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Molecular analysis of bacterial population structure and dynamics during cold storage of untreated and treated milk

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ABSTRACT

Spoilage bacteria in milk are controlled by treatments such as thermization, microfiltration and addition of carbon dioxide. However, little information is known about the changes in microbial communities during subsequent cold storage of treated milk. Culture-dependent methods and a direct molecular approach combining 16S rRNA gene clone libraries and quantitative PCR (Q-PCR) were applied to obtain a better overview of the structure and the dynamics of milk microbiota. Raw milk samples were treated by the addition of carbon dioxide (CO_2), thermization (TH) or microfiltration (MF) and stored at 4 °C or 8 °C up to 7 d. Untreated milk (UT) was used as a control. Psychrotrophic and staphylococci bacteria were enumerated in the milk samples by culture methods. For the molecular approach, DNA was extracted from milk samples and 16S rRNA gene was amplified by PCR with universal primers prior to cloning. The Q-PCR method was used to evaluate the dynamics of dominant bacterial species revealed by clone library analysis of 16S rRNA gene. Comparison of the 16S rRNA gene sequence indicated that the two most abundant operational taxonomic units (OTU), determined at 97% identity, belonged to the class *Gammaproteobacteria* (40.3% of the 1415 sequences) and *Bacilli* (40%). Dominant bacterial species in UT, CO_2 and TH milk samples at day 3 were affiliated with *Staphylococcus*, *Streptococcus*, *Clostridia*, *Aerococcus*, *Facklamia*, *Corynebacterium*, *Acinetobacter* and *Trichococcus*. Dominant bacterial species detected in MF milk were *Stenotrophomonas*, *Pseudomonas* and *Delftia*, while *Pseudomonas* species dominated the bacterial population of UT, CO_2 and MF milk samples at day 7. *Staphylococcus* and *Delftia* were the dominant bacterial species in thermized milk. Q-PCR results showed that populations of *S. aureus, A. viridans, A. calcoaceticus, C. variabile* and *S. uberis* were stable during 7 d of storage at 4 °C. Populations of *P. fluorescens, S. uberis* and total bacteria increased in UT and CO_2 milk samples during 7 d of storage at 8 °C and were noticeable from day 3. This study shows new microbial species which can develop during cold storage after milk treatment and contributes to identifying causes of reduced shelf life and deterioration of technological properties of milk during storage.

1. Introduction

Milk is an excellent growth medium for microorganisms found in the food environment and their numbers can increase rapidly during storage under inappropriate conditions. The microbial community of raw milk contributes to the sensory richness and variety of traditional cheese (Beuvier et al., 1997; Duthoit et al., 2005), but the multiplication of contaminating bacteria can affect the technological properties of milk. The quality of raw milk has been improved markedly by refrigeration on farms and in processing plants. However, cold storage of raw milk creates selective conditions for the growth of psychrotrophic bacteria (Champagne et al., 1994). Psychrotrophs account for less than 10% of initial raw milk microbiota and dominate the microbial community during cold storage (Cousin, 1982).

Several technologies have been proposed to maintain the technological ability and quality of milk over longer conservation periods. Treatments such as thermization (TH) can be applied to control or to inhibit bacterial growth in raw milk. The thermization process consists of heating milk around 65 °C with a holding time between 10 and 20 s. This heat treatment is effective in reducing mesophilic and psychrotrophic bacterial growth (Champagne et al., 1994). However, thermization cannot destroy heat resistant proteolytic and lipolytic enzymes produced by psychrotrophic bacteria (Griffiths et al., 1981). Heat treatment can activate the germination of spores which reduces the shelf life of thermized milk (Griffiths et al., 1986; Hanson et al., 2005). Alternative treatments to thermization include carbon dioxide (CO_2) which has been used to preserve the quality of milk (Rowe, 1988). The addition of CO_2 to refrigerated raw milk reduces the final pH to 6.2 (Roberts and Torrey, 1988), inhibits the growth of psychrotrophs and coliforms (Espie and Madden, 1997), reduces enzyme production (Rowe, 1988) and extends raw milk shelf life (Ma et al., 2003). Microfiltration (MF) is also an alternative method to heat treatment for

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reducing the microbial population of milk by mechanical separation without heat-induced chemical alterations (Maubois, 2002; Elwell and Barbano, 2006; Hoffmann et al., 2006). Microfiltration has been used commercially to produce milk products with a refrigerated shelf life of 32 d (Elwell and Barbano, 2006).

Many studies have been carried out to control psychrotrophic bacteria in milk by the use of thermization, microfiltration and addition of CO_2. However, little information is known about the changes in microbial communities during subsequent cold storage of treated milk (TH, MF, and CO_2). The study of the structure and the dynamics of milk microbiota acquired by a polyphasic approach is of great importance for selecting the appropriate treatment for preserving the quality and technological properties of milk.

Traditionally, the bacterial community of raw milk has been described by culture-dependent methods, which are generally time consuming and give only partial information on the composition of the microbiota. Furthermore, culture-independent methods have been useful to monitor changes in microbial communities. Culture-independent studies based on molecular fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) (Ercolini et al., 2004; Lafarge et al., 2004), temporal temperature gradient electrophoresis (TTGE) (Ogier et al., 2002; Lafarge et al., 2004), single strand conformation polymorphism (SSCP) (Duthoit et al., 2003) have been used to characterize bacterial diversity in raw milk. The 16S rRNA gene library-based molecular strategy is a powerful approach to identify members of a community and to assess their abundance without the limitations imposed by culture-dependent methods and biochemical identification (Amann et al., 1995; Schloss and Handelsman, 2005, 2006). A dual approach combining a culture-dependent method and direct recovery of 16S rRNA gene sequences has been used to describe the composition of bacterial communities in milk (Delbès et al., 2007; Hantsis-Zacharov and Halpern, 2007). Furthermore, quantitative PCR (Q-PCR), which shows very high diagnostic sensitivity and specificity as well as high repeatability, was used for the detection of *Staphylococcus aureus* in milk (Graber et al., 2007). The Q-PCR method can accurately quantify the target species and can be used to further validate results from other molecular methods.

The aim of this study was to assess the impact of thermization, microfiltration and the addition of carbon dioxide on the structure and the dynamics of milk microbiota during cold storage using culture-dependent methods combined with a molecular approach. In this strategy, microbial community composition and relative abundance were measured through 16S rRNA gene clone libraries while Q-PCR was used to quantify the dominant members of the microbial community in treated and untreated milk samples monitored over seven days of storage at 4 °C and 8 °C.

2. Materials and methods

2.1. Milk treatments

Three batches of 400 L of raw milk (1 day old) maintained at 4 °C were obtained from Agropur (Granby, Qc, Canada) over a period of 4 mo. Four treatments (untreated raw milk, carbon dioxide addition, thermization and microfiltration) were applied the same day of raw milk reception. For milk treated with carbon dioxide (CO_2), 65 L of raw milk at 4 °C was acidified to pH 6.2 by direct injection (bubbling) of CO_2 (Air Liquid Canada, Inc., Montreal, Qc, Canada) into raw milk. The CO_2 injected in milk was filtered with 0.2 µm pore size (Millex-FG 50 mm, Millipore Canada, Ltd, Nepean, On, Canada). The thermized milk (TH) was obtained by heating 75 L of raw milk at 65 °C for 16 s then stored immediately at 4 °C after heat treatment. The microfiltered milk (MF) was obtained by using the Bactocatch procedure as described by Trouvé et al. (1991). Raw milk (200 L) was skimmed at 50 °C in a centrifuge (Alfa-Laval, type 6 2181 m-60, Uppsala, Sweden). The skim milk was microfiltered at 50 °C using a MF pilot cross-flow unit (Alfa-Laval, type MFS-7, Uppsala, Sweden) equipped with a Sterilox membrane (pore 1.4 µm, total surface 1.4 m^2). The cream obtained after centrifugation was heated at 85 °C for 16 s and cooled at 50 °C before mixing with skimmed MF milk to obtain the microfiltered milk containing the same fat content as raw milk. After mixing, MF milk was stored immediately at 4 °C. Remaining raw milk (60 L) was used as control and designated as untreated milk (UT). Untreated and treated milk samples were poured into two sets of milk cans (30 L) previously cleaned with a 200 ppm chloride solution and rinsed with hot water (80 °C). For each treatment, milk samples were stored at 4 °C and 8 °C for 7 d. Milk samples (200 ml) were taken after 1, 3 and 7 d of storage. Microbial analysis was done directly after sampling (50 ml) while milk samples for molecular analysis (150 ml) were stored at −20 °C. Microbial analysis and quantitative PCR were done on all milk samples but clone libraries were only created from milk samples stored at 4 °C.

