and CO₂ milk decreased over storage time. Band b3 was the most intense band found in UT and CO₂ milk samples stored during 7 days at both storage temperatures (4 °C and 8 °C). Band b3 migrated to the same position as clone reference L6 corresponding to *Pseudomonas* sp., and the sequence was strongly related (similarity 0.99%) to the *Pseudomonas* genus. This band was particularly detected at day 1 in batch B. Bands b5 and b6 related to *Acinetobacter* sp. and *T. flocculiformis*, respectively, were detected in UT and CO₂ milk samples at day 7. Band b1 affiliated with *Weissella paramesenteroides* was found only in UT milk at day 7. Band b11 affiliated with *S. maltophilia* was mostly found in MF milk. Other bands such as b12, b18, and b19 related to *Rothia* sp., *Bifidobacterium crudilactis*, *E. faecium*, respectively, appeared occasionally in MF milk with relatively low intensities. An intense band corresponding to *S. aureus* (b14) was detected in the three batches of untreated and some treated (CO₂, TH) milk samples. This band was found to be particularly dominant in milk samples from

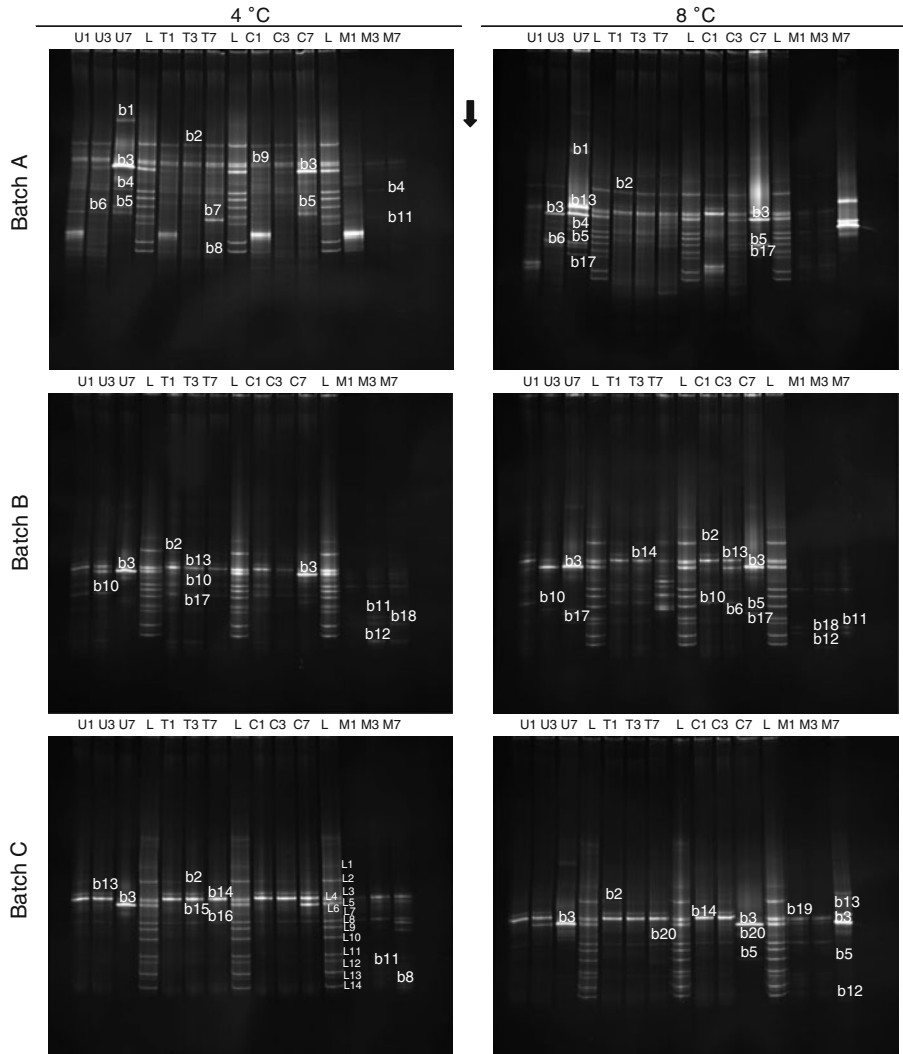


Fig. 4 PCR-DGGE profiles of PCR-amplified 16S rRNA gene fragments obtained from three independent batches (a, b and c) of untreated (U), CO₂ acidified (C), thermized (T), and microfiltered (M) milk samples at days 1, 3, and 7. Milk samples were stored at 4 °C and 8 °C. Lane designation indicates the four treatments at three times of sampling (U1, U3, U7, T1, T3, T7, C1, C3, C7, M1, M3, and M7) and the ladder (L). Bands were identified by comparison with the bands in the ladder (L) and bands from identified clones (Table 1). The arrow indicates the direction of electrophoresis

batches B and C. However, this band was missing or appeared with low intensity in MF milk. *S. aureus* (b14) and *Streptococcus agalactiae* (b13) were the main representative bands in untreated and treated (CO₂, TH) milk samples from batch C. Bands b2 and b9 related to *Aerococcus* sp. and *Facklamia tabacinasalis*, respectively, were noticeable in all milk but both bands showed the highest intensity in milk samples from batch A. A band corresponding to *Delftia acidovorans* (b7) was detected among major bands in the PCR-DGGE profile of TH milk at day 7. A

