International Journal of Food Microbiology 153 (2012) 192–202

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

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

The fate of indigenous microbiota, starter cultures, *Escherichia coli*, *Listeria innocua* and *Staphylococcus aureus* in Danish raw milk and cheeses determined by pyrosequencing and quantitative real time (qRT)-PCR

Wafa Masoud [a,*], Finn K. Vogensen [a], Søren Lillevang [b], Waleed Abu Al-Soud [c], Søren J. Sørensen [c], Mogens Jakobsen [a]

[a] Department of Food Science, Food Microbiology, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark
[b] Research and Development Department, Innovation Centre Brabrand, Arla Foods Amba, Rørdrumvej 2, 8220 Brabrand, Denmark
[c] Department of Biology, Molecular Microbial Ecology Group, University of Copenhagen, Sølvgade 83H, 1307K Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 6 July 2011
Received in revised form 12 October 2011
Accepted 13 November 2011
Available online 20 November 2011

Keywords:
Raw milk
Raw milk cheese
Pyrosequencing
Quantitative real time (qRT)-PCR

ABSTRACT

The purpose of this work was to study the bacterial communities in raw milk and in Danish raw milk cheeses using pyrosequencing of tagged amplicons of the V3 and V4 regions of the 16S rDNA and cDNA. Furthermore, the effects of acidification and ripening starter cultures, cooking temperatures and rate of acidification on survival of added *Escherichia coli*, *Listeria innocua* and *Staphylococcus aureus* in cheeses at different stages of ripening were studied by pyrosequencing and quantitative real time (qRT)-PCR.
A high diversity of bacterial species was detected in raw milk. *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus casei* and *Lactobacillus rhamnosus* were the main bacteria detected in raw milk and cheeses. Bacteria belonging to the genera *Brevibacterium*, *Staphylococcus*, *Escherichia*, *Weissella*, *Leuconostoc*, *Pediococcus* were also detected in both 16S rDNA and cDNA obtained from raw milk and cheeses. *E. coli*, which was added to milk used for production of some cheeses, was detected in both DNA and RNA extracted from cheeses at different stages of ripening showing the highest percentage of the total sequence reads at 7 days of ripening and decreased again in the later ripening stages. Growth of *E. coli* in cheeses appeared to be affected by the cooking temperature and the rate of acidification but not by the ripening starter cultures applied or the indigenous microbiota of raw milk. Growth of *L. innocua* and *S. aureus* added to milks was inhibited in all cheeses at different stages of ripening. The use of 16S rRNA gene pyrosequencing and qRT-PCR allows a deeper understanding of the behavior of indigenous microbiota, starter cultures and pathogenic bacteria in raw milk and cheeses.

1. Introduction

Raw milk cheeses are often characterized by richer and stronger flavor intensity than cheeses made from pasteurized milk, which leads to an increase demand for such cheeses. *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Enterococcus* spp., *Streptococcus* spp., *Micrococcus* spp., *Staphylococcus* spp., *Arthrobacter* spp., *Corynebacterium* spp., *Brevibacterium* spp., *Enterobacter* spp., *Citrobacter* spp. and *Acinetobacter* spp., which have been detected in raw milk cheeses (Casalta et al., 2009; Desmasures et al., 1997; Desmasures and Gueguen, 1997; Duthoit et al., 2003; Morea et al., 1999; Randazzo et al., 2002; Verdier-Metz et al., 2009), are assumed to play a role in the maturation and flavor development of raw milk cheeses. However, raw milk cheeses in some cases might be regarded as unsafe due to the possible contamination by pathogenic bacteria, mainly *Listeria monocytogenes* (Meyer-Broseta et al., 2003), enterotoxin producing strains of *Staphylococcus aureus* (Lindqvist et al., 2002) and *Escherichia coli* O157:H7 (Reitsma and Henning, 1996). The source of contamination can be the raw milk as delivered from the farm or the environmental conditions in the dairy i.e. post-process contamination (Rudolf and Scherer, 2001). It has been reported that the spontaneous micropopulations of raw milk cheeses are inhibitory to *L. monocytogenes* (Eppert et al., 1997; Millet et al., 2006). However, the composition of the bacterial communities responsible for inhibition or inactivation of pathogens and the underlying mechanisms are not known.

Recently, pyrosequencing of tagged amplicons of specific regions of the 16S rRNA gene has successfully been used to monitor microbial communities in various ecosystems like sputum (Armougom et al., 2009), gut (Andersson et al., 2008; Larsen et al., 2010), soil (Acosta-Martinez et al., 2008), wounds (Dowd et al., 2008), fermented foods (Humblot and Guyot, 2009; Roh et al., 2010) and raw milk cheese (Masoud et al., 2011). In a recent study, we investigated the microbial communities in

* Corresponding author. Tel.: +45 35 28 32 87; fax: +45 35 28 32 14.
E-mail address: wm@life.ku.dk (W. Masoud).

0168-1605/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2011.11.014

Danish raw milk cheeses using PCR denaturating gradient gel electrophoresis (PCR-DGGE) of the V3 region of the 16S rRNA gene and pyrosequencing of tagged 16S rRNA gene amplicons from DNA and RNA extracted from raw milk cheeses at different stages of ripening (Masoud et al., 2011). Pyrosequencing was able to provide an in depth detection of major and minor bacterial populations in raw milk cheese, which was not detected by PCR-DGGE.

In the present study, it was the main aim to characterize the bacterial communities in raw milk as well as in raw milk cheeses at different stages of ripening. Furthermore, the effects of different mixtures of starter cultures, cooking temperatures and rate of acidification on survival of *E. coli*, *L. innocua* and *S. aureus* in Danish semi hard raw milk cheeses were investigated. The bacterial diversity in raw milk and cheeses was investigated by tag-encoded amplicon pyrosequencing of 16S rRNA gene in both DNA and RNA extracted from raw milk and cheeses. Furthermore, the starter cultures, *E. coli*, *L. innocua* and *S. aureus*, were quantified by quantitative real time (qRT)-PCR.

2. Materials and methods

2.1. Media and cultures

Tryptone Soya Broth (TSB) (Oxoid, UK) was prepared by dissolving 30.0 g in 1 l distilled water. For de Man, Rogosa and Sharpe (MRS) broth, 52 g of MRS broth (Oxoid) were dissolved in 1 l distilled water. The diluent saline peptone (SPO) was prepared by dissolving 8.5 g NaCl (Merck, Germany), 0.30 g disodium hydrogen phosphate $Na_2HPO_4 \cdot 12H_2O$ (Merck) and 1.0 g Bactopeptone (Difco, USA) in 1 l distilled water. SPO was adjusted to pH 5.6 by the addition of 1 M NaOH. All media were autoclaved at 121 °C for 15 min.

Bacteria used in this study were obtained from Arla Foods, Denmark and they included the acidification starter cultures *Lactococcus lactis* L005, *Lactobacillus rhamnosus* BGP-2 and the ripening starter cultures *Brevibacterium linens* 004-0001 and *Microbacterium lacticum* M009. Furthermore, the non-pathogenic *E. coli* ATCC 43888, *S. aureus* ATCC 29213 and *Listeria innocua* 073.002, which were added to cheeses, were used as model microorganisms.

With the exception of *Lb. rhamnosus*, bacteria were inoculated in 25 ml of TSB broth at 30 °C for 24 h. *Lb. rhamnosus* was inoculated in MRS broth and incubated at 30 °C under anaerobic conditions for 24 h. After propagation cells were harvested by centrifuging (Ronbio Scientific, Shanghai, China) at 3000 × *g* for 10 min, and resuspended in SPO. DNA was isolated using Genelute Bacterial Genomic Kit (Sigma Aldrich, St. Louis, Mo, USA) according to the manufacturer instructions.

2.2. Raw milk and cheese samples

Cheeses were manufactured at Arla Foods. Cheeses were produced by heating the raw milk to 30 °C before adding the starter cultures and the model microorganisms, then the milk was coagulated by adding calf rennet and left for 30 min. After coagulation, the curd was cooked at 39 or 50 °C for 2 min and left to cool down. Cooking the curd at 39 °C resulted in a slower acidification rate i.e. 8 h to reduce the pH from 6.70 to 5.40 compared to the acidification rate at cooking temperature of 50 °C i.e. 4 h to reduce pH from 6.70 to 5.40. Finally the curd was pressed and left to ripen at 8 °C. For cheese treated with cooking temperature of 39 °C, four batches were produced with different mixtures of starter cultures at a concentration of 10^9 cfu/g of each starter and 10^6 cfu/g of each model microorganisms of the pathogenic bacteria (Table S1). The four cheeses produced included: cheese batch 16-3-1 with addition of the starter culture *L. Lactis*, cheese batch 16-3-2 with addition of *L. lactis* and a mixture of *E. coli*, *L. innocua* and *S. aureus* (mix 1), cheese batch 16-3-3 with addition of *L. lactis* and a mixture of *B. linens* and *M. lacticum* (mix 2) and cheese batch 16-3-4 with addition of *L. lactis*, mix 1 and mix 2. Another four batches of cheese (16-4-1, 2, 3, 4), which were treated by cooking at 50 °C for 2 min, were produced with the same mixtures of bacteria added to cheeses treated at 39 °C as well as *Lb. rhamnosus* to the four cheese batches (Table S1). Samples of raw milk, inoculated milk and cheeses obtained from a mixture of cheese surface and core at 0, 7, 14, 28 and 56 days of ripening were collected and stored at −80 °C until analyzed.