2.2. Microbial analyses

The psychrotrophic bacteria were evaluated on Plate Count Agar (Difco, Detroit, USA) after incubation at 7 °C for 10 d. Total staphylococci were determined on Baird Parker Agar supplemented with Bacto EY tellurite (Difco, Detroit, USA) after incubation at 37 °C for 24 h. Typical colonies of *S. aureus* are black, shiny and convex. *Bifidobacteria* were isolated on de Man-Rogosa-Sharpe medium (MRS, EMD Biosciences, San Diego, CA) containing 0.05% (w/v) L-cysteine-HCl (Calbiochem, San Diego, CA, USA), mupirocin (50 mg/L, GSK) and 1.5% (w/v) agar (Difco Laboratories, Detroit, MI) after incubation at 37 °C for 48 h in jars with an Anaerogen (Oxoid, Basingstoke, Hampshire, UK). Identification of isolates was done by 16S rRNA gene sequencing.

2.3. DNA extraction

The method of DNA extraction was adapted from that used by Vincent et al. (1998) and Grattepanche et al. (2005). Milk samples (25 ml) were clarified by addition of 5 ml ethylenediamine tetra-acetic acid (EDTA, 500 mM, pH 8.0) followed by centrifugation at 6500×*g* at 4 °C for 30 min. Samples were frozen at −20 °C for 10 min and the supernatant, containing cream and soluble milk proteins, was discarded. Cell pellets were washed twice with 1 ml sucrose buffer (sucrose 12% (w/v), 25 mM Tris–HCl pH 8.0) then suspended in 400 µL sucrose buffer containing 2 U mutanolysin (Sigma, Oakville, ON, Canada) and 800 µg lysozyme (Sigma). The suspension was incubated 1 h at 37 °C. Sodium dodecyl sulphate (SDS, 5 µL; 10% w/v), 250 mM EDTA pH 8.0 (12 µL), 20 mg/ml proteinase K (5 µL, Sigma) and sucrose buffer (78 µL) were added to the suspension and incubated 1 h at 55 °C. After this second enzyme digestion, 65 µL sucrose buffer and 135 µL NaCl (5 M) were added to the suspension. Protein material was eliminated by extracting twice with 700 µL phenol/chloroform/isoamyl alcohol (25:24:1). The upper aqueous phase was collected after centrifugation at 18,000×*g* for 15 min at 4 °C and further extracted with an equal volume of chloroform. The aqueous phase was collected and cold isopropanol (−20 °C, 700 µL) and 20 µg glycogen (Roche Diagnostics) were added. The suspension was held overnight at −20 °C for DNA precipitation. DNA pellets were obtained after centrifugation at 18,000×*g* for 30 min and washed twice with 70% ethanol. The air-dried DNA was suspended in 50 µL sterile deionized water. RNase (Roche Applied Science, Laval, QC, Canada, 1 µL) was added to 25 µL of DNA suspension and the sample was incubated for 30 min at 37 °C. The concentration of purified DNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and stored at -20 °C.

2.4. PCR amplification of 16S rRNA genes

The 16S rRNA genes were amplified from purified DNA using the primers F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and R788 (5′-GGACTACCAGGGTATCTAA-3′) to generate an amplicon of ca. 800 bp (Therese et al., 1998; Hartmann et al., 2005). The PCR mixture (50 µL per reaction) contained 1X PCR buffer (New England Biolabs (NEB), Mississauga, ON, Canada), 0.2 mM concentration of each deoxynucleoside triphosphates (Invitrogen, Burlington, ON, Canada), 300 nM of each primer (Integrated DNA Technologies, Coralville, IA), 2.5 U of Taq polymerase (NEB) and 400 ng of genomic DNA. The PCR amplification was carried out with a Tgradient thermocycler (Biometra, 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% (wt/vol) agarose gel containing 0.5 g/ml 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 PCR products was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Purified PCR products were stored at −20 °C.

2.5. 16S rRNA gene clone library construction

In order to identify the community members in milk microbiota, sixteen clone libraries were constructed from partial 16S rRNA gene amplified from DNA isolated from two batches of treated and untreated milk after 3 and 7 d of storage at 4 °C. The purified PCR products were ligated into the pGEM-T-Easy vector system (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. Competent *Escherichia coli* JM109 (Promega) were transformed with the ligation mixture to give the 16S rRNA gene clone libraries. White colonies were randomly picked from Luria-Bertani agar with ampicillin (100 µg/ml, Sigma, Oakville, ON, Canada), X-Gal (80 µg/ml, VWR, West Chester, PA, USA), and IPTG (0.5 Mm, VWR), transferred into 1 ml Luria–Bertani broth with ampicillin (100 µg/ml) in 96-well microtitre plates and grown at 37 °C with agitation for 24 h. Plasmid DNA purification was carried out using the Montage Plasmid Miniprep 96 kit (Millipore, Millipore Corporation, Billerica, MA, USA). Plasmid inserts were sequenced bidirectionnally using the F27 and R788 primers. Sequencing reactions were performed using the BigDye terminator v3.1 ready reaction cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and were analyzed on an automated DNA sequencer 3100 Genetic Analyzer (Applied Biosystems). Sequence assembly and further editing were performed with Sequencing analysis 5.1.1 (Applied Biosystems) and BIOEDIT Sequence Alignment editor software, version 7.0.5.2.

2.6. Phylogenetic analysis of 16S rRNA gene sequences

Phylogenetic affiliation of each sequence was attributed using the BLASTn program (http://blast.ncbi.nlm.nih.gov/) and the ribosomal database project (http://rdp.cme.msu.edu/). Tools (Bellerophon, NAST and distance matrix) available at the Greengenes website (http://greengenes.lbl.gov/) were used to analyze the set of sequences. Potential chimeras were detected using the Bellerophon tool (Huber et al., 2004) and NAST (Nearest Alignment Space Termination) tool was used to align sequences to a core set of sequences (Desantis et al., 2006). A distance matrix file was generated from the NAST alignment file by using the distance matrix tool.

The distance matrix file was used as input for the DOTUR program (Schloss and Handelsman, 2005) with a similarity cut-off of 97% used to define an OTU. Sequences were then assigned to major groups by using RDP Classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp). A phylogenetic tree of representative sequences from each OTU was constructed with MEGA version 4.0 using the neighbor-joining algorithm (Tamura et al., 2007). The Shannon–Weaver diversity index and Chao1 richness estimates calculated by DOTUR program were used to estimate microbial diversity and richness. The Shannon–Weaver diversity index (H') was used to determine the diversity of bacteria present in the clone libraries by the following equation: where S is the number of species (species richness) and p_i is the proportion of the species i in sample s. The index measures diversity by incorporating both the richness and the distribution (evenness) of types. Chao1 is the species richness estimator and assesses the number of species present.

The SONS program, which uses the "list" output file from the DOTUR program (Schloss and Handelsman, 2006), was used to calculate J_{abund} and the θ similarity indices with their standard errors to compare community membership and structure of raw milk microbiota. Cluster analysis on Euclidean distance using the average linkage method was performed with SYSTAT 10.