band affiliated to *Jeotgalicoccus psychrophilus* (b10) was found mainly in milk samples from batch B whereas the band related to *Lactobacillus kefiranofaciens* (b16) was found to be dominant in milk samples from batch C. Some bands observed in PCR-DGGE profiles could not be identified by using reference clones (Table 1).

3.4 Cluster analysis of milk microbiota by PCR-DGGE

PCR-DGGE profiles of untreated and treated milk samples were distributed into eight different clusters at the 43% similarity level (Fig. 5). Four clusters grouped a large number of milk samples. The first cluster was mostly composed of MF milk samples from batch B. MF milk samples at day 7 from the three batches were not grouped in the same cluster. A second cluster included MF milk samples from batches A and C. The largest cluster was mostly composed of treated (CO₂, TH) and untreated (UT) milk samples at days 1 and 3. The last cluster was mainly composed of UT and CO₂ milk samples stored for 7 days. In contrast, TH milk samples at day 7 were clustered with UT and CO₂ milk samples from days 1 and 3. Moreover, differences between milk batches stored at both temperatures (4 °C and 8 °C) were more pronounced than differences between treatments (UT, CO₂, and TH) at days 1 and 3.

3.5 Comparison of PCR-DGGE and T-RFLP analysis for the description of milk microbiota

During 7 days of storage at 4 °C and 8 °C, no significant difference was found between diversity indices calculated from T-RFLP and PCR-DGGE profiles of milk samples. However, the diversity index calculated from T-RFLP profiles was significantly lower ($P < 0.05$) for MF milk than for UT, CO₂, and TH milk samples. Overall, the Shannon diversity index (H') calculated from T-RFLP profiles was significantly higher ($P < 0.05$) than those calculated from PCR-DGGE profiles.

Two-way ANOSIM on T-RFLP and DGGE profiles confirmed that the structure of the bacterial community in milk samples at 8 °C was influenced by the storage duration and the treatment applied (Table 3). A significant difference was observed