2.3. Isolation of DNA and RNA from raw milk and cheeses

For isolation of DNA or RNA from raw milk and cheese samples, 2 g of milk or cheese were added to 20 ml of 2% w/v sodium citrate (Merck, Darmstadt, Germany) and mixed in a stomacher (Lab Blender 400, Seward, London, UK) at normal speed for 2 min. The mixture was then centrifuged (Ronbio Scientific) at 300 × *g*, 4 °C for 15 min. 10 ml of the supernatant were centrifuged at 5000 × *g*, 4 °C for 15 min and the pellet was used for further isolation of DNA or RNA.

For isolation of DNA, the pellet was resuspended in 1.50 ml of sterile 0.90% w/v NaCl and centrifuged (Ronbio Scientific) at 10,000 × *g*, 4 °C for 2 min. The resulting pellet was kept at −20 °C for 30 min to increase the recovery of DNA. DNA was isolated using Genelute Bacterial Genomic Kit (Sigma Aldrich) according to the manufacturer instructions.

RNA was isolated using the Total RNA Isolation Kit (Promega, Madison, WI, USA) according to the manufacturer instructions. Each sample of the isolated RNA was treated with Turbo DNA Free Kit (Applied Biosystem, Carlsbad, CA, USA) to get rid of any residual DNA. Each RNA sample was checked for the presence of residual DNA by performing PCR amplification and analyzing by electrophoresis in 1% w/v agarose gel (FMC BioProducts, Rockland, ME, USA) in 1× Tris–Acetate-EDTA buffer (Sigma) containing 0.50 μg/ml ethidium bromide (Sigma). In case of the presence of positive signals, RNA samples were treated once more with Turbo DNA Free Kit to eliminate the rest of the DNA. RNA was stored at −80 °C.

2.4. Reverse transcription (RT)-PCR

Reverse transcription was performed in 100 μl volume containing 10 μl of the total RNA, 28.50 μl of RNase-free water (Promega), 22 μl of 25 mM $MgCl_2$ (Promega), 20 μl of 1.25 μM dNTP mixture (Promega), 5.0 μl of a random hexamer (Promega), 2 μl of RNase inhibitor (Promega), 10 μl of 10× TaqMan RT Buffer (Promega) and 2.50 μl of Multiscribe Reverse Transcriptase (Promega). The reverse transcription incubation conditions were: pre-incubation at 25 °C for 10 min, reverse transcription at 48 °C for 30 min and finally inactivation of transcription at 95 °C for 5 min. cDNA was stored at −80 °C.

2.5. Pyrosequencing of DNA and cDNA extracted from raw milk and cheese

Tag-encoded amplicon pyrosequencing was conducted for 64 samples, which included DNA and reverse transcribed RNA extracted from raw milk, milk inoculated with the different mixtures of bacteria as described above and cheeses at 7, 28 and 56 days of ripening. Pyrosequencing of tagged amplicons of the V3 and V4 regions of the 16S rDNA and cDNA extracted from milk and cheeses was performed as previously described (Masoud et al., 2011). The sequence reads, were sorted and trimmed using the Pipeline Initial Process at the RDP's Pyrosequencing Pipeline (http://rdp.cme.msu.edu/). The RDP Classifier at the RDP's Pyrosequencing Pipeline was used to assign 16S rRNA gene sequences to the new phylogenetically consistent higher-order bacterial taxonomy with a confidence threshold of 80%. The sequences were also uploaded into the Meta Genome Rapid Annotations using Subsystem Technology (MG-RAST) (http://metagenomics.anl.gov/metagenomics.cgi) to be identified at the species level.

2.6. Quantitative real time (qRT)-PCR

Starter cultures used in production of the raw milk cheeses as described above as well as *E. coli*, *L. innocua* and *S. aureus*, which were added to some cheeses, were quantified in raw milk and cheeses by qRT-PCR. Both DNA and RNA, which were extracted from raw milk and cheeses, were amplified. qRT-PCR was set up in MicroAmp™Fast Optical 96-well Reaction Plates with Barcode, 0.10 ml (Applied Biosystems). Primers and probes for target genes were used for each bacterium (Table S2). With the exception of *B. linens*, TaqMan-Probe assays was performed to quantify bacterial species in raw milk and cheeses, amplification of DNA and cDNA was done in triplicates in a total volume of 20 μl containing 5 μl template, 10 μl of TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and 5 μl of primer-probe mix containing 900 nM of each primer and 200 nM probe (Applied Biosystems). Amplification of PCR products was done with a 7500 Fast Real Time PCR System (Applied Biosystems) using the following program: 1 cycle at 95 °C for 20 s and 40 cycles at 95 °C for 15 s followed by 60 °C for 30 s. For *B. linens*, the amplification was done using Power SYBR Green Master Mix (Applied Biosystems) in a reaction mixture of 5 μl template, 10 μl Power SYBR Green Master Mix and 5 μl of primers mix containing 900 nM of each primer. The amplification program of PCR products composed of 1 cycle at 50 °C for 2 min, an activation step at 95 °C for 10 min, 40 cycles at 95 °C for 15 s followed by 60 °C for 1 min and finally 1 cycle at 95 °C for 15 s, 60 °C for 1 min followed by 95 °C for 15 s.

Quantification was performed by interpolation in a standard regression curve of threshold cycle (C_T) values generated from DNA extracted from pure cultures with different concentrations of cells (10^2 to 10^6 cfu/ml). The C_T values were plotted against cell numbers to obtain standard curves.

3. Results

3.1. Characteristics of sequence reads in raw milk and cheeses

A total of 557,515 sequence reads were obtained from the 32 samples of DNA extracted from raw milk and cheeses with a range of 11,507 to 26,721 sequence reads per sample. For the 32 samples of RNA extracted from raw milk and cheeses, the total number of sequence reads was 590,967 with a total number of reads for each sample ranged from 14,595 to 24,213. The average sequence length for all the 16S rDNA and cDNA libraries was 328 bp. In all samples investigated, more than 99% of the sequence reads were found to belong to the bacterial domain.

3.2. Indigenous microbiota of raw milk

The classifications of sequences obtained from the 16S rDNA and cDNA libraries of the non inoculated raw milk, which was used for production of cheese batches 16-3-1, 2, 3 and 4 as well as the non inoculated raw milk used for production of cheese batches 16-4-1, 2, 3 and 4, are shown in Table 1. In the first sample of raw milk, 211 and 93 bacterial species were detected in the 16S rDNA and cDNA libraries, respectively (results not shown). Only the bacterial species that comprised 0.1% or more of the total sequence reads are shown in Table 1. *Streptococcus thermophilus* constituted 43.70 and 42.10% of the total sequence reads in the 16S rDNA and cDNA, respectively. The rest of *Streptococcus* species, which were found in the raw milk in less than 0.1% of the total sequence reads, included *Streptococcus suis*, *Streptococcus parauberis* and *Streptococcus mitis*. *L. lactis* was the next largest species present in the DNA and cDNA of raw milk comprising 19.0 and 26.20% in the 16S rDNA and cDNA libraries, respectively. The remaining sequence reads of the genus *Lactococcus*, which was present in the DNA and cDNA extracted from raw milk in less than 0.1% of the total sequence reads, were *Lactococcus piscium* and *Lactococcus chungangensis*. The other bacterial species, which were detected in percentages of 1–6.50% of the total sequences present in DNA and/or RNA extracted from this sample of raw milk included *B. linens*, *Acinetobacter* sp., *Aeromonas* sp., *Corynebacterium casei*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus sakei*, *L. rhamnosus*, *Marinomonas* sp., *Methylobacterium* sp., *Pseudomonas* sp., *Rothia mucilaginosa* and *Staphylococcus saprophyticus*.

Phylogenetic classification of sequences obtained from DNA and RNA extracted from the second sample of raw milk showed that the total numbers of bacterial species present in the 16S rDNA and cDNA libraries were 256 and 98, respectively (results not shown). Similar to the first raw milk sample, *St. thermophilus* was the main bacterial species in both the DNA and RNA comprising percentages of 46.70 and 53.90 of

Table 1
Phylogenetic classification of sequences obtained from the 16S rDNA and cDNA libraries extracted from the non inoculated raw milk, which was used to produce cheese batches 16-3-1, 2, 3 and 4 treated with 39 °C (RM1) and from the non inoculated raw milk used to produce cheeses batches 16-4-1, 2, 3 and 4 treated with 50 °C (RM2).

