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Characterization of bacterial populations in Danish raw milk cheeses made with different starter cultures by denaturating gradient gel electrophoresis and pyrosequencing

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ABSTRACT

The bacterial populations in Danish raw milk cheeses were identified using denaturating gradient gel electrophoresis (DGGE) of PCR amplicons of the V3 region of the 16S rRNA gene and pyrosequencing of tagged amplicons of the V3 and V4 regions of the 16S rRNA gene. Both DNA and RNA extracted from cheeses were studied in order to determine the metabolically active bacteria. The main bacteria, which included *Lactococcus*, *Lactobacillus* and *Streptococcus*, were detected by pyrosequencing and DGGE in both 16S rDNA and cDNA obtained from cheeses indicating their viability and contribution to cheese ripening. Other bacteria like *Corynebacterium*, *Halomonas*, *Pediococcus*, *Micrococcus* and *Staphylococcus*, which were encountered in some cheese samples at low percentages compared with the total bacterial populations, were only detected by pyrosequencing. 16S rRNA gene pyrosequencing is an efficient method for deep sequencing of microbial communities and it expands our knowledge of the bacterial diversity in raw milk cheese.

1. Introduction

The production of raw milk cheeses has long been known in Europe, particularly in France, Italy and Switzerland. Raw milk cheeses are often characterized by richer and stronger flavor intensity than cheeses made from pasteurized milk (Buchin et al., 1998) and they are seen as more natural. The indigenous microbiota of raw milk include among others *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. (Casalta, Sorba, Marina, & Ogier, 2009; Desmasures, Bazin, & Gueguen, 1997; Desmasures & Gueguen, 1997; Duthoit, Godon, & Montel, 2003; Morea, Baruzzi, & Cocconcelli, 1999; Randazzo, Torriani, Akkermans, de Vos, & Vaughan, 2002; Verdier-Metz, Michel, Delbes, & Montel, 2009), which are assumed to play a significant role in the maturation and flavor development of raw milk cheeses. However, the function of each bacterium present in raw milk cheeses and its role in sensory characteristics is not known.

Various PCR-based molecular methods for analysis of microbial communities in cheeses, such as denaturating gradient gel electrophoresis (DGGE) (Bonetta, Bonetta, Carraro, Rantsiou, & Cocolin, 2008; Randazzo et al., 2002), temperature gradient gel electrophoresis (TGGE) (Abriouel, Martin-Platero, Maqueda, Valdivia, & Martinez-Bueno, 2008), single-strand conformation polymorphism (SSCP) (Callon et al., 2007; Delbes, Ali-Mandjee, & Montel, 2007; Duthoit et al., 2003) and terminal restriction fragment length polymorphism (T-RFLP) (Rademaker, Peinhopf, Rijnen, Bockelmann, & Noordman, 2005), have been developed in the last decade. These culture independent techniques are often based on 16S rRNA gene analysis and are facilitated by the recent sequencing of a large number of bacterial species. Those molecular fingerprinting techniques were reported to be a valuable tool for monitoring microbial dynamics in cheeses but they were able to reveal only the dominant species present in the substrates (Jany & Barbier, 2008).

Pyrosequencing is a second-generation DNA sequencing platform based on the detection of pyrophosphate released during nucleotide incorporation, which was developed by Margulies et al. (2005). Recently, this technique has successfully been used to

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monitor microbial communities in various ecosystems like sputum (Armougom et al., 2009), gut (Andersson et al., 2008; Larsen et al., 2010), soil (Acosta-Martinez, Dowd, Sun, & Allen, 2008), wounds (Dowd et al., 2008) and fermented foods (Humblot & Guyot, 2009; Roh et al., 2010). To our knowledge, no studies have utilized pyrosequencing to investigate the microbial diversity in cheese.

The main aim of this study was to characterize the bacterial populations in raw milk cheeses using PCR-DGGE of the V3 region of the 16S rRNA gene and to identify the metabolically active bacteria during the ripening process by reverse transcription (RT)-PCR-DGGE. Furthermore, bacterial diversity in raw milk cheeses was investigated by tag-encoded amplicon pyrosequencing of 16S rRNA gene in both DNA and RNA extracted from raw milk cheeses and compared with identification results of DGGE.

