Diversity of thermophilic bacteria in milk assessed by culturing, PCR-DGGE, and pyrosequencing

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Abstract

Thermophilic lactic acid bacteria (LAB) species, such as *Streptococcus thermophilus*, *Lactobacillus delbrueckii*, and *Lactobacillus helveticus* are economically-important, because they used worldwide as industrial starters in dairy. The presence and relative abundance of these bacterial types in samples of raw milk (RM), pasteurized milk (PM) and pasteurized milk incubated at 42°C for 24 h (PFM) was assessed by PCR-DGGE. One RM and two PFM samples were further analyzed by pyrosequencing of 16S rDNA amplicons. Then, thermophilic species from all three types of samples were selectively recovered by culturing. The results obtained by culture-dependent and culture-independent techniques agree well in both type and numbers of the dominant populations, but differ in the minority components. DGGE profiles of RM and PM samples were shown to be highly similar and the most prominent bands belonged to different *Lactococcus*, *Leuconostoc* and *Streptococcus* species. In contrast, a reduced number of three DGGE bands was obtained in the PFM samples, two of which were assigned to *S. thermophilus*. As expected, the diversity found by pyrosequencing was much higher than that obtained by DGGE. Ninety eight operational taxonomic units (OTUs) were encountered at 3% of sequence divergence among 6,168 high-quality pyrosequencing reads. *Leuconostoc citreum* was identified by pyrosequencing as the dominant microorganism in the RM sample, followed among others by *Lactococcus plantarum*, *Streptococcus* spp., *Lactococcus raffinolactis*, and *Lactococcus lactis*. *S. thermophilus* accounted only for 0.2% of the sequences in the RM sample. In contrast, in both PFM samples the latter species accounted for more than 98% of the sequences. It was not surprising to find that pasteurization killed all members of the phylum *Proteobacteria* and most mesophilic LAB species detected by culture-independent techniques, which then were not recovered by culturing. *Enterococcus* spp. survived

pasteurization, but they were shown to be overgrown during fermentation by true thermophilic LAB species such as *S. thermophilus* and *L. delbrueckii*. In addition to LAB, isolates of *Streptococcus* spp., *Bacillus* spp., and *Clostridium* spp. were occasionally recovered among the thermophilic bacteria. The procedure followed in this study is considered a good strategy to analyze the bacterial diversity in milk and a suitable method for the isolation of new thermophilic LAB strains, among which adequate starters can surely be selected.

1. Introduction

Strains of the moderate thermophilic species *Streptococcus thermophilus*, *Lactobacillus delbrueckii*, and *Lactobacillus helveticus* are among the most industrially-important lactic acid bacteria (LAB) for dairy (Parente and Cogan, 2004; Mills et al., 2010). Carefully selected strains are used world-wide as starters and adjunct cultures to either take over the control of the fermentation or to provide aroma and taste compounds (Helinck et al., 2004; Smit et al., 2005). These species belong to the group of thermophilic (surviving pasteurization) and aciduric (living and multiplying in acidic environments) bacteria, which are dominant in natural fermentations subjected to a heating (cooking) step (from 55 to 85°C) before (such as in yogurt) or during manufacture (such as in some Italian and Swiss cheese varieties) (Hébert et al., 2000; Mora et al., 2002; Mauriello et al., 2003; Callanan et al., 2005). The heating destroys the dominant mesophilic species in milk, which otherwise are advantaged competitors of the thermophilic LAB. In natural, starter-free dairy fermentations, thermophilic LAB may come from the raw materials (including milk) and the manufacturing environment, which becomes enriched through the process. The microbial typing of such products has

76 allowed the identification and selection of new strains and strains with new properties,
77 which are intended for complementing and replacing currently-in-use thermophilic
78 starters (Hébert et al., 2000; Mora et al., 2002; Rossetti et al., 2008). The search for new
79 starters is particularly important for *S. thermophilus*, whose strains form a relatively
80 coherent and homogenous group with a low level of phenotypic and genetic
81 polymorphism (Rasmussen et al., 2008; Delorme et al., 2010).

82 Thermophilic bacteria from raw milk are assumed to be present in traditional cheeses
83 not subjected to heating, although they may constitute minority populations, which are
84 surpassed by a faster development of mesophiles. However, the ecosystem of such
85 products is not usually screened for thermophilic organisms (Cogan et al., 1997). Yet
86 DNA belonging to thermophilic species has been repeatedly detected by the recent use
87 of culture-independent microbial techniques (Ogier et al., 2004; Flórez and Mayo, 2006;
88 Alegría et al., 2009; Alegría et al., 2011; Edalatian et al., 2012). Moreover, traditional
89 cheese ecosystems might constitute a natural source for identification of improved/new
90 thermophilic LAB starter candidates (Wouters et al., 2002; Jensen et al., 2009).