Bacteria	Percentages (%) of bacterial species to the total sequence reads in the 16S rDNA and cDNA libraries of RM1		Percentages (%) of bacterial species to the total sequence reads in the 16S rDNA and cDNA libraries of RM2	
	DNA (11,970)*	cDNA (15,102)*	DNA (12,997)*	cDNA (16,194)*
Actinomyces radicidentis	0.5	1.4	0	0.6
Acinetobacter sp.	2.9	0.6	1.6	0.3
Alistipes finegoldii	0.1	0	0	0
Anaerococcus octavius	0.1	0	0	0
Aerococcus sp.	0.8	0.4	1.6	0.2
Aeromonas hydrophila	0.3	0.1	0	0
Aeromonas popoffii	0.8	0.8	0	0
Aeromonas sp.	2	1.4	0.9	0.5
Bacillus subtilis	0.1	0	0	0
Bifidobacterium pseudolongum	0.4	0.1	0	0.1
Bifidobacterium sp.	0	0	1.1	0
Brevibacterium linens	1.9	3.2	2.2	2.8
Caulobacter crescentus	0.1	0	0	0
Chryseobacterium piscium	0.1	0	0	0
Corynebacterium casei	2.3	1	3.8	0.6
Carnobacterium sp.	0.1	0	0	0
Empedobacter brevis	0.2	0	0	0
Enterobacter cloacae	0.4	0.4	0	0
Enterococcus faecalis	0.1	0.4	0.9	0.3
Escherichia coli	0.5	0.3	2	0.9
Facklamia tabacinasalis	0.3	0.1	0	0
Jeotgalicoccus psychrophilus	0.1	0.3	0	0
Kurthia gibsonii	0.1	0	0	0
Lactobacillus casei	3.2	1.4	1.9	2.6
Lactobacillus helveticus	0.9	0.5	0	0
Lactobacillus plantarum	1.3	0.2	1.5	0.3
Lactobacillus sakei	0.8	0	1.9	0
Lactobacillus rhamnosus	2.4	1.6	2.1	1.7
Lactococcus lactis	19	26.2	22.3	19.4
Leptotrichia hofstadii	0.2	0	0	0
Macrococcus equipercicus	0.1	0	0	0
Marinomonas	3.7	1.3	0	0
Methylobacterium extorquens	2.5	1.4	0	0
Meiothermus sp.	0.1	0	0	0
Methylobacterium sp.	0	0	1.7	5.5
Paracoccus carotinifaciens	0.1	0.3	0	0
Pediococcus pentosaceus	0.2	0.1	0	0
Pseudoalteromonas agarivorans	1.6	0.8	0	0
Pseudomonas gessardii	1.7	0.9	0	0
Pseudomonas sp.	0	0	1	0.5
Rothia mucilaginosa	0	6.5	0	5.1
Ruminococcus flavefaciens	0.1	0	0	0
Staphylococcus saprophyticus	2.8	5.4	1.3	2
Staphylococcus succinus	0	0	2.7	2
Streptococcus thermophilus	43.7	42.1	46.7	53,9
Stenotrophomonas maltophilia	0.2	0	0	0
Weissella hellenica	0.1	0.2	0.9	0.3

* Total number of sequence reads in the 16S rDNA and cDNA libraries.

the total sequence reads for DNA and cDNA, respectively (Table 1). *L. lactis* was present in 22.30 and 19.40% of the total sequence reads obtained from the 16S rDNA and cDNA libraries, respectively. *Acinetobacter* sp. *Aerococcus* sp., *B. linens*, *Bifidobacterium* sp., *C. casei*, *Lb. casei*, *Lb. plantarum*, *Lb. rhamnosus*, *Methylobacterium* sp., *Pseudomonas* sp., *R. mucilaginosa*, *S. saprophyticus* and *Staphylococcus succinus* were found in the 16S rDNA and cDNA libraries of raw milk comprising percentages of 1–5.50% of the total sequence reads.

3.3. Microbial communities in raw milk cheeses

The phylogenetic classification of sequences obtained from DNA and RNA extracted from milk and cheese batches 16-3-1, 2, 3 and 4, cooked at 39 °C (slow acidification rate) are shown in Tables 2 and 3. It was observed that *L. lactis*, which was added as starter culture to the 4 batches of cheese, was the main bacterial species present in the inoculated milk and in cheeses at later stages of ripening comprising 49.30 to 95.40% and 69.80 to 90.80% of the total sequence reads in the 16S rDNA and cDNA libraries as shown in Tables 2 and 3, respectively. *B. linens* and *M. lacticum* were detected in both DNA and RNA extracted from the 4 cheese batches with higher percentages in batches 16-3-3 and 4 to which they were added. The remaining bacteria, which were present in both 16S rDNA and cDNA libraries in most cheese samples, included *St. thermophilus*, *Lb. casei*, *Lb. rhamnosus*, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, *Methylobacterium sp.*, *S.*

Table 2
Phylogenetic classification of sequences obtained from DNA extracted from samples of cheese batch 16-3-1 in the inoculated milk (M), at 7 days (7D) and 28 days (28D) of ripening, cheese 16-3-2 at 7D, 28D and 56D of ripening and cheeses 16-3-3 and 4 in the inoculated milk, 7D, 28D and 56D of ripening. All cheeses were cooked at 39 °C.

Bacteria	Percentages (%) of bacterial species to the total sequence reads in the 16S rDNA libraries													
	M 16-3-1	7D 16-3-1	28 D 16-3-1	7D 16-3-2	28D 16-3-2	56D 16-3-2	M 16-3-3	7D 16-3-3	28D 16-3-3	56D 16-3-3	M 16-3-4	7D 16-3-4	28D 16-3-4	56D 16-3-4
Bifidobacterium sp.	0	0	0	0.1	0	0.1	0	0	2	0.2	0	0	0.2	0.2
Brevibacterium linens	0	0.2	0.6	0	0.1	0.2	14.9	5.7	7.2	7.3	10.2	4.3	3.9	2.6
Corynebacterium stationis	0.1	0.1	0	0	0	0	0.1	0	0	0.3	0.1	0.1	0	0
Corynebacterium variabile	0.1	0	0	0.1	0.1	0	0.1	0	0	0	0	0	0	0
Corynebacterium xerosis	0.2	0	0	0.1	0.1	0	0	0	0.6	0	0.1	0.3	0	0
Escherichia coli	0.1	0.4	0	10.3	1.6	1.6	0	0.1	0.2	0.1	2.4	8.2	1.7	0.9
Enterococcus italicus	0	0	0.2	0.1	0.1	0	0	0	0.2	0	0.1	0	0	0
Streptococcus thermophilus	2.2	0.6	0.8	3.5	0.6	0.5	0.1	0.6	1	0.6	4.8	6.6	3.8	0.8
Lactobacillus acidipiscis	0	0	1.0	0	2.3	0.2	0	0	0.8	1.4	0	0	0.1	0.1
Lactobacillus casei	0.2	0.4	2.3	0	0.7	4.4	0.4	0	3.1	4.2	0.1	0.5	0.2	1.1
Lactobacillus coryniformis	0	0	0	0	0	0	0	0.1	2.8	2.4	0.4	0	0.8	1.8
Lactobacillus fuchuensis	0	0	0	0	0	0	0	0	3.8	1.1	0.2	0	0.1	0.1
Lactobacillus helveticus	0	0	0.2	0	0.6	0	0	0	0	0	0	0	0	0
Lactobacillus plantarum	0	0	0	0	0.5	0.5	0	0.2	1.9	2	02	0.1	0.3	0.1
Lactobacillus rhamnosus	0.2	0.1	0.4	0.5	2.9	4.4	0.2	0.6	3.7	1.7	0.2	1.3	0.3	1.9
Lactobacillus sakei	0	0	0.6	0.2	0.3	3.2	0.2	0.2	1.5	0.9	0	0.4	0.2	0.9
Lactococcus chungangensis	19.4	0.4	1.8	0.5	0.1	0.1	5	0.4	0.3	0.2	5.5	0.3	0.1	0.1
Lactococcus lactis	76.2	95.4	80	81.6	74.3	79.9	72.2	80.6	49.3	65	61.8	71.4	83.7	83
Leuconostoc mesenteroides	0.4	0.1	0.7	0.2	0	0	0.2	0.3	0.8	0.2	0.7	0.1	0	0.5
Listeria innocua	0	0	0	0.4	0.5	0.1	0	0	0	0	1.9	0.1	0	0
Methylobacterium sp.	0.1	1.3	0.4	0	0	0	0.9	0.3	0.1	0.3	0.3	0.4	0.2	0.1
Microbacterium lacticum	0	0	0	0	0	0	5.1	0.5	0.4	1	4.2	0.6	0.3	0.2
Pediococcus pentosaceus	0.1	0.2	0.7	0.3	1.2	0.1	0	0.1	3.2	3.7	0.2	0.2	0.5	0.2
Pseudomonas fluorescens	0.1	0	0.1	0	0	0	0	0	0	0	0	0	0	0
Rothia mucilaginosa	0.3	0.1	2.8	0	0	0.2	0	0	0	0	0	0.1	0.1	0
Staphylococcus aureus	0	0	0	0.6	0.5	0.1	0	0	0	0	0.2	0.2	0.3	0.1
Staphylococcus saprophyticus	0.1	0.5	3.9	0.8	5.3	1.8	0.4	10.2	8.3	6.4	2.4	1.1	0.4	1.3
Staphylococcus succinus	0	0	0.1	0.2	1.6	0.2	0	0	0.3	0.2	0.1	0.3	0.4	0.5
Staphylococcus xylosus	0.2	0.1	0.3	0.2	1.3	0.3	0	0.1	0.6	0	0.1	0.7	0.2	0.8
Weissella hellenica	0	0	2.8	0.1	5.2	1.3	0	0	7.6	0.3	0.2	0.7	0.4	0.5
Weissella paramesenteroids	0	0.1	0.3	0.2	0.1	0.8	0.2	0	0.3	0.5	1.8	2	1.8	2.2

Table 3
Phylogenetic classification of sequences obtained from RNA extracted from samples of cheese batch 16-3-1 in the inoculated milk (M), at 7 days (7D) and 28 days (28D) of ripening, cheese 16-3-2 at 7D, 28D and 56D of ripening and cheeses 16-3-3 and 4 in the inoculated milk, 7D, 28D and 56D of ripening. All cheeses were cooked at 39 °C.