2.7. Quantitative PCR (Q-PCR)

Q-PCR specific primers were designed to monitor six species (*Acinetobacter calcoaceticus*, *Aerococcus viridans*, *Staphylococcus aureus*, *Streptococcus uberis*, *Pseudomonas fluorescens*, and *Corynebacterium variabile*) identified as abundant in the clone libraries (Supplemental material Table S1). Q-PCR primers were selected from the consensus sequences of clones from each species using Primer Express 2.0 (Applied Biosystems) with optimized conditions for SYBR Green detection (Supplemental material Table S1). Primer secondary structures and dimer formation were controlled with Oligo Analyzer V 3.1 software (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Q-PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA). To estimate Q-PCR efficiencies of primer sets, selected plasmid DNA was 10-fold serially diluted in sterile water and the calibration curve slope was used in the following equation to estimate reaction efficiency: $E = 10^{(-1/\text{slope})}$ (Supplemental material Table S2). The specificity of the newly designed primers was verified by a PCR assay using the sequenced clone recombinant plasmids as target DNA (Supplemental material Table S1).

Q-PCR was performed with the ABI PRISM 7500 system (Applied Biosystems). PCR products were detected with SYBR Green fluorescent dye and amplified according to the following protocol: initial hold for 10 min at 95 °C, followed by 40 two-step cycles at 95 °C for 15 s and 60 °C for 60 s. Each PCR reaction contained the following: 12.5 µL 2X SYBR Green PCR master mix with ROX passive reference dye (Applied Biosystems), 2 µL of specific plasmid DNA calibration standard or appropriately-diluted DNA, each of the forward and reverse primers at 100 nM, and a nuclease-free ultrapure distilled water (Invitrogen) was added to obtain a final volume of 25 µL. In each run, negative controls without DNA for each primer set were included. After amplification, dissociation curve analysis was performed by increasing the temperature by 1 °C every 20 s from 65 °C to 94 °C, to confirm primer specificity. Q-PCR was done in triplicate for each sample. For accurate quantification of unknown samples, specific plasmid calibration curves were created in each run. For this purpose, primer sets specific to the 16S rRNA gene plasmid inserts identified to each targeted species were used (Supplemental material Table S2). The concentration of genomic DNA from three batches of milk samples and specific plasmids were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). A 10-fold serial dilution series of specific plasmid DNA, ranging from 4.8×10^1 to 4.8×10^6 copies, was used to construct the standard curves. A coefficient of variation (CV) based on C_t for each triplicate sample was calculated at different concentrations from calibration curves developed for each primer set within the same run to evaluate the precision (intra-assay variation) and at least ten independent PCR runs to evaluate reproducibility (inter-assay variation) of Q-PCR.

Gene copy number in specific plasmid calibration curves or in unknown samples were calculated with the following equation: *Copies per nanogram* $= \frac{(n \times mw)}{N_A \times 10^{-9}}$, where n is the length of PCR product in bp, mw is the molecular weight per bp assuming average molecular masses of 660 Da for 1 bp of double-stranded DNA and N_A is the Avogadro constant (6.02×10^{23} molecules per mol). Cell densities were approximated using a conversion of 16S rRNA gene copies per cell of specific target species and total bacteria (Supplemental material Table S2) obtained from the rrnDB website (http://ribosome.mmg.msu.edu/rrndb/index.php, Lee et al., 2009). Statistical analyses were performed with JMP Version 7.0 (SAS Institute Inc, Cary, NC, USA). Analysis of variance (ANOVA) was done to verify if there was a significant difference in the population of target species in untreated and treated milk samples. Data were analyzed at the 5% level of significance.

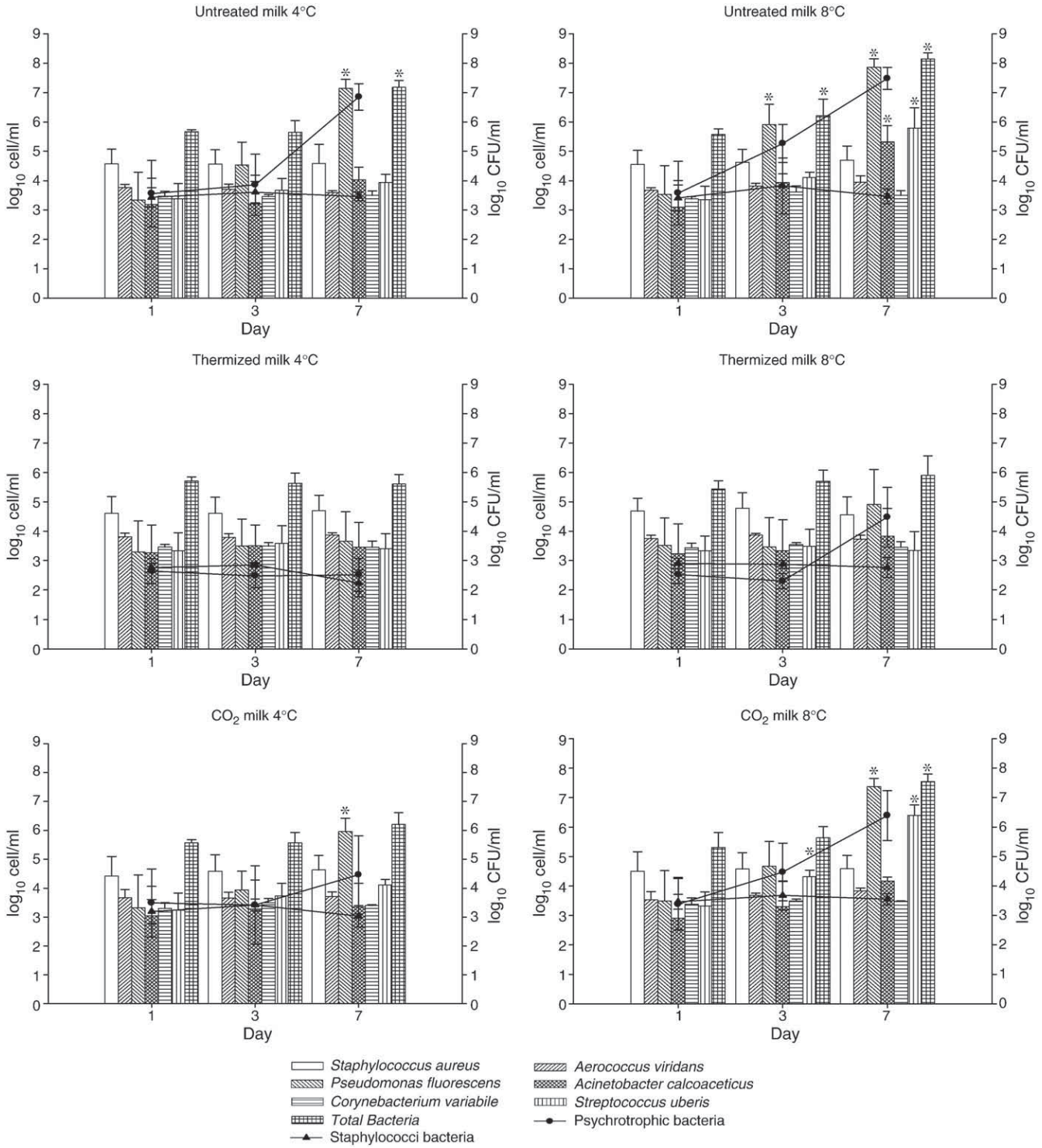

Fig. 1. Dynamics of target species in untreated milk, CO_2 and thermized milk samples. Error bars represent the standard deviation of the mean $\log_{10}$ cell/mL milk obtained from three batches of milk. *: Variations are considered significant ($p \leq 0.05$) when target species population from a given day was different from Day 1. Psychrotrophs and staphylococci were counted by viable count and the six dominant species were quantified by Q-PCR.

2.8. Nucleotide sequence accession numbers

Clone library 16S rRNA gene sequences are available in GenBank under accession numbers GQ466808 to GQ468222.