91 Since culturing methods have proven to be unreliable for a complete microbial
92 characterization of different ecosystems, including those of food fermentations (Giraffa
93 and Neviani, 2001; Jany and Barbier, 2008), some PCR-based, culture-independent
94 microbial techniques, such as denaturing gradient gel electrophoresis (DGGE) (Cololin
95 et al., 2004; Ercolini et al., 2004; Ogier et al., 2004; Giannino et al., 2009), temporal
96 temperature gradient gel electrophoresis (TTGE) (Ogier et al., 2002), single stranded
97 conformation polymorphisms (SSCP) and construction and analysis of libraries of
98 conserved genes such as the 16S rRNA gene (Duthoit et al., 2003; Delbès et al., 2007;
99 Rasolofo et al., 2010), have recently been applied to study the microbial diversity and
100 population dynamics in milk and dairy ecosystems (for a recent comprehensive review

see Quigley et al., 2011). Pyrosequencing, an automated high-throughput parallel sequencing technique, has also begun to be applied to the study of dairy products (Dobson et al., 2011; Alegría et al., 2012; Leite et al., 2012; Masoud et al., 2012). This technique enables a rapid and accurate analysis of thousands of nucleotide sequences, which can then be used to analyze the population structure, gene content, and metabolic potential of the microbial communities in an ecosystem. By means of these culture-independent microbial techniques, cultured species have all been detected, but also a vast array of previously unnoted microorganisms (Dobson et al., 2011; Alegría et al., 2012; Leite et al., 2012; Masoud et al., 2012).

In the search for new thermophilic LAB strains, the bacterial diversity of thermophilic and aciduric species present in milk was approached by means of culture-independent techniques such as DGGE and pyrosequencing. Culturing methods were then applied to recover the cultivable bacteria from samples after pasteurization and pasteurization and fermentation. Results of the culturing and culture-independent methods were then compared among themselves and to those in the recent literature. In addition, the culturing technique allowed the recovery of thermophilic LAB isolates, which, after appropriate functional characterization, might be of interest for dairy.

2. Material and Methods

2.1. Sampling and treatment of milk samples

Milk samples were collected from different farms in Northern Spain and transported to the laboratory under refrigerated conditions. Samples were left untreated (raw milk, RM samples) or subsequently subjected to pasteurization at 63°C for 30 min (PM samples) and fermentation at 42°C for 24 h (PFM samples).

2.2. Isolation of total microbial DNA from milk

Total microbial DNA from milk samples was isolated by using the QIAamp DNA stool minikit (Qiagen, GmbH, Hilden, Germany) following the optimized protocol reported by Martín et al. (2007) with an additional step of bacterial enzymatic lysis. This modification consisted in a previous treatment of the cells with 30 mg/ml of lysozyme and 20 units of mutanolysin (both from Sigma-Aldrich, Saint Louis, MO, USA) at 37°C for 60 min. Purified DNA was measured using an Epoch micro-volume spectrophotometer (BioTek Instruments, Winooski, VT, USA).

2.3. PCR-DGGE analysis of milk samples

In order to assess the composition and dynamics of the dominant bacterial populations in milk samples, a PCR-DGGE analysis was performed using bacterial universal primers, as follows.

2.3.1. PCR amplification of 16S sequences

Total DNA from ten samples each of RM, PM, and PMF was subjected to PCR-DGGE analysis. For this, the V3 region of the bacterial 16S rRNA gene was amplified with F357-GC (5'–CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG–GGGTACGGGAGGCAGCAG–3' and R518 (5'–ATTACCGCGGCTGCTGG–3') primers, as reported by Muyzer et al. (1993). PCR was performed in 50 µl reaction volumes using Taq-DNA polymerase with ~100 ng of each milk DNA sample as a template and 0.2 mM of each primer. PCR conditions were as follows; 95°C for 2 min, 35 cycles of 95°C for 30 s, 56°C for 40 s and 72°C for 1 min, and a final extension step at 72°C for 5 min.

2.3.2. Electrophoresis conditions and identification of bands

DGGE was performed by using a DCode apparatus (Bio-Rad, Richmond, CA, USA) at 60°C and employing 8% polyacrylamide gels with a denaturing range of 40-60%. Electrophoresis was performed at 75 V for 16 h. Bands were visualized under UV light after staining with ethidium bromide (0.5 µg ml⁻¹) and photographed.

Bands were initially identified by comparison with those of a DGGE ladder constructed as reported elsewhere (Flórez and Mayo, 2006), using chromosomal DNA of pure cultures of the species *Weissella confusa*, *Macrococcus caseolyticus*, *Streptococcus infantarius*, *Lactococcus lactis*, *S. thermophilus* and *Brevibacillus brevis*. In addition, representative bands were excised from the acrylamide gels and their DNA was eluted overnight in 50 µl of sterile water at 4°C. The DNA was re-amplified with the same primer pair without the GC-clamp, and sequenced by cycle extension in an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were compared to those in GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the Ribosomal Database Project (RDP) (<http://rdp.cme.msu.edu/index.jsp>) databases using online tools. The identity of the bands was determined on the basis of the highest score.