Bacteria	Percentages (%) of bacterial species to the total sequence reads in the 16S cDNA libraries													
	M 16-3-1	7D 16-3-1	28 D 16-3-1	7D 16-3-2	28D 16-3-2	56D 16-3-2	M 16-3-3	7D 16-3-3	28D 16-3-3	56D 16-3-3	M 16-3-4	7D 16-3-4	28D 16-3-4	56D 16-3-4
Bifidobacterium sp.	0	1.7	0	0	0.1	0.4	0	0.1	0	0	0	0.1	0	0.1
Brevibacterium linens	2.2	2.3	2.3	1.2	1.5	0.9	4.3	2.4	1.1	1.4	4.7	2.3	3.6	3.2
Corynebacterium sp.	0.4	0	0.2	0	0.1	0	0	0	01	0.1	0.1	0.1	0	0.1
Escherichia coli	0.8	0.8	0.8	2.9	2.4	2.2	2.1	0.1	0.7	0	1	3.8	1.6	2
Enterococcus faecalis	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0
Lactobacillus casei	2.9	1.9	1.2	3.6	2.4	1.8	3.1	3.2	1.9	0.8	2.9	3.6	2.1	1.4
Lactobacillus plantarum	1.1	0	0	0	0	0	0	0	0.2	0.1	0	0.5	0	0
Lactobacillus rhamnosus	1.9	1.4	0.8	2	1.8	3.5	2.9	0	1.8	1.4	4.9	5.4	3.7	3.9
Lactobacillus sakei	0.6	0.8	0.7	0	0	0	2.4	0	0	0	0	1.3	0.4	0
Lactococcus lactis	69.8	85.1	90.8	86.6	87	80.6	73.1	91	90.5	88.4	77.7	70.9	81.7	83.7
Leuconostoc mesenteroides	0	0	0	0.4	0.3	0.2	0	0	0.2	0.1	0	0.6	0	0
Methylobacterium sp.	2.9	1.4	1.2	0	0	0	1.7	0	0.4	0.2	0.8	0.9	0.1	0.3
Microbacterium lacticum	0	0	0	0	0	0	2.3	1.7	0.6	1	3.7	1.9	2.2	0.8
Pediococcus pentosaceus	0.2	0.2	0.1	0.2	0.4	0.7	0	0.2	0.1	0	0	0.7	0.2	0.1
Pseudomonas sp.	0.2	0	0	0	0	0	0.3	0	0.2	0.2	0.3	0	0	0
Rothia mucilaginosa	9.5	3.3	0.3	0	0	2.1	2.3	0	0	0	0.2	1.2	0	0
Staphylococcus saprophyticus	1	0.7	0.6	0.9	1	0.7	0.8	0.3	0.6	1.3	0.7	2	0.8	0.3
Staphylococcus succinus	0	0	0	0	0	0	0	0	0	0.7	0	0.3	0	0
Staphylococcus xylosus	0.6	0.4	0.3	0.4	0.3	0.1	0	0	0.1	1.6	0	1.8	0.7	0.3
Streptococcus thermophilus	5.9	1.7	0.7	1.1	2.4	6.6	4.6	1	0.3	2.7	2.9	0.7	1.2	2.6
Weissella hellenica	0	0	0	0.4	0.2	0.1	0.1	0	0.2	0	0	0.9	1.3	0.9
Weissella paramesenteroids	0	0	0	0.3	0.1	0.1	0	0	0.1	0	0	1	0.4	0.3

saprophyticus and *Staphylococcus xylosus*. *E. coli* was also detected in both DNA and RNA extracted from milk and all samples of cheeses to which it was added as well as in most samples of milk and cheeses to which this bacterium was not added. However, the percentages of *E. coli* from the total sequence reads were less than 1% in milk and cheeses to which *E. coli* was not added. Furthermore, in the inoculated milk and cheeses to which *E. coli* was added i.e. 16-3-2 and 4, the percentages of *E. coli* showed an increase at 7 days of ripening but decreased drastically later in the ripening (Tables 2 and 3). On the other hand, *S. aureus* was only detected in the DNA extracted from the inoculated milk and cheeses 16-3-2 and 4 to which it was added but not in the RNA extracted from same samples suggesting that this bacterium was metabolically inactive. With the exception of DNA extracted from cheese 16-3-4 at 28 days of ripening, *L. innocua* was detected in the DNA extracted from cheeses 16-3-2 and 4 at the different stages of ripening but not in the RNA extracted from same samples. Some bacterial species like *L. chungangensis*, *Lactobacillus helveticus*, *Lactobacillus coryniformis* and *Lactobacillus acidipiscis* were only detected in the 16S rDNA libraries but not in the cDNA of the same samples suggesting a metabolic inactive state.

For cheese batches 16-4-1, 2, 3 and 4, which were cooked at 50 °C (fast acidification rate) and to which both *L. lactis* and *Lb. rhamnosus* were added, *St. thermophilus*, *L. lactis*, *Lb. casei* and *Lb. rhamnosus* were the main bacteria detected in both DNA and RNA extracted from all samples of inoculated milk and cheeses at different stages of ripening with *St. thermophilus* showing the highest percentages (Tables 4 and 5). *B. linens* and *M. lacticum* were also detected in both 16S rDNA and cDNA libraries of inoculated milk and cheeses but at lower percentages than in cheeses treated with 39 °C.

S. saprophyticus, *Staphylococcus epidermidis* and *Staphylococcus cohnii* were also detected in the majority of the 16S rDNA and cDNA libraries extracted from inoculated milk and cheeses but at lower percentages. A similar behavior of *E. coli* in cheeses 16-3-1, 2, 3 and 4 was observed in cheeses 16-4-1, 2, 3 and 4 but with lower percentages (Tables 4 and 5). Again, *S. aureus* and *L. innocua* were only detected in the DNA extracted from inoculated milk and cheeses to which they were added but not in the RNA extracted from same samples.

3.4. Quantification of bacteria in raw milk and cheeses by qRT PCR

Quantification of the starter cultures and the model bacteria added to cheeses was determined by qRT-PCR targeting specific gene for each bacterium. The quantities of *L. lactis*, *Lb. rhamnosus*, *B. linens*, *M. lacticum*, *E. coli*, *L. innocua* and *S. aureus* in raw milk and cheeses 16-3-1, 2, 3, 4, cooked at 39 °C, which were detected in the extracted DNA and RNA are shown in Figs. 1 and 2, respectively. For *L. lactis*, the number of colony forming units detected in the raw milk was in averages of 5.0×10^3 cfu/g and 2.0×10^3 cfu/g in DNA and RNA, respectively. In the inoculated milk and in the 4 cheese batches at all stages of ripening, to which *L. lactis* was added as a starter culture, *L. lactis* was present in quantities ranged from 2.50×10^7 to 8.80×10^8 cfu/g and 2.40×10^5 to 7.10×10^5 cfu/g, in DNA and RNA, respectively. After 7 and 14 days of ripening, the numbers of colony forming units increased to more than 10^7 cfu/g, which decreased again at later stages of ripening reaching an amount of about 1.40×10^4 cfu/g after 56 days of ripening (Fig. 2). *Lb. rhamnosus* was not added as a starter culture to cheese batches 16-3-1, 2, 3 and 4, but it was detected in the DNA and RNA extracted from raw milk as well as in all cheeses at the different stages of ripening (Figs. 1 and 2). The number of colony forming units of *Lb. rhamnosus* found in both DNA and RNA extracted from raw milk was about 2.0×10^2 cfu/g, which increased to a range of 4.0×10^4 to 5.0×10^5 cfu/g in the DNA and to about 8.0×10^4 cfu/g in the RNA after 7 days of cheeses ripening and decreased again later in the ripening process. The ripening starter cultures *B. linens* and *M. lacticum*, which were added to cheese batches 16-3-3 and 4 but not to cheeses 16-3-1 and 2, were detected in both DNA and RNA extracted from the raw milk and the four cheese batches (Figs. 1 and 2). The numbers of colony forming units of *B.*

Table 4
Phylogenetic classification of sequences obtained from DNA extracted from samples of cheese batch 16-4-1 in the inoculated milk (M), at 7 days (7D) and 28 days (28D) of ripening and cheeses 16-3-2, 3 and 4 in the inoculated milk, 7D, 28D and 56D of ripening. All cheeses were cooked at 50 °C.