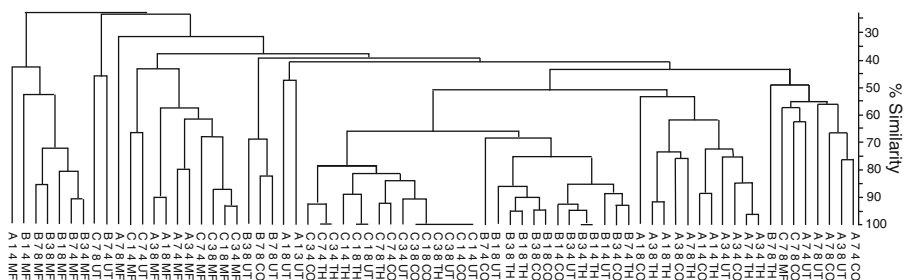


Fig. 5 Cluster analysis of PCR-DGGE profiles obtained from three independent batches (a, b and c) of untreated (UT), acidified (CO), thermized (TH), and microfiltered (MF) milk samples and carried out using the clustering algorithm of Ward with Dice's coefficient of similarity. Milk samples were stored at 4 °C and 8 °C for 1, 3, and 7 days

Table 3 Two-way ANOSIM analysis based on similarity matrices derived from T-RFLP and DGGE profiles of milk samples

Method	Temperature	Source of variation*			
		Day		Treatment	
		<i>R</i> value	<i>P</i> value	<i>R</i> value	<i>P</i> value
T-RFLP	4 °C	0.014	0.411	0.264	0.003
	8 °C	0.216	0.016	0.417	< 0.0001
DGGE	4 °C	0.043	0.304	0.239	0.004
	8 °C	0.181	0.036	0.254	0.001

R ANOSIM statistic; *P* probability level

Values were considered significantly different if $R > 0.5$ and $P < 0.05$.

in the structure of bacterial populations between treated and untreated milk samples stored at 4 °C. Moreover, one-way ANOSIM on T-RFLP and DGGE profiles shows that the bacterial population at day 1 was significantly different from the population at day 7. The bacterial population from MF milk samples was significantly different compared with UT, TH, and CO₂ milk samples (Table 4).

Comparison of the Euclidian matrices obtained from PCR-DGGE and T-RFLP analysis revealed a significant correlation using the Mantel test ($r=0.69$, $P=0.001$), which indicates that both techniques provide similar results.

Table 4 One-way ANOSIM analysis based on similarity matrices derived from T-RFLP and DGGE profiles of milk samples

Parameters	Source of variation*							
	T-RFLP				DGGE			
	4 °C		8 °C		4 °C		8 °C	
	<i>R</i>	<i>P</i>	<i>R</i>	<i>P</i>	<i>R</i>	<i>P</i>	<i>R</i>	<i>P</i>
1-day versus 3-day	0.001	1.000	-0.031	1.000	0.001	1.000	-0.014	1.000
1-day versus 7-day	0.224	0.011	0.271	0.002	0.155	0.046	0.206	0.002
3-day versus 7-day	0.052	0.487	0.125	0.084	0.128	0.085	0.091	0.166
UT versus CO ₂	-0.090	1.000	-0.021	1.000	-0.063	1.000	-0.012	1.000
UT versus TH	0.090	0.584	0.220	0.059	0.052	1.000	0.129	0.386
UT versus MF	0.621	0.000	0.565	0.000	0.562	0.001	0.416	0.004
CO ₂ versus TH	0.042	1.000	0.108	0.571	-0.034	1.000	0.001	1.000
CO ₂ versus MF	0.705	0.000	0.664	0.001	0.604	0.000	0.468	0.002
TH versus MF	0.726	0.001	0.668	0.001	0.630	0.001	0.458	0.001

Bold values were considered significantly different ($R > 0.5$ and $P < 0.05$)

R ANOSIM statistic; *P* probability level

Labels refer to UT=untreated milk, CO=CO₂ milk, TH=thermized milk, MF=microfiltered milk

4 Discussion

In this study, the structure and the alterations of milk microbiota during cold storage were assessed by PCR-DGGE and T-RFLP analysis. Molecular fingerprinting methods were used because they provided an overview of microbial community composition. Conventional microbiological methods or culture-dependent methods detected only a small fraction of the microbial community and were not suitable for exhaustive characterization (Jany and Barbier 2008). For the first time, a comparative analysis of the bacterial community in untreated and treated (TH, CO₂, and MF) milk samples during cold storage was carried out using the T-RFLP and PCR-DGGE techniques.