2.4. Pyrosequencing analysis of milk samples

Amplicons of 16S rDNA sequences from three milk samples were subjected to pyrosequencing. Of these samples, one was an RM sample, which was analyzed in order to assess the overall bacterial diversity in milk. The other two corresponded to PFM samples, which were selected to determine the diversity of thermotolerant and aciduric bacteria after fermentation.

2.4.1. Primers and 16S rRNA gene amplification conditions

Two universal primers, Y1 (5'-TGGCTCAGGACGAACGCTGGCGGC-3') (position 20-43 on 16S rRNA gene of *Escherichia coli*) and Y2 (5'-CCTACTGCTGCCTCCCGTAGGAGT-3') (positions 361-338) (Young et al., 1991), were used to amplify by PCR a 348-bp stretch of DNA embracing the V1 and V2 hypervariable regions of the prokaryotic 16S rDNA. The universal primers were enlarged at the 5' end with a 10-bp sample-specific barcode and forward (5'-CGTATCGCCTCCCTCGCGCCATCAG-3') and reverse (5'-CTATGCGCCTTGCCAGCCCGCTCAG-3') 454-adaptors. Amplifications were carried out using the following PCR conditions: 95°C for 5 min, 25 cycles of 94°C for 30 s, 52°C for 40 s and 72°C for 30 s, and a final extension step at 72°C for 10 min.

Amplicons were purified through PCR Clean-Up columns, and DNA concentration and quality was measured. Equal amounts of DNA from the three samples were pooled, for a total amount of 100 ng. Pooled DNA was subsequently amplified in PCR-mixture-oil emulsions and sequenced in different lanes of a PicoTiterPlate on a 454 Genome Sequencer 20 system (Roche, Basel, Switzerland).

2.4.2. Sequence treatment and bioinformatics analysis

The raw sequences were processed by using Mothur v.1.26.0 (Schloss et al. 2009). The flowgrams were submitted to Pyronoise (Quince et al. 2011) to reduce error in the retained data set. Additionally, all sequences missing the forward primer and/or having a length smaller than 300 bases were removed. The high quality sequences were then aligned using Silva reference database and the chimeras were detected and eliminated. The resultant alignments file with only high quality sequences served as an input to construct the distance matrix and to cluster the sequences into OTUs. To perform the taxonomic assignment high-quality partial 16S rDNA sequences were submitted to the RDP-II classifier using an 80% confidence threshold to obtain the taxonomic

assignment and the relative abundance of the different bacterial groups (Wang et al., 2007). The clusters were constructed at a 3% dissimilarity cut-off and served as OTUs for generating predictive rarefaction models and for determining the ACE and Chao1 richness (Chao and Bunge, 2002) and Shannon diversity indices (Shannon and Weaver, 1949). The MOTHUR program was also used to select representative sequences of each OTU. In an attempt to give the OTUs a taxonomic assignment down to the genus level, representative reads of the more abundant OTUs were compared against the RDP using the Seqmatch option. Representative sequences and their nearest neighbors in RDP were also aligned with MEGA 5.0 software (Tamura et al., 2011) and the Kimura 2-parameter model for the construction of a phylogenetic tree using the neighbor-joining method. The equivalent sequence of the Archaea *Pyrolobus fumarii* (X99555) was used as an outgroup. Finally, manual sequence comparisons against both RDP and GenBank databases were also performed.

2.4.3. Nucleotide sequence accession numbers.

The pyrosequencing data of this work were deposited in the NCBI Sequence Read Archive (SRA) and are available under accession numbers SRS349940, SRR521141, SRR521142 and SRR521143.

2.5. Analysis of milk by culturing

Twenty two milk samples were collected from different farms in Northern Spain and transported to the laboratory under refrigerated conditions. To select for thermotolerant and aciduric organisms, samples were sequentially subjected to pasteurization at 63°C for 30 min on a water bath and then incubated at 42°C for 24 h. Aliquots of raw milk (RM), pasteurized milk (PM) and pasteurized milk incubated at 42°C (PMF) were serially diluted in Maximum Recovering Diluent (Scharlab, Barcelona, Spain) and

plated on Plate Count Agar with 1% of skim milk (PCAM; Merck, Darmstadt, Germany) and Brain Heart Infusion agar (BHI; Oxoid, Basingstoke Hampshire, UK), both media were added with 0.5% each of lactose and glucose. For total, mesophilic counts RM samples were incubated at 30°C for 48 h, and to select for thermophilic bacteria PM and PMF samples were incubated at 42°C for 24 h.