Bacteria	Percentages (%) of bacterial species to the total sequence reads in the 16S rDNA libraries														
	M 16-4-1	7D 16-4-1	28 D 16-4-1	M 16-4-2	7D 16-4-2	28D 16-4-2	56D 16-4-2	M 16-4-3	7D 16-4-3	28D 16-4-3	56D 16-4-3	M 16-4-4	7D 16-4-4	28D 16-4-4	56D 16-4-4
Bifidobacterium sp.	0	0	0	0.1	0	0.6	0.1	0.1	0	0	0.1	0.8	0	0	0
Brevibacterium linens	0.9	1.2	0.8	1.1	0.8	1.2	0.3	7.1	3.6	2.5	1.4	6.7	5.3	2.2	0.6
Corynebacterium suicordis	0	0	0.2	0.1	0	0	0	0.2	0	0	0.1	0.1	0.2	0	0.1
Corynebacterium xerosus	0	0	0	0.1	0	0	0	0	0	0	0	0.2	0	0	0
Escherichia coli	0.7	0.1	0.1	3.4	0.4	0.3	0.1	0.6	0	0.1	0	0.4	1.4	0.7	0.5
Enterococcus italicus	0	0.1	0.2	0	0	0.1	0	0	0	0.2	0	0.1	0	0	0
Lactobacillus acidipiscis	0	0	0	0.1	0.1	0.6	0.7	0.3	0	0	0	0	1.3	0.4	0
Lactobacillus casei	2.2	3.1	2.7	2.1	0.1	4.8	2.1	0.8	1.9	5.3	3.4	1.5	5.8	2.7	1.9
Lactobacillus plantarum	0.9	0.5	0.2	0.2	0	1.5	0.2	0.4	0	0	0	0	0	0	0
Lactobacillus rhamnosus	19.9	21	19.6	13.8	13.6	14.6	7.4	11.8	10.3	16.8	8	3.5	12.7	10.7	7.4
Lactococcus chungangensis	5.4	0.1	1.8	0.8	0.1	0	0.1	0.8	0.4	0.3	0.2	5.8	0.3	0.1	0.1
Lactococcuss lactis	32.3	32.7	16.9	28.9	16.4	9.5	8.9	29.7	27.3	17.2	5.2	31.5	20.4	15.5	5.3
Leuconostoc mesenteroides	0	0	0	0.1	1	0	0	0.1	0	0	0	0.2	0.1	0	0
Listeria innocua	0	0	0	0.8	0.1	0.1	0.1	0	0	0	0	0.1	0	0.1	0
Methylobacterium sp.	0.6	0.1	0.3	0	0	0	0	0	0.3	0.2	0	0	0	0	0
Microbacterium lacticum	0.3	0.4	1.3	0.9	0.5	0.3	0.1	0.3	0.5	1.6	1.5	2.1	1.8	0.7	0.3
Pediococcus pentosaceus	0	0	0.2	0.2	0.4	0.1	0.2	0.1	0	0	0.2	0	0.1	0	0
Pseudomonas lundensis	0.1	0	0	0.1	0	0	0	0.1	0	0	0	0.1	0	0	0
Rothia mucilaginosa	0.1	0.1	0.1	0.2	0	0	0	0	0.6	0.1	0	0	0	0	0
Staphylococcus aureus	0	0	0	1.7	0.4	0.8	0.1	0.1	0	0	0	0.7	0.2	0.1	0.1
Staphylococcus cohnii	0.2	0.2	0	0	0.1	0	0	0	0.7	0.8	0.4	1.5	0.9	0.3	0.6
Staphylococcus epidermidis	0.2	0.2	0.1	0	0.9	0.5	0	0.1	1	1	1.2	0.6	1.7	0.8	0.6
Staphylococcus saprophyticus	0.4	3.4	4.2	4.5	1	2.4	0.6	0.1	1.6	1.3	3.5	1.6	7	3.2	2.6
Streptococcus thermophilus	35.8	36.6	51.2	40.5	65.1	62.1	76.7	46.6	51.3	52.3	74.8	42.4	40.5	62.3	79.7
Weissella hellenica	0	0.1	0.1	0.1	0	0	0	0	0	0	0	0	0.2	0.1	0.1
Weissella paramesenteroids	0	0.1	0	0.2	0.1	0.5	2.3	0.7	0.5	0.3	0	0.1	0.1	0.1	0.1

Table 5
Phylogenetic classification of sequences obtained from RNA extracted from samples of cheese batch 16-4-1 in the inoculated milk (M), at 7 days (7D) and 28 days (28D) of ripening and cheeses 16-3-2, 3 and 4 in the inoculated milk, 7D, 28D and 56D of ripening. All cheeses were cooked at 50 °C.

Bacteria	Percentages (%) of bacterial species to the total sequence reads in the 16S cDNA libraries														
	M 16-4-1	7D 16-4-1	28 D 16-4-1	M 16-4-2	7D 16-4-2	28D 16-4-2	56D 16-4-2	M 16-4-3	7D 16-4-3	28D 16-4-3	56D 16-4-3	M 16-4-4	7D 16-4-4	28D 16-4-4	56D 16-4-4
Brevibacterium linens	1	0.6	0.3	0.6	0.3	0.2	1.5	3.1	3.2	2.1	1.2	3.2	3.1	2	1.2
Corynebacterium xerosis	0.2	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0
Escherichia coli	0.3	0.6	0.1	0.4	1.2	0.7	0.5	0.7	0.3	0.2	0.1	0.2	1	0.4	0.2
Lactobacillus casei	6.2	5	4.5	5.1	11.4	3.6	1	10	2.7	2.4	1.8	10.1	5.5	2.4	1.8
Lactobacillus plantarum	0.8	0.7	0.5	0	0	0.2	0.2	0	0	0	0	0	0	0	0
Lactobacillus rhamnosus	14.6	14	18.4	9.7	10.4	9.1	8.3	2.2	7.5	18.3	12.8	2.2	7.4	18.2	12.7
Lactococcus lactis	37.1	43.2	6.6	45.1	14	1.9	1.9	32.4	30.6	22.5	8.1	29.9	29	24.6	8
Methylobacterium sp.	0.3	0	0	0	0.8	0.1	0.5	0.3	0.3	0.2	0	0.5	0.8	0.1	0.3
Microbacterium lacticum	0.2	0.2	0.1	0.3	0.1	0.1	0.1	1.1	0.7	0.5	0.2	13	0.7	0.5	0.3
Pseudomonas sp.	0.2	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0
Rothia mucilaginosa	0.2	0.1	0.1	0.3	0	0	0.8	0.3	0.1	0.1	0	0.1	1	0	0
Staphylococcus cohnii	0.5	0.2	0	0	0	0	0.6	0	0	1.3	0.6	0	0	1.3	0.6
Staphylococcus epidermidis	0.6	0.3	0	0.1	0.2	0.1	0.5	0	0.2	0.7	4.1	0	0.2	0.7	4.1
Staphylococcus saprophyticus	0.8	0.4	0	0.3	0	0.1	0.8	0.6	0.5	1.3	4.6	0.6	0.5	1.4	4.6
Streptococcus thermophilus	36.9	34.7	69.4	37.7	61.6	83.9	83.2	49.2	53.3	50.2	66.5	40.2	50.8	48.4	66.2
Weissella hellenica	0	0	0	0.1	0	0	0.1	0.1	0.5	0	0	0	0	0	0
Weissella paramesenteroids	0.1	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0

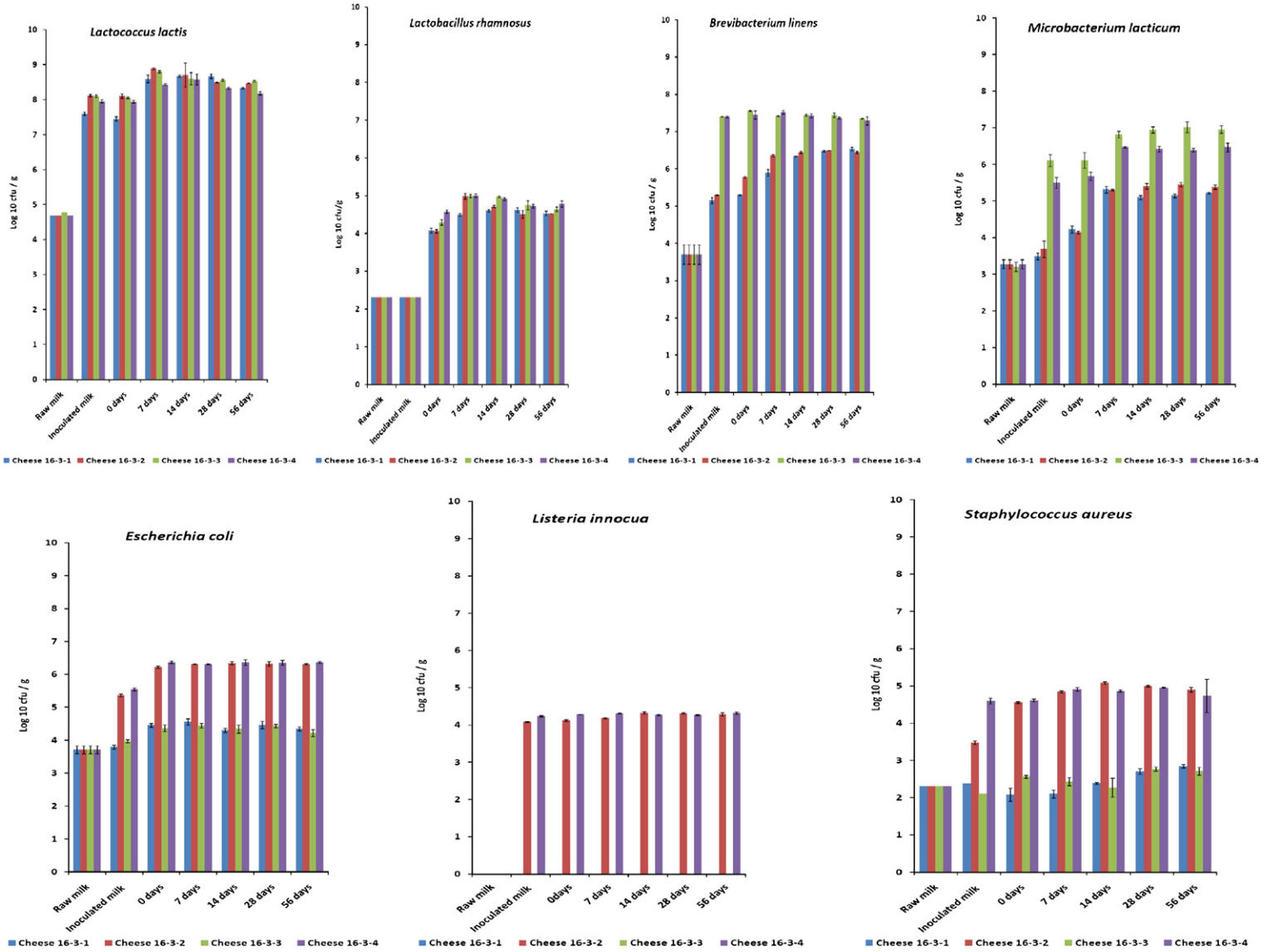

Fig. 1. Quantities of *Lactococcus lactis*, *Lactobacillus rhamnosus*, *Brevibacterium linens*, *Microbacterium lacticum*, *Escherichia coli*, *Listeria innocua* and *Staphylococcus aureus* determined by quantitative real time PCR (qRT-PCR) of DNA extracted from raw milk, inoculated milk, cheeses 16-3, 1, 2,3 and 4 cooked at 39 °C at 0, 7, 14, 28 and 56 days of ripening.