2. Materials and methods

2.1. Milk and cheese samples

Samples of raw milk and semi hard raw milk cheeses at different stages of ripening were obtained from Arla Foods, Denmark. The manufacture of cheeses was done in the same manner as artisanal cheese but with addition of four different starter cultures to four batches of cheese (6-2-1, 6-2-2, 6-3-1 and 6-3-2) made from one batch of raw milk. The starter cultures added were as follows: *Lactobacillus nodensis* and *Lactococcus lactis* to cheese 6-2-1, *Lactobacillus helveticus* to cheese 6-2-2, *Lactobacillus plantarum* and *Lc. lactis* to cheese 6-3-1 and *Lb. helveticus* and *Lactobacillus delbrueckii* to cheese 6-3-2. The cheeses were produced by heating the milk to 30 °C before adding the starter cultures, whereafter the milk was coagulated by adding calf rennet and left for 30 min. After coagulation, the curd was heated to 50 °C for 2 min and left to cool down. Finally the curd was pressed, cut into pieces, brined and left to ripen at 8 °C. Samples of raw milk and cheeses at 0, 7, 14, 28 and 56 days of ripening were collected and stored at −80 °C until analyzed.

2.2. Isolation of DNA and RNA from raw milk and cheese

Two milliliters of raw milk or 2 g of cheese, which was a mixture of cheese taken from surface and core were suspended in 4 mL of 4 M guanidine thiocyanate (Sigma–Aldrich, St. Louis, Mo, USA) – 0.1 M Tris–HCl, pH 7.5 (Bio-Rad, Hercules, CA, USA), and 250 μL 10% *N*-lauroylsarcosine (Sigma–Aldrich) as previously described (Bonaiti, Parayre, & Irlinger, 2006). The suspensions were vortexed for approximately 3 min. From this raw extract, 350 μL was transferred into a 2 mL tube, then stored at (−80 °C) or used directly for DNA extraction. Genomic DNA extraction from raw milk cheese was performed by using the Maxwell® 16 automated sample purification system (Promega, Madison, WI, USA). The yield of genomic DNA was estimated to be about 30 μg DNA g^{-1} cheese or milk. The quality of the DNA was visually estimated on 1% (w/v) agarose in 1 × Tris–Acetate–EDTA buffer containing 0.5 μg mL^{-1} ethidium bromide (Sigma–Aldrich).

For isolation of RNA from raw milk and cheese samples, 2 mL of milk or 2 g of cheese were added to 20 mL 2% sodium citrate and mixed in a stomacher (Lab Blender 400, Seward, London, UK) at normal speed for 2 min. The mixture was then centrifuged at 300 × *g* for 15 min. Ten mL of the supernatant were centrifuged at 5000 × *g* for 15 min and the pellet was used for further isolation of RNA. RNA was isolated using the Total RNA Isolation Kit (Promega) according to the manufacturer instructions. Each sample of 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 in 1 × Tris–Acetate–EDTA buffer containing 0.5 μg mL^{-1} ethidium bromide (Sigma). In case of the presence of positive signals, RNA samples were treated again with Turbo DNA Free Kit to eliminate the rest of the DNA.

2.3. PCR amplification and reverse transcription (RT)-PCR

DGGE samples were prepared by performing PCR with the RoboCycler® Gradient 96 Temperature Cycler with Hot Top Assembly (Stratagene, La Jolla, CA, USA). Approximately 200 bp fragment of the V3 region of the 16S rDNA was amplified using the primers PRBA338f with a GC clamp (5′CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG′3) (Walter et al., 2000) and PRUN558r (5′-ATTACCGCGGCTGCTGG-3′) (Ercolini, Hill, & Dodd, 2003). PCR mixtures (50 μL) contained 20 ng of DNA template, 0.2 μM of the primers, 0.25 mM of dNTP's, 2.5 mM $MgCl_2$, 5 μL PCR buffer and 1.25 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) were prepared. The PCR amplification was performed under the following conditions: 5 min of initial denaturation at 95 °C; 35 cycles of denaturation (30 s at 95 °C), annealing (30 s at 60 °C), extension (40 s at 72 °C); and a subsequent final extension at 72 °C for 10 min. The PCR products were analyzed on 2% (w/v) agarose gel in 1 × Tris–Acetate–EDTA buffer containing 0.5 μg mL^{-1} ethidium bromide (Sigma).