3. Results

3.1. Microbial analysis of untreated and treated milk samples by culture-dependent methods

The population of psychrotrophic bacteria increased in UT and CO_2 milk samples at both storage temperatures (Fig. 1). However, at 8 °C, the increase of psychrotrophic bacteria in UT and CO_2 milk samples was already noticeable after 3 d of storage. The addition of CO_2 in milk was not efficient in reducing the initial population of psychrotrophic bacteria, but the number of psychrotrophic bacteria at day 7 was lower in CO_2 milk compared to UT milk. The population of psychrotrophic bacteria in TH milk was reduced approximately to 2 $\log_{10}$ cfu/ml by thermization and was stable during storage at 4 °C but an increase was observed at day 7 when TH milk was stored at 8 °C. Moreover, no increase was observed in the population of staphylococci present regardless of treatment or storage temperature applied to milk samples. However, the initial population of staphylococci was reduced by 1 $\log_{10}$ cfu/ml in TH milk while it was reduced by 3 $\log_{10}$ cfu/ml in MF milk (data not shown).

3.2. Evaluation of milk microbial community composition using 16S rRNA gene clone libraries

In order to emphasize the microbial community of milk samples, clone libraries were constructed from samples taken after 3 and 7 d of storage at 4 °C. The 1415 sequences in clone libraries were placed into 202 operational taxonomic units (OTU) by the furthest-neighbor method and a similarity threshold of 97% in the DOTUR program (Table 1). One hundred eighteen OTUs (8.3%) contained only one sequence and 1297 sequences (91.7%) were 97% identical to at least one other sequence.

OTUs and sequences identified in clone libraries were grouped into nine classes (Table 1). The two most populated OTUs belonged to the class *Gammaproteobacteria* and *Bacilli*. Members of the *Bacilli* class were distributed over 54 OTUs and were mainly recovered from UT, CO_2 and TH milk samples with *Staphylococcus* (19.3%) and *Streptococcus* (5%) being the dominant species. Two hundred seven sequences (14.6%) were identified as *Staphylococcus aureus*. Members of the *Gammaproteobacteria* class were distributed over 19 OTUs and were mainly recovered from UT, CO_2 and MF milk samples with the dominant group being affiliated with the *Pseudomonas* genus (30.2%).

Table 1
Global composition of the 16S rDNA gene clone libraries from untreated (UT), CO_2, thermized (TH) and microfiltered (MF) milk samples after 3 and 7 d of storage at 4 °C.

Class	UT	CO_2	TH	MF	Number of sequences	Relative abundance (%)	OTUs[a]
Alphaproteobacteria	1	4	8	21	34	2.4	17
Betaproteobacteria	1	1	23	30	55	3.9	7
Gammaproteobacteria	202	122	25	221	570	40.3	19
Bacilli	112	180	233	41	566	40	54
Clostridia	16	23	36	11	86	6.1	52
Actinobacteria	22	14	20	20	76	5.4	29
Bacteroidetes	2	9	8	4	23	1.6	19
Acidobacteria	0	1	0	0	1	0.1	1
TM7	1	0	0	1	2	0.1	2
Unclassified	0	0	0	2	2	0.1	2
Total	357	354	353	351	1415	100	202

[a] OTU is defined as a 16S rDNA sequence similarity of ≥97% (Gevers et al., 2005).

Seven OTUs were affiliated with the *Betaproteobacteria* class and were mainly recovered from TH and MF milk samples where *Delftia* (2.8%) was the dominant member. Seventeen OTUs were affiliated with *Alphaproteobacteria* and were mainly recovered from MF milk in which *Ochrobactrum* (0.5%) was the dominant genus. Classes of *Clostridia*, *Actinobacteria* and *Bacteroidetes* had a low relative abundance but these classes had a diversified community (52, 29 and 19 OTUs, respectively). *Actinobacteria* were mainly recovered from all milk samples in which *Corynebacterium* (2%) and *Microbacterium* (0.8%) were the dominant member. Moreover, *Clostridia* were mainly recovered from TH milk and *Bacteriodetes* were mainly recovered from CO_2 and TH milk samples. The rare OTUs recovered from MF milk belong to *Acidobacteria* and TM7.

3.2.1. Clone library structure and composition

The 16S rRNA gene clone library structure and composition from untreated and treated milk samples is summarized in Supplemental material Table S3, while the most abundant OTUs are presented in Fig. 2. *Staphylococcus aureus* was dominant in UT, CO_2 and TH milk samples while *S. haemolyticus* and *S. saprophyticus* were subdominant in TH milk. *Streptococcus uberis* was dominant in all milk samples at day 3 while *S. parauberis* and *S. macedonicus* were subdominant in CO_2 milk at day 7. *Lactococcus lactis* was subdominant in UT and CO_2 milk samples, respectively at day 3 and 7. *Enterococcus faecium*, *E. italicus* and *Weissella thailandensis* were subdominant in UT and TH milk samples at day 3. *Trichococcus floculiformis*, *Nostocoida limicola* I and *Ruminococcus* sp. were dominant in CO_2 milk. *Clostridium disporicum*, *Facklamia tabacinasalis*, *Jeotgalicoccus psychrophilus* and *Lactobacillus kefiranofaciens* were subdominant in TH milk. *Aerococcus viridans* was dominant in CO_2 and TH milk samples. *Bacillus cereus* was detected in UT, CO_2 and TH milk samples at day 3. *Lysinibacillus fusiformis* was dominant in MF milk at day 3. *Bifidobacterium crudilactis* was found in UT, CO_2 and TH milk samples but this species was mostly detected in TH milk. *Corynebacterium* sp. was subdominant in UT milk. *Microbacterium phylosphaerae* was dominant in MF milk at day 7. *Ochrobactrum, Brevundimonas* and *Rhizobiales* were dominant in MF milk. *Delftia acidovorans* was dominant in MF and TH milk samples while *Acidovorax* and *Comamonas* were subdominant in MF milk. *Pseudomonas mendocina* and *P. pseudoalcaligenes* were dominant in MF milk at day 3. In contrast, *P. fluorescens* and *P. synxantha* were dominant in UT and CO_2 milk samples at day 7 while *P. putida* and *P. syringae* were dominant in MF milk. Moreover, *P. aeruginosa* was only detected in MF milk. *Stenotrophomonas maltophilia* was dominant in MF milk. *Acinetobacter calcoaceticus* was dominant in all milk samples at day 3 but was dominant in TH milk at day 7. *Bacteroides* and *Chryseobacterium* were subdominant in CO_2 and TH milk samples.

3.2.2. Effect of milk treatment on bacterial diversity

The Shannon diversity index (H') was similar (3.4) for CO_2, thermized and untreated milk at day 3. During subsequent storage at 4 °C, CO_2 and thermization treatment had no effect on microbial diversity (Table 2). Microfiltration had eliminated the majority of bacterial cells, which is shown by the decrease in the number of OTUs. However, the Shannon index of MF milk (3.0) was close to that of untreated milk at day 3. Bacteria in MF milk were diversified in spite of the physical removal of the majority of microorganisms. The Shannon index (H') decreased to 0.3 at day 7 in UT milk (Table 2). The decrease is less marked in TH and MF milk samples (2.8 and 2.1, respectively), showing that milk treatments had different effects on the various species in TH and MF milk samples. Microbial diversity was lower in UT and CO_2 milk samples, but remained higher in TH and MF milk samples. However, with regards to the difference between observed OTUs and estimated OTUs Chao1 in milk studied, the diversity might have been underestimated.

3.2.3. Relative abundance in treated and untreated milk samples during cold storage

At day 3, microfiltered milk had different community structure compared to CO_2, TH and UT milk samples as the dominant bacteria detected were *Stenotrophomonas*, *Pseudomonas*, *Delftia* and *Streptococcus*. The relative abundance of *Staphylococcus* in CO_2 and TH milk samples was similar to UT milk, but it decreased to 3.5% in MF milk. The relative abundance of *Acinetobacter* and *Streptococcus* in CO_2, TH and MF milk samples was similar to UT milk (Table 3).