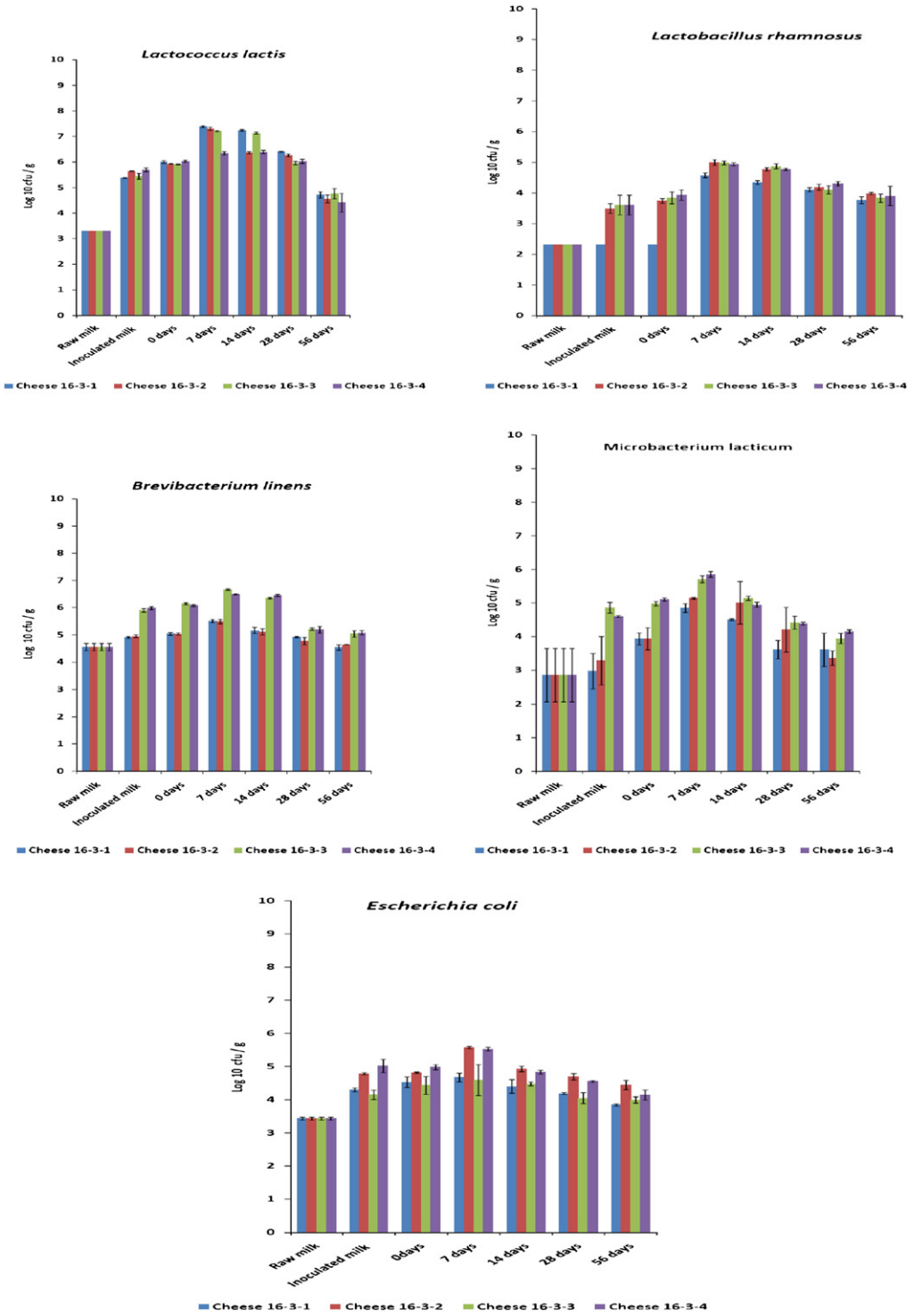

Fig. 2. Quantities of *Lactococcus lactis*, *Lactobacillus rhamnosus*, *Brevibacterium linens*, *Microbacterium lacticum* and *Escherichia coli* determined by quantitative real time PCR (qRT-PCR) of RNA extracted from raw milk, inoculated milk, cheeses 16-3, 1, 2,3 and 4 cooked at 39 °C at 0, 7, 14, 28 and 56 days of ripening.

linens and *M. lacticum* present in the DNA extracted from raw milk were about 5.50×10^3 cfu/g and 1.90×10^3 cfu/g, respectively. In the RNA extracted from raw milk, the numbers of colony forming units of *B. linens* and *M. lacticum* were about 3.60×10^4 and 8.50×10^2 cfu/g, respectively. In cheeses at different stages of ripening, *B. linens* and *M. lacticum* were present in ranges of 2.20×10^5 to 3.70×10^7 cfu/g and 5.50×10^3 to 1.0×10^7 cfu/g, respectively. Both bacteria showed an increase in the numbers of colony forming units in the four batches of cheese after 7 days of ripening and decreased at later ripening stages. With regard to *E. coli*, *L. innocua* and *S. aureus*, which were added to cheese batches 16-3-2 and 4, *E. coli* was detected in the DNA extracted from raw milk as well as in the four batches of cheese in a range of 3.70×10^3 to 2.40×10^6 cfu/g. *E. coli* was also detected in the RNA extracted from raw milk and cheeses 16-3-1, 2, 3 and 4 comprising average 2.80×10^3 cfu/g in the raw milk, which increased later in the ripening process, reaching its peak at 7 days of ripening. On the other hand, *L. innocua* was not detected in DNA extracted from raw milk or cheeses 16-3-1 and 3 to which it was not added. The number of colony forming units of *L. innocua* in the DNA extracted from cheeses 16-3-2 and 4 was in an

average of 1.80×10^4 cfu/g. *S. aureus* was detected in the DNA extracted from raw milk and the four cheese batches at the different stages of ripening comprising about 2.10×10^2 cfu/g in the raw milk and an average of 6.10×10^4 cfu/g in cheeses 16-3-2 and 4 to which it was added. *L. innocua* and *S. aureus* were not detected in any of the RNA extracted from raw milk and cheeses.

A similar behavior of *L. lactis*, *B. linens*, *M. lacticum* and the model bacteria in cheeses 16-3-1, 2, 3 and 4 was observed in cheese batches 16-4-1, 2, 3 and 4 cooked at 50 °C (results not shown) except that the numbers of colony forming units of these bacteria, which were detected in cheeses 16-4-1, 2, 3 and 4, were lower than those found in cheeses 16-3-1, 2, 3 and 4. Unsurprising, *L. rhamnosus*, which was added as a starter culture to cheeses 16-4-1, 2, 3 and 4 showed higher numbers of colony forming units in these cheese batches than in cheese batches 16-3-1, 2, 3 and 4. Furthermore, for *L. lactis*, *Lb. rhamnosus* and *B. linens*, the highest numbers of colony forming units in cheeses 16-4-1, 2, 3 and 4 were obtained at 14 days of cheese ripening, but not at 7 days of ripening as observed for cheeses 16-3-1, 2, 3 and 4.

