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Microbial population profile during ripening of Protected Designation of Origin (PDO) Silter cheese, produced with and without autochthonous starter culture

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Abstract –

An autochthonous starter culture was used in the production of Protected Designation of Origin (PDO) Silter raw milk cheese, and compared to a control cheese in order to study its influence on microbial population dynamics during ripening. Curd and cheese at different ripening periods (0, 30, 60 and 200 days) from two independent dairies were analysed combining culture-based microbiological analysis, Length Heterogeneity-PCR and 16S microbiome profiling.

The autochthonous starter determined higher lactic acid bacteria (LAB) levels for the first 30 days of ripening, without interfering with secondary microbiota that determines the tipicality of this cheese. Only a few genera and species persisted in the cheese despite the microbial richness of the curd. In addition, the high levels of different LAB reduced harmful microorganisms. The various analytic methods used resulted in discrepancies in the proportions of *Enterococcus*, *Lactococcus* and *Lactobacillus* spp., but the pivotal role of *Streptococcus* and *Lactococcus* genera was evident. The dominant species included those selected to formulate the starter (*St. thermophilus*, *Lc. lactis*, *Ln. mesenteroides*) as well as *Lb. paracasei.*

The addition of autochthonous starter proved to be effective in controlling the first phases of the cheese-making, without compromising cheese typicality.

Keywords: autochthonous starter, cheese, microbial dynamics, Next Generation Sequencing, raw milk

1. Introduction

Protected Designation of Origin (PDO) Silter cheese is a traditional half-fat cheese produced on the alpine pastures and in the valleys in the province of Brescia (Northern Italy) since the end of the 17th century. This hard cheese, suitable for eating or grating, is made with raw milk mainly from typical mountain breeds (Brown Swiss, Grey Alpine, Italian Red Spotted), using selected

autochthonous starter cultures or natural milk or whey cultures which are produced locally, and its ripening lasts for at least 100 days. Silter technology involves no heat treatment of the milk (MIPAF, 2015), thus preserving the microbial richness (intra-sample diversity) and variability (inter-sample diversity) of the raw milk, which is strictly connected to the area of origin and artisanal cheese-making practices and equipment (Montel et al., 2014). Actually, traditional raw milk cheeses represent an excellent source of bacterial strains with physiological and biochemical properties of technological interest. On the other hand, sensory defects in raw milk cheeses frequently occur due to microbial and compositional changes in the milk. The addition of autochthonous starters for PDO cheeses represents a possible way of optimizing the fermentative process during cheese-making, minimizing flavour and texture defects or health risks associated with some members of the raw milk complex community, without compromising the typicality of each cheese (Bassi, Puglisi, & Cocconcelli, 2015). In addition, knowledge of changes in the microbial community throughout the production process permits the management and improvement of microbial processes that contribute to cheese quality and microbiological safety (Justé, Thomma, & Lievens, 2008). Therefore, a key issue in cheese microbiology is to identify and characterize the individual components of cheese microbiota and their proportion, as well as to follow their evolution during ripening.

In order to investigate the microbial composition of cheese, a wide range of techniques is available, including methods depending on cultivation followed by phenotypic or molecular characterization and methods entirely relying on molecular characterization (Quigley et al., 2011).

Besides culture-based methods, which are useful for understanding the physiological potential of isolated organisms (Orphan, Taylor, Hafenbradl, & Delong, 2000), culture-independent approaches nowadays offer a fast and promising alternative, as the recovery of viable, non-viable, damaged/lysed/permeabilized and non-cultivable bacterial cells is possible in a single step. Consequently, these methods can promote a greater understanding of overall community structure and activity over time, providing a picture of cheese microbiota dynamics and allowing a deeper

77 comprehension of the different pathways involved in defining cheese varieties (O'Sullivan, Giblin,
78 McSweeney, Sheehan, & Cotter, 2013; Pogačić, Kelava, Zamberlin, Dolenčić- Špehar, &
79 Samaržija, 2010).
80 This study aimed at assessing the effectiveness of one autochthonous starter culture in leading the
81 PDO Silter cheese-making process and its impact on the diversity and dynamics of the bacterial
82 population during ripening (up to 200 days), which is strictly connected with the safety, quality and
83 identity of this traditional raw milk cheese. To this end, the conventional culture approach was
84 combined with two culture-independent tools, i.e. LH-PCR and Illumina Next Generation
85 Sequencing to compare cheese produced with the addition of the autochthonous starter culture to
86 that made with no starter culture.
87
88 **2. Materials and methods**
89
90 *2.1. Autochthonous starter culture*
91 In a previous study (Vanoni, 2007), 50 isolates from different dairy products (milk, curd and
92 cheese) collected throughout the PDO Silter production process had been identified and
93 technologically characterized according to Morandi, Brasca, & Lodi (2011). These strains were
94 combined to formulate different starter cultures, comprising the most representative species with a
95 dominant role in the ripening process of this cheese. The best performing starter culture was scaled-
96 up to industrial level and selected for this work. The lyophilized starter composition was as follows:
97 *Lactococcus lactis* subsp. *lactis* ST87, *Leuconostoc pseudomesenteroides* ST23 and *Ln.*
98 *mesenteroides* ST32, *Streptococcus thermophilus* ST56 and *S. thermophilus* ST182. All the
99 Streptococci and Lactococci strains were added at a level of 10^6 cfu mL^{-1} and *Leuconostoc* strains at
100 a level of 10^4 cfu mL^{-1} into milk
101
102 *2.2. Cheese production and sampling*

Two independent dairies (B and R) were chosen in order to represent independent biological replicates. A total of four batches of Silter cheese was manufactured by B and R: for each producer, a batch with the addition of the selected autochthonous starter culture in the vat (experimental, E) and a batch with no starter (control, C) were simultaneously prepared from the same bulk raw milk, following the production specification (Figure 1; MIPAF, 2015) and using brine salting.

The curd was sampled immediately after moulding and samples of cheese were collected at different ripening periods (30, 60 and 200 days) and transferred to the laboratory under refrigerated conditions. Microbiological analyses were performed within 24 h of sample arrival.

