Impact of thistle rennet from Carlina acanthifolia All. subsp. acanthifolia on bacterial diversity and dynamics of a specialty Italian raw ewes' milk cheese

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Dear Editor,
would you please consider the manuscript titled "**Impact of thistle rennet from *Carlina acanthifolia* All. subsp. *acanthifolia* on bacterial diversity and dynamics of a specialty Italian raw ewes' milk cheese"** for publication as short communication in International Journal of Food Microbiology.

Caciofiore della Sibilla is an Italian specialty soft cheese manufactured with *Sopravissana* raw ewes' milk and thistle rennet prepared with young fresh leaves and stems of *Carlina acanthifolia* All. subsp. *acanthifolia*, according to an ancient tradition deeply rooted in the territory of origin (mountainous hinterland of the Marche region, Central Italy). In this study, the impact of thistle rennet on the bacterial dynamics and diversity of *Caciofiore della Sibilla* cheese was investigated by applying a polyphasic approach based on culture and DNA-based techniques (Illumina sequencing and PCR-DGGE). A control cheese manufactured with the same batch of ewes' raw milk and commercial animal rennet was analyzed in parallel. Overall, a large number of bacterial taxa were identified, including spoilage, environmental and pro-technological bacteria, primarily ascribed to *Lactobacillales*. Thistle rennet was observed clearly to affect the early bacterial dynamics of *Caciofiore della Sibilla* cheese with several bacterial groups (*Lactobacillus alimentarius*/*paralimentarius* and *Lactobacillus plantarum*/*paraplantarum*/*pentosus*) being detected in the phyllosphere of *C. acanthifolia* All, thistle rennet and curd obtained with thistle rennet. Other bacterial taxa, hypothetically originating from the vegetable coagulant (*Enterococcus faecium, Lactobacillus brevis, Lactobacillus delbrueckii, Leuconostoc mesenteroides/pseudomesenteroides*), were exclusively found in *Caciofiore della Sibilla* cheese by PCR-DGGE. At the end of the maturation period, Illumina sequencing demonstrated that both cheeses were dominated by *Lactobacillales*; however curd and cheese produced with thistle rennet were co-dominated by *Lactobacillus* and *Leuconostoc*, whereas *Lactoccous* prevailed in curd and cheese produced with commercial animal rennet followed by *Lactobacillus*. Differences in the bacterial composition between the two cheeses at the end of their maturation period were confirmed by PCR-DGGE analysis.

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Highlights

- *Caciofiore della Sibilla* cheese and a control cheese were analyzed in parallel
- A polyphasic approach based on culture and DNA-based techniques was applied
- Thistle rennet affected early bacterial dynamics of *Caciofiore della Sibilla* cheese
- The two cheeses differed in their bacterial composition at the end of ripening

Impact of thistle rennet from *Carlina acanthifolia* All. subsp. *acanthifolia* on bacterial diversity and dynamics of a specialty Italian raw ewes' milk cheese

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Abstract

Caciofiore della Sibilla is an Italian specialty soft cheese manufactured with *Sopravissana* raw ewes' milk and thistle rennet prepared with young fresh leaves and stems of *Carlina acanthifolia* All. subsp. *acanthifolia*, according to an ancient tradition deeply rooted in the territory of origin (mountainous hinterland of the Marche region, Central Italy). In this study, the impact of thistle rennet on the bacterial dynamics and diversity of *Caciofiore della Sibilla* cheese was investigated by applying a polyphasic approach based on culture and DNA-based techniques (Illumina sequencing and PCR-DGGE). A control cheese manufactured with the same batch of ewes' raw milk and commercial animal rennet was analyzed in parallel. Overall, a large number of bacterial taxa were identified, including spoilage, environmental and pro-technological bacteria, primarily ascribed to *Lactobacillales*. Thistle rennet was observed clearly to affect the early bacterial dynamics of *Caciofiore della Sibilla* cheese with several bacterial groups (*Lactobacillus alimentarius*/*paralimentarius* and *Lactobacillus plantarum*/*paraplantarum*/*pentosus*) being detected in the phyllosphere of *C. acanthifolia* All, thistle rennet and curd obtained with thistle rennet. Other bacterial taxa, hypothetically originating from the vegetable coagulant (*Enterococcus faecium, Lactobacillus brevis, Lactobacillus delbrueckii, Leuconostoc mesenteroides/pseudomesenteroides*), were exclusively found in *Caciofiore della Sibilla* cheese by PCR-DGGE. At the end of the maturation period, Illumina sequencing demonstrated that both cheeses were dominated by *Lactobacillales*; however curd and cheese produced with thistle rennet were co-dominated by *Lactobacillus* and *Leuconostoc*, whereas *Lactoccous* prevailed in curd and cheese produced with commercial animal rennet followed by *Lactobacillus*. Differences in the bacterial composition between the two cheeses at the end of their maturation period were confirmed by PCR-DGGE analysis.

Keywords: *Sopravissana* raw ewes' milk cheese, vegetable milk coagulant, bacterial biota, culture, PCR-DGGE, high-throughput sequencing

1. Introduction

Caciofiore della Sibilla is a specialty soft cheese manufactured in a restricted central Italian geographical area using *Sopravissana* raw ewes' milk and thistle rennet obtained from young fresh leaves and stems of *Carlina acanthifolia* All. subsp. *acanthifolia* according to an ancient local tradition.

The term "thistle" refers to plants belonging to the tribe *Cynareae* (synonym: *Cardueae*) especially ascribed to the genera *Carduus*, *Cirsium*, and *Onopordum*. However, plants outside this tribe are sometimes considered thistles, including those within the genera *Cynara*, *Scolymus*, *Silybum*, *Onopordum*, and *Carlina*. In the Mediterranean area, the exploitation of thistle rennet, especially that obtained from *Cynara* spp., is particularly widespread in western Africa (García et al., 2012; Cardinali et al., 2016), Italy, and the Iberian peninsula, the latter boasting a large number of Protected Designation of Origin (PDO) cheeses manufactured with this peculiar coagulant (Aquilanti et al., 2011; Cardinali et al., 2016). Cheeses coagulated with thistle rennet are generally manufactured at family-run or artisan dairy farms, most often located in marginal areas (e.g., high altitude pastures, dry lands or islands) using raw ewes' or goats' milk, or a mixture of both. Though these cheeses are greatly appreciated by consumers for their unique, distinctive flavor, their manufacturing is generally seasonal, mainly due to limitations on the availability of young leaves or flowers from spontaneously growing thistles.

Caciofiore della Sibilla cheese undoubtedly falls within this cheese category. The manufacturing technology of this cheese, which had been lost for more than 50 years in the original area of production, has very recently been revived by two local family-run dairies located in Pieve Torina and Belforte del Chienti (Macerata district), respectively. The cheese produced by these local dairies from late spring to early summer is 3-4 cm tall, has an average weight of 0.2 to 0.8 kg and is characterized by a very thin straw-white outer rind and a cream-white soft core, with a sweetish buttery smell, a delicate but incisive flavor with a scent of wild herbs, and a slightly acidulous, pleasant taste.

To date, few studies have been conducted to identify the bacterial biota harboured by thistle-rennet cheeses (Sousa and Malcata, 1997; Vioque et al., 2000; Gómez et al., 2001; Roseiro et al., 2003; Fernández-Salguero et al., 2002; Tejada and Fernández-Salguero, 2003; Aquilanti et al., 2011; Galán et al., 2012; Ordiales et al., 2013) and almost all of these investigations have been focused on Protected Designation of Origin (PDO) or specialty cheeses manufactured with thistle coagulants obtained from *Cynara* spp.

Based on these premises, this study uses a polyphasic molecular approach based on culture and DNA-based techniques to assess the impact of an unexplored milk coagulant obtained from *Carlina acanthifolia* All. subsp. *acanthifolia* on the bacterial dynamics and diversity of *Caciofiore della Sibilla* cheese. To this end, two cheese manufactures produced with

the same batch of *Sopravissana* raw ewes' milk and coagulated with either thistle rennet or commercial powdered animal rennet were analyzed in parallel and the results comparatively evaluated.

2. Materials and Methods

2.1. Cheese-making process and sampling

Caciofiore della Sibilla cheese was made in a family-run dairy farm located in Pieve Torina (Italy) following an ancient local manufacturing method without any addition of starter cultures.

The sole *Caciofiore della Sibilla* cheese manufacture produced by the dairy farm during spring and summer 2015 was sampled and analysed. Raw milk obtained from *Sopravissana* ewes during one milking day was filtered and separated into two batches; three cheese wheels were produced from each batch. The first batch (labelled "C") was coagulated with commercial powdered calf rennet (Caglificio Clerici, Cadorago, CO, Italy; 1:10,000), whereas the second batch (labelled "Cf") was coagulated with a crude aqueous extract prepared from *C. acanthifolia* All. subsp. *acanthifolia*. The traditional preparation of the crude aqueous extract based on the use of fresh young leaves and stems - containing a considerable quantity of latex - of plants spontaneously growing in the high altitude pastures (>1000 m a.s.l.) of Monti Sibillini National Park has previously been detailed by Cardinali et al. (2016). Milk coagulants were added to raw milk pre-heated at 35 °C and gently stirred. After clotting (ca. 30'), the curds were manually broken into rice-sized grains, transferred into plastic perforated molds (8 x 8 cm) and manually pressed to remove the whey. Molded cheeses were held at 12-13 °C for 4 h, dry salted and ripened for 20 days under controlled conditions (12-13 °C and 70 % relative humidity).