4. Discussion

Identification of microbial communities in raw milk and cheeses at different stages of ripening was carried out using the culture independent technique pyrosequencing of tagged 16S rRNA gene amplicons. To our knowledge, this is the first study for determination of indigenous microbiota in raw milk using pyrosequencing. The sequence reads in the 16S rDNA libraries of raw milk samples showed a high biodiversity in bacterial species. The numbers of bacterial species detected in DNA extracted from the first and second sample of raw milk were 211 and 256, respectively. On the other hand, the numbers of bacterial species, which were detected in the RNA extracted from the first and second raw milk samples, were reduced to 93 and 98, respectively, suggesting that more than half of the bacterial species present in raw milks were metabolically inactive. Those high numbers of bacterial species in raw milk has not previously been reported by other culture dependent and independent methods (Abriouel et al., 2008; Callon et al., 2007; Hantsis-Zacharov and Halpern, 2007; Holm et al., 2004; Randazzo et al., 2002; Sanjuan et al., 2003), which usually detect only the dominant species. The main bacteria, which were detected in the raw milk samples, included *L. lactis* and *St. thermophilus*. The role of *L. lactis* and *St. thermophilus* in milk acidification and cheese ripening is well documented (Centeno et al., 1996; Cogan et al., 1997; Hannon et al., 2003; Marino et al., 2003; Randazzo et al., 2002, 2010). In both DNA and RNA extracted from raw milk samples the detected *Lactobacillus* species included *Lb. casei*, *Lb. plantarum*, *Lb. rhamnosus* (Table 1), as well as *Lactobacillus coryniformis* and *Lactobacillus delbrueckii*, which were detected in less than 0.1% of the total sequence reads (results not sown). Those bacteria are well known in their contribution to cheese aroma and flavor development (Di Cagno et al., 2010; Randazzo et al., 2007). The bacterial genera *Acinetobacter*, *Actinomyces*, *Aerococcus*, *Aeromonas*, *Arthrobacter*, *Bifidobacterium*, *Brevibacterium*, *Corynebacterium*, *Marinomonas*, *Methylobacterium*, *Pseudomonas*, *Rothia* and *Staphylococcus* were identified in raw milk samples in percentages of 1–6% of the total sequence reads in the 16S rDNA and cDNA libraries. Some of these bacterial genera were detected previously in milk and cheeses and were suggested to cause milk and cheese spoilage (Ercolini et al., 2009; Giannino et al., 2009). The apparent diversity of bacterial species in the inoculated milks and in cheeses at different stages of ripening was observed to decline. It appears that many of the viable bacterial species detected in the raw milk died due to milk acidification, cooking temperature or to other micro environmental conditions during cheese ripening, which might decrease their contribution to cheese flavor. The more intense flavor of raw milk cheeses compared to cheeses produced from pasteurized milk might be due to the fact that milk pasteurization induced partial or total inactivation of indigenous milk enzymes as well as partial denaturation of milk proteins.

The main bacteria detected in cheeses at the different stages of ripening included *Lactobacillus*, *Lactococcus* and *Streptococcus*. In a recent study, pyrosequencing and DGGE analysis of DNA and RNA extracted from Danish raw milk cheeses has also showed that these 3 bacterial genera constituted the main bacteria found at the different stages of ripening (Masoud et al., 2011). *L. lactis*, which was added as a starter culture to all cheeses, was the main bacterial species found in the 16S rDNA and cDNA libraries in cheese batches 16-3-1, 2, 3 and 4. *L. lactis* was also detected in the DNA and RNA extracted from cheese batches 16-4-1, 2, 3 and 4 but at lower percentages. This might be due to the higher cooking temperature used in production of those cheeses i.e. 50 °C. The numbers of colony forming units of *L. lactis* in the milk and cheeses, which were determined by qRT-PCR, showed a decrease in the numbers of colony forming units of *L. lactis* in cheeses treated with 50 °C compared to cheeses treated with 39 °C, which agrees with the pyrosequencing data. In contrast, *S. thermophilus*, was present at higher percentages in cheeses treated with 50 °C than in cheeses treated with 39 °C. It seems that the higher temperature induces growth of *St. thermophilus* in cheeses. *L. casei* and *Lb. rhamnosus* were also detected in the DNA and RNA extracted from milk and all cheeses at different stages of ripening indicating their important roles in cheese ripening. *Lb. rhamnosus* was added to cheese batches 16-4-1, 2, 3 and 4, which explains its higher percentages and numbers in these cheeses than in cheeses 16-3-1, 2, 3 and 4. The remaining bacteria, which were detected in the raw milk as well as in the majority of cheeses, included *B. linens*, *E. coli*, *L. mesenteroides*, *Methylobacterium sp.*, *P. pentosaceus*, *S. saprophyticus*, *S. succinus*, *S. xylosus*, *S. cohnii*, *S. epidermidis*, *W. hellenica* and *W. paramesenteriodes*. Some of these bacterial species might have a positive role on cheese while others might have a negative role on cheeses quality and safety.

For the purpose of investigating the effects of starter cultures, cooking temperatures and acidification rate on growth of pathogenic bacteria in raw milk cheese, the model bacteria *E. coli*, *L. innocua* and *S. aureus* were added to 4 cheese batches. Their occurrence in milk and cheeses was determined by pyrosequencing and qRT-PCR. *E. coli*, which was found in the 16S rDNA and cDNA libraries extracted from raw milk samples, was also detected in all cheeses at different stages of ripening reaching the highest percentages at 7 days of ripening, whereafter it decreased at later stages of ripening. *E. coli* showed the same behavior in milk and cheeses to which *B. linens* and *M. lacticum* were or were not added indicating that these two bacteria did not have inhibitory effect on growth of *E. coli*. In addition, the percentages of *E. coli* in cheeses cooked at 39 °C were higher than those treated with 50 °C indicating that the higher temperature seems to reduce growth of *E. coli* in cheeses. This agrees with the numbers of colony forming units of *E. coli* in raw milk and cheeses determined by qRT-PCR. It has been reported that some strains of *E. coli* are acid-resistant and can withstand pH values as low as 3 (Jordan et al., 1999). Furthermore, *E. coli* was found to survive during the manufacturing process of Camembert, Feta and Cheddar cheeses (Ramsaran et al., 1998; Reitsma and Henning, 1996). *E. coli* can be a food safety hazard, therefore, it is very essential to find preventive means for inhibiting growth and survival of *E. coli* in cheeses.

Pyrosequencing and qRT-PCR showed that *L. innocua* and *S. aureus* were only detected in the DNA extracted from cheeses to which they were added but not in the cDNA extracted from same samples indicating their unviable states. It was observed that *L. innocua* and *S. aureus* showed the same behavior in cheeses with and without the addition of the ripening starters *B. linens* and *M. lacticum*. It appears that *L. innocua* and *S. aureus* were not inhibited by *B. linens* or *M. lacticum*. Inhibition of *L. innocua* and *S. aureus* might be due to acidification or to other environmental conditions during cheese ripening. It has been reported that *L. innocua* was not able to grow in Turkish white cheese in a pH range of 4.92–5.20 (Ozturkoglu et al., 2006). Rogga et al. (2005) found that *L. monocytogenes* was able to grow in soft cheese only at pH above 5.50. In addition, pH values below 5.80 or 6.30 were reported to reduce

growth of *S. aureus* in 2 types of French raw milk semi hard cheeses (Delbes et al., 2006). *Listeria innouca* and *S. aureus* might also be inhibited due to production of antibacterial components by bacteria present in milk and cheeses. Production of antimicrobial compounds mainly, bacteriocins by bacteria like *St. thermophilus* (Ward and Somkuti, 1995), *L. lactis* (Ayad et al., 2001), *Lb. plantarum* (Xie et al., 2011), *Lb. sakei* (Todorov et al., 2011), *B. linens* (Motta and Brandelli, 2008) has been reported.

From the present study, it can be concluded that pyrosequencing of tagged amplicons of the V3 and V4 regions of the 16S rDNA and cDNA in raw milk was able to detect a high diversity of bacterial species. Furthermore, a very good agreement was observed between the pyrosequencing of 16S rDNA and cDNA and qRT-PCR for detection and quantification of starter cultures, *E. coli*, *L. innocua* and *S. aureus* in raw milk and cheeses. *B. linens* and *M. lacticum* seem not to affect growth of *E. coli*, *L. innocua* and *S. aureus* during cheese ripening. *L. innocua* and *S. aureus* were not detected in the 16S cDNA libraries extracted from milk and cheeses to which they were added indicating their death. On the other hand, *E. coli* was found in the 16S rDNA and cDNA libraries extracted from milk and cheeses. More studies are needed to fully understand the occurrence and survival of undesired and pathogenic bacteria during raw milk cheese ripening, which will help in finding means that can prevent survival of such microorganisms in raw milk cheeses.

Supplementary materials related to this article can be found online at doi:10.1016/j.ijfoodmicro.2011.11.014.

Acknowledgments

This study was funded by The Danish Dairy Research Foundation and The Danish Ministry of Food, Agriculture and Fisheries.

References

Abriouel, H., Martin-Platero, A., Maqueda, M., Valdivia, V., Martinez-Bueno, M., 2008. Biodiversity of the microbial community in Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. International Journal of Food Microbiology 127, 200–208.

Acosta-Martinez, V., Dowd, S., Sun, Y., Allen, V., 2008. Tag-encoded pyrosequencing analysis of bacterial diversity in a single soil type as affected by management and land use. Soil Biology and Biochemistry 40, 2762–2770.

Andersson, A.F., Lindberg, M., Jakobsson, H., Backhed, F., Nyren, P., Engstrand, L., 2008. Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS One 3, e2836.

Armougom, F., Bittar, F., Stremler, N., Rolain, J.M., Robert, C., Dubus, J.C., Sarles, J., Raoult, D., La Scola, B., 2009. Microbial diversity in the sputum of a cystic fibrosis patient studied with 16S rDNA pyrosequencing. European Journal of Clinical Microbiology and Infectious Diseases 28, 1151–1154.

Ayad, E.H.E., Verheul, A., Wounters, J.T.M., Smith, G., 2001. Population dynamics of lactococci from industrial, artisanal and non-dairy origin in defined strain starters for Gouda-type cheese. International Dairy Journal 11, 51–61.