2.6. Identification of bacteria

Representative colonies from the counting plates were picked at random, purified twice in the same medium of isolation, and a single colony was inoculated in the corresponding liquid medium and incubated during 24 h at either 30 or 42°C. Cultures were preserved frozen at -80°C in liquid media containing 15% of glycerol. Cryocultures were thawed, recovered in the medium of isolation, and identified by partial amplification of their 16S rRNA genes, sequencing and comparison of the sequences in databases. Cell extracts were obtained by a mechanical procedure in a Mini-Beadbeater apparatus (BioSpec Products, Inc., Bartlesville, OK, USA). For that, a single colony was suspended in 100 µl of sterile water and mixed with 50 mg of sterile glass beads (Sigma-Aldrich). Cell extracts (between 2 and 4 µl) were then used as a source of template DNA for PCR amplification. Amplifications were performed with the universal primers 27YMF (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR was done in 50 µl reaction volumes using a Taq-DNA polymerase master mix (Ampliqon, Skovlunde, Denmark) and 0.2 mM of each primer. PCR conditions were as follows: 95°C for 5 min, 35 cycles of 94°C for 30 s, 50°C for 45 s and 72°C for 2 min, and a final extension step at 72°C for 5 min. Amplicons were then purified through GenElute PCR Clean-Up columns (Sigma-Aldrich) and sequenced. Isolates were assigned to a given species as above.

3. Results

3.1. PCR-DGGE analysis of bacterial communities

A PCR-DGGE analysis of the bacterial 16S rRNA gene was conducted in 10 milk samples with universal primers to obtain an overview of the community structure of the dominant bacteria in raw milk (RM) and to evaluate the microbial changes occurring after pasteurization (PM) and incubation (PFM). The results are depicted in Figure 1. The number of bands in the profiles ranged between 6 to 10 in the RM and PM samples, while only three to four bands were found in the PFM samples. Band patterns of RM and PM samples were almost identical. In general, the whole profiles of PM samples seemed to be weaker than those corresponding to RM samples. Moreover, punctual bands, such as the uppermost bands in milk C (RM sample, Panel A, line 7) and milk H (RM sample, Panel B, line 7), are hardly visible in the corresponding PM samples. Occasionally, the contrary is also true, as it happens with the uppermost bands in milk G (PM sample, Panel B, line 5) and milk H (PM sample, Panel B, lane 8).

In total 52 bands were identified by reamplification, sequencing and sequence comparison (bands with a number on the top in Figure 1). Most of them were identified down to the species level, and these were assigned to 15 different species, while seven bands were only identified at the genus levels, and two bands (migrating at the same position) (bands 16) could not be identified (their DNA did not reamplify). Prominent bands in RM and PM were present in most samples and belonged to *Lactococcus garvieae* (band 2), *Weissella confusa/cibaria* (band 3), *Lactococcus raffinolactis* (band 5), *Streptococcus viridians* (band 7), *Lc. lactis* (band 8), and *S. thermophilus* (bands 9). It is noteworthy the migration at the same position of bands corresponding to *Lc.*

garvieae and *Weissiella confusa/cibaria*; although apparently they were never present in the same sample, because the six bands purified from this position could be identified as belonging to either one of these species. DGGE profiles of PFM samples were clearly dominated by a thick band corresponding to *S. thermophilus*. As an exception, bands corresponding to *Brevibacillus brevis* and *Clostridium perfringens* were the most prominent in samples F (PFM) and G (PFM), respectively.

3.2. Bacterial composition determined by pyrosequencing

16S rDNA amplicons from the RM sample D (RM) and those of sample C (PFM) and D (PFM) were subjected to pyrosequencing. A total of 42,951 raw reads were obtained, including 3,794 reads from sample D (RM), 20,417 from sample D (PFM), and 18,740 from sample C (PFM). Of these, a total of 23,057 corresponded to high-quality partial 16S rDNA sequences longer than 300 bp of samples of samples D (RM) (2,056 reads), D (PFM) (10,432 reads), and C (PFM) (10,569 reads). To avoid the bias of analyzing the data with different sequences numbers, we performed all the analyses (with exception of the taxonomic assignment for relative abundance) with a normalized number of sequences for all treatments.

Figure 2 shows the number of OTUs and the diversity richness indexes of Chao, Ace, and Jackknife of the three samples. It is evident that the bacterial diversity present in the RM sample is much higher than that in the PFM samples. Similar results were obtained by the Shannon index at 97% similarity, with values of 1.95, 0.13, and 0.07 for samples D (RM), D (PFM), and C (PFM), respectively. Congruently, rarefaction curves of RM and PFM samples were shown to be rather dissimilar (Figure 3). Rarefaction curves of the normalized sequences showed that the sequence coverage was very good for all

libraries. They also showed that the diversity in D (RM) sample is higher than that in the two PFM samples [C (PFM) and D (PFM)].

Sequences were assigned at the RDP classifier to five different phyla, *Firmicutes* (22,763), *Proteobacteria* (261), *Deinococcus-Thermus* (12), *Bacteroidetes* (9), and *Actinobacteria* (8). Of these *Bacteroidetes* and *Actinobacteria* sequences were never encountered among the PFM samples. The *Firmicutes* were the most abundant in all three samples. Moreover, the percentage of the sequences of this phylum rose from 87% in the raw milk sample to 99.8% in the PFM samples. Members of the class *Bacilli* (22,678 reads) were dominant among the *Firmicutes*. The relative abundance at the genus level of reads belonging to the class *Bacilli* is depicted in Figure 4. Mesophilic LAB (*Leuconostoc*, *Lactococcus*), present in the RM sample were entirely replaced by *Streptococcus (thermophilus)* in both PFM samples. Reads belonging to *Proteobacteria* (of classes *Gamma*-, *Alpha*-, and *Beta*-*proteobacteria*) were mostly found in sample D (RM), although a few were also retrieved from the PFM samples.