2.3. Lactic acid bacteria (LAB) enumeration and isolation

For microbiological analyses, curd and cheese (10 g) samples were homogenized in 90 mL of a 2% (w/v) sterile K_2HPO_4 buffer solution (Sigma-Aldrich, St. Louis, MO USA) for 1 min in a Stomacher BagMixer (Interscience, St. Nom, France). Samples were serially diluted in quarter-strength Ringer's solution (Scharlau Microbiology, Barcelona, Spain) and inoculated into the following culture media: de Man – Rogosa – Sharpe (MRS) agar (Biolife Italiana, Milano, Italy) under anaerobic conditions (AnaerocultA, Merck, Darmstad, Germany) at 37 °C for 72 h for total lactic acid bacteria; M17 agar (Biolife Italiana) at 37 °C for 48 h for Lactococci and Streptococci; Mayeux, Sandine and Elliker (MSE) agar (Biolife Italiana) at 30 °C for 5 days for *Leuconostoc* spp.; Kanamycin Aesculin Azide (KAA) agar (Scharlau Microbiology) at 37 °C for 48 h for Enterococci. Duplicate analyses were performed on each sample. All colonies with different morphologies were selected from the highest dilution plates of samples produced with the addition of the autochthonous starter culture. The dilution factor and number of colonies with the different morphologies were considered. The purity of the isolates was checked by streaking repeatedly on

Homofermentative-Heterofermentative Differential (HHD) agar and sub-culturing using the same isolation media and temperatures. Isolates were examined for Gram staining, catalase production and cell morphology. All Gram-positive, catalase-negative, non motile isolates were stored in Litmus milk (Biolife Italiana) at -18 °C.

2.4 RAPD-PCR typing and 16S rRNA sequencing

For the identification of isolates, a polyphasic approach was used as previously described (Morandi, Silvetti, Miranda Lopez, & Brasca, 2015). DNA was extracted from overnight bacterial cultures by the Microlysis kit (LaboGen, Rho, Italy) following the manufacturer's instructions. For preliminary identification, RAPD-PCR was performed with primer M13 (5'-GAGGGTGGCGGTTCT-3') (Huey & Hall, 1989) and the resulting fingerprints were compared with the BioNumeric 5.0 software package (Applied Maths, Sint-Martens-Latem, Belgium), using the UPGMA (unweighted pair group method with arithmetic averages) cluster analysis. The reproducibility value of the RAPD-PCR assay, calculated from two repetitions of independent amplification of type strains, was higher than 90%. The genomic DNA of representative strains of each cluster was afterwards sequenced in order to identify the isolated strains. 16S rRNA sequencing was performed with specific primers p8FPL (5'-AGTTTGATCCTGGCTCAG-3') and p806R (5'-GGACTACCAGGGTATCTAAT-3') generating an amplicon of approximately 800 bp (McCabe, Khan, Zhang, Mason, & McCabe, 1995). Amplification products were sent to Macrogen Europe (Amsterdam, the Netherlands) for sequencing and sequences were analyzed with NCBI BLAST search (NCBI, 2017). Species names were assigned whenever the degree of homology was higher than 97%.

The LAB strains composing the autochthonous starter were analyzed by RAPD-PCR reaction performed with primers M13, D11344 (5'-AGTGAATTCGCGGTCAGATGCCA-3') and D8635 (5'-GAGCGGCCAAAGGGAGCAGAC-3') (Akopyanz, Bukanov, Westblom, Kresovich, & Berg,

1992); amplification conditions, as well as electrophoresis and analysis of the amplification products, were as previously described by Morandi, Silvetti, Miranda Lopez, & Brasca, 2015.

2.5 Development of an LH-PCR database and LH-PCR of cheese isolates

To set up an appropriate LH-PCR database, a total of 78 bacterial strains belonging to 71 species were selected from different culture collections (Table 1). The genomic DNA of the strains was extracted from an overnight culture as described above as well as directly from grated and homogenized cheese (100 g) following the procedure described by Cremonesi et al. (2007). The LH-PCR reaction was performed as previously reported by Brusetti and co-workers (2006) with the primer 27F (AGAGTTTGATCCTGGCTCAG), labelled at its 5' end with phosphoramidite dye (6-FAM), and 338R (GCTGCCTCCCGTAGGAGT) (Ritchie, Schutter, Dick, & Myrold, 2000). The 500 LIZ (Thermo Fisher Scientific) was used as internal size standard. The samples were run on the ABI Prism 310 Genetic Analyser for 28 min at 15 kV, with an injection time of 5 s at 15 kV. The LH-PCR data were analysed by Gene-Mapper 4.0 software (Thermo Fisher Scientific) and a threshold of 100 RFU (Relative Flurescence Units) was fixed. The repeatability of LH-PCR analysis was assessed by three repetitions of independent amplifications of different bacterial DNAs. Analyzing the replicates of the same sample, we found a maximum variation in size of 0.3 bp for peaks referring to a specific species, therefore peaks with sizes diverging by 0,3 bp or more were attributed to different strains.

2.6 Genomic DNA extraction for Illumina analysis

For the analyses, 45 mL of 2% (w/v) K_2HPO_4 buffer solution (Sigma-Aldrich) were added to five grams of each sample; the sample was then mixed for 1 min and 30 s in a Stomacher machine (PBI, Milan, Italy). The DNA was extracted starting with 800 µL of the homogenized sample following

the protocol described in Cremonesi et al. (2006) with some modifications. Briefly, 400 µL of lysis buffer (3 mol L^{-1} guanidine thiocyanate, 20 mmol L^{-1} EDTA, 10 mmol L^{-1} Tris-HCl, pH 6.8, 40 mg mL^{-1} Triton X-100, 10 mg mL^{-1} dithiothreitol) and 300 µL of binding solution (40 mg mL^{-1} silica from Sigma Aldrich, directly suspended in the lysis buffer) were added to the sample and vortexed for 30 s to obtain an emulsified solution. Then the sample was incubated for 5 min at room temperature. After centrifugation for 30 s at 550 × g, the supernatant was discarded and the silica-DNA pellet obtained was subsequently washed twice with 500 µL of lysis buffer, twice with 500 µL of washing solution (25% absolute ethanol, 25% isopropanol, 100 mmol L^{-1} NaCl, 10 mmol L^{-1} Tris-HCl, pH 8) and once with 500 µL of absolute ethanol. After every washing and vortexing, the silica-DNA pellet was centrifuged for 30 s at 550 × g and the supernatant was discarded. The pellet was then vacuum-dried for 10 min. After the addition of 100 µL of elution buffer (10 mmol L^{-1} Tris-HCl, pH 8, 1 mmol L^{-1} EDTA), the silica-DNA pellet was gently vortexed and incubated for 10 min at 65 °C. After a 5 min centrifugation at 550 × g, the supernatant containing the DNA was recovered and stored at −20 °C. DNA concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, De., USA).