Reverse transcription was performed in 100 μL volume containing 10 μL total RNA, 28.5 μL RNase-free water, 22.0 μL 25 mM $MgCl_2$, 20.0 μL 1.25 μM dNTP mixture, 5.0 μL of a random hexamer (Promega), 2.0 μL RNase inhibitor (Promega), 10 μL 10 × TaqMan RT Buffer and 2.5 μL 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. Three microlitres of the cDNA was used for the PCR amplification as described above.

2.4. DGGE analysis

The PCR products of the 16S DNA and cDNA were analyzed by DGGE using a DCode system apparatus (Bio-Rad, Hercules, CA, USA). Polyacrylamide gels (8%, w/v, acrylamide–bisacrylamide) were prepared with a Bio-Rad Gradient Delivery System (Model 475, Bio-Rad) using solutions containing 30 and 65% denaturant (100% denaturant corresponds to 7 M urea and 40%, v/v, formamide). Gels were run at 60 °C for 16 h at 70 V. The amplified fragments were visualized by SYBR-GOLD (Molecular Probes, Eugene, OR, USA) staining and UV transillumination.

2.5. Sequencing of DGGE fragments

For identification of the specific microbial population observed as DGGE bands, the square root of the number of bands with similar migration on the gel was sequenced. The selected bands were excised from the DGGE gels with a sterile scalpel and the DNA was eluted in 50 μL distilled water overnight at 4 °C. To confirm their electrophoretic mobility relative to the fragment from which they were excised, DNA from bands was amplified and the PCR products were analyzed by DGGE as described above. In case of observing other bands on the gel, the target band was again excised from the gel and analyzed by DGGE until a single band was obtained. PCR products that migrated as the original bands were purified using QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. The purified PCR products were sequenced at Macrogen (Seoul, Korea). Sequences obtained from the DGGE bands were aligned to the 16S rRNA gene

sequences in the National center for Biotechnology Information (NCBI) GenBank, using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast/) (Altschul et al., 1997).

2.6. Pyrosequencing of DNA and RNA extracted from cheese

Tag-encoded amplicon pyrosequencing was conducted for 22 samples, which included DNA and reverse transcribed RNA extracted from cheese batch number 6-2-1 at 0 and 56 days of ripening and from cheese batches 6-2-2, 6-3-1 and 6-3-2 at 0, 14 and 56 days of ripening. The concentrations of DNA and cDNA were measured with a GeneQuant spectrometer (Pharmacia LKB, Biochrom Ltd, Cambridge, UK) and the DNA and cDNA concentrations were adjusted to 5 ng μL^{-1}. The primers used for pyrosequencing were PRK341F (5′CCTACGGGRBGCASCAG-3′) and MPRK806R (5′GGACTACNNGGGTATCTAAT-3′) (Yu, Lee, Kim, & Hwang, 2005) flanking the V3 and V4 region of the 16S rRNA gene. The first PCR amplification was done by adding 1 μL of the diluted DNA or cDNA to 40 μL reaction mixture containing 1 × Phusion HF buffer (Finnzymes, Oy, Espoo, Finland), 2.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.8 U Phusion Hot Start DNA Polymerase (Finnzymes), 0.5 μM of each primer (TAG Copenhagen A/S, Denmark). The PCR was performed by the following cycle conditions: an initial denaturation at 98 °C for 30 s, followed by 30 cycles of denaturation at 98 °C for 5 s, annealing at 56 °C for 20 s and elongation at 72 °C for 20 s, and final elongation step at 72 °C for 5 min. After PCR amplification, the samples were held at 60 °C for 3 min and then placed on ice before the PCR products were analyzed on 1% (w/v) agarose gel with ethidium bromide and visualized with UV-Illumination. Bands of the PCR products were cut from agarose gel and purified by QIAEX II Gel Extraction Kit (QIAGEN). The second PCR amplification was performed as described above, except that the primers PRK341F and MPRK806R with an adapter (CCTAYGGGRBGCASCAG) and 22 barcodes of 10 nucleotides length were used. Furthermore, the number of cycles for denaturation, annealing and elongation was reduced to 15. The PCR products were analyzed on 1% (w/v) agarose gel with ethidium bromide and visualized with UV-Illumination and the bands of PCR products were cut from agarose gel and purified by the Montage Gel extraction kit (Millipore). The amplified fragments with adapters and Tags were quantified using Qubit™ fluorometer (Invitrogen) and mixed in approximately equal concentrations (4×10^5 copies μL^{-1}) to ensure equal representation of each sample. A two-region 454 sequencing run was performed on a GS FLX Standard PicoTiterPlate (70 × 75) using a GS FLX pyrosequencing system according to the manufacturer's instructions (Roche).