Ewes' raw milk, fresh young leaves and stems of *C. acanthifolia* All. subsp. *acanthifolia*, thistle rennet, curds and cheeses (after 1, 3, 6, 10 and 20 days of ripening) were collected in triplicate. Samples were transported to the laboratory under controlled temperature (4 °C) and processed within 24 h. Triplicate samples were pooled before viable counting and molecular analysis.

2.2. pH measurements

The pH of samples collected during cheese-making was measured using the pH meter 300 (Hanna Instruments, Padova, Italy), equipped with a solid electrode (HI2031, Hanna Instruments). Three independent measurements were performed for each sample, and the mean values ± standard deviations were calculated.

2.3. Microbial counts and bulk formation

For the microbial counts, 10 grams of each solid sample were accurately homogenized in 90 mL of sterile aqueous citrate (2 % w/v) for 2 min at 260 rpm using a Stomacher apparatus (400 Circulator, VWR International PBI, Milan, Italy). Aliquots of decimal dilutions of raw milk, thistle rennet and the homogenates were inoculated in duplicate on opportune solid media to determine the load of (i) presumptive lactococci, thermophilic cocci and lactobacilli; (ii) coagulase-negative cocci; (iii) total mesophilic aerobes; (iv) enterococci; (v) and finally *Enterobacteriaceae* (Garofalo et al., 2017). Viable counts were expressed as log colony forming units (log cfu) per gram or mL of sample ± standard deviations. For all samples but cheeses, bulk cells were prepared by harvesting confluent colonies from both the lowest MRSA and M17 dilution plates and the countable plates with a number of colonies ranging from 30 to 300 (Garofalo et al. 2015). For cheeses, the sole colonies grown on the countable plates were harvested. Harvested cells were suspended in 2 mL of sterile saline solution added with glycerol, and stored at -20 °C prior to DNA extraction (Garofalo et al., 2015).

2.4. DNA extraction from cheese samples and bulks

For raw milk, thistle rennet, curd and cheese homogenates, total microbial DNA was extracted using a commercial kit (PowerFood™ Microbial DNA Isolation Kit, Mo Bio Laboratories, Carlsbad, USA), as previously elucidated (Cardinali et al. 2016). For bulk cells, DNA extraction was performed using the method proposed by Hynes et al. (1992) with some modifications as reported by Osimani et al. (2015). Assessment of quantity and purity of DNA extract was carried out as described by Osimani et al. (2016).

2.5. PCR amplification and DGGE analysis

The universal primers 338F (5'-ACT CCT ACG GGA GGC AGC AGC AG-3'), added with the GC clamp at the 5' end (Ampe et al. 1999), and 518R (5'-ATTACC GCG GCT GCT GG-3') were used to amplify the V3 region of the 16S rRNA gene (Alessandria et al., 2010).

Approximately 100 ng of bacterial DNA were amplified as previously reported by Osimani et al. (2015) using the thermal cycler My Cycler (Bio-Rad Laboratories, Segrate, Italy). DGGE runs were performed as reported by the same authors. Sequencing of DNA eluted from selected DGGE bands was performed in accordance to Taccari et al. (2016). Sequences were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/) using

155 the Basic Local Alignment Search Tool (BLAST). A sequence identity ≥97 % was chosen as a threshold for
156 unambiguous assignment into species.
157
158 *2.6. DNA amplification and Illumina sequencing*
159
160 The extracted DNA was used to study the bacterial diversity of the samples (raw milk, thistle rennet, curds and cheeses)
161 by 16S rRNA amplicon Illumina sequencing. A 464-nucleotide sequence of the V3-V4 region of the 16S rRNA gene
162 was analyzed as previously described by Alfonzo et al. (2017).
163
164 *2.7. Illumina data analysis and sequence identification by QIIME*
165
166 Sequences obtained from Illumina sequencing were processed using Quantitative Insights Into Microbial Ecology
167 (QIIME) software package version 1.9 (Caporaso et al., 2010) as previously detailed by Alfonzo et al. (2017). The data
168 generated by Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under
169 Ac. No. PRJNA340351.
170
171 *2.8. Statistical analysis*
172
173 For each cheese batch, viable counts recorded after 1, 3, 6, 10 and 20 days of maturation were analyzed by an ANOVA
174 and a Tukey's test (α= 0.05) for means separation. At each sampling time, data from the two batches "C" and Cf" were
175 compared using the Student's t-test (α=0.05). A principal component analysis (PCA) with a correlation matrix was
176 carried out on standardized data to visually identify bacterial dynamics over time. All the statistical analyses were
177 performed using JMP statistical software v. 11.0 (SAS Institute, Cary, NC, USA).
178
179 **3. RESULTS**
180
181 *3.1. pH measurement and viable counting*
182
183 Raw ewes' milk and thistle rennet were characterized by pH mean values of 6.39±0.10 and 3.79±0.05, respectively; the
184 results of pH measurements carried out in curds and cheeses sampled at different time points are reported in Fig. 1. As

185 revealed by ANOVA, curds and cheeses coagulated with thistle and animal rennet significantly differed in their pH
186 values up until the 10th day of ripening, whereas after 20 days of maturation, both cheeses reached similar pH values.
187 The results of viable counting are shown in Table 1. The phyllosphere of *C. acanthifolia* All. was characterized by high
188 levels of total mesophilic aerobes, lactococci, *Enterobacteriaceae* and coagulase-negative cocci and, conversely, by low
189 levels of presumptive thermophilic cocci, enterococci and lactobacilli. Low bacterial loads were seen in thistle rennet.
190 No significant differences were observed between raw ewes' milk and curds in the viable counts of presumptive
191 lactobacilli, total mesophilic aerobes and enterococci, whereas significant differences were observe in the loads of
192 presumptive lactococci, termophilic cocci and coagulase-negative cocci.
193 Viable counts of lactobacilli, total mesophilic aerobes, enterococci and *Enterobacteriaceae* increased progressively
194 during the ripening of *Caciofiore della Sibilla* cheese; moreover, at the end of the maturation period (20 days), the two
195 cheeses differed significantly in the load of all the bacterial groups assayed, except for lactobacilli and
196 *Enterobacteriaceae*.
197 When PCA was computed, the first component accounted for 85.7 % of total variability whereas the second component
198 accounted for an additional 5.3 % (Fig. 2). As clearly evidenced by the plot, the C*aciofiore della Sibilla* cheese had a
199 greater range of variation in the bacterial loads than the control cheese, especially up until the third day of maturation.
200
201 *3.2. PCR-DGGE analyses*
202
203 The DGGE profiles obtained by analyzing the DNA extracted from both the samples and the bulk cells harvested from
204 selected dilution plates are shown in Fig. S1 and S2, and the results of multiple alignments of DNA sequences from
205 selected DGGE bands are shown in Table S1. These results are summarized in Tables 2 and 3. In the phyllosphere of *C.*
206 *acanthifolia* All., the closest relatives to *Pantoea agglomerans, Pseudomonas alcaliphila Acinetobacter baumannii*, the
207 *Lactobacillus alimentarius/paralimentarius* group, the *Lactobacillus graminis/curvatus group*, *Enterobacter* spp.,
208 *Pseudomonas* spp., and *Bacillus* spp. were found. Members of the latter genus were also detected in thistle rennet, along
209 with members of the *Lactobacillus plantarum/paraplantarum/pentosus* group and the *Lactobacillus*
210 *alimentarius*/*paralimentarius* group.
211 In raw ewes' milk, the following taxa were detected: *Acinetobacter johnsonii, Enterobacter cloacae, Enterobacter*
212 *hormaechei, Pseudomonas* spp. and *Lactococcus lactis.* The latter species was detected at both the lowest and highest
213 dilution.
214 Completely different bacterial compositions were seen in the two curds: curd obtained with commercial animal rennet
215 was dominated by *Enterobacteriaceae* bacteria, while curd obtained from thistle rennet was dominated by lactic acid

bacteria. In general, the bacterial diversity of cheeses during ripening was higher than that of raw milk, thistle rennet and curds. Furthermore, if the two cheeses were comparatively evaluated, a few taxa, such as *Enterococcus faecium*, *Lactobacillus brevis*, *Leuconostoc mesenteroides* and *Weisella* spp., were exclusively found in *Caciofiore della Sibilla* cheese, whereas other taxa, such as *Bifidobacterium dentium*, *Citrobacter* spp., *Enterobacter hormaechei*, *Erwinia chrysanthemi* and the *Lact. alimentarius/paralimentarius* group, were found only in the control cheese. By contrast, the following bacteria were identified in both cheeses, namely, *Enterococcus feacalis*, *L. lactis*, the *Lactobacillus casei/paracasei/rhamnosus* group, the *Lact. plantarum/paraplantarum/pentosus* group and *Staphylococcus sciuri*. With the exception of *Bacillus subtilis*, *Acinetobacter johnsonii* and *Enterobacter cloacae*, all of the remaining taxa were detected in the highest dilution plates, thereby suggesting a load of at least 10^5-10^8 cfu/g.