2.7 Library preparation and bioinformatic analysis

The genomic DNA extracted from cheese samples was amplified using primers described in literature (Caporaso et al., 2011; Klindworth et al., 2013), which target the V3-V4 hypervariable regions of the 16S rRNA gene.

All PCR amplifications were performed by using 1x HotStarTaq Master Mix (Qiagen) following the manufacturer's instructions, with 0.2 µL of each primer (100 µM) and 2 µL of genomic DNA (5 ng μL^{-1}). The amplicons cycling conditions were 15 min at 94 °C, followed by 25 cycles of 1 min at 94 °C, 1 min at 58 °C and 1 min at 72 °C, and finally 7 min at 72 °C. Finally amplicons were cleaned up with Agencourt® AMPure® XP (Beckman, Coulter Brea, CA) following 16S

Metagenomic Sequencing Library Preparation protocol (Illumina, 2013) and checked for amplicon size with 2100 Bioanalyzer Instruments (Agilent Technologies, Santa Clara, CA). Libraries were prepared by a second PCR amplification step using Nextera XT Index 1 Primers FC-131-1002), following 16S Metagenomic Sequencing Library Preparation protocol. The 16 libraries obtained were quantified by Real Time PCR with KAPA library Quantification Kits (KapaBiosystems, Inc. MA, United States) pooled in equimolar proportion and sequenced in one MiSeq run (Illumina) with 300-base paired-end reads. After obtaining the data, Illumina raw reads were trimmed using Trimmomatic v0.32 (Bolger et al. 2014) requiring a minimum base quality of 20 (Phred scale) and a minimum read length of 36 nucleotides. Only trimmed reads were included in the downstream analysis. For long amplicons with non-overlapping paired ends, the first paired read was concatenated to the reverse complement of second paired end read, separated with a single N base. For partial overlapping reads, the consensus was performed using fastq-join tool (Aronesty, 2011; Aronesty, 2013). Joined reads were dereplicated according to USEARCH pipeline (Edgar et al. 2011), with a threshold ≥2 filtering out reads without replicates. For OTU selection and identification, reads were clustered at 97% identity using VSEARCH version 1.1.3 (Rognes et al., 2016). Each cluster was identified using BLASTN against the 16S formatted SILVA database (Silva111, 99% clustered version) (Quast et al., 2013). A standard overall OTU table was generated and converted into BIOM file format (McDonald et al., 2012) using QIIME1.8.0 utilities (Caporaso et al., 2010). Organism taxonomy was adapted according to QIIME taxonomy standards with custom Ruby scripts. The coverage of bacteria alpha-diversity associated with each specimen was investigated through rarefaction curves obtained by plotting observed species vs. simulated sequencing, using QIIME pipeline. Principal Component analyses (PCA) were carried out to provide spatial illustrations of community structure across soils (beta diversity), using QIIME pipeline.

Finally, MiSeq Reporter software (MSR) was used for classifying organisms from V3 and V4 amplicon using the Greengenes database (http://greengenes.lbl.gov/) and the output of this

233 workflow was a classification of reads at several taxonomic levels (kingdom, phylum, class, order,
234 family, genus, and species).
235
236 **3. Results**
237
238 *3.1 LAB enumeration*
239
240 Culture-dependent LAB content in both C and E cheeses from dairies B and R is summarized in
241 Table 2. For both dairies, E cheese showed a higher level of LAB up to 30 days compared to C
242 cheese, but afterwards LAB predominated in C cheese. Lactococci and Streptococci determined in
243 M17 agar dominated the LAB community throughout ripening. However, the LAB count in MRS
244 agar exceeded coccus population count after 200 days, thus highlighting the numerical superiority
245 of Lactobacilli. The use of the autochthonous starter culture led to a greater content of *Leuconostoc*
246 spp. determined in MSE Agar, especially for sample R. Simultaneously, *Enterococcus* genus in
247 KAA agar decreased. Enterococci count initially differed among samples and was lower in both
248 samples B, but, although exhibiting different growth evolution, they reached the same level in the
249 final phase of the ripening period (5.61 $\log_{10}$ cfu g^{-1}), except for the C sample produced by dairy R,
250 which had a greater value (7.01 $\log_{10}$ cfu g^{-1}).
251
252 *3.2 LAB community description and assessment of autochthonous LAB persistence*
253
254 Microbiological analyses of curd and cheeses were also performed to evaluate the persistence of the
255 selected autochthonous LAB strains during 200 days of ripening. The dynamics of indigenous raw
256 milk microbiota were simultaneously monitored.
257 One hundred and six LAB isolates (66 from B and 40 from R) were picked up from the 3 culture
258 media (MRS agar, M17 agar, and MSE agar) after microbiological analyses of samples derived

from milk added with the selected autochthonous starter culture at different ripening times. All the LAB strains considered in this study were characterized by RAPD-PCR amplification profiles obtained with one primer. Genotypic heterogeneity and variability among *Lactobacillus*, *Streptococcus* and *Lactococcus* strains was evident (Figure S-1). The sequences of partial 16S rRNA gene from the 98 LAB isolates showed high degrees of similarity to those of the GenBank reference strains (between 98.6 and 99.8%) and thus were assigned to a specific microbial species, whereas 8 isolates were not identified. As shown in Table 3, the genera *Lactobacillus* (43.9%), *Lactococcus* (17.3%), *Streptococcus* (13.3%), *Enterococcus* (13.3%), and *Leuconostoc* (12.2%) were detected. Considering both producers, LAB were classified into 10 different species, mainly represented by cocci (7 species). Rod-shaped strains mostly consisted of *Lb. paracasei* (44.1 and 23.1% for B and R, respectively), but a few strains belonging to *Lb. plantarum* (3.4 and 12.8% for B and R, respectively) were also found. Among cocci, *Ln. mesenteroides* (10.2 and 15.4% for B and R, respectively), *Lc. lactis* (11.9 and 17.9% for B and R, respectively) and *St. thermophilus* (15.3 and 5.1% for B and R, respectively) were the dominant species. Strains ascribable to *Enterococcus* genus were also abundant (8.5 and 20.5% for B and R, respectively) and occurred during the whole ripening period as well as *St. thermophilus*. The coccus population dominated in curd samples, whereas Lactobacilli prevailed in cheese during ripening; in particular, *Lb. paracasei* was detected in all samples at 60 and 200 days of ripening and markedly increased throughout the ripening of cheese collected from producer B. *Lc. garviae* was sporadically present in curd samples exclusively. The presence of *St. uberis* and *St. equinus*, derived by a contamination of animal origin, was recognized in samples of cheese B (Table 3).