Sorting and trimming of sequences, which were of an average of 239 bp were done by 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 50%. The sequences were also uploaded into the Meta Genome Rapid Annotations using Subsystem Technology (MG-RAST) (http://metagenomics.nmpdr.org/metagenomics.cgi) to be identified at the species level.

3. Results

3.1. Identification of bacteria from raw milk cheese by DGGE

Identification of the bacterial populations in the raw milk and cheese samples at different ripening stages was done by PCR-DGGE targeting the V3 region of the 16S rRNA gene. *Leuconostoc lactis*, *Lb. plantarum*, *Streptococcus bovis*, *Jeotgalicoccus psychrophilus*, *Staphylococcus warneri*, *Chryseobacterium* spp., *Staphylococcus xylosis* and *Lc. lactis* were detected in the DGGE profile of the raw milk sample (results not shown). With the exception of *Lc. lactis* and *Lb. plantarum*, all bacteria found in the raw milk were absent in the DGGE profiles of the 4 batches of cheeses (Fig. 1).

Fig. 1a shows the DGGE profiles of the DNA and RNA extracted from cheese samples for batch number 6-2-1 at different stages of ripening. *Lb. nodensis*, *Lc. lactis* and *Streptococcus thermophilus* were found in both DNA- and RNA- profiles of all samples investigated indicating their viability. *Lactobacillus casei* was detected in the DNA-DGGE profile as strong bands, but were very weak in the DGGE profile of extracted RNA, which suggests that this bacterium might not be metabolically active.

The main bacteria found in the DGGE profiles of the DNA and RNA extracted from cheese samples of batch number 6-2-2 are shown in Fig. 1b. *Lb. helveticus* and *S. thermophilus* were found in the DNA- and RNA-DGGE profiles of all samples. However, in the DGGE derived from the RNA extracted from cheese at 14, 28 and 56 days of ripening, the intensity of the bands corresponding to *Lb. helveticus* became weaker and that was the case for *S. thermophilus* at 28 and 56 days of ripening.

Lc. lactis was found as weak bands in the DGGE of the DNA extracted from this cheese at all stages of ripening but not in the DGGE of the RNA extracted from same samples. *Lb. delbrueckii* was detected in the DNA-DGGE profiles at 14, 28 and 56 days of ripening but not in the RNA-DGGE profiles of the same samples. On the other hand, *Lactobacillus fermentum* and *Lb. casei* were found in the RNA-DGGE profile at 14, 28 and 56 days of ripening but not in the DNA-DGGE of the same samples. *Brevibacterium stationis* and *Corynebacterium tuscaniae* were only detected in the RNA extracted from cheese after 28 days of ripening but not in samples at other stages of ripening.

As shown in Fig. 1c, the DNA- and RNA-DGGE profiles showed the presence of *S. thermophilus* and *Lc. lactis* in cheese 6-3-1 at all stages of ripening. *Lb. plantarum* was also present in both the DNA- and RNA- profiles of this cheese except in the RNA-DGGE at 0 day of ripening (Fig. 1c).

S. thermophilus and *Lb. helveticus* were detected in both DNA- and RNA-DGGE profiles of cheese 6-3-2 (Fig. 1d). However, the intensity of the bands representing *Lb. helveticus* was weaker on the RNA-DGGE profile of the same cheese. *Lb. delbrueckii* was detected on both DNA- and RNA-DGGE profiles of same cheese at 28 and 56 days of ripening. *Lc. lactis* was only detected in the DNA-DGGE at all ripening stages but only at 0 day of ripening in the RNA-DGGE of this batch of cheese. On the other hand, *Lb. fermentum* was only detected in the RNA-DGGE profiles of cheese 6-3-2 at 14, 28 and 56 days of ripening.

3.2. Identification of bacteria from raw milk cheese by pyrosequencing

A total of 1 57 380 reads were generated from the 22 samples analyzed by pyrosequencing. The 22 samples included both 16S rDNA and cDNA libraries of cheese batch number 6-2-1 at 0 and 56 days of ripening and for cheese batches 6-2-2, 6-3-1 and 6-3-2 at 0, 14 and 56 days of ripening. Of these, 33 024 sequences were obtained from the 11 16S rDNA libraries and 1 24 356 sequences were obtained from the 11 16S cDNA libraries. The average sequence length was 239 bp.