3.3. Illumina sequencing

The DNA extracted from ewes' raw milk, curds and cheeses was successfully amplified in the bacterial V3-V4 16S rRNA gene region. After splitting and quality trimming of raw data, 62,772 reads remained for subsequent analysis. After alignment, OTUs were clustered at a 3 % distance and the doubles and singletons (OTUs counting only two or one reads, respectively) were discarded by a filter script implemented in QIIME. Chao1 estimator predicted an average of 688 and 231 ± 43 OTUs in milk and cheese samples, respectively, and the average observed counts were 661 and 176 ± 40 OTUs, respectively, suggesting that we were able to capture approximately 96 % and 76 % of the OTUs estimated as present in the milk and cheese bacterial populations, respectively.

In most samples, a number of OTUs belonged to chloroplasts. These OTUs were not very abundant in raw milk, curd produced with animal rennet, and *Caciofiore della Sibilla* cheese at 3 and 20 days of ripening, accounting for less than 1.2 % of total reads number, with the exception of *Caciofiore della Sibilla* cheese at 3 days of ripening, where they constituted 10.6 % of the total reads number. Conversely, chloroplast OTUs constituted the majority of the reads number in curd obtained with thistle rennet and *Caciofiore della Sibilla* cheese collected after 1 day of ripening, accounting for 81.9 % and 78.5 % of the total reads number, respectively. The relative abundance (%) of the different bacterial groups identified in raw milk, curds and cheeses are reported in Fig. 3. Only the groups with an incidence of 0.1 % were considered. The majority of milk OTUs belonged to three different groups: *Lactobacillales*, *Enterobacteriaceae* and *Clostridiales*, which together accounted for 60 % of the total number of bacterial OTUs in raw milk. Various minority taxa were also identified, including *Staphylococcus*, *Planococcaceae* and *Bacteroidales*.

As the bacterial dynamics were comparatively evaluated along with the two cheese-making processes, the following evidence emerged. In curd obtained with commercial animal rennet, *Planococcaceae* and *Enterobacteraceae* prevailed,

247 followed by *Lactobacillales*, *Staphylococcus* and *Macrococcus*. *Enterobacteraceae, Lactobacillales* and
248 *Staphylococcus* were also found in curd obtained with thistle rennet. At the end of the maturation period, both cheeses
249 were dominated by *Lactobacillales*, which accounted for approximately 65 % of the total number of bacterial OTUs;
250 however, different minority taxa were found, namely, *Enterobacteriaceae* in control ewes' milk cheese, and both
251 *Macrococcus* and *Acidobacteria* in *Caciofiore della Sibilla* cheese.
252 In Fig. 4, a finer taxonomic composition within the order *Lactobacillales* is shown. In raw ewes' milk, OTUs
253 corresponding to *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Staphylococcus*, *Carnobacterium*, *Leuconostoc* and
254 *Weissella* were found, though for the latter two taxa, a relative abundance lower than 0.1 % was seen. In curd and
255 cheese obtained with thistle rennet, *Lactobacillus* and *Leuconostoc* co-dominated, whereas *Lactococcus* prevailed in
256 curd and cheese produced with commercial animal rennet, followed by *Lactobacillus*. The two cheese manufactures
257 also differed in the composition of minority taxa, with *Leuconostocs* and *Pediococci* occurring at low levels in both the
258 control and *Caciofiore della Sibilla* cheese, respectively.
259
260 **4. Discussion**
261
262 To the best of the authors' knowledge, this report is the first to describe the contribution of thistle rennet obtained from
263 *C. acanthifolia* All. subsp. *acanthifolia* to cheese bacterial composition and dynamics. Moreover, no previous
264 investigations have been conducted on *Caciofiore della Sibilla* cheese, a Mediterranean soft ewes' raw milk cheese
265 manufactured according to a revived local ancient technology.
266 As far as raw *Sopravissana* ewes' milk was analysed, viable counts of presumptive lactobacilli, lactococci, thermophilic
267 cocci, total mesophilic aerobes, *Enterobacteriaceae* and coagulase-negative cocci were within the range reported by
268 other authors for raw ewes' milk (Quigley et al. 2013).
269 Regarding thistle rennet, viable counts of total mesophilic aerobes and presumptive lactobacilli were comparable to
270 those found by other authors (Aquilanti et al. 2011; Barbosa et al., 1981) in thistle rennets obtained from *Cynara* spp.
271 dried flowers; by contrast, slightly lower counts of presumptive lactococci and thermophilic cocci were found compared
272 to those reported by the same authors (Aquilanti et al. 2011; Barbosa et al. 1981).
273 At the end of ripening (20 days) *Caciofiore della Sibilla* cheese showed viable counts of total mesophilic aerobes and
274 presumptive lactobacilli comparable with those reported by Aquilanti et al. (2013) in a raw cow's milk *Caciotta* cheese
275 manufactured with an aqueous extract of *C. cardunculus*, whereas the counts of coagulase-negative cocci and
276 *Enterobacteriaceae* were higher. Higher counts of *Enterobacteriaceae* in cheeses at the end of their ripening might
277 suggest a lack of hygiene during milking and cheese-making. However, the metabolic activity of members of this

bacterial family has been positively correlated to high proteolysis and lipolysis in artisanal cheeses, resulting in the production of volatile aroma compounds (Tabla et al., 2016).

When DNA-based techniques were applied to the profiling of the bacterial biota, numerous taxa were identified, including starter lactic acid bacteria (LAB), namely *Lactococcus lactis*, which is known to lower the pH and control fermentation rapidly) and a wide variety of adventitious microorganisms (such as environmental, spoilage and non-starter lactic acid bacteria (NSLAB), which gain access to the cheese at any stage of the manufacturing process). NSLAB are mainly responsible for key physico-chemical transformations, e.g., proteolysis and lipolysis, which greatly impact basic cheese characteristics, such as flavor, appearance and texture (Gobbetti et al., 2015). Illumina sequencing allowed both the majority and minority taxa to be identified, whereas single bacterial species were unambiguously recognized by PCR-DGGE. When the latter technique was applied, discrepancies were seen in the fingerprints obtained by the bulk and the direct approach, in terms of both the number of taxa identified and their relative abundance; this finding was in agreement with a recent finding by the same authors on semi-hard cheese models (Aquilanti et al., 2016)

In *Sopravissana* raw ewes' milk, PCR-DGGE allowed the sole species *L. lactis* to be identified among LAB. Illumina sequencing confirmed the occurrence of *Lactococcus* spp. in raw ewes' milk, though as a minority taxon with respect to the members of the genus *Lactobacillus*. The presence of both lactococci and lactobacilli in raw ewes' milk has previously been documented, as reviewed by Quigley et al. (2013). The presence of *L. lactis* was also evidenced in two previous studies (Aquilanti et al., 2011; 2013) carried out onto raw cow's milk *Caciotta* cheeses manufactured with aqueous extract of *Cynara cardunculus* dried flowers. The occurrence of additional genera, including *Aerococcus Leuconostoc, Streptococcus* and *Enterococcus,* has previously been found in raw ewes' milk by next-generation sequencing (Quigley et al., 2013).

In both the phyllosphere of *C. acanthifolia* All. and thistle rennet, culture-dependent PCR-DGGE identified various LAB species, as well as both spoilage and environmental microorganisms. To date, there is a paucity of data on the bacterial biota colonizing the aerial surfaces of thistles and thistle rennets. In 1968, Mundt and Hammer (1968) first enumerated and identified lactobacilli on plant surfaces; in such a pioneering systematic study, *L. plantarum*, L. *fermenti* and L. *brevis* were the species most frequently isolated, whereas *L. casei*, *L. viridescens*, *L. cellobiosus*, *L. salivarius,* and *L. buchneri* occurred at lower frequencies. A widespread but sporadic distribution of low counts of lactobacilli in the phyllosphere of higher plants was also highlighted by the same authors. In accordance with data collected in the present study, Widnyana and Javandira (2016) recently reported the presence of *Bacillus* spp. and *Pseudomonas* spp. on plant surfaces, where these microorganisms exert a plant-growth stimulating activity as well as an inhibitory activity towards plant pathogens.