Strains constituting the autochthonous starter culture differed in their persistence from raw milk to ripened cheese. In fact, *St. thermophilus* ST56 possessed the highest survival and was also detected at the end of the ripening period. An opposite trend was shown by *St. thermophilus* ST182 and *Ln. pseudomesenteroides* ST23, which did not grow even in curd. *Ln. mesenteroides* ST32 was isolated up to 60 days from its addition to milk. All these strains exhibited the same behaviour during

285 cheese-making B and R. On the contrary, *Lc. lactis* ST87 propagation continued until the late
286 ripening period for cheese R, while its presence in cheese B occurred within the first 30 days of
287 ripening.
288
289 *3.3 Community structure study by culture-independent LH-PCR*
290
291 The different fragment sizes in the LH-PCR profiles were attributed to bacterial species according
292 to the LH-PCR database (Table 1), which included all isolated species. Amplified fragments varied
293 in the range 310-381 ± 0.5 bp. Table 4 shows all the peaks observed after LH-PCR analysis of the
294 16 curd and cheese samples. Clearly LH-PCR analysis did not reflect the expected richness of raw
295 milk cheeses. Further, different species often had the same-length amplicon; for instance, the peak
296 at 353 ± 0.5 bp, which was detected in all samples, is attributable to *Lc. lactis* subsp. *lactis* or
297 *Paenibacillus polimixa* or *Staphylococcus aureus* or *S. xylosus* or *St. macedonicus*.
298 With regard to dairy R, the E curd, and the 30 and 60 day cheeses showed a similar bacterial
299 composition. Electropherograms presented only the peak at 350 ± 0.5 bp (attributable either to *Ln.*
300 *citreum* or to *Ln. mesenteroides* subsp. *mesenteroides)* in addition to the 353 ± 0.5 bp peak. But, the
301 E 200 day cheese was characterized by a peak at 351 ± 0.5 bp (attributable to *Lc. lactis* subsp.
302 *cremoris* or *Lysinibacillus fusyformis* or various *Leuconostoc* species), which was also found in all
303 C samples. In C 30 and 60 day cheeses another peak, presumably corresponding to *Enterobacter*
304 *cloacae* or *Propionibacterium freudenreichii* (345 ± 0.5 bp), was detected. In C 60 day cheese, the
305 fragments of 362± 0.5 and 365 ± 0.5 bp also revealed the presence of *Enterococcus* spp., namely *E.*
306 *durans* or *E. faecium*, and *Lb. helveticus*, respectively.
307 As regards to producer B, all C samples showed the peak at 351 ± 0.5 bp, as observed for producer
308 R. The electropherogram of C curd presented a major bacterial diversity and included the following
309 peaks: 342 ± 0.5 (ascribable to *Clostridium tyrobutyricum* or *Pseudomonas fragi*), 345 ± 0.5, 346 ±
310 0.5 (ascribable to *Citrobacter freundi*), and 360 ± 0.5 (ascribable to *E. italicus* or *E. casseliflavus*)

bp, whereas the fragment of 365 ± 0.5 bp was found in all C cheeses. In electropherogram of E curd fragments of 342 ± 0.5, 344 ± 0.5 (ascribable to *Cl. butyricum* or *Kokuria rosea*) and 350 ± 0.5 bp were identified. In the E 30 day cheese the peak attributable to *E. cloacae* or *P. freudenreichii* appeared (345 ± 0.5 bp) together with the 351 ± 0.5 bp peak. The LH-PCR profile referring to E 60 and 200 day cheeses showed the same peaks: 350 ± 0.5 and 366± 0.5 (ascribable to *Lactobacillus acidophilus*) bp. In the E 60 day cheese, a peak at 344 ± 0.5 was detected as well.

Evaluation of data referring to peak height and area indicated that the 353-bp fragment is the greatest in all samples, confirming the relevance of *Lc. lactis* subsp. *lactis* in this cheese. In addition, the importance and the successful survival of *Leuconostoc* spp. (351-bp fragment) in the first phases of cheese-making and up to 60 days of ripening were confirmed by LH-PCR analysis (Table 4).

3.4 Taxonomic distributions among samples with 16S microbiome profiling

From the Miseq run, a total of 20.928.344 raw reads were obtained; rarefaction curve analysis, which assesses species richness from sampling, suggested that our data were sufficient for a complete description of the biodiversity of cheese samples. However, the distribution of the microbial community (beta diversity) did not distinguish samples according to their ripening stage or starter addition (Supplementary material 1).

After assigning sequences to a taxonomic lineage using the Greengenes database (Supplementary material 2), the annotated reads revealed the predominance of *Firmicutes* (> 98%) in cheese samples at 30, 60 and 200 days of ripening and the co-presence of *Firmicutes*, *Proteobacteria* and *Bacteriodetes* in curd samples (for batches E and C, for both producers).