Among the normalized 6,168 high-quality reads, 98 OTUs at the 97% sequence identity were obtained. In an attempt to identify reads at the species level, representative sequences of the more abundant OTUs (those with more than 10 sequences) were manually compared against the RDP and GenBank databases. The results of these assignments are summarized in Table 1. Sequences from the several LAB species accounted for 80% of the RM reads and more than 99.5% of the sequences from the PFM samples 7.46% of the reads. As it happened for the genera (Figure 4), mesophilic LAB species are a majority in the RM sample (*Leu. citreum*, *Lc.lactis*, *Lactobacillus plantarum*), but they are completely replaced by *S. thermophilus* in the PFM samples. It is intriguing the large number of *Vibrio cholerae*-related sequences, of which 3 were retrieved from PFM samples.

3.3. Culturing analysis of milk samples

As the presence of thermophilic LAB species had been unequivocally found to be present among the analyzed milk samples, these and another 12 milk samples were subjected to the same treatments (pasteurization, pasteurization and fermentation) and dilutions of the RM, PM, and PFM samples were plated in the two non-selective, rich media utilized in this work (PCAM and BHI) for the recovery of the total cultivable bacteria. Representative colonies were then identified as indicated.

Microbial counts of the 22 raw milk samples at 30°C for 48 h ranged between 2.38×10^4 to 6.82×10^5 colony forming units (cfu)/ml. In most cases, counts on PCAM were slightly higher than those in BHI, though differences did never reach a logarithmic unit. Counts of 20 out of the 22 RM samples were within the tolerated levels in Spain for cheese milk ($<1.0 \times 10^5$ cfu/ml; RD 1728/2007). Counts in PM samples lowered between two to three log units (average 3.19×10^2 cfu/ml). Aliquots of the pasteurized samples were further incubated at 42°C for 24 h (PFM samples). Counts of PFM samples surpassed 1.0×10^9 ufc/ml in all analyzed milk samples. Fermentation resulted in coagulated milk with good appearance and a mild lactic flavor in all but two samples. Similarly, the pH of the PFM samples ranged from 4.25 to 4.86; except for the referred odd samples, which showed a pH of 6.35 and 6.68, respectively. These two samples have a strong putrefactive smell and, therefore, were not analyzed further by culturing. Colonies from the counting plates of all other 20 samples of RM (164), PM (110), and PFM (78), were randomly selected from PCAM and BHI media, purified and stocked. Colonies were molecularly identified by partial amplification of the 16S rRNA gene, sequencing and sequence comparison. The results obtained are summarized in Table 2. In terms of the number of species, the diversity was maintained in RM (13 species), PM

(11 species) and PFM (15 species) samples, although the species profiles of the two latter samples were completely different to those of the RM (Table 1). In RM samples near 90% of the isolates belonged to *Lactococcus* species (146 isolates), while these and other mesophilic species were replaced by thermophilic organisms in PM and PFM samples. In fact, only two species *S. thermophilus* and *Enterococcus durans/faecium* were encountered in all three types of samples. A majority of the species surviving the pasteurization step (PM) could also be recovered after incubation at 42°C (PFM). Only a few species were exclusively identified in the PFM samples. These belonged mainly to the genera *Bacillus*, *Lactobacillus*, and *Streptococcus*), suggesting they were minority components, which survived pasteurization and grew in milk at the incubation temperature. *S. thermophilus* was found as the single thermophilic bacterium in five PFM samples. Conversely, four different thermophilic species were identified in six PFM samples, and five and seven distinct species in one sample each (data not shown). Thus, a great variability of thermophilic bacteria exists at the species level in the analyzed milk samples.

4. Discussion

In this work, the diversity of thermophilic and aciduric bacteria present in milk has been investigated by culturing and by the culture-independent techniques of DGGE and pyrosequencing of 16S rDNA amplicons. Among these organisms, mild thermophilic LAB species (such as *S. thermophilus*, *L. delbrueckii*, and *L. helveticus*) are used worldwide as industrial starters for many fermented dairy commodities (Parente and Cogan, 2004; Mills et al., 2010). Milk samples were obtained in Northern Spain, a geographical area in which selective procedures for these types of organisms have never

been used traditionally. Therefore, though to constitute subdominant populations, these thermophilic species represent those dwelling normally in milk, as they had never been enriched by technological (evolutive) pressure. Number and types of thermoduric/aciduric bacteria were then compared with the dominant mesophilic species present in raw milk.