Lactobacilli ascribed to the *Lact. alimentarius/paralimentarius* group and the *Lact, plantarum/paraplantarum/pentosus* found by PCR-DGGE in both the phyllosphere of *C. acanthifolia* All. and thistle rennet were also detected in curd obtained by milk coagulation with thistle rennet, thus strongly suggesting a role of the coagulating agent in the early bacterial dynamics of *Caciofiore della Sibilla* cheese. By contrast, the occurrence of *P. acidilactici* in the sole curd obtained by milk coagulation with thistle rennet is more likely related to an environmental contamination, since this species was apparently absent in ewes' raw milk, thistle phyllosphere and thistle rennet.

A different picture emerged in curd obtained by milk coagulation with commercial animal rennet. In this case, LAB were found to be the minority by Illumina sequencing; among these microorganisms, *Lactococcus*, likely derived from raw ewes' milk, was predominant. PCR-DGGE confirmed the occurrence of *L. lactis* as the sole pro-technological species, along with various spoilage and pathogenic bacteria.

As far as the bacterial dynamics are considered, the stable presence of *L. lactis* in both the *Caciofiore della Sibilla* and control cheeses suggested by the results of PCR-DGGE might be, at least in part, ascribed to the contribution of raw ewes' milk, where lactococci and *L. lactis* have been revealed by llumina sequencing and culture-dependent PCR-DGGE, respectively. It is worth noting that when this latter technique was applied, closest relatives to *L. lactis* were found in the bulk cells harvested from both the lowest and the highest dilution plates, thus suggesting a load of *L. lactis* $\geq 10^5$ ufc/mL. *L. lactis* has a wide ecological distribution and is mostly associated with the milk environment and dairy products (Cavanagh et al., 2015). As recently elucidated by Ruggirello et al. (2016), this species can persist in late ripening of cheese in both the viable and the viable but nonculturable (VNC) states, shifting its catabolism to peptide and amino acid consumption.

Besides *Lactococcus*, bacteria ascribed to *Leuconostoc* and *Lactobacillus* dominated during ripening of *Caciofiore della Sibilla* cheese; both genera have been detected from t1 to t20 by both Illumina sequencing and PCR-DGGE analysis.

The dominance of *Leuconostoc* during the maturation of *Caciofiore della Sibilla* cheese might be explained by the higher initial pH of the curd obtained with thistle rennet compared to that produced with commercial animal rennet. Indeed, as reported by Hemme and Foucaud-Scheunemann (2004), *Leuconostoc* can be affected by the acidification of the growth medium. Interestingly, *Leuconostoc* was indicated by Illumina sequencing as a minority bacterial taxon in control ewes' milk cheese; however, no closest relatives to this genus were identified by PCR-DGGE in this cheese manufacture in either of the two approaches, thus strongly emphasizing the usefulness of a combined analytical strategy. Moreover, since apparently neither raw ewes' milk nor thistle and thistle rennet harboured leuconostocs, as revealed by PCR-DGGE, the occurrence of these microorganisms in *Caciofiore della Sibilla* cheese might be tentatively ascribed to a contamination from the dairy environment. However, members of the genus *Leuconostoc* have previously been detected in a raw cow's milk Caciotta cheese manufactured with thistle rennet from *Cynara cardunculus* dried

flowers (Aquilanti et al., 2011), as well as in other cheeses produced with vegetable coagulants, such as *Serra da Estrela* PDO cheese and *La Serena* PDO cheese (Macedo et al., 2004). Based on these latter evidences, it might be also hypothesized that in thistle, thistle rennet and curd obtained with thistle rennet, leuconostcocs occurred at levels below the limit of detection of the PCR-DGGE technique.

In addition, *Lactobacillus delbrueckii*, *Lactobacillus brevis*, and *Enterococcus faecium* were retrieved by PCR-DGGE exclusively in *Caciofiore della Sibilla* cheese throughout its maturation. Since these species could not be detected by PCR-DGGE before t1, again it might be hypothesized that they originate from the vegetable coagulant, where they occur at very low levels. *Lactobacillus delbrueckii* includes a subspecies that can be positively affected by the addition of vegetable extracts to the dairy matrix (Joung et al., 2016); even *Lactobacillus brevis* has been retrieved in vegetable-based matrices (Ruiz Rodriguez et al., 2016). Finally, enterococci are NSLAB that are naturally present in the gastrointestinal tract of humans and animals; they can enter the dairy environment via cross-contaminations, where they play a crucial role during cheese ripening, due to their high adaptation to the cheese environment (e.g., high salt concentration and low pH) (Giraffa 2003) and to acknowledged technological traits, such as the production of bacteriocins (İspirli et al., 2017).

Finally, members of two additional bacterial groups, namely, *Lactobacillus casei*/*paracasei*/*rhamnosus* and *Lactobacillus plantarum*/*pentosus*/*paraplantarum*, were identified by PCR-DGGE in both cheese-manufactures; since these microorganisms could not be detected in either raw ewes' milk or thistle rennet, once again, a contamination from the dairy environment might be hypothesized.

An important piece of evidence addresses the occurrence of *Enterobacteriaceae* in the two cheese manufactures: a higher occurrence of this microbial group was seen in control ewes' milk cheese by applying both DNA-based techniques. However, this finding was not supported by viable counts; indeed, at the end of the ripening period, comparable loads of *Enterobacteriaceae* were found in the two cheeses by culturing in selective VRBA medium, thus suggesting the need for an integration between culture-dependent and culture-independent techniques. It is worth remembering that Illumina sequencing provides a relative but not absolute abundances of single microbial taxa, whereas PCR-DGGE allows a qualitative or, at most semi-quantitative, overview of heterogeneous microbial communities.

5. Conclusions

Overall, the combined analytical approach enabled differences and similarities in the bacterial biota and dynamics of the two cheeses under study to be defined. The integration of data from viable counting, Illumina sequencing and PCR-DGGE provided the detection of major and minor bacterial components (Illumina sequencing and viable counting), as

well as the identification of single bacterial species (PCR-DGGE) that could not be revealed with the first two techniques. The primary result confirmed the association of lactobacilli, lactococci and leuconostocs with cheese and the dairy environment; moreover, the vegetable coagulant was demonstrated to affect the early bacterial dynamics of *Caciofiore della Sibilla* cheese, and at the end of its maturation, the composition of the bacterial biota significantly differed from the control ewes' milk cheese.

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Figure legends

Fig. 1. Results of pH measurements of curd obtained from milk coagulation with commercial rennet, curd obtained from milk coagulation with thistle rennet, *Caciofiore della Sibilla* and control ewes' milk cheese during cheese-making and ripening.

* pH values of curd obtained from milk coagulation with commercial rennet (◊) and from milk coagulation with thistle rennet (Δ).

Fig. 2. Loading and score plot of principal components analysis (PCA) based on viable counts evolution over time of *Caciofiore della Sibilla* (dots) and control ewes' milk cheese (triangles).

Fig. 3. Relative abundances (%) of bacterial groups and vegetable genome identified by MySeq Illumina in raw ewes' milk (rm); curd obtained by milk coagulation with commercial animal rennet (cc); curd obtained by milk coagulation with carline thistle rennet (ct); control raw ewe's milk cheese and *Caciofiore della Sibilla* cheese collected at 1, 3, and 20 days of ripening (C_1, C_3, C_{20} and Cf_1, Cf_3, Cf_{20} respectively).
"Other" represents all genera that were unidentified, belonged to uncultured or whose abundance was less than 0.1 %.

Fig. 4. Relative abundances (%) of the different LAB genera identified by MySeq Illumina in raw ewes' milk (rm); curd obtained by milk coagulation with commercial animal rennet (cc); curd obtained by milk coagulation with carline thistle rennet (ct); control raw ewe's milk cheese and *Caciofiore della Sibilla* cheese collected at 1, 3, and 20 days of ripening (C_1, C_3, C_{20} and Cf_1, Cf_3, Cf_{20} respectively).
Each percentage is related to the total amount of LAB found in each sample.