Firmicutes was the most abundant phylum and was dominated by members of the class Bacilli belonging to the order *Bacillales* and *Lactobacillales* with the following families: *Staphylococcaceae, Enterococcaceae, Leuconostocaceae, Lactobacillaceae* and the predominance

of *Streptococcaceae*. As reported in Figure 2, for cheesemaker B *Lactococcus* spp. decreased from 40% (curd C) to 4.3%, while *Streptococcus* spp. increased from 7.3% up to 77% after 200 days of ripening. For producer R a similar result was obtained, except for the concentration of *Lactococcus* spp. that increased to 42% in cheese C at 60 days of ripening. In curd E a higher presence of *Streptococcus* spp. (36% for producer B and 85.7% for producer R) and a lower prevalence of *Lactococcus* spp. (8.7% for producer B and 1.1% for producer R) was found. *St. thermophilus* was the dominant species in Silter cheese samples with an increase during curd fermentation and no differences between samples with or without the autochthonous starter. *Bacteriodetes* were represented by *Flavobacteriaceae*, while *Proteobacteria* by *Moraxellaceae* and *Pseudomonodaceae*. Other bacteria, namely *Pseudomonas* spp., *Haloanella* spp., *Fructobacillus* spp., *Melissococcus* spp., *Acinetobacter* spp. and *Raoultella* spp. were detected in curd sample C representing 22% of the 26.5% and 18% of the 32.2% of the other genera detected in this sample from producers B and R, respectively. Among the 40% of reads classified as others for curd E, *Acinetobacter* spp., *Pseudomonas* spp., *Haloanella* spp., *Raoultella* spp., and *Simidua* spp. represented 35% of the genera found (producer B), while only 2.5% of the reads in producer R were classified as others for the curd sample. Further undesirable bacteria present in samples from both producers consisted of *Staphylococcus* spp., *Chryseobacterium* spp., *Corynebacterium* spp. and diverse *Enterobacteriaceae* genera. Indeed, of all sequences, more than 20% for producer B and 5% for producer R could not be assigned at the genus level, and were thus assigned as unclassified sequences.

4. Discussion

A crucial step toward protecting microbial diversity in artisanal cheeses is to make an in-depth investigation of the indigenous microbiota during cheese manufacturing and ripening (Fuka et al., 2013). In the present study, the influence of a selected autochthonous starter culture on the

363 microbial ecosystem was explored. For this purpose, a detailed determination of the complex
364 microbial community of Silter cheese and its dynamic changes during 200 days of ripening was
365 made using both culture-dependent and -independent tools. Control samples exhibited greater LAB
366 count variability than the experimental, thus it was found that the use of the autochthonous starter
367 culture was successful in minimizing divergences in product quality.
368 The experimental curd presented an increase in lactococcal and streptococcal numbers, which
369 remained practically constant throughout ripening, thus supporting the hypothesis that they are
370 determinant in the ripening processes of Silter cheese. Early in the ripening process acidifying
371 Lactococci and Streptococci predominate. Indeed, *St. thermophilus* persisted from curd to mature
372 cheese. These findings are similar to those reported by Marino, Mainfreni, & Rondinini (2003) in
373 raw milk PDO Montasio cheese, in which *St. thermophilus* plays a crucial role in biochemical
374 processes related to product ripening. Later on, after the concentrations of major carbohydrates and
375 free water decrease, and the sodium chloride load increases, mesophilic Lactobacilli prevail. The
376 other dominant species throughout the ripening period of Silter cheese is *Ln. mesenteroides*. Instead
377 *Leuconostoc* spp. are generally non-starter LAB (NSLAB), being adventitious contaminants that
378 grow later during cheese ripening due to the lack of sufficient proteolytic activity. However, these
379 heterofermentative LAB are used in the dairy industry, in synergistic functional relationship with
380 acid-producing Lactococci, in the manufacturing of some cheese varieties, due to their ability to
381 form holes by gas (CO_2) production and to form volatile flavour compounds like ethanol,
382 acetaldehyde, diacetyl, acetoin and 2,3-butanediol (Cibik & Chapot-Chartier, 2000; Quigley et al.,
383 2013). In the present work, the addition of the selected autochthonous strains resulted in an increase
384 in the *Leuconostoc* spp. in E cheeses compared with controls. This was evident in the E cheeses of
385 both producers between 30 and 60 days of ripening, when these microorganisms reached and
386 maintained their maximal levels (> 6,00 $\log_{10}$ cfu g^{-1}), though their loads at the end of the ripening
387 period were similar or inferior to those in the C cheeses. Commonly, the growth rate and final
388 biomass of the *Leuconostoc* population in cheese is limited, between 10^4 and 10^7 cfu g^{-1}, because of

389 the competition for peptides or amino acid in milk with other LAB such as *Lactococcus* (Hemme
390 D., 2012). The adjunct *Leuconostoc* seemed to influence the enterococcal growth for up to 60 days
391 of ripening. In fact, a higher level of *Leuconostoc* spp. coincided with a lower number of
392 *Enterococcus* spp. These results support the earlier evidence of *Leuconostoc* strains with an
393 inhibitory effect against enterococcal species (Morandi, Cremonesi, Silvetti, & Brasca, 2013).
394 Enterococci counts differed among the producers. This variability was probably a reflection of the
395 qualitative heterogeneity of the raw milk and differing hygienic practices during milking and
396 storage (Randazzo, Vaughan, & Caggia, 2006). But Enterococci, which are not negligible
397 components of the adventitious microbiota of several artisanal raw milk cheeses (De Pasquale, Di
398 Cagno, Buchin, De Angelis, & Gobbetti, 2014), were present at the same level (5.60 $\log_{10}$ cfu g^{-1}) in
399 the late ripening of 3 of the 4 samples analysed. Their presence during the late stages of maturation
400 also implies that they are of utmost importance in cheese ripening, contributing to the sensory
401 profile of the final product (Marino et al., 2003). Thus, the autochthonous starter culture did not
402 seem to interfere with the indigenous NSLAB that determines the tipicality of each type of cheese.
403 Other microbial species occurred at low incidence or sporadically, some affecting cheese-ripening
404 processes. Among them, *Lc. garvieae* is associated with raw milk, but is also employed as a
405 secondary adjunct culture as it contributes to the development of distinctive sensory traits (Settanni
406 et al., 2012). *St. uberis* is an environmental bacterium responsible for bovine mastitis, able to
407 secrete extracellular streptokinase which is involved in accelerating cheese proteolysis due to the
408 increase in plasmin activity (Upadhyay et al., 2004).

409 RAPD-PCR and 16S rRNA sequencing were used to control whether selected
410 autochthonous LAB starters can sustain viability throughout ripening. In both the B and R cheeses
411 all the starter strains acted in the same way, except for *Lc. lactis* ST87 where the ability to survive
412 in the B and R samples differed. Adjunct strains were not detected at all, not even at the beginning
413 of the ripening. These results can be attributed to the strain-specific stress response to physio-
414 chemical modifications in the different curd-cheese environments. Indeed, the decrease in pH,

changes in the a_w values correlated with an increasing level of salt, as well as the presence of prophages, all influence LAB survival during ripening (Lepeuple et al., 1998; Serrazzanetti, Gottardi, Montanari, & Gianotti, 2013). This does not exclude a contribution of the adjunct strains to the maturation of Silter cheese.