Pioneering application of the DGGE technique to traditional cheeses showed that, at least in some cheese systems, some dominant and metabolically active microorganisms had never been recovered by culturing (Ercolini et al., 2001; Randazzo et al., 2002). In that sense, our group has detected DGGE bands of thermophilic LAB species in cheeses made from either raw milk without added starters (Flórez and Mayo, 2006; Alegría et al., 2009) or from pasteurized milk inoculated with mesophilic starters (Alegría et al., 2011). The DGGE profiles of RM and PM samples were found to be identical or very similar. Thus, even though most mesophilic species are dead (as shown by the ensuing culturing results), they still contribute to the DNA pool which is subsequently amplified and resolved by the PCR-DGGE technique. Co-migration and double-banding are well recognized limiting factors of the DGGE (von Wintzingerode et al., 1997; Becker et al., 2000), as well as the formation of uncharacterized artifacts, hampering the identification of specific bands. In this work, bands for *Lc. garvieae* and *W. confusa/cibaria* were shown to migrate to the same position in the gels; but the two species were never present on the same sample. Twenty two different species were identified by DGGE among the 52 bands purified, reamplified and sequenced; this includes all intense bands. Intensity of an individual band is thought to be a semi-quantitative measure of the abundance of this sequence in the population (Muyzer et al., 1993). This statement is supported by the general agreement between the results obtained in this work by culturing and DGGE. As concern the RM samples, the predominant species determined

by both techniques were the same, *Lc. garvieae*, *Lc. raffinolactis*, *Lc. lactis*, and *Streptococcus* spp. However, in the PFM samples less diversity was found with the DGGE technique as compare to conventional culturing. Moreover, of the three bands observed in these samples, two of them (the upper and lower bands) belonged to a single species: *S. thermophilus* (bands 9). The remaining band (band 10) -thought supposed to belong to *L. delbrueckii*- could not be unequivocally identified. The sequence of this band showed some unspecificity; probably because of DNA contamination from the upper *S. thermophilus* band.

The bacterial diversity uncovered by pyrosequencing was much higher than that established with the culture-dependent approach and the PCR-DGGE method. Not surprisingly, pyrosequencing is very powerful tool for the analysis of structure and dynamics of the microbial populations from different ecosystems. It has already been applied to dairy products, including grains and beverages of kefir (Dobson et al., 2011; Leite et al., 2012) and the manufacturing and ripening of traditional (Alegría et al., 2012) and industrial cheeses (Masoud et al., 2012). In this work, only one RM and two PFM samples were subjected to pyrosequencing. Analysis of the rarefaction curves suggests that the bacterial community was well represented in all three samples. The diversity found by pyrosequencing was high at the phylum (5 phyla) and genus (53) levels. This technique identified *Leuc. citreum* as the dominant microorganism in sample D (RM), followed by *Lc. lactis*, *Lc. raffinolactis*, and *S. thermophilus*. Except for *Leuc. citreum*, all other three species gave intense bands by DGGE and were recovered from the cultures. By the DGGE technique the most intense band in this sample was shown to belong to *W. confusa/ciabaria*. *Weissiella* species were included until recently among the phylogenetically-related genus *Leuconostoc* (Collins et al., 1993). Misidentification may be due to the short DNA (180 bp) sequence (and at a

different position within the 16S rDNA) as compared to that obtained by pyrosequencing (>300 bp). In fact, although of biological significance, identification down to the species level by comparison of partial 16S rRNA gene sequences is tentative (Stackebrandt and Goebel, 1994; Palys et al., 1997). Thus, diversity indices and species richness should be more appropriately considered in terms of OTUs. In fact, sequence differences in OTUs assigned to the same species (Table 3) are considered to be due mostly to the arbitrary cutoff of 0.03%, although pyrosequencing errors may also contribute to this assignment (Sundquist et al., 2007). Confident identification at the genus/species level (95% and 97% pairwise sequence identities, respectively) will still require higher specificity of DNA amplification and more accurate database identification (Sundquist et al., 2007; Petrosino et al., 2009).

From a bacteriological point of view, most milk samples were encountered to be within the legal limits. The high number of isolates from different LAB species (and particularly that of *Lc. lactis*) indicates the milk is appropriate for cheesemaking, even for the manufacturing of raw milk cheeses without added starters. In addition, although some mastitis-causing organisms were retrieved (such as *Streptococcus agalactiae*), true human pathogens were never detected by culturing. As expected, except for *Enterococcus* spp., pasteurization killed most mesophilic LAB species, including all *Lactococcus* and some *Streptococcus* species. Enhanced thermotolerance of *Enterococcus* as compared to *Lactococcus* has been used as a differential phenotypic property to distinguish between species of these two genera (Devriese et al., 1995). The same bacterial species were recovered by culturing in PM and PFM samples, which strongly suggests that species surviving the pasteurization in milk (thermoduric) can all grow in milk at 42°C and resist alive at the low pH reached by fermentation (aciduric). Therefore, these two selective methods are considered adequate for the recovering of