Fig. S1. Bacterial DGGE profiles of the DNA extracted from the bulk of colonies harvested from selected plates and the DNA extracted directly from the samples of raw ewes' milk (rm); curd obtained by milk coagulation with carline thistle rennet (ct) (panel **a)**; fresh young leaves of *Carlina acanthifolia* All. (ca); carline thistle rennet (tr) (panel **b);** curd obtained by milk coagulation with commercial animal rennet (cc) (panel **c**). Lanes I_H and I_L indicate DNA extracted from the bulk of colonies harvested from the MRS Agar plates spiked with the highest and lowest dilutions, respectively. Lanes II_H and II_L indicate DNA extracted from the bulk of colonies harvested from the M17 Agar plates incubated at 22 °C spiked with the highest and lowest dilutions, respectively. Lanes III_H and III_L indicate DNA extracted

522 from the bulk of colonies harvested from the M17 Agar plates incubated at 45 °C spiked with the highest and lowest
523 dilutions, respectively. Lane m indicates DNA extracted from the samples. Mix' refers to the mixture of species: a -
524 *Lactobacillus plantarum* DSMZ 2601*;* b - *Pediococcus pentosaceus* DSMZ 20336^{T}; c - *Lactobacillus parabuchneri*
525 DSMZ 5708; d - *Lactobacillus buchneri* DSMZ 20057; e - *Lactobacillus paracasei* NRRL 4560^{T}*;* f - *Lactobacillus*
526 *casei* NCIMB 4114.
527
528 **Fig. S2**. Bacterial DGGE profiles of the DNA extracted directly from the samples of control raw ewes' milk cheese
529 (batch C) and *Caciofiore della Sibilla* cheese (batch Cf) (panel **a**) and the DNA extracted from the bulk of colonies
530 harvested from selected plates of control raw ewes' milk cheese (panel **b**) and *Caciofiore della Sibilla* cheese (panel **c**)
531 collected at 1, 3, 6, 10 and 20 days of ripening (C_1, C_3, C_6, C_{10}, C_{20} and Cf_1, Cf_3, Cf_6, Cf_{10}, Cf20, respectively).
532 Lane I indicates DNA extracted from the bulk of colonies harvested from the MRS Agar plates spiked with the highest
533 dilutions. Lane II indicate DNA extracted from the bulk of colonies harvested from the M17 Agar plates incubated at 22
534 °C spiked with the highest dilutions. Lane III indicate DNA extracted from the bulk of colonies harvested from the M17
535 Agar plates incubated at 45 °C spiked with the highest dilutions. Mix' refers to the mixture of species: a - *Lactobacillus*
536 *plantarum* DSMZ 2601*;* b - *Pediococcus pentosaceus* DSMZ 20336^{T}; c - *Lactobacillus parabuchneri* DSMZ 5708; d -
537 *Lactobacillus buchneri* DSMZ 20057; e - *Lactobacillus paracasei* NRRL 4560^{T}*;* f - *Lactobacillus casei* NCIMB 4114.

Table 1

Table 1 Results of viable counting of bacteria in *Caciofiore della Sibilla* and control ewes' milk cheese.

Sample	***Caciofiore della Sibilla* cheese**	**Control cheese**		***Caciofiore della Sibilla* cheese**	**Control cheese**		***Caciofiore della Sibilla* cheese**	**Control cheese**	
	Lactococci (M17 22°C)			Thermophilic cocci (M17 45°C)			Lactobacilli (MRSA)		
Raw milk	5.00 ± 0.06			4.08 ± 0.06			4.78 ± 0.02		
Carlina acanthifolia All.	6.68 ± 0.03			4.88 ± 0.04			3.54 ± 0.10		
Thistle rennet	3.13 ± 0.14			2.10 ± 0.28			4.01 ± 0.00		
Curd	4.11 ± 0.00	4.82 ± 0.12		4.67 ± 0.00	4.28 ± 0.02		4.90 ± 0.06	4.74 ± 0.11	
1 day of ripening	5.56 ± 0.02^{d}	8.57 ± 0.01^{c}	<.0001*	4.68 ± 0.00^{e}	7.50 ± 0.06^{b}	0.0002*	5.06 ± 0.05^{d}	8.57± 0.00^{b}	0.0001
3 days of ripening	7.30 ± 0.00^{c}	7.98 ± 0.04^{d}	0.0016*	6.32 ± 0.01^{d}	7.37 ± 0.08^{bc}	0.0028*	8.08 ± 0.00^{c}	8.89± 0.01^{ab}	<.0001
6 days of ripening	8.62 ± 0.13^{b}	9.00 ± 0.14^{b}	0.1098	8.26 ± 0.00^{b}	8.18 ± 0.08^{a}	0.3251	8.66 ± 0.09^{b}	9.03 ± 0.01^{a}	0.0312*
10 days of ripening	9.12 ± 0.18^{a}	9.14 ± 0.06^{b}	0.9113	8.31 ± 0.00^{a}	8.00 ± 0.02^{a}	0.0012*	8.68 ± 0.01^{b}	8.96 ± 0.01^{a}	0.0009*
20 days of ripening	8.90 ± 0.05^{ab}	9.52 ± 0.06^{a}	0.0079*	7.42 ± 0.03^{c}	7.24 ± 0.05^{c}	0.0450*	9.05 ± 0.03^{a}	8.87 ± 0.22^{ab}	0.3557

Sample	***Caciofiore della Sibilla* cheese**	**Control cheese**		***Caciofiore della Sibilla* cheese**	**Control cheese**		***Caciofiore della Sibilla* cheese**	**Control cheese**		***Caciofiore della Sibilla* cheese**	**Control cheese**	
	Coagulase-negative cocci (MSA)			Total mesophilic aerobes (PCA)			Enterococci (Slanetz-Bartley Agar)			*Enterobacteriaceae* (VRBGA)		
Raw milk	4.01 ± 0.03			5.14 ± 0.16			4.30 ± 0.06			3.93 ± 0.06		
Carlina acanthifolia All.	5.24 ± 0.01			7.27 ± 0.04			3.97 ± 0.16			5.34 ± 0.03		
Thistle rennet	3.06 ± 0.03			4.07 ± 0.04			3.60 ± 0.01			1.00 ± 0.00		
Curd	5.06 ± 0.08	4.52 ± 0.04		4.99 ± 0.01	5.13 ± 0.07		4.37 ± 0.01	4.23 ± 0.05		1.10 ± 0.00	3.39 ± 0.16	
1 day of ripening	5.16 ± 0.02^{d}	6.75 ± 0.10^{e}	0.0022*	5.37 ± 0.02^{e}	8.45 ± 0.03^{a}	<.0001*	4.60 ± 0.05^{e}	6.09 ± 0.05^{e}	0.0011*	1.74 ± 0.06^{e}	7.55 ± 0.05^{a}	<.0001*
3 days of ripening	6.34 ± 0.02^{c}	7.97 ± 0.04^{d}	0.0003*	8.15 ± 0.08^{d}	8.87 ± 0.02^{a}	0.0056*	5.14 ± 0.08^{d}	7.12 ± 0.02^{c}	0.0099*	2.19 ± 0.02^{d}	7.30 ± 0.01^{b}	<.0001*
6 days of ripening	7.47 ± 0.04^{b}	8.91 ± 0.19^{a}	0.0086*	8.58 ± 0.00^{c}	8.71 ± 0.73^{a}	0.8181	7.78 ± 0.00^{c}	7.55 ± 0.01^{b}	0.0007*	5.41 ± 0.02^{c}	7.13 ± 0.07^{b}	0.0009*
10 days of ripening	8.72 ± 0.02^{a}	8.40 ± 0.03^{b}	0.0059*	8.82 ± 0.01^{b}	9.10 ± 0.03^{a}	0.0073*	8.26 ± 0.01^{b}	7.90 ± 0.01^{a}	0.0004*	6.17 ± 0.05^{b}	7.07 ± 0.09^{b}	0.0073*
20 days of ripening	7.44 ± 0.01^{b}	7.34 ± 0.01^{d}	0.0074*	9.20 ± 0.02^{a}	8.87 ± 0.00^{a}	0.0017*	8.51 ± 0.07^{a}	6.94 ± 0.04^{d}	0.0012*	6.40 ± 0.00^{a}	6.32 ± 0.03^{c}	0.0597

Viable counts of lactococci, thermophilic cocci, lactobacilli, coagulase-negative cocci, total mesophilic aerobes, enterococci and *Enterobacteriaceae* expressed as mean values ± st. dev. of samples collected during ripening of *Caciofiore della Sibilla* and control ewes' milk cheese. Mean separation test throughout ripening: different letters on the same column indicate significant differences over time according to Tukey's test ($\alpha = 0.05$). Least significant difference (LSD) is reported.
*Occurrence of significant differences due to cheese batch (C or Cf) according to Student's t test ($\alpha = 0.05$).

Table 2

Table 2 Bacterial species identified by PCR-DGGE analysis in raw ewe's milk, *Carlina acanthifolia* All. subsp. *acanthifolia*, thistle rennet, curd obtained from milk coagulation with thistle rennet and curd obtained from milk coagulation with commercial rennet.