At day 7, the genus *Pseudomonas* dominated the microbial community of untreated, CO_2 and MF milk samples (Table 3). Relative abundance of *Pseudomonas* reached 94.2% in UT milk while they represented 56.6% and 55.6% in CO_2 and MF milk samples, respectively. In TH milk, *Pseudomonas* was almost absent (0.6%). *Microbacterium* (6.7%) was only found in MF milk at day 7. The population of *Delftia* (12.7%) was the second dominant OTU in thermized milk after *Staphylococcus*. However, the proportion of *Delftia* in MF milk decreased to 1.1% at day 7. Relative abundance of *Staphylococcus* in UT and CO_2 milk samples decreased to 1.6% and 12.6%, respectively. However, it increased to 41% in TH milk and remained low (1.1%) in MF milk. Relative abundance of *Stenotrophomonas* in MF milk decreased to 13.5% while that of *Pseudomonas* increased to 55.6%, suggesting a growth competition between the two genera. Even though *Acinetobacter* was dominant at day 3, the proportion of this species decreased in UT, CO_2 and TH milk samples. This population was not detected in MF milk. The relative abundance of *Trichococcus* (6.6%) increased in CO_2 and TH milk samples and decreased in UT milk. The relative abundance of *Streptococcus* and *Lactococcus* decreased in UT and TH milk samples but it remained stable in CO_2 milk. *Lactobacillus* was not detected in UT and MF milk samples, while the proportion of *Lactobacillus* was low in CO_2 milk (1.1%) and remained stable in TH milk with a relative abundance of 2.9%.

3.2.4. Community membership and structure of milk microbiota

Community membership considers the list of OTUs while community structure takes into account the list and abundance of OTUs. These measures provide different images of the similarity between milk treatments over storage time. The abundance-based Jaccard (J_{abund}) is a measure of community overlap which is defined as the probability that a randomly selected OTU is found in both communities. A J_{abund} value of 1 implies that the two communities share all OTUs whereas a value of 0 implies that both communities do not share any OTUs. At day 3, community membership of CO_2 and TH milk samples was not different compared to UT milk with J_{abund} values of 0.96 (standard error (SE) = 0.11) and 0.84 (SE = 0.11), respectively. MF milk had different community membership with a J_{abund} value of 0.13 (SE = 0.06) compared to UT milk (Fig. 3A). At day 7, community membership of CO_2 milk (Fig. 3A) was similar compared to UT milk with a J_{abund} value of 0.81 (SE = 0.21). Thermized milk had different community membership but this community was comparable to that of UT milk at day 3 with a J_{abund} value of 0.55 (SE = 0.13). Community structure of treated milk (Fig. 3B) was evaluated by calculating the Θ value, where a value of 1 implies identical community structure while a value of 0 implies dissimilar community structure. The community structure of CO_2 and TH milk samples at day 3 were identical compared to UT milk with a Θ value of 0.86 (SE = 0.04) and 0.87 (SE = 0.05), respectively. The community structure of UT and CO_2 milk samples at day 7 were similar with a Θ value of 0.76 (SE = 0.05). The community structure of MF milk at day 3 and 7 was completely different compared to untreated milk (Fig. 3B). These results show that treatments (CO_2 and thermization) applied to raw milk stored at 4 °C had no effect on microbial community structure and membership at day 3. MF milk communities were very different in structure from other treated or untreated milk samples. At day 7, community structure and community membership changed in all milk samples. The change in microbial community over time was noticeable in CO_2 and UT milk samples, which were similar in structure. Moreover, the community structure in TH milk was stable at day 7, as it was similar to those of UT, CO_2 and TH milk samples at day 3.

3.3. Quantification of the abundance of six species by quantitative PCR (Q-PCR)

Populations of *Staphylococcus aureus, Aerococcus viridians, Acinetobacter calcoaceticus, Corynebacterium variabile* and *Streptococcus uberis* were stable during 7 d of storage at 4 °C in all milk samples analyzed with means of 4.59, 3.73, 3.37, 3.45, and 3.60 $\log_{10}$ cell/ml, respectively. However, populations of *A. calcoaceticus* and *S. uberis* increased at day 7 in UT and CO_2 milk samples when stored at 8 °C. The increased population of *S. uberis* was already noticeable at day 3 and this population reached 6.4 $\log_{10}$ cell/ml at day 7 in CO_2 milk stored at 8 °C (Fig. 1). Populations of *P. fluorescens* and total bacteria increased in UT and CO_2 milk samples during 7 d of storage (Fig. 1). The population of *P. fluorescens* in UT milk stored at 4 °C was low (3.34 $\log_{10}$ cell/ml) at day 1 but increased rapidly and reached 4.53 $\log_{10}$ cell/ml and 7.15 $\log_{10}$ cell/ml at day 3 and 7, respectively. The increase of *P. fluorescens* in UT was more pronounced at 8 °C and reached 7.86 $\log_{10}$ cell/ml at day 7. However, the Q-PCR cell count of *P. fluorescens* in CO_2 milk at day 7 was lower (5.96 $\log_{10}$ cell/ml) compared to UT milk stored at 4 °C. In contrast, CO_2 treatment had little effect on *P. fluorescens* at 8 °C and this population reached 7.37 $\log_{10}$ cell/ml at day 7. Populations of total bacteria increased to 7.18 $\log_{10}$ cell/ml and 8.15 $\log_{10}$ cell/ml at 4 °C and 8 °C, respectively at day 7. The increase of total bacteria was noticeable at day 3 in UT milk stored at 8 °C. However, the population of total bacteria in CO_2 milk at both storage temperatures was lower (6.20 $\log_{10}$ cell/mL and 7.54 $\log_{10}$ cell/ml at 4 °C and 8 °C, respectively) compared to UT milk. In thermized milk at 4 °C, the populations of *P. fluorescens* and total bacteria were stable during 7 d of storage with means of 3.48 and 5.65 $\log_{10}$ cell/ml, respectively. However, an increase in the population of *P. fluorescens* was noticeable at day 7 in TH milk stored at 8 °C.

To validate the results obtained by the Q-PCR method, the Q-PCR cell counts and the relative abundance of clones of the target species were compared with bacterial counts (cfu/ml) from culture-dependent methods. Significant correlation between the cell count of total bacteria and *P. fluorescens* in Q-PCR and the bacterial count of psychrotrophic bacteria was observed in UT and CO_2 milk samples with the correlation coefficients (R^2) of 0.93 ($p < 0.0001$) and 0.92 ($p < 0.0001$), respectively (data not shown). Moreover, no correlation was found between *S. aureus* cell counts by Q-PCR and by plating but both methods showed that this population was stable in UT, CO_2 and TH milk samples. In addition, a significant correlation between the relative abundance of the six target species in Q-PCR compared to the clone libraries was found with the correlation coefficient (R^2) of 0.95 ($p < 0.0001$) (data not shown).

Analysis of variance on the six target species quantified by Q-PCR shows the significant effects of treatments (CO_2 and TH), temperature (4 °C and 8 °C) and storage time (1, 3 and 7 d) on six members of the microbial community of milk (Table 4). Storage temperature significantly affected the total bacteria in milk, as the estimate of total bacteria was higher at 8 °C. Time of storage significantly affected the population of *P. fluorescens, A. calcoaceticus, S. uberis* and total bacteria. These species increased in UT and CO_2 milk samples during storage. The interaction between temperature and time of storage significantly affected *A. viridans, P. fluorescens*, *A. calcoaceticus*, *S. uberis* and total bacteria. The increase of these species during storage was more pronounced at 8 °C. The interaction between treatment and time of storage significantly affected *P. fluorescens* and the total bacteria. These species were affected by CO_2 treatment but an increase of their population was observed during storage. The triple interaction among milk treatments, temperature and time of storage