The classifications of sequences obtained from the 16S rDNA and cDNA libraries are summarized in Tables 1 and 2, respectively. In both 16S rDNA and cDNA libraries, it was found that bacteria belonging to the genera *Streptococcus*, *Lactococcus* and *Lactobacillus* constituted the largest group present in cheese samples. In the 22 cheese samples, *Streptococcus* represented percentages ranging

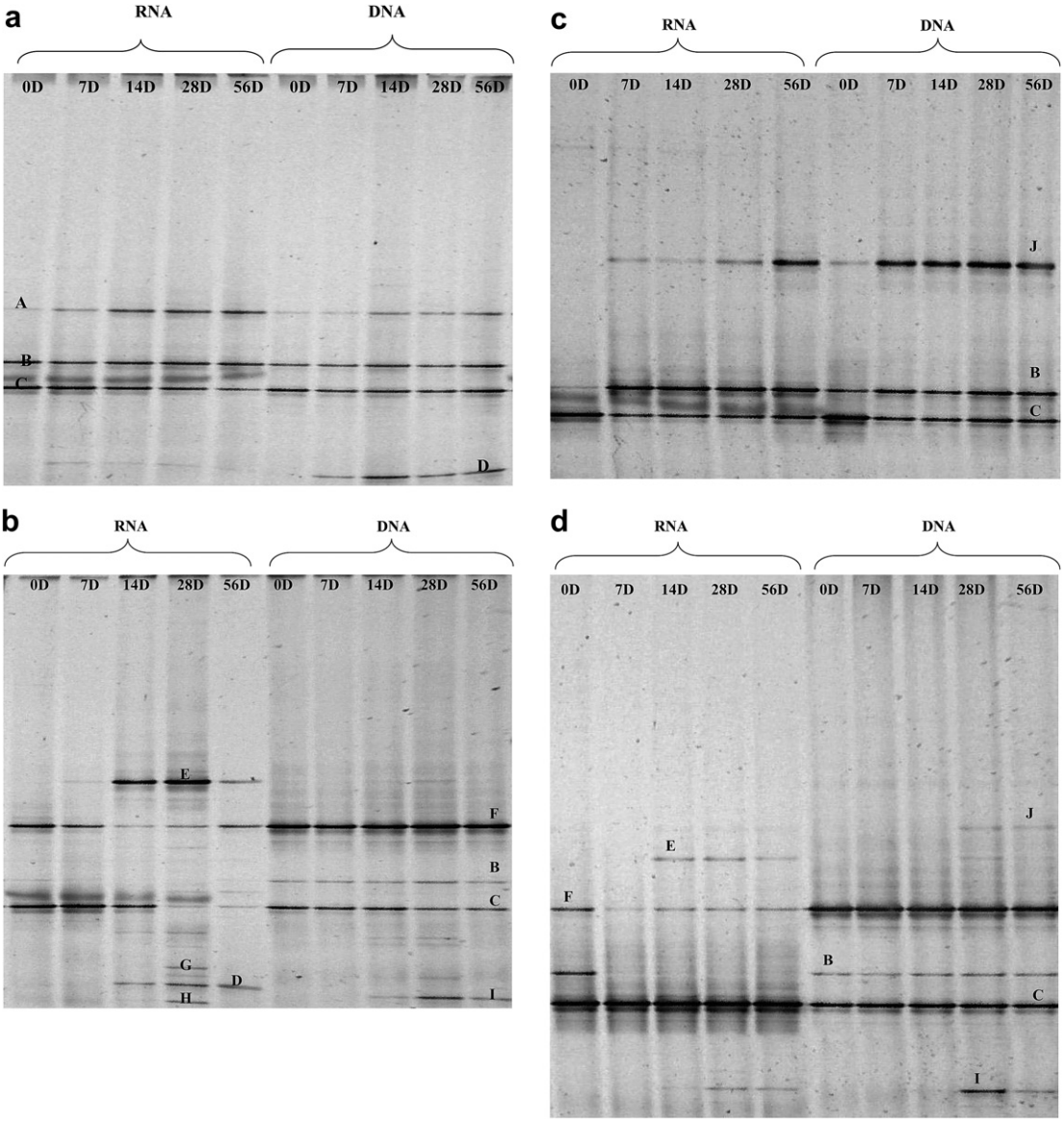

Fig. 1. DGGE profiles for 16S rDNA and RNA of cheese batch no. 6-2-1 (a), 6-2-2 (b), 6-3-1 (c) and 6-3-2 (d) at 0 (0D), 7 (7D), 14 (14D), 28 (28D) and 56 (56D) days of ripening. Bands were identified as A: *Lactobacillus nodensis*, B: *Lactococcus lactis*, C: *Streptococcus thermophilus*, D: *Lactobacillus casei*, E: *Lactobacillus fermentum*, F: *Lactobacillus helveticus*, G: *Corynebacterium tuscaniae*, H: *Brevibacterium stationis*, I: *Lactobacillus delbrueckii* and J: *Lactobacillus plantarum*.