thermophilic LAB from milk belonging to the most technologically-relevant species. *S. thermophilus* isolates were a majority in both PM and PFM samples, followed by *L. delbrueckii* and *S. macedonicus* (this last species has been recently reclassified as *Streptococcus gallolyticus* subsp. *macedonicus*; Schlegel et al., 2003). At different percentages, these microorganisms are usually recovered from both whey starters and the corresponding cooked cheeses (Pacini et al., 2006; Rossetti et al., 2008). The only discrepancy is *L. helveticus*, which in cooked cheese is usually found in a higher proportion. *L. helveticus* is one of the more aciduric LAB (Slattery et al., 2010), suggesting this species might be enriched through repeated manufacturing. As for the mesophilic LAB species (Delgado and Mayo, 2004; Alegría et al., 2009), high phenotypic and genetic diversity has been found among the thermophilic isolates (data not shown), which is indicative that adequate thermophilic starter candidates can surely be identified. It was surprising to find that, in terms of the number of species, the diversity was not reduced in PM and PFM samples as compared to RM samples. In fact, although the number of colonies identified in the PFM samples was lower (78), the number of species detected was higher (15).

In general, the results obtained by all three techniques utilized in this work agree well. They all identified mesophilic LAB as dominant among RM samples and thermophilic LAB (especially *S. thermophilus*) as predominant among PM and PFM samples. Discrepancies between results of culturing and culture-independent surveys have been repeatedly reported (Ercolini et al., 2001; Randazzo et al., 2002; Duthoit et al., 2003; Flórez and Mayo, 2006; Delbés et al., 2007; Alegría et al., 2009; Alegría et al., 2012), and may result from a number of biases, like differences in the lysis efficiency during DNA isolation, preferential PCR amplification (which may also be different for different primer pairs), or interspecies 16S rRNA operon copy number. As

seen in this and other works (Edalatian et al., 2012), detection of non cultivable and dead cells by the DNA-based techniques may also account for further differences.

5. Conclusions

Culturing and two culture-independent methods were used to analyze the thermophilic bacterial diversity present in milk from a geographical area in which thermophils are not selected by the traditional cheese technology. Our results clearly indicate the usefulness of a polyphasic combination of culture-dependent and culture-independent methods. These two approaches produce complementary information, which can be used for a more accurate microbial characterization of milk-associated environments. The dominant populations were investigated by culturing and PCR-DGGE, while pyrosequencing of 16S rDNA amplicons was used to obtain a deep inventory of all bacterial types. The results obtained with all three microbial techniques agree rather well in both type and numbers of the dominant populations, but differ in the minority components. The technological significance of the high bacterial diversity present in the milk samples and the sample-specific variation detected is unclear. It might well be related with the consubstantial batch to batch variation of fermented dairy products. The biochemical, genetic and technological properties of the wild isolates from this work belonging to *S. thermophilus*, *L. delbrueckii*, and *L. helveticus* species is currently under way. The results will then be compared with those obtained with industrial, well-recognized starter strains.

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Table 1.- Identification at the species level of representative pyrosequencing reads with the SeqMatch tool in the RDP and by BLAST analysis against GenBank.

OTU code	No. of reads	% of reads of each sample ^a			Closest cultivable species
		D (RM)	D (PFM)	C (PFM)	
OTU1	4,053	0.2	98.0	99.0	<i>Streptococcus thermophilus</i>
OTU2	1,292	61.2	1.0	0.6	<i>Leuconostoc citreum</i>
OTU3	97	4.4	0.3	>0.1	<i>Lactococcus plantarum</i>
OTU4	87	4.1	>0.1	0.1	<i>Vibrio cholerae</i>
OTU5	77	3.7	-	-	<i>Lactococcus plantarum</i>
OTU6	70	3.3	>0.1	>0.1	<i>Streptococcus</i> spp.
OTU7	56	2.7	-	>0.1	<i>Lactococcus raffinolactis</i>
OTU8	55	2.6	>0.1	-	<i>Lactococcus lactis</i>
OTU9	41	1.9	>0.1	-	<i>Citrobacter</i> spp.
OTU10	40	1.8	0.1	>0.1	<i>Lactococcus lactis</i>
OTU11	37	1.8	-	>0.1	<i>Weissella confusa</i>
OTU12	26	1.3	-	-	<i>Acinetobacter johnsonii</i>
OTU13	21	1.0	>0.1	-	<i>Geobacillus toebii</i>
OTU14	20	1.0	-	-	<i>Acinetobacter calcoaceticus</i>
OTU15	15	0.7	-	-	<i>Lactobacillus delbrueckii</i>
OTU16	15	0.7	-	-	<i>Methylobacterium</i> spp.
OTU17	15	0.7	-	-	<i>Methylobacterium populi</i>
Others (81)	151	6.9	0.4	-	<i>Veillonella</i> spp.

^aRM, raw milk; PM, pasteurized milk (63°C for 30 min) milk; PFM, pasteurized milk incubated for 24 h at 42°C.

Table 2.- Number of isolates and their frequency of appearance (percentage in parenthesis) of the different genera and species identified in milk by culturing.