LH-PCR, based on natural variations in the length of 16S rRNA gene fragments, was successfully applied for the analysis of whey starter cultures and hard, long- ripened cheeses (Pogačić et al., 2010). However the authors in this study made no mention of LAB richness and the diversity typical of artisanal raw milk cheeses. Furthermore, no temporal changes in the bacterial community profiles emerged. LH-PCR was thus proven to be an ineffective tool for investigating complex bacterial consortia, and the level of phylogenetic resolution proved it to be a weak point of this method, as already suggested by other authors (Ritchie et al., 2000). In fact, members of more than one taxonomic group presented LH-PCR products of the same size. The 353-bp fragment length, which recurred in all samples, was attributed to microbial species that have a different impact on cheese quality, such as *P. polimixa*, *S. aureus*, *S. xylosus*, *St. macedonicus*, and *Lc. lactis* subsp. *lactis*. These last 2 microorganisms contribute to artisanal cheese flavour and microbial quality during ripening (Cavanagh, Fitzgerald, & McAuliffe, 2015; Vendramin et al., 2014), but also *S. xylosus* can play an important role in the ripening process of some cheese varieties (Delbès & Montel, 2005); *P. polymyxa* is a gas-producing spore-former (Cremonesi, Vanoni, Silvetti, Morandi, & Brasca, 2012) and *S. aureu*s can cause food-borne illness due to the production of heat-stable enterotoxins (Quigley et al., 2013). Similarly, the 351-bp fragment was attributed to both LAB species of technological relevance (*Leuconostoc* spp and *Lc. lactis* subsp. *cremoris*), but also to the spore-forming *Lysinibacillus fusiformis* possessing harmful proteolytic activity (De Jonghe et al., 2010). Other researchers have highlighted LH-PCR limitations, including the inability to resolve complex amplicon peaks and the underestimation of diversity, as phylogenetically distinct taxons may produce same-length amplicons (Rastogi & Sani, 2011). Moreover, only prevalent, active members of the community might be amplified, whereas low abundant members may not even be

441 detected (Lazzi, Rossetti, Zago, Neviani, & Giraffa, 2003). All these drawbacks point to the need to
442 combine LH-PCR with traditional cultivation methods, as already suggested by other authors
443 (Ndoye, Rasolofo, LaPointe, & Roy, 2011). In any case, peaks referable to *Leuconostoc* spp.
444 recurred in all samples. This corroborates the results of other methods applied in this research, i. e.
445 the increase in *Leuconostoc* proportion consequent to the presence of these LAB in the starter. On
446 the other hand, the technique is valuable for microbiologically simple and controlled ecosystems,
447 where the dominant species composition is known (Lazzi et al., 2003).

448 The use of a metagenomic approach has revolutionized the field of microbial study,
449 particularly for bacteria that are difficult to culture and/or are present in low abundance in a specific
450 environment. This technique has already been described in dairy products for microbial diversity in
451 cheeses and, more specifically, artisanal cheeses (Aldrete-Tapia et al., 2014; Fuka et al., 2013;
452 Quigley et al., 2012), validating the application of this technology. In this study, using the V3-V4
453 region of the 16S rRNA, the microbiota of Silter cheese during ripening with or without a selected
454 autochthonous starter culture was studied, allowing the identification of microbial communities and
455 bacteria that could be potentially present in this product. As shown for other dairy products
456 (Aldrete-Tapia et al., 2014; Fuka et al., 2013; Quigley et al., 2012), this analysis revealed, in the
457 microbiota, a significant abundance of *Lactobacillales* composed especially of *Streptococcus* spp.,
458 and *Lactococcus* spp., *Leuconostoc* spp., *Lactobacillus* spp., *Enterococcus* spp., that play a pivotal
459 role in the formation of typical organoleptic properties of cheese. From curd to ripened cheese two
460 genera were found in higher proportions: *Streptococcus* and *Lactococcus*, and the most
461 representative species were *St. thermophilus*, *St. vestibularis*, *Lc. lactis* and *Lc. raffinolactis*. The
462 commensalism of *Lc. lactis* and *Lc. raffinolactis* was recently reported to have positive organoleptic
463 implications in dairy products (Kimoto-Nira et al., 2012). Although metagenomic analysis
464 evidenced *Lc. lactis* persistence throughout the time of cheese ripening, and traditional plating on
465 M17 medium led to loads ranging between 6.85 and 8.76 $\log_{10}$ cfu g^{-1}, *Lc. lactis* was essentially
466 identified in the early stage of ripening, most likely because this species is autolytic or uncultivable.

Recently, other authors pointed out that Lactococci are able to grow on M17 medium when they are abundant and not stressed, e.g. during milk and curd fermentation, and they are present in a viable but nonculturable state during cheese ripening (Mangia, Fancello, & Deiana, 2016; Ruggirello, Dolci, & Cocolin, 2014). Enterococci have been reported in natural starters of different cheeses (Aldrete-Tapia et al., 2014; Giannino, Marzotto, Dellaglio, & Feligini, 2009); despite their presence at high cell numbers as demonstrated through cultivation, Illumina sequencing detected *Enterococcus* spp. sporadically (B) and in low or a slightly higher level (R), but in samples without the autochthonous starter. Actually, for *Enterococcus* spp. the outcome of pyrosequencing studies is significantly influenced by DNA extraction procedures and primer sets as demonstrated by Starke, Vahjen, Pieper, & Zentek (2014). Discrepancies in the *Lactobacillus* species revealed by culture-dependent and -independent methods were also observed. A culture-independent method underscored the increasing content of *Lactobacillus* during ripening as well as remarkable intra-species diversity (53 detected species), but the numerical importance of *Lb. paracasei* was not highlighted, as shown by a culture-dependent approach. The ineffective recovery of other species through culture media might be explained as the inability to grow at 37 °C as described for *Lb. helveticus* in previous studies (Dolci, Alessandria, Rantsiou, Bertolino, & Cocolin, 2010; Mangia et al., 2016). In addition, metagenomics analysis of curd has shown the presence of several undesirable bacteria, including *Acinetobacter, Pseudomonas, Staphylococcus, Serratia, Chryseobacterium, Corynebacterium,* and *Enterobacter*. These subdominant or minor genera were presumably the resident microbiota of dairy processing plants or connected with the hygienic practices of the farm. Interestingly, some species of *Acinetobacter* and *Enterobacteriaceae* were recognized as contributing to the flavour of traditional cheeses through citrate catabolism and lipolytic or proteolytic activity (Pangallo et al., 2014). It is worth noting that 16S microbiome profiling evidenced, in cheese produced without starter addition, the presence of a larger number of unclassified species that might represent novel bacteria, and thus of interest for further investigation

(Figure 2). This observation corroborates the evidence that a starter is effective in controlling microbial evolution in cheese.