Table 1
Phylogenetic classification of sequences obtained from DNA extracted from four batches of cheeses (6-2-1, 6-2-2, 6-3-1 and 6-3-2) at 0 days (0D), 14 days (14D) and 56 days (56D) of ripening.

Cheese	Percentages (%) of bacterial genera to the total sequence reads in the 16S rDNA libraries								
	Streptococcus	*Lactococcus*	*Lactobacillus*	*Corynebacterium*	*Brevebacterium*	*Pseudomonas*	*Halomonas*	*Pediococcus*	*Staphylococcus*
0D 6-2-1	80.1	18.7	1.2	0	0	0	0	0	0
56D 6-2-1	58.2	18.1	23.0	0.5	0	0.1	0	0.1	0
0D 6-2-2	28.2	1.1	70.2	0.3	0	0.2	0	0	0
14D 6-2-2	20.3	1.5	74.2	2.7	0.1	0	0.8	0.1	0.3
56D 6-2-2	9.1	1.4	87.6	1.9	0	0	0	0	0
0D 6-3-1	93.5	6.2	0.3	0	0	0	0	0	0
14D 6-3-1	50.7	29.3	17.3	2.1	0.1	0	0.5	0	0
56D 6-3-1	52.4	32.6	14.9	0.1	0	0	0	0	0
0D 6-3-2	30.6	1.1	67.9	0.2	0.2	0	0	0	0
14D 6-3-2	38.4	1.6	59.4	0.5	0	0	0.1	0	0
56D 6-3-2	34.8	2.3	62.8	0.1	0	0	0	0	0

Table 2
Phylogenetic classification of sequences obtained from RNA extracted from four batches of cheeses (6-2-1, 6-2-2, 6-3-1 and 6-3-2) at 0 days (0D), 14 days (14D) and 56 days (56D) of ripening.

Cheese	Percentages (%) of bacterial genera to the total sequence reads in the 16S cDNA libraries[a]														
	Str.	*Lc.*	*Lb.*	*Co.*	*Bre.*	*Pse.*	*Hal.*	*Ped.*	*Staph.*	*Mic.*	*Ac.*	*Vao.*	*Wei.*	*Bif.*	*Ent.*
0D 6-2-1	83.7	15.4	0.9	0	0	0	0	0	0	0	0	0	0	0	0
56D 6-2-1	31.9	44.7	22.2	0.9	0.1	0.1	0	0	0	0	0.1	0	0	0	0
0D 6-2-2	81.2	0.2	18.1	0.4	0.1	0	0	0	0	0	0	0	0	0	0
14D 6-2-2	55.6	0.1	40.2	3.3	0.1	0	0.1	0.1	0.3	0.1	0	0.1	0	0	0
56D 6-2-2	50.6	1.7	43.8	3.1	0	0	0.1	0.1	0.3	0	0	0.1	0.1	0.1	0
0D 6-3-1	99.2	0.8	0	0	0	0	0	0	0	0	0	0	0	0	0
14D 6-3-1	31.1	68.2	0.6	0.1	0	0	0	0	0	0	0	0	0	0	0
56D 6-3-1	42.3	52.3	5.2	0.1	0	0	0	0	0	0	0.1	0	0	0	0.1
0D 6-3-2	83.1	0.6	16.2	0.1	0	0	0	0	0	0	0	0	0	0	0
14D 6-3-2	85.8	0.1	14.0	0.1	0	0	0	0	0	0	0	0	0	0	0
56D 6-3-2	86.6	0.1	13.1	0.1	0.1	0	0	0	0	0	0	0	0	0	0