Species	Analyzed sample ^a			TOTAL
	RM	PM	PFM	
<i>Streptococcus thermophilus</i>	4 (2.44)	62 (56.36)	40 (51.28)	106
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	58 (35.36)	-	-	58
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	57 (34.75)	-	-	57
<i>Lactobacillus delbrueckii</i> subsp. <i>delbrueckii</i>	-	15 (13.63)	14 (17.94)	29
<i>Lactococcus garvieae</i>	28 (17.07)	-	-	28
<i>Enterococcus durans</i> /E. <i>faecium</i>	1 (0.60)	11 (10.00)	1 (1.28)	13
<i>Streptococcus macedonicus</i>	-	3 (2.72)	9	12
<i>Streptococcus bovis</i> /S. <i>equinus</i> /S. <i>suis</i>	-	8 (7.27)	2 (2.56)	10
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	-	5 (4.54)	2 (2.56)	7
<i>Streptococcus agalactiae</i>	6 (3.65)	-	-	6
<i>Lactococcus raffinolactis</i>	4 (2.44)	-	-	4
<i>Enterococcus</i> spp.	-	2 (1.81)	1 (1.28)	3
<i>Bacillus licheniformis</i>	-	-	2	2
<i>Enterococcus faecalis</i>	-	1 (0.90)	1 (1.28)	2
<i>Acinetobacter</i> spp.	1 (0.60)	-	-	1
<i>Bacillus circulans</i>	-	1 (0.90)	-	1
<i>Bacillus</i> spp.	-	-	1 (1.28)	1
<i>Brevibacterium</i> spp.	1 (0.60)	-	-	1
<i>Enterobacter</i> spp.	1 (0.60)	-	-	1
<i>Hafnia alvei</i>	1 (0.60)	-	-	1
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>	-	-	1 (1.28)	1
<i>Lactobacillus helveticus</i>	-	-	1 (1.28)	1
<i>Leuconostoc pseudomesenteroides</i>	1 (0.60)	-	-	1
<i>Lysinibacillus fusiformis</i>	-	-	1 (1.28)	1
<i>Paenibacillus lautus</i> /P. <i>ginsengagri</i>	-	1 (0.90)	-	1
<i>Streptococcus salivarius</i>	1 (0.60)	-	-	1
<i>Streptococcus sanguinis</i>	-	-	1 (1.28)	1
<i>Streptococcus</i> spp.	-	-	1 (1.28)	1
<i>Virgibacillus proomii</i>	-	1 (0.90)	-	1
TOTAL	164 (100)	110 (100)	78 (100)	352
Number of species detected	13	11	15	29

^aRM, raw milk; PM, pasteurized (63°C for 30 min) milk; PFM, pasteurized milk incubated for 24 h at 42°C

Figure1.pptx

Figure 2

Figure 3

Figure 4

Figure Legends

Figure 1.- DGGE profiles of the V3 variable region of the bacterial 16S rRNA gene of 10 samples (A through J, in Panels A and B) of raw milk (RM), pasteurized milk at 63°C for 30 min (PM), and pasteurized milk incubated at 42°C for 24 h (PFM). M, DGGE markers used as a control of the electrophoresis and composed of amplicons from isolated strains, as follows: a, *Weissella confusa*; b, *Macrococcus caseolyticus*; c, *Streptococcus infantarius*; d, *Lactococcus lactis*; e, *Streptococcus thermophilus*; and f, *Brevibacillus brevis*. Bands with a number on the top were identified by sequencing and sequence comparison. Identification key: 1, *Streptococcus* spp. (*S. infantarius*, *S. equinus*, *S. gallolyticus*); 2, *Lactococcus garvieae*; 3, *W.confusa/cibaria*; 4, *Leuconostoc citreum*; 5, *Lactococcus raffinolactis*; 6, *Lactobacillus brevis*; 7, *Streptococcus viridans*; 8, *Lc. lactis*; 9, *S. thermophilus*; 10, *Lactobacillus* spp., 11, *Streptococcus salivarius*; 12, *Pseudomonas putida*; 13, *Pseudomonas fragi*; 14, *Pseudomonas fluorescens*; 15, *Macrococcus* spp.; 16, unidentified band; 17, *Lactococcus plantarum*; 18, *S. infantarius*; 19, *Bacillus* spp.; 20, *Brevibacillus* spp., 21, *Brevibacillus brevis*; 22, *Clostridium perfringens*, 23, *Clostridium mesophilum*.

Figure 2.- Estimated OTU richness and diversity index for 16S rRNA genes at 3% of divergence distance of the three pyrosequenced samples: milk D (RM), milk D (PFM) and milk C (PFM).

Figure 3.- Rarefaction curves of partial sequences of the bacterial 16S rRNA gene from the samples milk D (RM), milk D (PFM) and milk C (PFM).

Figure 4.- The relative abundance at the genus level of the reads belonging to phylum *Firmicutes*, class *Bacilli* of the three analyzed milk samples using RDP-Classifer.