Consequently, (and not surprisingly) culture-dependent and –independent analyses often produce contrasting results when these two approaches are combined to investigate cheese microbiota (Pangallo et al., 2014).

5. Conclusion

As shown by our analysis, the biodiversity of samples with or without the autochthonous starter culture during ripening was similar, emphasizing the fact that the use of the autochthonous starter had no marked effect on the LAB cheese microbiota. Nevertheless, the genomic profiles suggested that the autochthonous starter culture helps control cheese-making by reducing undesirable bacteria in curd and unclassified bacteria during cheese ripening, without interfering with the indigenous NSLAB. The preservation of the cheese microbiota composition ensures PDO Silter typicality, which represents a cultural heritage, but it is also a value-adding strategy that can help small dairy producers and the sustainability of rural areas.

The application of complementary culture-dependent and -independent techniques highlighted the differences and disparities in the overall picture of the microbiota of Silter cheese, corroborating the efficacy of a polyphasic strategy for a more accurate and comprehensive investigation of the structure and evolution of microbial ecosystems. The present approach, which lends itself for use in other food matrices, offsets the weaknesses inherent in using each technique singly.

On the one hand, genomic profiles, by representing rare community components, provide an enlarged image of microbial biodiversity, which is still underexplored and partially uncharacterized; conversely, culture-dependent methods, though underestimating microbial diversity, still remain crucial to fully accounting for any shift in community dynamics.

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721 Figure 1: PDO Silter cheese flow chart.

722 Figure 2: Genus level abundance profiles for the microorganisms of interest using 16S rRNA
723 sequence classification during ripening (B-C and R-C samples without autochthonous starter for
724 producers B and R, respectively; B-E and R-E samples with the autochthonous starter for producers
725 B and R, respectively).

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727

Table 1. Length heterogeneity (LH)-PCR fragment length database of different microbial species of dairy interest. The values between the brackets, after the Fragments lengths, represent the secondary peaks present in the fingerprint of different species.

Species		Source	Fragment lengths (bp)*
Acetobacter	*pasteurianus*	CNR-ISPA	317.3
Bacillus	spp.	CNR-ISPA	346.9
Bacillus	*badius*	CNR-ISPA	353.8
	licheniformis	CNR-ISPA	349.2 (351.4)
	subtilis	CNR-ISPA	349.1
Brevibacillus	*borstelensis*	CNR-ISPA	338.7
Citrobacter	*freundi*	CNR-ISPA	346.1
Clostridium	*baratii*	DSM 601^T	349.1
	beijerinkii	DSM 791^T	342.9
	butyricum	DSM 10072^T	343.9
	sporogenes	ATCC 3584	343.2
	tyrobutyricum	CNR-ISPA	342.1
Enterobacter	*cloacae*	CNR-ISPA	344.8
Enterococcus	*casseliflavus*	CNR-ISPA	360.1
	durans	DSM 20633^T	362.2
	faecalis	ATCC 23655	361.2
	faecium	DSM 20477^T	362.2 (361.1)
	gilvus	CNR-ISPA	360.9
	hirae	CNR-ISPA	363.7
	italicus	CNR-ISPA	359.9
	lactis	DSM 23655T	363.0
Lactobacillus	*acidophilus*	CNR-ISPA	366.1
	brevis	CNR-ISPA	369.2
	buchneri	DSM 20057^T	377.3 (375.3)
	casei	CNR-ISPA	367.2 (369.1)
	curvatus	CNR-ISPA	371.2
	delbrueckii subsp. *bulgaricus*	DSM 20081^T	361.1 (342.6)
	delbrueckii subsp. *delbrueckii*	DSM 20074^T	362.9
	delbrueckii subsp. *lactis*	CNR-ISPA	363.0
	fermentum	DSM 20052^T	375.5 (373.2)
	helveticus	DSM 20075^T	365.2 (368.1)
	parabuchneri	CNR-ISPA	378.7
	paracasei subsp. *paracasei*	ATCC 25303	363.9 (341.6)
	plantarum	CNR-ISPA	367.2 (365.0)
	reuteri	ATCC 55730	375.4
	rhamnosus	CNR-ISPA	369.3
	sakei subsp. *sakei*	CNR-ISPA	374.3
	sanfranciscensis	DSM 20663^T	379.2
Lactococcus	*garvieae*	CNR-ISPA	354.9
	lactis subsp. *cremoris*	CNR-ISPA	351.2
	lactis subsp. *lactis*	CNR-ISPA	352.9

	raffinolactis	DSM 20433^{T}	361.0
Leuconostoc	*citreum*	CNR-ISPA	350.2
	lactis	CNR-ISPA	350.9
	mesenteroides	CNR-ISPA	351.1
	mesenteroides subsp. *cremoris*	DSM 2346^{T}	351.1
	mesenteroides subsp. *mesenteroides*	DSM 20343^{T}	350.1 (341.7, 348.9)
	pseudomesenteroides	CNR-ISPA	351.1
Lysinibacillus	*fusyformis*	CNR-ISPA	350.9
	sphaericus	CNR-ISPA	353.7
Paenibacillus	*polymyxa*	CNR-ISPA	353.1
Pediococcus	*acidilactici*	CNR-ISPA	377.9
	damnosus	DSM 20331	381.2
	pentosaceus	CNR-ISPA	378.1 (338.1)
Pseudomonas	*chloraphis*	CNR-ISPA	339.2
	fluorescens	CNR-ISPA	341.0
	fragi	ATCC 4973	341.9
	putida	ATCC 12633	341.4
	syringae	ATCC 19310	343.1
Propionibacterium	*freudenreichii*	CNR-ISPA	345.1
	jensenii	CNR-ISPA	342.8 (338.2)
	thonei	CNR-ISPA	342.9
Sphingomonas	spp.	CNR-ISPA	309.8
Staphylococcus	*aureus*	ATCC 19095	352.8
	aureus	CNR-ISPA	352.9
	epidermidis	CNR-ISPA	351.7
	pasteuri	CNR-ISPA	351.9
	xylosus	DSM 20266^{T}	353.1
Streptococcus	*macedonicus*	CNR-ISPA	353.2
	thermophilus	CNR-ISPA	361.2 (341.7, 353.1)
Kocuria	*rosea*	CNR-ISPA	344.1
Weissella	*confusa*	CNR-ISPA	380.1