Restriction enzyme BstUI was used in T-RFLP analysis because the T-RFLP profiles from this enzyme were well correlated with the microbial structure found in clone library analysis (Rasolofo et al. 2010) and concurs with the study reported by Engebretson and Moyer (Engebretson and Moyer 2003). Of the 18 restriction endonucleases examined, BstUI, DdeI, Sau96I, and MspI had the highest frequency of resolving single populations in model communities (Engebretson and Moyer 2003). The determination of an accurate fragment size is of great importance in the inference of a plausible community composition. Sizes of T-RFs in a profile are then matched with *in silico* T-RF sizes from the 16S rRNA gene sequences of phylotypes in a database (Schutte et al. 2008). In this study, the identification of the peaks in T-RFLP profiles was done by comparison with the T-RF of identified clones from clone libraries (Rasolofo et al. 2010) and by matching the T-RF from the other restriction enzymes to the TAP T-RFLP database (Marsh 2000). The T-RFLP analysis of milk samples reveals the most dominant sequence, but the fluorescent signal of a subdominant sequence could be confused with the background noise. Some discrepancies appeared during the assignment of the peaks in T-RFLP profiles with regard to the migration of reference clones. Particularly, peak p361 could not be unambiguously identified even with analysis of the clone library database and the T-RFs from other restriction enzymes. This peak could be a pseudo T-RF which may be formed during PCR (Egert and Friedrich 2003), which is a possible occurrence with all PCR-based molecular methods. Further identification of this peak remains a challenge because T-RFs cannot be isolated by the T-RFLP method as yet. Moreover, we observed up to a 7 bp difference between the theoretical and actual T-RFs in our study. Different fluorescein dyes (Tu et al. 1998) and purine content of the T-RF (Kaplan and Kitts 2003) can affect the electrophoretic mobility of fragments causing discrepancies in apparent fragment size. Discrepancies between *in silico* and experimental T-RF sizes may reduce specific identification. Accordingly, precaution should be taken in the assignment of peaks in T-RFLP profiles because closely related sequences may yield different T-RF sizes while sequences from different phylotypes may yield similar T-RF sizes (Hartmann and Widmer 2006) and one peak can contain numerous terminal restriction fragments (T-RFs) differing only by a few base pairs (Dunbar et al. 2001).

Minor bacterial species or phylotypes were not detected by PCR-DGGE analysis due to the limit in sensitivity. The great advantage of PCR-DGGE is that differentiating bands can be excised and identified by other molecular approaches to lead to the phylogenetic affiliation of the ribotypes in each band (Giraffà and

Neviani 2001; Raats et al. 2011). However, in our study, the affiliation of dominant bands was done by comparison with band position obtained from identified clones selected from clone libraries (Rasoloflo et al. 2010). Dominant species recovered from clone libraries were confirmed by PCR-DGGE analysis. Cluster analysis of PCR-DGGE profiles shows that all MF milk and UT and CO₂ milk samples sampled at day 7 were clustered into two distinct clusters. MF milk samples were clustered because of the presence of *Stenotrophomonas* while UT and CO₂ milk samples at day 7 were clustered due to the presence of *Pseudomonas* and *Trichococcus*. According to the Mantel test, both fingerprinting techniques provided similar results for the bacterial community composition of the milk analyzed but PCR-DGGE was less efficient in discriminating between treatments. Differences in the Shannon diversity index were not significant among all milk samples when calculated with either of the molecular methods used in this study. Although a decrease of diversity index was observed in UT and CO₂ milk, care should be taken in the interpretation of the results because the T-RFLP method was reported as not suitable for evaluating certain indices of community diversity such as richness or evenness (Blackwood et al. 2007). Moreover, microbial diversity may not be affected by changes in microbial community structure because modifications in the richness of some species may be compensated by changes of others. Species richness may exhibit less variability in response to environmental factors than species composition (Ernest and Brown 2001). Even though this method does not give a complete estimate of bacterial diversity, T-RFLP was efficient in this study to demonstrate the effect of treatment on the structure and the dynamics of milk microbiota during cold storage.