Callon, C., Duthoit, F., Delbes, C., Ferrand, M., De Frileux, Y., Le Cremoux, R., Montel, M. C., 2007. Stability of microbial communities in goat milk during a lactation year: molecular approaches. Systematic and Applied Microbiology 30, 547–560.

Casalta, E., Sorba, J.-M., Marina, A., Ogier, J.-C., 2009. Diversity and dynamics of the microbial community during the manufacture of Calenzana, an artisanal Corsican cheese. International Journal of Food Microbiology 133, 243–251.

Centeno, J.A., Cepeda, A., Rodriguez-Otero, J.L., 1996. Lactic acid bacteria isolated from Arzua cow's milk cheese. International Dairy Journal 6, 65–78.

Cogan, T.M., Barbosa, M., Beuvier, E., Bianchi-Salvadori, B., Cocconcelli, P.S., Fernandes, I., Gomez, J., Gomez, R., Kalantzopoulos, G., Ledda, E., Medina, M., Rea, M.C., Rodriguez, E., 1997. Characterization of the lactic acid bacteria in artisanal dairy products. The Journal of Dairy Research 64, 409–421.

Delbes, C., Alomar, J., Chougui, N., Martin, J.F., Montel, M.C., 2006. *Staphylococcus aureus* growth and enterotoxin production during the manufacture of uncooked, semi-hard cheese from cows' raw milk. Journal of Food Protection 69, 2161–2167.

Desmasures, N., Gueguen, M., 1997. Monitoring the microbiology of high quality milk by monthly sampling over 2 years. The Journal of Dairy Research 64, 271–280.

Desmasures, N., Bazin, F., Gueguen, M., 1997. Microbiological composition of raw milk from selected farms in the Camembert region of Normandy. Journal of Applied Microbiology 83, 53–58.

Di Cagno, R., De Pasquale, I., De Angelis, M., Calasso, M.P., Buchin, S., Gobbetti, M., 2010. Contribution of selected adjunct or attenuated non-starter lactobacilli to ripening of Italian caciotta cheese. Australian Journal of Dairy Technology 65, 189–191.

Dowd, S.E., Sun, Y., Secor, P.R., Rhoads, D.D., Wolcott, B.E., James, G.A., Wolcott, R.D., 2008. Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosomal shotgun sequencing. BMC Microbiology 8, 43.

Duthoit, F., Godon, J.J., Montel, M.C., 2003. Bacterial community dynamics during production of registered designation of origin Salers cheese as evaluated by 16S rRNA gene single-strand conformation polymorphism analysis. Applied and Environmental Microbiology 69, 3840–3848.

Eppert, I., Valde-Stauber, N., Gotz, H., Busse, M., Scherer, S., 1997. Growth reduction of *Listeria* spp. Caused by undefined industrial red smear cheese cultures and bacteriocin-producing *Brevibacterium linens* as evaluated in situ on soft cheese. Applied and Environmental Microbiology 63, 4812–4817.

Ercolini, D., Russo, F., Ferrocino, I., Villani, F., 2009. Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. Food Microbiology 26, 228–231.

Giannino, M.L., 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. International Journal of Food Microbiology 130, 188–195.

Hannon, J.A., Wilkinson, M.G., Delahunty, C.M., Wallace, J.M., Morrissey, P.A., Beresford, T.P., 2003. Use of autolytic starter systems to accelerate the ripening of cheddar cheese. International Dairy Journal 13, 313–323.

Hantsis-Zacharov, E., Halpern, M., 2007. Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. Applied and Environmental Microbiology 73, 7162–7168.

Holm, C., Jepsen, L., Larsen, M., Jespersen, L., 2004. Predominant microflora of downgraded Danish bulk tank milk. Journal of Dairy Science 87, 1151–1157.

Humblot, C., Guyot, J.P., 2009. Pyrosequencing of tagged 16SrRNA gene amplicons for rapid deciphering of the microbiomes of fermented foods such as pearl millet slurries. Applied and Environmental Microbiology 75, 4354–4361.

Jordan, K.N., Oxford, L., O'Byrne, C.P., 1999. Survival of low-pH stress by *Escherichia coli* O157: H7: correlation between alterations in the cell envelope and increased acid tolerance. Applied and Environmental Microbiology 65, 3048–3055.

Larsen, N., Vogensen, F.K., Van Den Berg, F.W.J., Nielsen, D.S., Andreasen, A.F., Pedersen, B.K., Abu-Al-Soud, W., Sørensen, S.J., Hansen, H.L., Jakobsen, M., 2010. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. PLoS One 5, e9085.

Lindqvist, R., Sylven, S., Vågsholm, M., 2002. Quantitative microbial risk assessment exemplified by *Staphylococcus aureus* in unripened cheese made from raw milk. International Journal of Food Microbiology 78, 155–170.

Marino, M., Maifreni, M., Rondinini, G., 2003. Microbiological characterization of artisanal Montasio cheese: analysis of its indigenous lactic acid bacteria. FEMS Microbiology Letters 229, 133–140.

Masoud, W., Takamiya, M., Vogensen, F.K., Lillevang, S., Abu Al-Soud, W., Sørensen, S.J., Jakobsen, M., 2011. Characterization of bacterial populations in Danish raw milk cheeses made with different starter cultures by denaturating gradient gel electrophoresis (DGGE) and pyrosequencing. International Dairy Journal 21, 142–148.

Meyer-Broseta, S., Diot, A., Bastian, S., Riviere, J., Cerf, O., 2003. Estimation of low bacterial concentration: *Listeria monocytogenes* in raw milk. International Journal of Food Microbiology 80, 1–15.

Millet, L., Saubusse, Didienne, R., Tessier, L., Montel, M.C., 2006. Control of *Listeria monocytogenes* in raw-milk cheeses. International Journal of Food Microbiology 108, 105–114.

Morea, M., Baruzzi, F., Cocconcelli, P.S., 1999. Molecular and physiological characterization of dominant bacterial populations in traditional Mozzarella cheese processing. Journal of Applied Microbiology 87, 574–582.

Motta, A.S., Brandelli, A., 2008. Properties and antimicrobial activity of the smear surface cheese coryneform bacterium *Brevibacterium linens*. European Food Research and Technology 227, 1299–1306.

Ozturkoglu, S., Gurakan, G.C., Alpas, H., 2006. Behavior and control of *Listeria innocua* during manufacture and storage of Turkish white cheese. European Food Research and Technology 222, 614–621.

Ramsaran, H., Chen, J., Brunke, B., Hill, A., Griffiths, M.W., 1998. Survival of bioluminescent *Listeria monocytogenes* and *Escherichia coli* O157: H7 in soft cheeses. Journal of Dairy Science 81, 1810–1817.

Randazzo, C.L., Torriani, S., Akkermans, A.D.L., de Vos, W.M., Vaughan, E.E., 2002. Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. Applied and Environmental Microbiology 68, 1882–1892.

Randazzo, C.L., De Luca, S., Todaro, A., Restuccia, C., Lanza, C.M., Spagna, G., Caggia, C., 2007. Preliminary characterization of wild lactic acid bacteria and their abilities to produce flavour compounds in ripened model cheese system. Journal of Applied Microbiology 103, 427–435.

Randazzo, C.L., Pitino, I., Ribbera, A., Caggia, C., 2010. Pecorino Crotonese cheese: study of bacterial population and flavour compounds. Food Microbiology 27, 363–374.

Reitsma, C.J., Henning, D.R., 1996. Survival of enterohemorrhagic *Escherichia coli* O157: H7 during the manufacture of cheddar cheese. Journal of Food Protection 59, 460–464.

Rogga, K.J., Samelis, J., Kakouri, A., Katsiari, M.C., Savvaidis, I.N., Kontominas, M.G., 2005. Survival of *Listeria monocytogenes* in Galotyri, a traditional Greek soft acid-curd cheese, stored aerobically at 4 °C and 12 °C. International Dairy Journal 15, 59–67.

Roh, S.W., Kim, K.H., Nam, Y.D., Chang, H.W., Park, E.J., Bae, J.W., 2010. Investigation of archaeal and bacterial diversity in fermented seafood using barcoded pyrosequencing. The ISME Journal 4, 1–16.

Rudolf, M., Scherer, S., 2001. High incidence of *Listeria monocytogenes* in European red smear cheese. International Journal of Food Microbiology 63, 91–98.

Sanjuan, S., Rua, J., Garcia-Armesto, M.R., 2003. Microbial flora of technological interest in raw ovine milk during 6 °C storage. International Journal of Dairy Technology 56, 143–148.

Todorov, S.D., Rachman, C., Fourrier, A., Dicks, L.M.T., van Reenen, C.A., Prevost, N., Dousset, X., 2011. Characterization of bacteriocin produced by *Lactobacillus sakei* R1333 isolated from smoked salmon. Anaerobe 17, 23–31.

Verdier-Metz, I., Michel, V., Delbes, C., Montel, M.C., 2009. Do milking practices influence the bacterial diversity of raw milk? Food Microbiology 26, 305–310.
Ward, D., Somkuti, G.A., 1995. Characterization of a bacteriocin produced by *Streptococcus thermophilus* St 134. Applied Microbiology and Biotechnology 43, 330–335.
Xie, Y., An, H.R., Hao, Y.L., Qin, Q.Q., Huang, Y., Lou, Y.B., Zhang, L.B., 2011. Characterization of an anti-Listeria bacteriocin produced by *Lactobacillus plantarum* LB-B1 isolated from koumiss, a traditionally fermented dairy product from China. Food Control 22, 1027–1031.