Bacterial species	Raw ewes' milk							*Carlina acanthifolia* All.							Thistle rennet							Curd obtained from milk coagulation with thistle rennet							Curd obtained from milk coagulation with commercial rennet						
	MRS		M17 22°C		M17 45°C		M	MRS		M17 22°C		M17 45°C		M	MRS		M17 22°C		M17 45°C		M	MRS		M17 22°C		M17 45°C		M	MRS		M17 22°C		M17 45°C		M
	H	L	H	L	H	L		H	L	H	L	H	L		H	L	H	L	H	L		H	L	H	L	H	L		H	L	H	L	H	L	
Acinetobacter baumannii												•	•																						
Acinetobacter johnsonii				•		•	•																						•	•	•				
Bacillus cereus																	•		•																
Bacillus sp.												•	•					•	•					•		•									
Bacillus subtilis																	•									•									
Enterobacter cloacae					•	•																										•	•		
Enterobacter hormaechei					•	•																										•	•		
Enterobacter sp.										•	•	•																				•	•		
Escherichia coli																																	•		
Kurthia gibsonii																																•	•	•	
Lactobacillus alimentarius/paralimentarius group								•	•													•		•	•	•									
Lactobacillus graminis/curvatus group								•	•																										
Lactobacillus plantarum/paraplantarum/ pentosus group															•	•						•	•		•		•								
Lactococcus lactis	•	•																											•	•	•	•			
Pantoea agglomerans										•																									
Pediococcus acidilactici																						•		•											
Pseudomonas alcaliphila											•																								
Pseudomonas sp.			•	•	•	•				•	•	•	•																						
Staphylococcus chromogenes																																•	•	•	

• DGGE bands resulting from the analysis of the DNA extracted from the bulk of colonies harvested from high (H) and low (L) dilution agar plates used for viable counting from selected plates or the DNA extracted directly from samples showing ≥97% of similarity with the sequences of the closest relatives found by a BLAST search in the GenBank. M indicate the DNA extracted directly from a cheese samples matrix.

Table 3

Table 3 Bacterial species identified during the manufacture and ripening of control ewe's milk cheeses and *Caciofiore della Sibilla* by PCR-DGGE analysis.

Bacterial species	t_1				t_3				t_6				t_{10}				t_{20}			
	MRS	M17 22°C	M17 45°C	M	MRS	M17 22°C	M17 45°C	M	MRS	M17 22°C	M17 45°C	M	MRS	M17 22°C	M17 45°C	M	MRS	M17 22°C	M17 45°C	M
Bifidobacterium dentium				■				■				■				■				■
Citrobacter sp.							■				■				■				■	
Enterobacter hormaechei				■				■												
Enterobacter sp.			■				■													
Enterococcus faecalis	●		●■						■	●■	●■		■	■	■				●■	
Enterococcus faecium	●	●	●				●			●	●		●	●	●	●	●		●	●
Erwinia chrysanthemi				□				□				□				□				□
Lactobacillus alimentarius/paralimentarius group				■				■				■				■				■
Lactobacillus brevis	●				●	●											●			
Lactobacillus casei/ paracasei/rhamnosus group				●				●									■			●
Lactobacillus delbrueckii	●	●	●			●			●	●			●	●				●		
Lactobacillus plantarum/ pentosus/paraplantarum group																	●■	●		
Lactococcus lactis	●■	●■	●■	■	■	●■		■	●■	●■	■	●■	●■	●■		●■	■	●■	■	■
Leuconostoc mesenteroides/pseudomesenteroides								●										●		●
Staphylococcus sciuri		●	●■				●■		●■	■	■		■	■	■		■			
Weissella sp.					●															

● DGGE bands resulting from the analysis of the DNA extracted from the bulk of colonies harvested from selected plates or the DNA extracted directly from *Caciofiore della Sibilla* cheese samples showing ≥97% of similarity with the sequences of the closest relatives found by a BLAST search in the GenBank; ■ DGGE bands resulting from the analysis of the DNA extracted from the bulk of colonies harvested from selected plates or the DNA extracted directly from control ewe's milk cheese samples showing ≥97% of similarity with the sequences of the closest relatives found by a BLAST search in the GenBank; □ DGGE bands resulting from the analysis of the DNA extracted from the bulk of colonies harvested from selected plates or the DNA extracted directly from control ewe's milk cheese samples showing ≤97% of similarity with the sequences of the closest relatives found by a BLAST search in the GenBank;

t1: cheese sampled after 1 day of maturation; t3: cheese sampled after 3 days of maturation; t6: cheese sampled after 6 days of maturation; t10: cheese sampled after 10 days of maturation; t20: cheese sampled after 20 days of maturation.

M: DNA extracted directly from a cheese samples matrix.

Figure 1

Fig. 1.

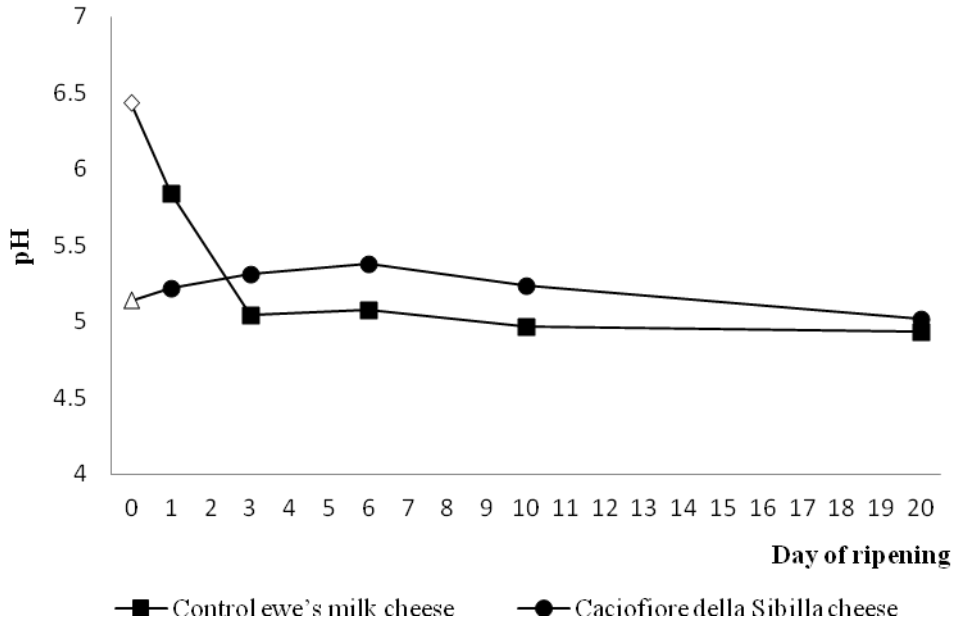


Figure 2

Fig. 2.

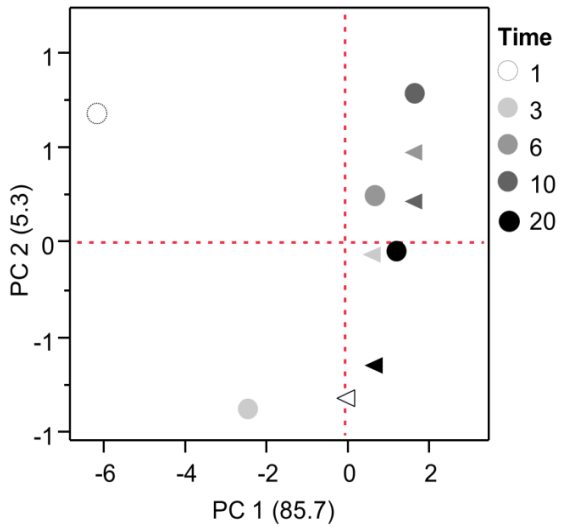


Figure 3

Fig. 3.

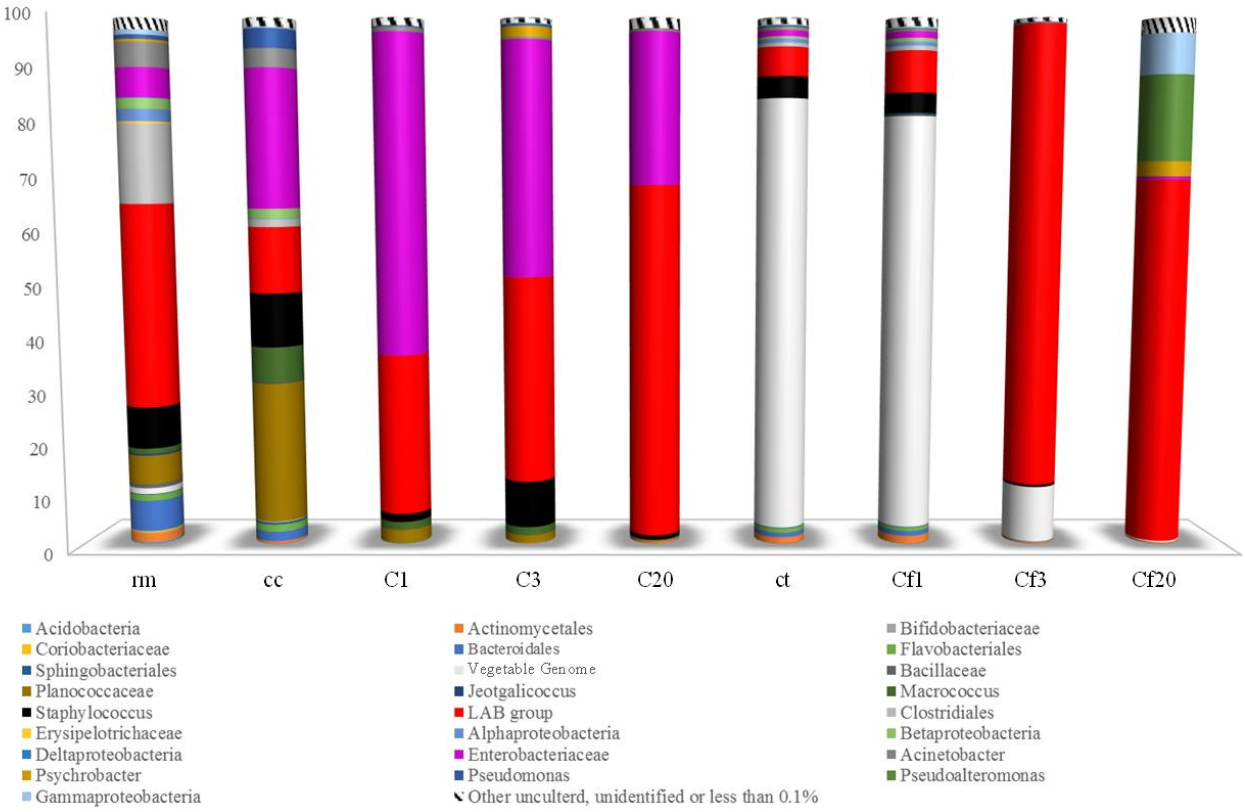


Figure 4

Fig.4.