Variable region V3 is generally used in PCR-DGGE analysis because of its length and species-specific variability and the availability of ribosomal sequences in the public database (Delbès et al. 2007). In this study, the V1-V3 variable region yielded DNA fragment lengths after amplification of ca. 500 bp, but we were able to obtain a good resolution and good separation of DNA fragments. Using six restriction enzymes in T-RFLP analysis did not necessarily increase the resolution (data not shown). Nevertheless, the T-RFLP method did show higher resolution than PCR-DGGE even though results from both techniques were correlated.

Both PCA analysis and ANOSIM on T-RFLP profiles revealed that the bacterial community structure in MF milk was different from those in UT, CO₂, and TH milk samples. Even though microfiltration treatment eliminates the majority of microorganisms, the diversity of the population remaining in MF milk samples was revealed by the T-RFLP method, thus demonstrating the sensitivity of this high throughput approach. PCA analysis of the T-RFLP peak profiles provided more information about the effect of milk treatment and showed that the composition of bacterial communities gradually and continuously changed from day 1 to day 7 at both storage temperatures (4 °C and 8 °C). This study demonstrates that T-RFLP analysis efficiently highlighted the dynamics and the structure of bacterial community in milk samples. Microfiltration appears to standardize milk so that batch and storage time no longer affect sample grouping. In addition, although cold storage has been shown to diminish *Actinobacteria* (Raats et al. 2011), the microfiltration-treated milk shows an increase in *Actinobacteria* during cold storage (Rasoloflo et al. 2010), perhaps related to absence of normally dominant species.

Although *Staphylococcus* contributes significantly to the community over 3 days of cold storage, this species is superseded by *Pseudomonas* by 7 days for all treatments except thermization, where *Staphylococcus* remains dominant at the end of the storage period. Thus, by day 7, batch differences were no longer evident, but treatment remained a major source of community variation. Microbiota composition after CO₂ treatment most closely resembled untreated milk samples by day 7. This treatment delays microbial growth, but does not substantially alter community composition compared with untreated milk. Carbon dioxide can inhibit the growth of microorganisms in raw milk and can inhibit microbial enzyme activities (Rowe 1988).

T-RFLP and PCR-DGGE analysis revealed a relatively complex bacterial community in the milk samples studied. Some bacterial species were common in treated milk, indicating that milk is a preferential niche for those bacteria during cold storage. Particularly, *Stenotrophomonas* was mainly found in MF milk and *Pseudomonas* and *Trichococcus* were mostly found in UT milk at day 7 while *Streptococcus* was found in CO₂ milk. The presence of *Streptococcus* in CO₂ milk samples is not surprising because carbonation decreased the pH of the milk, and *Streptococcus* is an acid-tolerant bacterium (Russell 1991). *Trichococcus flocciformis* and *Prevotella* spp. were found among major species at day 7 in UT and CO₂ milk samples by both methods. The genus *Trichococcus* has been detected in raw milk and dairy waste (Hantsis-Zacharov and Halpern 2007; McGarvey et al. 2007) while *Prevotella* sp. was found among the dominant bacteria in the rumen (Sundset et al. 2007). Moreover, this species was found among dominant bacteria in gastric cancer microbiota (Dicksved et al. 2009). The presence of *Prevotella* can indicate fecal contamination of milk and is a concern because this species became dominant after storing UT and CO₂ milk samples at 8 °C.