[a] Abbreviations are: *Str.*, *Streptococcus*; *Lc.*, *Lactococcus*; *Lb.*, *Lactobacillus*; *Co.*, *Corynebacterium*; *Br.*, *Brevibacterium*; *Ps.*, *Pseudomonas*; *Hal.*, *Halomonas*; *Ped.*; *Pediococcus; Staph.*, *Staphylococcus*; *Mic.*, *Micrococcus*; *Ac.*, *Acinetobacter*; *Vao.*, *Vaococcus*; *Wei.*, *Weissella*; *Bif.*, *Bifidobacterium*; *Ent.*, *Enterococcus.*

from 9.1 to 93.5 and 31.1 to 99.2% of the sequence reads obtained from the 16S rDNA and cDNA libraries, respectively. All of the sequence reads that belong to the genus *Streptococcus* were identified by MG-RAST as *S. thermophilus*. In cheeses 6-2-1 and cheese 6-3-1, the genus *Lactococcus* comprised a range of 6.2–32.6 and 0.8–68.2% of the total sequence reads obtained from the 16S rDNA and cDNA libraries, respectively. On the other hand, in cheeses 6-2-2 and 6-3-2, the genus *Lactococcus* was present in percentages of only 1.1–2.3 and 0.1–1.7% of the sequence reads obtained from the 16S rDNA and cDNA libraries, respectively. All sequence reads of the genus *Lactococcus* were found to belong to *Lc. lactis*. In cheese batches 6-2-1 and 6-3-1, *Lactobacillus* comprised 0.3–23 and 0–22.2% of the total reads obtained from the 16S rDNA and cDNA libraries, respectively. In cheese 6-2-1, more than 95% of the sequence reads of the genus *Lactobacillus* were identified as *Lb. nodensis* and the rest were identified as *Lb. casei* and *Lactobacillus tucceti*. About 99% of the sequence reads, which belong to the genus *Lactobacillus* in cheese 6-3-1, were identified as *Lb. plantarum*.

The genus *Lactobacillus* was present in high percentages in cheese batches 6-2-2 and 6-3-2 comprising 59.4–87.6 and 13.1–43.8% of the sequence reads of the 16S rDNA and cDNA libraries, respectively. The majority of *Lactobacillus*, i.e., between 95 and 99% was identified as *Lb. helveticus* and the rest of sequences were belonging to *Lb. casei*, *Lb. delbrueckii* and *Lb. fermentum*.

With the exception of cheese 6-2-1 and 6-3-1 at 0 days of ripening, *Corynebacterium* was detected in both 16S rDNA and cDNA libraries of all cheese samples investigated. The occurrence of *Corynebacterium* in cheese samples comprised 0.1–2.7 and 0.1–3.3% of the total sequence reads obtained from 16S rDNA and cDNA libraries, respectively. The other bacteria, namely *Brevibacterium*, *Pediococcus*, *Pseudomonas*, *Halomonas* and *Staphylococcus*, were detected occasionally in some the DNA of some cheese samples comprising less than 1% of the total sequences. The same bacteria together with *Micrococcus*, *Acinetobacter*, *Vagococcus*, *Weissella* and *Enterococcus* were also found in less than 1% of the sequence reads obtained from the 16S cDNA libraries of some cheese samples.

4. Discussion

In the current study, pyrosequencing of tagged 16S rRNA gene amplicons was used, for the first time, to investigate the bacterial populations in cheese. Both DNA and cDNA extracted from cheese samples were analyzed to detect the metabolically active bacteria. Surprisingly, the numbers of sequences obtained from the 16S cDNA libraries were significantly higher than those obtained from the 16S rDNA libraries of the same samples, to which no explanation was found. This is in contrast to the findings of Humblot and Guyot (2009), who found that the number of sequence reads obtained from DNA extracted from an African fermented food were significantly higher than those obtained from cDNA extracted from the same samples. It was proposed that this could be due to a bias in PCR amplification or to imprecise quantification of the PCR products leading to strong deviation during pooling of samples (Humblot & Guyot, 2009). However, this is not the case for samples included in this study since all the 11 16S cDNA libraries yielded more sequences than the 16S rDNA libraries of cheese samples.