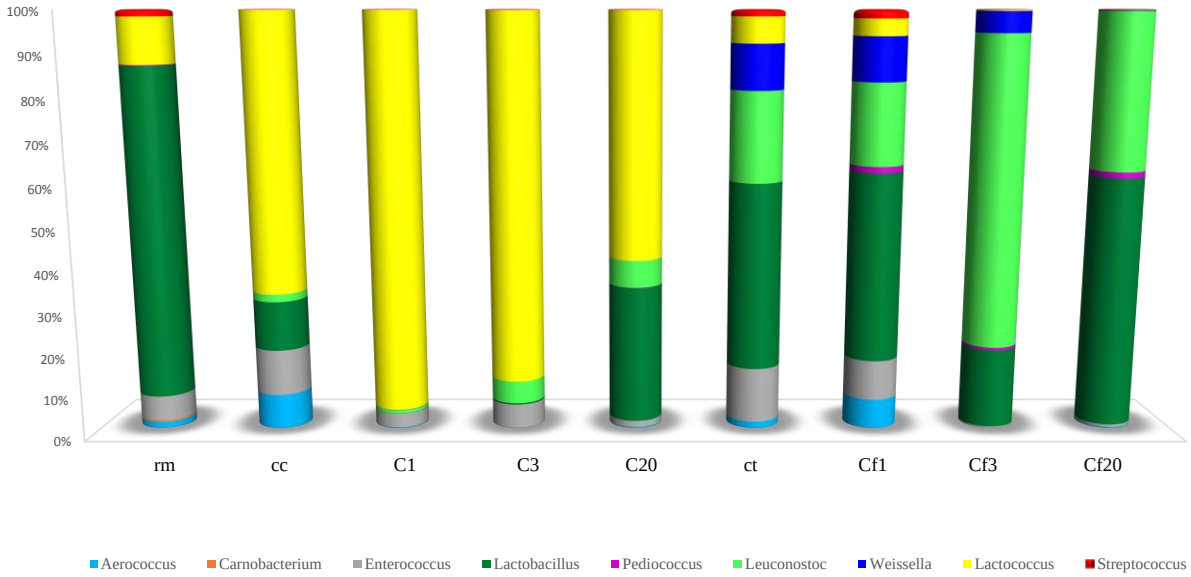


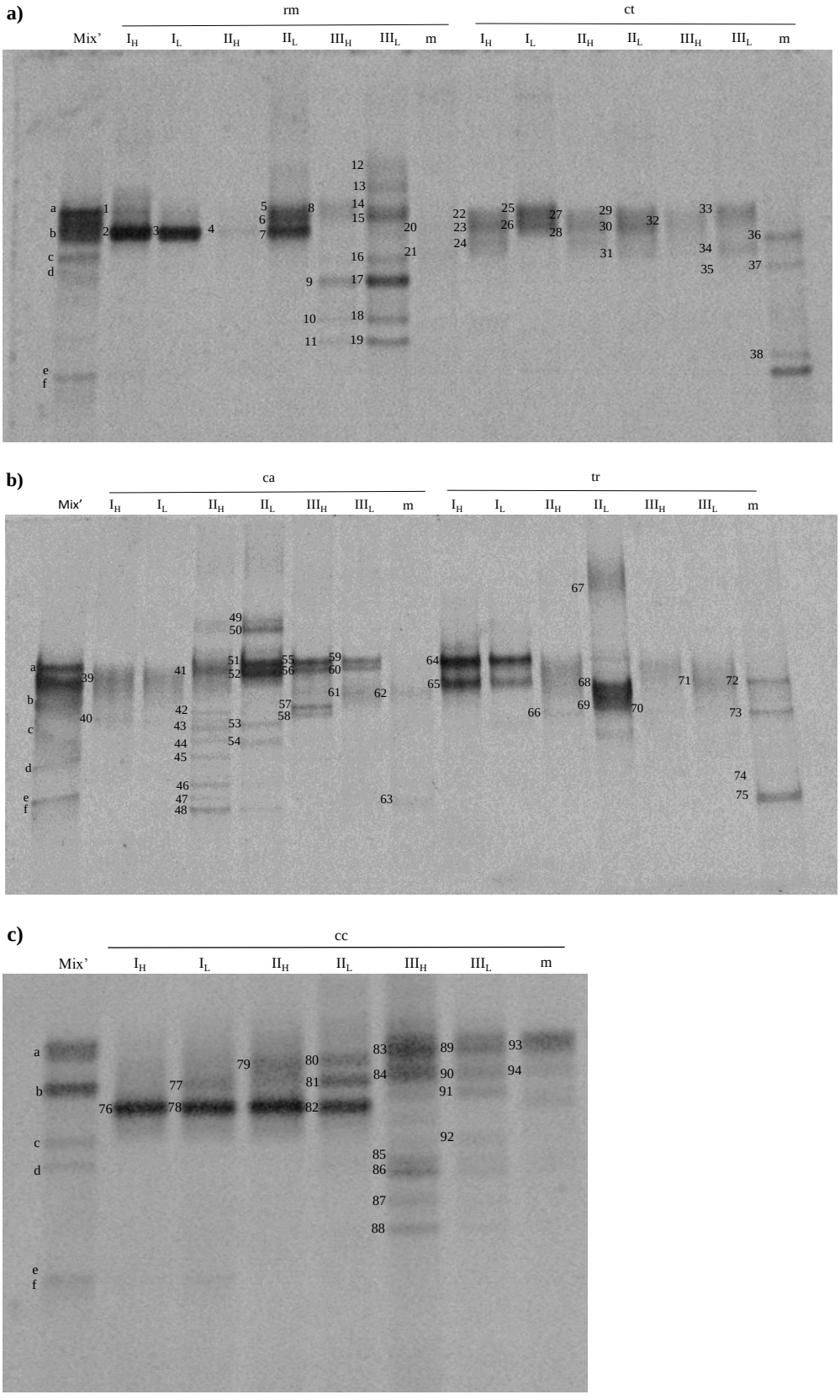
Table S1 Results from the sequencing of the bands cut from the DGGE gels.