Spoilage bacteria, related to *Pseudomonas* sp. and *Acinetobacter* sp., were found to be dominant only in UT and CO₂ milk samples at day 7. These bacteria are not dominant at the beginning of storage but their proteolytic and lipolytic activities may have a potential role in the typical properties of cheese made from raw milk (Hantsis-Zacharov and Halpern 2007; Raats et al. 2011). Major species in milk samples were detected by PCR-DGGE, for the most part. However, subdominant populations were identified in clone libraries in our previous study (Rasolofo et al. 2010). In order to facilitate the characterization of subpopulations in a complex microbiota, group-specific PCR-DGGE can be used when necessary (Marzotto et al. 2006). The variability among PCR-DGGE profiles could be attributed to the effect of treatments, but also to the presence of adventitious bacteria in different milk batches. Even within a 4-month period, community variation can be observed among batches of cold-stored raw milk from bulk tank of mixed farm origin. This variation could be due to the microbial diversity of farm milk or to the temperature or storage abuse in bulk tank. However, study of raw milk microbiota from three different European countries revealed that microbiota from farm tanks was found to have a greater diversity than those from dairy bulk tanks. Dairy bulk tank milk was enriched in LAB and Gram negative bacteria compared with farm milk, which was attributed to the longer refrigeration time (Fricker et al. 2010). Both fingerprinting methods indicated that bacterial species such as *Trichococcus*, *Prevotella*, and *Stenotrophomonas* can be used as marker organisms in all three batches of treated milk for temperature or storage abuse.

This study demonstrates that microbial species continue to grow during storage at low temperature after treatments applied to three different batches of bulk tank raw milk. Results obtained from culture-dependent methods demonstrate that thermization and microfiltration treatment were better than CO₂ treatment in reducing the initial population of microorganism studied. CO₂ treatment has no effect on initial population of psychrotrophic, proteolytic, and lipolytic microorganisms, but it affects microbial growth during milk storage at 4 °C. The destruction of psychrotrophic bacteria by thermization correlated with the development of thermophilic bacteria presumably through reduced competition. The removal of dominant bacteria in the microfiltered milk allowed the detection of subdominant bacteria which could be potentially pathogenic, including *S. maltophilia* and *Ochrobactrum anthrapi*. Addition of CO₂ to milk samples inhibited the growth of psychrotrophs but also helped to create an environment conducive to the development of other bacteria found in untreated milk. Thus, bacterial diversity in milk with added CO₂ is more like that of untreated milk. Furthermore, CO₂ treatment improved short-term storage duration, which is a considerable advantage in the dairy industry. Moreover, species that are not usually observed in milk were detected by molecular methods, supporting the benefit and usefulness of these methods in the study of microbial communities in food.

5 Conclusion

According to our knowledge, this is the first report of molecular fingerprinting methods describing the bacterial population structure and changes of treated (TH, CO₂, and MF) and untreated milk samples during cold storage. T-RFLP analysis demonstrated that the relative abundance of bacterial community members of milk varied during cold storage depending on the treatment applied while PCR-DGGE profiles revealed changes in the dominant population. Furthermore, PCA analysis of T-RFLP profiles suggested that the diversity and the evenness of the microbial community altered during cold storage, and these changes were strongly affected by treatment. Molecular methods based on mRNA were not used in the present study, but mRNA-based approaches may be able to emphasize active bacteria. We demonstrated in this study that the T-RFLP method was able to resolve the structure and the dynamics of milk microbiota, to differentiate the treatments and to discriminate specific bacteria which can be used as markers for a better control of milk quality during storage. Thus, T-RFLP is a suitable alternative to PCR-DGGE for comprehensive and accurate analysis of the bacterial community structure and composition in milk, especially when dealing with large sample numbers. Knowledge of temporal shifts in bacterial community structure of milk and the factors that govern these shifts could aid in developing strategies for improving the microbiological quality of milk.