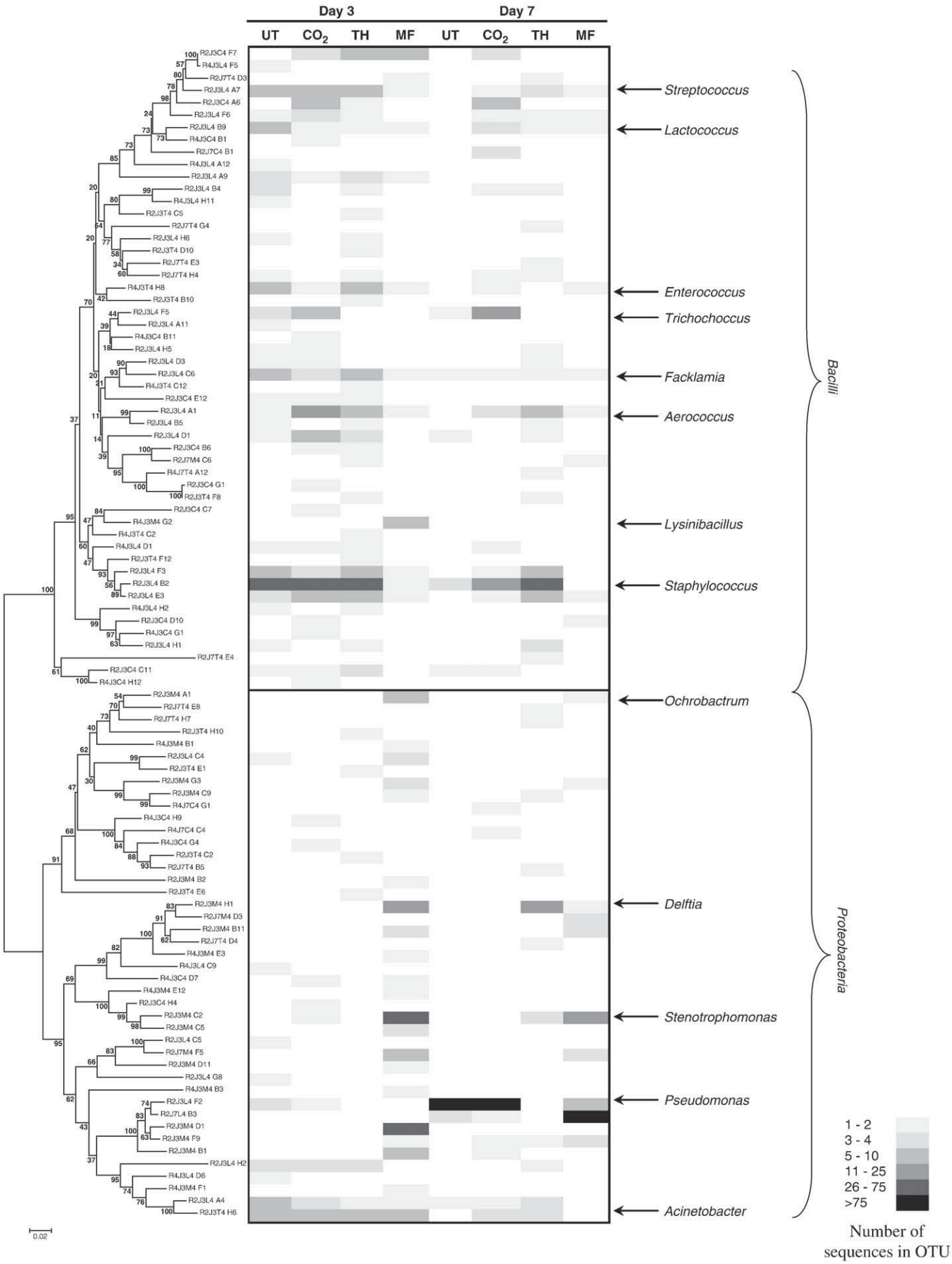

Table 2
Composition and diversity index of the sequenced clones from libraries of bacterial 16S rDNA isolated from untreated (UT), CO_2, thermized (TH) and microfiltered (MF) milk samples stored at 4 °C.

Day	Treatment	Number of clones	OTU[a]	Chao1 (95% CI)[b]		Shannon H' (95% CI)	
3	UT	168	63	161	(104–298)	3.4	(3.2–3.6)
	CO_2	172	67	312	(165–681)	3.4	(3.2–3.6)
	TH	180	62	110	(83–175)	3.4	(3.2–3.6)
	MF	173	42	81	(55–155)	3.0	(2.8–3.1)
7	UT	189	8	10	(8–24)	0.3	(0.2–0.5)
	CO_2	182	26	61	(36–152)	1.8	(1.6–2.0)
	TH	173	53	109	(76–190)	2.8	(2.6–3.1)
	MF	178	34	58	(42–105)	2.1	(1.9–2.4)

[a] OTU is defined as a 16S rDNA sequence similarity of ≥97% (Gevers et al., 2005).
[b] 95% confidence interval.

was significant for *P. fluorescens*, *S. uberis* and the total bacteria. The growth of these species during storage was affected by treatment applied on milk samples and the temperature of storage.

4. Discussion

The bacterial composition after milk treatment influences its quality and shelf life during storage. Many authors have reported that the population of psychrotrophic bacteria increased to high levels during cold storage of raw milk, which affected milk quality (Cousin, 1982; Griffiths et al., 1988; Roberts and Torrey, 1988; Champagne et al., 1994; Ma et al., 2003). In this study, the population of psychrotrophic bacteria increased in raw milk (UT) during storage at 4 °C and 8 °C which is in agreement with the literature.

To control the microbiological quality of milk, treatments such as thermization, the addition of CO_2 and microfiltration have been used to eliminate bacteria or to inhibit their growth in milk (Champagne et al., 1994). In this study, the addition of CO_2 to milk had no effect on the initial population of psychrotrophic bacteria in raw milk, as observed by Roberts and Torrey (1988) and Ma et al. (2003). Many authors have reported that CO_2 can inhibit the growth of psychrotrophs in raw milk (Rowe, 1988; Ma et al., 2003; Martin et al., 2003). In our study, culture-dependent methods show that the growth of psychrotrophs in CO_2 milk was lower compared to untreated milk during cold storage. The inhibition of psychrotrophs by the addition of CO_2 in milk was more efficient at a lower storage temperature, showing the impact of temperature abuse on storage time. Populations of psychrotrophic bacteria were reduced by thermization and remained stable at 4 °C during 7 d, indicating that this treatment was as efficient as pasteurization in controlling psychrotrophs (Ravanis and Lewis, 1995). However, at a higher storage temperature of 8 °C, thermization was not efficient for milk stored more than 3 d, as observed for pasteurized and UHT milk (Griffiths et al., 1988).

Many studies based on culture-dependent methods have been done to describe the structure of culturable portions of the milk microbiota (Ma et al., 2003; Munsch-Alatossava and Alatossava, 2006). The emergence of molecular methods provides a new way to obtain an accurate global view of microbial communities in milk (Ogier et al., 2002; Duthoit et al., 2003; Ercolini, 2004; Lafarge et al., 2004). However, due to bias introduced by PCR-based methods, the accuracy of the data obtained by molecular methods used in this study must be solidly supported by another method such as Q-PCR, which is sensitive and precise. Clone library sequencing was applied to assess the dynamics of the bacterial community in untreated and treated milk samples stored at 4 °C. The majority of sequences in the clone libraries was affiliated with the Class *Bacilli* and *Gammaproteobacteria* which is in agreement with Delbès et al. (2007) and Hantsis-Zacharov and Halpern (2007). Seven classes were found in culturable psychrotrophic communities from raw milk in which *Gammaproteobacteria*, *Bacilli* and *Actinobacteria* were the dominant classes (Hantsis-Zacharov and Halpern, 2007). When diversity of bacterial populations in raw milk was investigated by molecular methods, the dominant clones were affiliated with the class *Clostridia* and *Bacilli* whereas the subdominant populations were affiliated with the classes *Proteobacteria*, *Actinobacteria* and *Bacteriodetes* (Delbès et al., 2007). According to our knowledge, this study is the first to report clones affiliated with the classes *Acidobacteria* and TM7 in milk. *Acidobacteria* is an acidophilic bacterium that we found in CO_2 milk and TM7 was found in UT and MF milk samples. These genera have commonly been found in environmental samples (Brons and van Elsas, 2008; Chim Chan et al., 2008) and TM7 was reported in a study of dairy waste (McGarvey et al., 2007). The presence of these species in milk seems to indicate a contamination by microorganisms from the farm environment.