Band(s)	**Closest relative**	**% Ident.**[a]	**Acc.no.**[b]
1, 2, 3, **76, 78, 82, 98, 103, 106, 107, 115, 123, 124, 125, 126,** 129, 134, 135, **136,** 140, **153, 155,** 164, 168, 178, **180, 181**, 183, 184, 194, **199**, 218, 226, 237, 241	*Lactococcus lactis*	100%	KT429894
4**, 5, 7,** 8, 14, **41, 49, 50, 51, 52, 53, 55, 59**	*Pseudomonas sp.*	100%	HF546529
6, 15, **21, 77, 79, 80, 81**	*Acinetobacter johnsonii*	99%	LN774358
43, 44, 46, 47, 48, 57, 58, 86, 127, 131, 132, 133, 142, 146, 149, 150	*Enterobacter sp.*	100%	HQ439419
11, **12, 19, 85**	*Enterobacter cloacae*	99%	KP226566
13, 20, 36, 37, 38, 63, 66, 72, 73, 74, 75, 91, 100, 102, 105, 108, 114, 116, **161,** 165, 169, **137, 138, 139, 182, 186, 201, 253**	Failed		
9, **10, 16, 17,** 18, **87, 88, 95, 99**	*Enterobacter hormaechei*	98%	HM584024
22**, 25, 26,** 29, **33, 64, 65, 175, 176, 246, 247, 249**, 252	*Lactobacillus plantarum* */pentosus* */paraplantarum*	99%	KJ775808 KT634227 KU315098
23, 27, 30, **32, 40, 101**	*Lactobacillus alimentarius* */paralimentarius*	98%	M58804 AB626063
24, 28	*Pediococcus acidilactici*	99%	KM921945
31, 34, 61, 62, 70, 200	*Bacillus sp.*	99%	HM755812
35, 69	*Bacillus subtilis*	98%	GQ392055
39	*Lactobacillus graminis* */ curvatus*	96%	LC063167 KP117256
42, 45	*Pantoea agglomerans*	99%	AB681812
54	*Pseudomonas alcaliphila*	97%	EU144361
56, 60	*Acinetobacter baumannii*	100%	AY269241
67, 68,71	*Bacillus cereus*	100%	KF782833
83, 89, 93	*Kurthia gibsonii*	99%	KT165384
84, 90, 94	*Staphylococcus chromogenes*	99%	KR028439
92	*Escherichia coli*	99%	KC539468
96, 104	*Bifidobacterium dentium*	100%	AP012326
97	*Erwinia chrysanthemi*	96%	DQ123809
109, 113, 122, 179	*Lactobacillus casei* */paracasei* */rhamnosus*	97%	KM921936 KT626389 KT626387
110, 111, 112, **118**, 119, 120, **250**	*Leuconostoc mesenteroides* */pseudomesenteroides*	96%	KR137536 KJ186948
117, 121, 191, 195, **196**, 202, 206, **213**, 215, 219, **220**, 221, **222, 223, 227, 228**, 229, 230, 231, 233, 234, 235, 238, 239, 242, 243, 244, 245, 254, 255, **256**	*Enterococcus faecium*	98%	KM921922
130, 152, **157**, 163, 167, 171, 187, 192**,** 204, 207, **224,** 232, 257	*Enterococcus faecalis*	98%	KP298396
128, **141, 143**, 144, **145,** 151, **154,** 156, 162, 166, **170**, 177, 197, **203, 205**, 214, 216	*Staphylococcus sciuri*	99%	JQ511682
147, 148, 158, 159, 160, **172,173,174, 185, 188, 189**	*Citrobacter sp.*	99%	KP212094
190, 210, 211, **248**	*Lactobacillus brevis*	99%	KP221640
193, 198, 208, 212, 217, 225, 236, 240, 251	*Lactobacillus delbrueckii*	97%	JN969331
209	*Weissella sp.*	99%	KF598906

[a] Percentage of identical nucleotides in the sequence obtained from the DGGE band and the sequence of the closest relative found in the GenBank DNA database.
[b] Accession number of the sequence of the closest relative found by BLAST search.

Figure S1

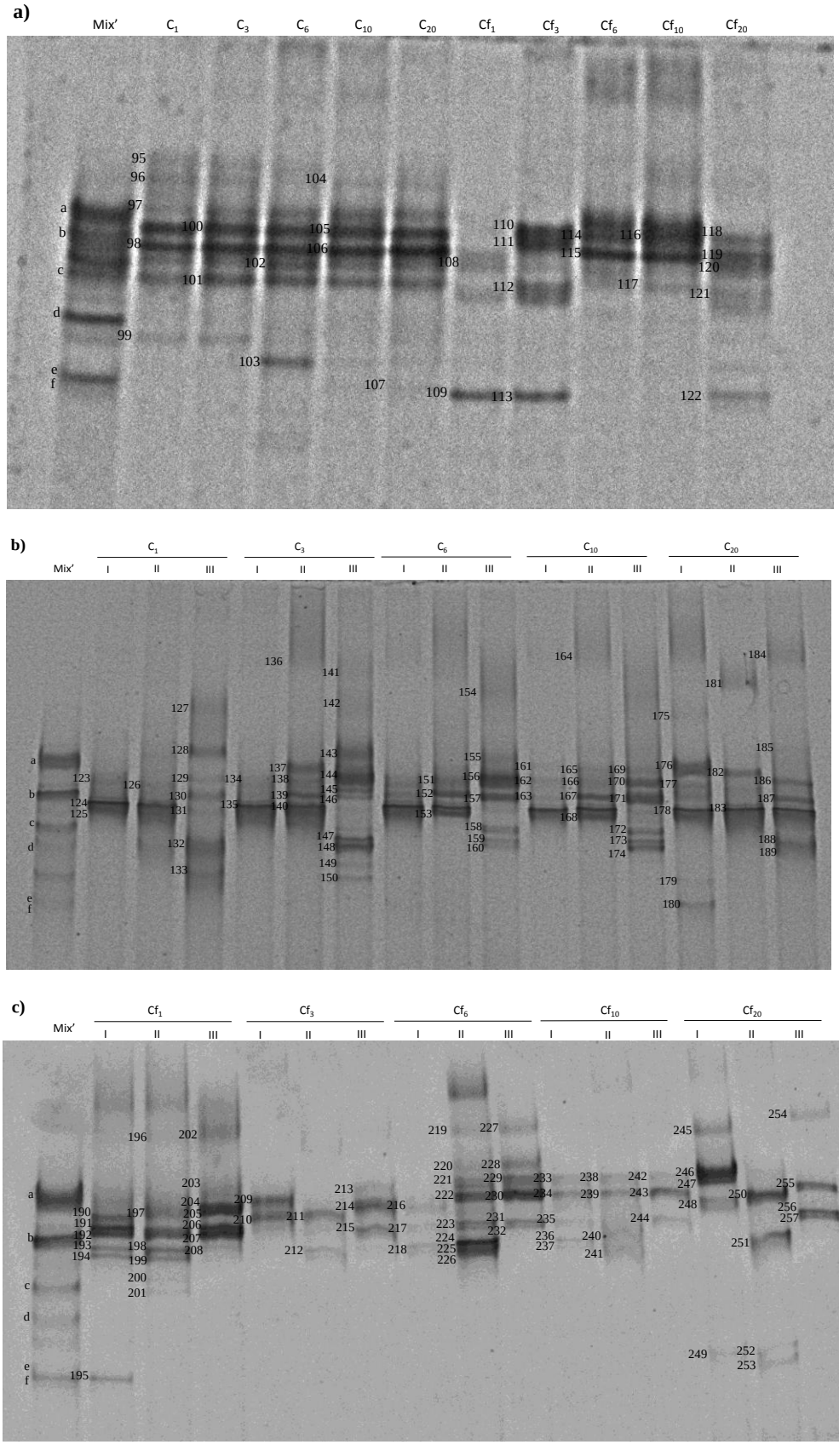
Fig. S1. Bacterial DGGE profiles of the DNA extracted from the bulk of colonies harvested from selected plates and the DNA extracted directly from the samples of raw ewe's milk (rm); curd obtained by milk coagulation with carline thistle rennet (ct) (panel **a)**; fresh young leaves of *Carlina acanthifolia* All. (ca); carline thistle rennet (tr) (panel **b);** curd obtained by milk coagulation with commercial animal rennet (cc) (panel **c**).



Lanes I_H and I_L indicate DNA extracted from the bulk of colonies harvested from the MRS Agar plates spiked with the highest and lowest dilutions respectively. Lanes II_H and II_L indicate DNA extracted from the bulk of colonies harvested from the M17 Agar plates incubated at 22°C spiked with the highest and lowest dilutions respectively. Lanes III_H and III_L indicate DNA extracted from the bulk of colonies harvested from the M17 Agar plates incubated at 45°C spiked with the highest and lowest dilutions respectively. Lane m indicate DNA extracted from the samples. Mix' refers to the mixture of species: a - *Lactobacillus plantarum* DSMZ 2601*;* b - *Pediococcus pentosaceus* DSMZ 20336^T; c - *Lactobacillus parabuchneri* DSMZ 5708; d - *Lactobacillus buchneri* DSMZ 20057; e - *Lactobacillus paracasei* NRRL 4560^T*;* f - *Lactobacillus casei* NCIMB 4114.

Figure S2

Fig. S2. Bacterial DGGE profiles of the DNA extracted directly from the samples of control raw ewe's milk cheese (batch C) and *Caciofiore della Sibilla* cheese (batch Cf) (panel **a**) and the DNA extracted from the bulk of colonies harvested from selected plates of control raw ewe's milk cheese (panel **b**) and *Caciofiore della Sibilla* cheese (panel **c**) collected at 1, 3, 6, 10 and 20 days of ripening (C_1, C_3, C_6, C_{10}, C_{20} and Cf_1, Cf_3, Cf_6, Cf_{10}, Cf_{20} respectively).



Lane I indicate DNA extracted from the bulk of colonies harvested from the MRS Agar plates spiked with the highest dilutions. Lane II indicate DNA extracted from the bulk of colonies harvested from the M17 Agar plates incubated at 22°C spiked with the highest dilutions. Lane III indicate DNA extracted from the bulk of colonies harvested from the M17 Agar plates incubated at 45°C spiked with the highest dilutions. Mix' refers to the mixture of species: a - *Lactobacillus plantarum* DSMZ 2601*;* b - *Pediococcus pentosaceus* DSMZ 20336^{T}; c - *Lactobacillus parabuchneri* DSMZ 5708; d - *Lactobacillus buchneri* DSMZ 20057; e - *Lactobacillus paracasei* NRRL 4560^{T}*;* f - *Lactobacillus casei* NCIMB 4114.