Acknowledgments This study was supported by a grant from Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT), Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ), Novalait, and Agriculture and Agri-Food Canada award to G. LaPointe, D. Roy, and D. St-Gelais and by the NSERC Canada Research Chair awarded to D. Roy. The authors thank D. St-Gelais, S. Turcot, S. Labrie, and L. Lagacé for their scientific advice and technical assistance.

References

- Blackwood CB, Hudleston D, Zak DR, Buyer JS (2007) Interpreting ecological diversity indices applied to terminal restriction fragment length polymorphism data: insights from simulated microbial communities. *Appl Environ Microbiol* 73:5276–5283
- Champagne CP, Laing RR, Roy D, Mafu AA, Griffiths MW (1994) Psychrotrophs in dairy products: their effects and their control. *Crit Rev Food Sci Nutr* 34:1–30
- Delbès C, Ali-Mandjee L, Montel MC (2007) Monitoring bacterial communities in raw milk and cheese by culture-dependent and -independent 16S rRNA gene-based analyses. *Appl Environ Microbiol* 73:1882–1891
- Dicksved J, Lindberg M, Rosenquist M, Enroth H, Jansson JK, Engstrand L (2009) Molecular characterization of the stomach microbiota in patients with gastric cancer and in controls. *J Med Microbiol* 58:509–516
- Dunbar J, Ticknor LO, Kuske CR (2001) Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. *Appl Environ Microbiol* 67:190–197
- Egert M, Friedrich MW (2003) Formation of pseudo-terminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure. *Appl Environ Microbiol* 69:2555–2562
- Elwell MW, Barbano DM (2006) Use of microfiltration to improve fluid milk quality. *J Dairy Sci* 89 (Suppl 1):E20–E30
- Engebretson JJ, Moyer CL (2003) Fidelity of select restriction endonucleases in determining microbial diversity by terminal-restriction fragment length polymorphism. *Appl Environ Microbiol* 69:4823–4829
- Enwall K, Hallin S (2009) Comparison of T-RFLP and DGGE techniques to assess denitrifier community composition in soil. *Lett Appl Microbiol* 48:145–148
- Ernest SKM, Brown JH (2001) Homeostasis and compensation: the role of species and resources in ecosystem stability. *Ecology* 82:2118–2132
- Frank JF, Hassan AN, Roginski H (2002) Microorganisms associated with milk. *Encyclopedia of Dairy Sciences*, Elsevier, Oxford, UK
- Fricker M, Skanseng B, Rudi K, Stessl B, Ehling-Schulz M (2010) Shift from farm to dairy tank milk microbiota revealed by a polyphasic approach is independent from geographical origin. *Int J Food Microbiol* 145 (Suppl 1):S24–S30
- Giannino ML, Marzotto M, Dellaglio F, Feligini M (2009) Study of microbial diversity in raw milk and fresh curd used for Fontina cheese production by culture-independent methods. *Int J Food Microbiol* 130:188–195
- Giraffà G, Neviani E (2001) DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. *Int J Food Microbiol* 67:19–34
- Hantsis-Zacharov E, Halpern M (2007) Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. *Appl Environ Microbiol* 73:7162–7168
- Hartmann M, Widmer F (2006) Community structure analyses are more sensitive to differences in soil bacterial communities than anonymous diversity indices. *Appl Environ Microbiol* 72:7804–7812
- Jany JL, Barbier G (2008) Culture-independent methods for identifying microbial communities in cheese. *Food Microbiol* 25:839–848
- Kaplan CW, Kitts CL (2003) Variation between observed and true Terminal Restriction Fragment length is dependent on true TRF length and purine content. *J Microbiol Methods* 54:121–125
- Kitts CL (2001) Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. *Curr Issues Intest Microbiol* 2:17–25
- Lafarge V, Ogier JC, Girard V, Maladen V, Leveau JY, Gruss A, Delacroix-Buchet A (2004) Raw cow milk bacterial population shifts attributable to refrigeration. *Appl Environ Microbiol* 70:5644–5650
- Legendre P, Gallagher E (2001) Ecologically meaningful transformations for ordination of species data. *Oecologia* 129:271–280
- Marsh TL (2000) Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Curr Opin Microbiol* 2:323–327
- Marzotto M, Maffei C, Paternoster T, Ferrario R, Rizzotti L, Pellegrino M, Dellaglio F, Torriani S (2006) *Lactobacillus paracasei* A survives gastrointestinal passage and affects the fecal microbiota of healthy infants. *Res Microbiol* 157:857–866