The genus *Pseudomonas* dominated the microbial community of UT, CO_2 and MF milk samples at day 7, but was most diversified in MF milk. In a previous study, psychrotrophic populations increased within 24 h of cold storage and bacterial dynamics showed considerable variation among samples (Lafarge et al., 2004). However, in our study, an increase in the population of *Pseudomonas* was noticeable at day 3 when UT and CO_2 milk samples were stored at 8 °C but not at 4 °C. Thus, CO_2 milk can be stored longer without a significant decrease in potential technological properties when proper temperature control is carried out. However, changes in population structure following CO_2 treatment appear to favor the increase of *Trichococcus* in milk samples. *Trichococcus floculiformis* was the dominant species in CO_2 and TH milk samples. *Trichococcus pasteurii* has been detected in dairy waste (McGarvey et al., 2007) and raw milk (Hantsis-Zacharov and Halpern, 2007).

Table 3
Relative abundance (% of total number) of 16S rDNA clones isolated from treated (CO_2, TH, MF) and untreated (UT) milk samples during storage at 4 °C.

Class	Genus	Day 3				Day 7			
		UT	CO_2	TH	MF	UT	CO_2	TH	MF
Bacilli	*Streptococcus*	4.8	9.3	7.2	5.2	0.0	8.2	4.0	1.7
	Lactococcus	3.0	1.7	1.1	0.6	0.0	3.8	0.6	1.1
	Lactobacillus	3.6	0.0	2.8	0.0	0.0	1.1	2.9	0.0
	Trichococcus	3.0	4.1	0.0	0.0	0.5	6.6	1.2	0.0
	Facklamia	5.4	3.5	6.7	0.6	0.5	0.5	1.7	0.6
	Aerococcus	1.8	6.4	4.4	0.6	0.0	1.6	4.6	1.1
	Staphylococcus	32.7	32.0	32.2	3.5	1.6	12.6	41.0	1.1
	Other *Bacilli*	8.3	9.3	16.1	4.6	1.1	1.6	5.2	2.8
Clostridia	*Clostridia*	9.5	11.6	12.2	2.9	0.0	1.6	8.1	3.4
Acidobacteria	*Acidobacteria*	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
Unclassified	Unclassified	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.6
Actinobacteria	*Corynebacterium*	6.0	2.9	2.2	0.0	1.1	1.1	1.7	1.1
	Microbacterium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.7
	Other *Actinobacteria*	6.0	3.5	3.3	1.7	0.0	0.5	4.0	1.7
Proteobacteria	*Delftia*	0.0	0.0	0.0	9.2	0.0	0.0	12.7	1.1
	Stenotrophomonas	0.0	0.6	0.0	19.1	0.0	0.0	1.7	13.5
	Pseudomonas	2.4	0.6	0.0	24.3	94.2	56.6	0.6	55.6
	Acinetobacter	6.5	4.7	5.6	4.0	1.1	2.7	4.0	0.0
	Other *Proteobacteria*	5.4	4.1	3.9	21.4	0.0	1.1	3.5	6.7
TM7	TM7	0.6	0.0	0.0	0.6	0.0	0.0	0.0	0.0
Bacteriodetes	*Bacteriodetes*	1.2	5.2	2.2	1.2	0.0	0.0	2.3	1.1

Fig. 2. Phylogenetic architecture of the two major classes of 16S rDNA clone libraries and their abundance in untreated, CO_2, thermized and microfiltered milk samples during 7 d of storage at 4 °C.

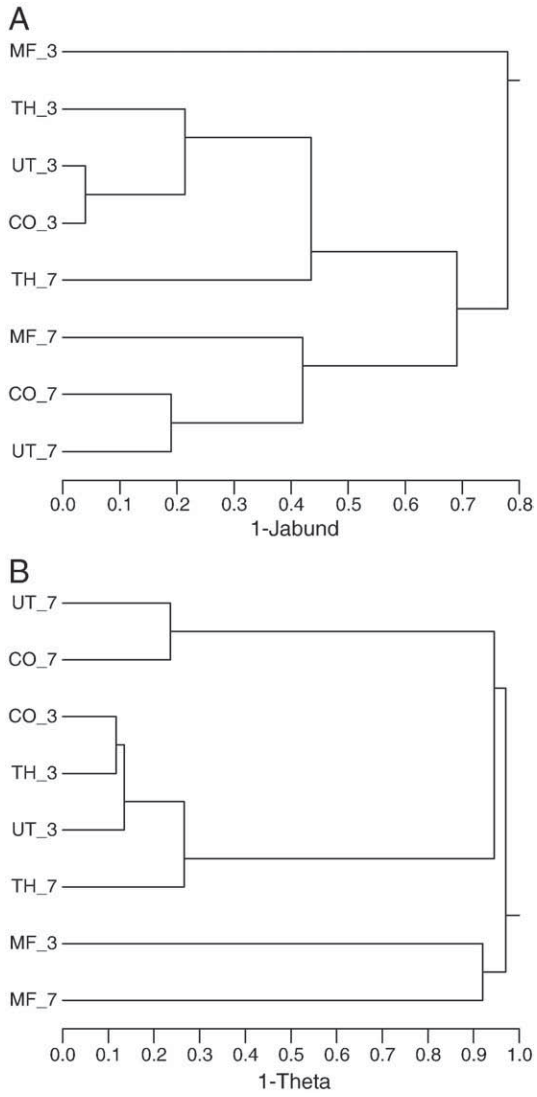

Fig. 3. (A) Dendrogram comparing community membership of OTUs from treated (TH, CO_2, MF) and untreated milk samples using average linkage of Euclidean distance (distance = $1 - J_{abund}$); (B) Dendrogram comparing community structure of OTUs from treated and untreated milk samples using average linkage of Euclidean distance (distance = $1 - \Theta$); UT = untreated milk; CO = CO_2 milk; TH = thermized milk; MF = microfiltered milk; 3 = milk samples stored during 3 d; 7 = milk samples stored during 7 d.

Staphylococcus aureus, which is commonly found in milk (Peles et al., 2007) and produces enterotoxins in contaminated milk leading to food poisoning (Asao et al., 2003), was dominant in UT, CO_2 and TH milk samples. The population of *S. aureus* remained stable in treated milk during storage at both temperatures. Thus, slight temperature abuse did not lead to increase in *S. aureus* even in the absence of competing microorganisms. Gilmour and Harvey (1990) reported that growth of *Staphylococcus* was limited by the low temperature during milk storage and *Staphylococcus* was not a good competitor in raw milk containing a high number of microorganisms.

Clostridium disporicum was found to be a dominant species in the *Clostridium* genus. Julien et al. (2008) reported that *C. disporicum* was an important member of clostridial populations transmitted to milk in western Québec. Occurrence of this species in milk could be due to lower silage quality (Julien et al., 2008). *Clostridia* are spore-forming anaerobes, thus they are resistant to heat and grow in anaerobic conditions, some under refrigeration conditions. The presence of *Clostridia* in milk studied here could be due to the contamination of the teats by the bedding or soil from the farm, which further contaminated the milk during the milking process. The reduction in abundance of *Clostridia* over storage time was most noticeable for UT and CO_2 milk samples.

Among lactic acid bacteria (LAB), *Enterococcus faecium* and *E. italicus* were dominant in UT and TH milk samples. *E. italicus* was reported to be a dominant species in artisanal Italian cheeses (Fortina et al., 2004) while *E. faecium* was a dominant member of LAB in traditional raw milk Salers cheese (Callon et al., 2004). *Lactobacillus* and *Lactococcus* species had a low relative abundance in milk samples, but their relative abundance in treated milk remained stable during storage. In contrast, Lafarge et al. (2004) reported that *Lactococcus lactis* was a major raw milk species and *Lactobacillus plantarum* was a minor species in raw milk in France. In their study, the population of *Lactococcus* decreased under refrigeration while that of *Lactobacillus* disappeared after incubation of raw milk at 4 °C for 24 h.