The main bacteria, which were detected in cheese samples by DGGE and pyrosequencing, included the genera *Lactococcus*, *Lactobacillus* and *Streptococcus*. With regard to the main bacteria identified from the sequence reads of the 16S rDNA and cDNA libraries as well as from the DNA and cDNA-DGGE profiles of the same cheese samples, a good agreement was observed indicating that those bacteria were metabolically active and contribute to the ripening of cheese. However, the percentages of the main bacteria from the total sequences were different between 16S rDNA and cDNA libraries of the same samples. For example, the genus *Lactobacillus*, which was found to belong to *Lb. helveticus*, was present in a range of 59.4–87.6% of the 16S rDNA libraries in cheeses 6-2-2 and 6-3-2, while the percentages of *Lactobacillus* sequences in 16S cDNA libraries of the same cheeses were 13.1–43.8. This agrees with the DNA and RNA-DGGE profiles of these two cheeses, in which *Lb. helveticus* was shown to have strong bands in the DNA-DGGE profiles but the intensity of the bands was weaker on the RNA-DGGE profiles of the same samples. *Lactobacillus helveticus* was part of the starter cultures added to this batch of cheese, which might explain the high abundance of this bacterium in the 16S rDNA libraries as well as in its DNA-DGGE profiles of this cheese. It seems that many of the added *Lb. helveticus* died because its percentages in the 16S cDNA libraries of same cheese samples decreased and the intensity of bands became weaker on the cDNA-DGGE profiles.

The genus *Streptococcus*, which was identified as *S. thermophilus*, was present in the 16S rDNA and cDNA libraries obtained from all cheeses. This agrees again with the presence of S. *thermophilus* in the rDNA and cDNA-DGGE profiles of all cheeses, which confirms that *S. thermophilus* seems to have an important role in cheese ripening. *S. thermophilus* was not identified in the DGGE profile of raw milk making it possible that this bacterium originates from the production environment. *S. thermophilus* has been isolated from cheeses and was found to contribute in milk acidification and cheese ripening (Marino, Maifreni, & Rondinini, 2003; Randazzo et al., 2002; Randazzo, Pitino, Ribbera, & Caggia, 2010).

Lc. lactis showed stronger bands intensities in the DNA and cDNA-DGGE profiles of cheese 6-2-1 and 6-3-1 than the DNA and cDNA-DGGE profiles of cheese 6-2-2 and 6-3-2. This is in accordance with the results obtained by pyrosequencing. *Lc. lactis* was part of the starter cultures added to cheese batches 6-2-1 and 6-3-1 but not to cheese 6-2-2 and 6-3-2. The presence of *Lc. lactis* in cheese 6-2-2 and 6-3-2 seemed to originate from the raw milk as it was detected there. The role of *Lc. lactis* in cheese ripening is well known (Centeno, Cepeda, & Rodriguez-Otero, 1996; Cogan et al., 1997; Hannon et al., 2003; Zarate, Belda, Perez, & Cardell, 1997).

With the exception of the cheese curds 6-2-1 and 6-3-1, *Corynebacterium* was detected by pyrosequencing in the 16S rDNA and the cDNA libraries of all cheese samples in low percentages. However, *Corynebacterium* was not found in the DGGE profile of any of the cheese samples except in the cDNA-DDGE profile of cheese 6-2-2 at 28 days of ripening where *C. tuscaniae* was detected. Other bacterial genera like *Halomonas*, *Pediococcus*, *Micrococcus*, *Pseudomonas*, *Staphylococcus* and others were also detected occasionally by pyrosequencing in some cheese samples at very low percentages. On the contrary, those bacteria were not detected in the DGGE profiles of any of the cheese samples. Pyrosequencing appears to be more sensitive in detecting minor bacterial groups in cheese present at low concentrations.

5. Conclusions

A very good agreement was observed between the tag-encoded amplicons pyrosequencing and PCR-DGGE methods targeting 16S rDNA in detecting the main bacteria present in the raw milk cheeses. The main bacterial genera, which included *Lactococcus*, *Lactobacillus* and *Streptococcus*, were detected by pyrosequencing and DGGE methods in both 16S rDNA and cDNA obtained from cheese samples at different stages of ripening indicating their viability and contribution to the ripening of cheeses. However, the minor bacterial populations present in raw milk cheeses were not detected by DGGE. Therefore, pyrosequencing was able to provide an in depth detection of minor bacterial populations in raw milk cheeses. In addition, while DGGE is a semi quantitative method in which the intensity of the bands correlates to the prevalence of bacteria in cheeses, pyrosequencing provided a more reliable estimate of the relative abundance of the individual bacteria by being able to calculate percentages of bacteria sequences to the total sequences obtained. Pyrosequencing appears to be a powerful and promising method, which will allow for more deep investigation of bacterial populations in cheese and their possible role in the ripening process.

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