- McCune B, Mefford MJ, PC-ORD (2006) Multivariate analysis of ecological data, Version 5.10, MjM Software, Glenden Beach, Oregon, USA
- McGarvey JA, Miller WG, Zhang R, Ma Y, Mitloehner F (2007) Bacterial population dynamics in dairy waste during aerobic and anaerobic treatment and subsequent storage. *Appl Environ Microbiol* 73:193–202
- Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59:695–700
- Ogier JC, Lafarge V, Girard V, Rault A, Maladen V, Gruss A, Leveau JY, Delacroix-Buchet A (2004) Molecular fingerprinting of dairy microbial ecosystems by use of temporal temperature and denaturing gradient gel electrophoresis. *Appl Environ Microbiol* 70:5628–5643
- Osborn AM, Moore ER, Timmis KN (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environ Microbiol* 2:39–50
- Park S, Ku YK, Seo MJ, Kim DY, Yeon JE, Lee KM, Jeong S-C, Yoon WK, Ham CH, Kim HM (2006) Principal component analysis and discriminant analysis (PCA-DA) for discriminating profiles of terminal restriction fragment length polymorphism (T-RFLP) in soil bacterial communities. *Soil Biol Biochem* 38:2344–2349
- Raats D, Offek M, Minz D, Halpern M (2011) Molecular analysis of bacterial communities in raw cow milk and the impact of refrigeration on its structure and dynamics. *Food Microbiol* 28:465–471
- Rasolofo EA, St-Gelais D, LaPointe G, Roy D (2010) Molecular analysis of bacterial population structure and dynamics during cold storage of untreated and treated milk. *Int J Food Microbiol* 138:108–118
- Rowe MT (1988) Effect of carbon dioxide on growth and extracellular enzyme production by *Pseudomonas fluorescens* B52. *Int J Food Microbiol* 6:51–56
- Russell JB (1991) Resistance of *Streptococcus bovis* to acetic acid at low pH: relationship between intracellular pH and anion accumulation. *Appl Environ Microbiol* 57:255–259
- Schutte UM, Abdo Z, Bent SJ, Shyu C, Williams CJ, Pierson JD, Forney LJ (2008) Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. *Appl Microbiol Biotechnol* 80:365–380
- Shannon CE, Weaver W (1963) The mathematical theory of communication. University of Illinois Press, Urbana, USA
- Sundset MA, Praesteng KE, Cann IK, Mathiesen SD, Mackie RI (2007) Novel rumen bacterial diversity in two geographically separated sub-species of reindeer. *Microb Ecol* 54:424–438
- Talbot G, Roy CS, Topp E, Beaulieu C, Palin MF, Masse DI (2009) Multivariate statistical analyses of rDNA and rRNA fingerprint data to differentiate microbial communities in swine manure. *FEMS Microbiol Ecol* 70:540–552
- Therese KL, Anand AR, Madhavan HN (1998) Polymerase chain reaction in the diagnosis of bacterial endophthalmitis. *Br J Ophthalmol* 82:1078–1082
- Tu O, Knott T, Marsh M, Bechtol K, Harris D, Barker D, Bashkin J (1998) The influence of fluorescent dye structure on the electrophoretic mobility of end-labeled DNA. *Nucleic Acids Res* 26:2797–2802