Interestingly, *Bifidobacterium crudilactis* was detected in untreated and treated milk (CO_2 and TH) by clone library and was also isolated from milk samples (data not shown). *B. crudilactis* seemed to be stimulated by thermization. This species was first isolated from raw milk in France (Delcenserie et al., 2007), and confirmed *B. crudilactis* isolates in this study showed 100% identity of their 16S rRNA gene sequences to those reported by Delcenserie et al. (2007). According to our knowledge, this is the first time that *B. crudilactis* has been detected by both molecular and culture-dependent methods in milk in North America.

The relative abundance of *Streptococcus* and *Corynebacterium* decreased during storage of thermized milk. Collins (1981) reported that *Streptococcus* and *Corynebacterium* are thermoduric microorganisms. Our results suggest that even though they may resist the initial heat treatment, these thermoduric bacteria do not persist during cold storage. Halophilic bacteria belonging to the genera *Jeotgalicoccus* and *Salinicoccus* were found in milk in this study, which corresponds with previous observations in cow and goat milk (Callon et al., 2007; Delbès et al., 2007). The relative abundance of *J. psychrophilus* in thermized milk increased over storage time, this species did not appear to be affected by the thermization process and was favored by changes in the microbial population following this treatment.

Facklamia species were detected in TH and UT milk samples, and some *Facklamia* species are known to be opportunistic pathogens (LaClaire and Facklam, 2000). *Facklamia tabacinasalis* was isolated from powdered tobacco (Collins et al., 1999), but has also been detected in raw milk (Delbès et al., 2007). *Facklamia sourekii,* a possible pathogen, was found in a lactating cow with hematuria and urodynia (Takamatsu et al., 2006). However, the occurrence of *F. sourekii* was low in untreated milk in this study. Only microfiltration reduced *Facklamia* in milk, although this genus decreased in abundance over cold storage, whether the milk was treated or not.

Microfiltration was efficient for removing *S. aureus* from milk, which is in agreement with Trouvé et al. (1991). Microfiltration treatment applied to raw milk eliminates the majority of microorganisms, but the bacterial reduction is independent of the initial population level (Trouvé et al., 1991). It is thus important to gain knowledge of the remaining bacteria in MF milk, which can be detected by the molecular methods used in our study. *Stenotrophomonas, Delftia* and *Ochrobactrum* were not eliminated by microfiltration and dominated MF milk. These species were previously detected in cow and goat milks (Callon et al., 2007; Hantsis-Zacharov and Halpern, 2007) as well as in bulk tank milk for *O. anthropi* (Jayarao and Wang, 1999). *Delftia* isolates showed high proteolytic activity (Hantsis-Zacharov and Halpern, 2007), and thus contribute to reducing the shelf life and technological properties of milk. These three genera could be considered as more specific indicators for the contamination level of microfiltered milk during packaging, storage and distribution, as the usual indicator organisms are absent. Hantsis-Zacharov and Halpern (2007) found that *Microbacterium* was ubiquitous in raw milk and has shown both lipolytic and proteolytic

Table 4
ANOVA of the effect of milk treatment, temperature and time of storage on six targeted species estimated by Q-PCR.

Source	*df*	Sum of squares						
		Staphylococcus aureus	*Aerococcus viridans*	*Pseudomonas fluorescens*	*Acinetobacter calcoaceticus*	*Corynebacterium variabile*	*Streptococcus uberis*	Total bacteria
Batch	2	10.3**	0.41**	22.63**	22.81**	0.3**	2.94**	3.48**
Temperature	1	0.01	0.04	0.18	0.03	0.001	0.001	0.19*
Treatment	2	0.06	0.03	0.003	0.08	0.06	0.03	0.03
Temperature*Treatment	2	0.01	0.004	0.002	0.01	0.03	0.01	0.03
Time	2	0.05	0.01	24.61**	0.92*	0.02	1.16*	2.93**
Temperature*Time	2	0.02	0.12*	1.95**	1.96**	0.01	4.51**	2.61**
Treatment*Time	4	0.04	0.06	9.92**	0.73	0.04	0.56	2.53**
Temperature*Treatment*Time	4	0.08	0.13	1.63*	0.49	0.05	2.39*	0.63**
Error	34	0.33	0.45	3.63	4.53	0.42	5.47	1.14

*$p \leq 0.05$; **$p \leq 0.01$.
Treatment = Untreated milk, carbon dioxide-treated milk and thermized milk.
Temperature = milk stored at 4 °C and 8 °C.
Time = milk stored during 1, 3 and 7 d.

activities. *Microbacterium* was detected only in microfiltered milk in our study, when dominating bacteria were eliminated, and increased in relative abundance over storage time. The analysis of microfiltered milk following cold storage revealed minor microbial contaminants that may contribute to milk spoilage, which have not previously been detected amongst a competing microbial population.

The Q-PCR method was used for the first time to assess the impact of milk treatment (TH and CO_2) on the dynamics of six dominant bacterial species (*A. calcoaceticus, A. viridans, S. aureus, S. uberis, P. fluorescens*, and *C. variabile*) in treated and untreated milk samples during cold storage. Furthermore, Q-PCR was used to validate measurements obtained with clone libraries and conventional methods for the population of psychrotrophs and *S. aureus*. *S. uberis* increased in CO_2 milk samples during storage at 8 °C. In contrast, Dogan and Boor (2004) found that *S. uberis* did not grow in milk stored at 4.4 and 7 °C and the high number of *S. uberis* in bulk tank milk reflected the high number of this bacterium in mastitic cows. To our knowledge, this is the first report demonstrating that the growth of *S. uberis* was stimulated by CO_2 during cold storage. Results observed by Q-PCR, especially for total bacteria, *P. fluorescens* and *S. aureus*, were in accordance with those obtained with conventional and molecular methods reported by different authors (Ma et al., 2003; Graber et al., 2007; Hantsis-Zacharov and Halpern, 2007; Gillespie et al., 2009). No correlation was found between bacterial and cell count of *S. aureus*. Moreover, the cell count of *S. aureus* by Q-PCR was 1.8 $\log_{10}$ cell/ml higher compared to the bacterial count of staphylococci. This was expected, as total accumulated biomass is measured by Q-PCR, not only live bacteria. Discrepancies between results of molecular methods and culture-dependent methods may be due to biases introduced during DNA isolation, PCR amplification and intragenomic heterogeneity in 16S rRNA gene operon copy number (Coenye and Vandamme, 2003; Frey et al., 2006). The use of universal bacterial primers and the presence of dominant bacteria may hide the variation of less dominant populations (Duthoit et al., 2003). Nevertheless, use of specific primers for the quantification of six dominant bacteria by the Q-PCR method was useful for quantifying those targeted species with high specificity and high repeatability in treated and untreated milk samples. Moreover, the efficacy of the treatment on dominant bacteria in milk samples was emphasized by Q-PCR especially for *P. fluorescens* and *S. uberis*. Particularly, the growth of those species during storage was affected by treatment applied whose efficacy was in turn affected by the temperature.

5. Conclusion

The structure and the dynamics of milk microbiota were revealed by clone libraries and specific members were quantified by Q-PCR. Bacterial community dynamics observed by culture-dependent methods were enhanced by the analysis of 16S rRNA gene clone libraries. Moreover, the dynamics of six targeted species revealed by Q-PCR was correlated with those in clone libraries, thus validating the results obtained by molecular methods. In our study, many species not commonly detected in milk were only revealed by culture-independent methods, further supporting the importance of using molecular methods in studies of microbial communities. Culture-dependent methods only detect what is culturable on the media selected, thus underestimating the diversity of the microbiota in food matrices. Despite the observed bias, molecular and culture-dependent methods produce complementary information on the structure and dynamics of milk microbiota. Further work should be done to study the pathogenicity and the part played by these newly detected species during cheese processing.

According to our knowledge, this is the first time that the effect of treatment (CO_2, thermization, microfiltration) on the structure and the dynamics of milk microbiota during cold storage were revealed by using molecular methods. The use of specific primers in Q-PCR was helpful for monitoring dominant bacteria affecting milk quality during cold storage. The molecular method used in our study is based on 16S rRNA gene, revealing total accumulated biomass. In the future, targeting 16S rRNA would reveal transcriptionally active populations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijfoodmicro.2010.01.008.

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