ATCC: American Type Culture Collection
DSM: Deutche Sammlung von Mikroorganismen und Zellkulturen
CNR ISPA: Italian National Research Council, Institute of Sciences of Food Production collection

The superscript "T" after the strain number indicates that the strain is the type strain of the species or subspecies

*Fragment lengths are reported as mean values of three repetitions of independent amplifications of different bacterial DNAs

Table 2. LAB enumeration results in different isolation media at various stages of Silter PDO ripening (data expressed in $\log_{10}$ cfu g^{-1}. Mean values of two determinations ± Standard Deviation).

Producer	Cheese-making	MRS 37°C				M17 37°C			
		curd	30 d	60 d	200 d	curd	30 d	60 d	200 d
B	control	6.30±0.06	6.56±0.10	8.19±0.07	9.21±0.13	6.85±0.18	8.20±0.08	8.46±0.03	8.76±0.14
	experimental	7.76±0.15	7.23±0.09	7.28±0.11	8.01±0.28	7.79±0.04	8.15±0.02	7.78±0.22	7.93±0.03
R	control	6.29±0.02	7.96±0.09	7.98±0.04	7.56±0.09	6.57±0.07	8.04±0.01	8.03±0.06	6.85±0.04
	experimental	5.95±0.09	8.22±0.10	7.65±0.20	7.52±0.13	7.15±0.16	8.06±0.09	7.90±0.05	7.08±0.09

Producer	Cheese-making	MSE 30°C				KAA 37°C			
		curd	30 d	60 d	200 d	curd	30 d	60 d	200 d
B	control	5.18±0.06	6.28±0.01	5.70±0.04	5.79±0.20	4.60±0.16	5.00±0.03	6.60±0.19	5.60±0.00
	experimental	4.48±0.17	6.85±0.11	6.18±0.11	5.00±0.07	4.60±0.03	4.00±0.11	5.48±0.05	5.61±0.01
R	control	4.15±0.05	4.48±0.01	4.00±0.03	4.70±0.07	5.82±0.00	6.48±0.10	7.54±0.18	7.01±0.09
	experimental	5.52±0.09	6.70±0.15	6.36±0.08	4.78±0.18	6.04±0.06	6.00±0.02	6.00±0.09	5.61±0.09

d = days of ripening

Table 3. Numbers of LAB strains isolated throughout Silter PDO cheese ripening.

LAB species	Producer							
	B				R			
	curd	30 d	60 d	200 d	curd	30 d	60 d	200 d
Lc. garviae	1				2			
Lc. lactis		7			1	2	3	1
Ln. mesenteroides		4	2			3	2	1
St. equinus				1				
St. uberis			1					
St. thermophilus	3	1		5				2
Lb. paracasei		4	6	16			5	4
Lb. plantarum			1	1		3	1	1
Lb. parabuchneri			1					
Enterococcus spp.		2		3	5	1		2
unclassified	1	4	2					1

d = days of ripening

Table 4. Numerical LH-PCR output of the 16SrRNA gene fragment generated after PCR from DNA isolated from curd and cheese samples. Values represent the main peaks (size of fragment in basepairs (bp)). In brackets peak heights and areas. Peak area is calculated by the software converting fluorescence data into electropherograms; the peaks represented fragments of different sizes. Peak height is given as relative fluorescence unit (RFU) and the areas under the peaks were the relative proportions of the fragments.

Cheese-making	Producer							
	B				R			
	Curd	30 d	60 d	200 d	Curd	30 d	60 d	200 d
Control	341.9 (633-12487) 344.8 (273-4159) 346.3 (127-1341) 351.2 (2387-43006) 353.1 (587-7261) 360.1 (566-8687)	351.1 (450-6317) 353.1 (1910-26570) 365.2 (222-3244)	351.1 (282-3339) 353.1 (920-13798) 365.3 (240-3916)	351.1 (273-3518) 353.0 (1961-24938) 365.2 (240-3916)	350.9 (371-3888) 353.1 (2870-32786)	344.9 (257-4796) 351.2 (515-5777) 352.9 (2750-33126)	344.8 (153-2740) 350.9 (1053-12112) 353.0 (2010-29901) 362.0 (135-2806) 365.2 (142-1802)	351.1 (371-3888) 353.0 (2870-32786)
Experimental	341.9 (398-5293) 344.0 (799-9210) 350.2 (546-7469) 353.1 (2566-31358)	344.9 (137-1520) 351.2 (320-4137) 353.1 (2579-30556)	344.0 (127-1219) 350.1 (566-7471) 352.9 (2768-30994) 366.0	350.1 (444-5209) 352.9 (3469-38338) 366.0	350.2 (760-8077) 352.9 (3235-34832)	350.2 (313-3000) 353.1 (4148-43520)	350.2 (306-3021) 352.9 (6702-69444)	351.2 (231-2607) 353.1 (3970-38833)

	(102-1798) (259-3432)
Fragment lenghts*	Species
341.9	*Cl. tyrobutyricum*; *Ps. fragi*
344.0	*Cl. butyricum*; *K. rosea*
344.8-344.9	*E. cloacae*; *Pr. freudenreichii*
346.3	*C. freundi*
350.1-350.2	*Ln. citreum*; *Ln. mesenteroides* subsp. *mesenteroides*
350.9-351.1-351.2	*Lc. lactis* subsp. *cremoris*; *Ly. fusyformis*; *Ln. lactis*; *Ln. mesenteroides* subsp. *cremoris*; *Ln. mesenteroides*; *Ln. pseudomesenteroides*
352.9-353.0-353.1	*Lc. lactis* subsp. *lactis*; *P. polimixa*; *S. aureus*; *S. xylosus*; *St. macedonicus*
360.1	*E. casseliflavus*; *E. italicus*
362.0	*E. durans*; *E. faecium*
365.2-365.3	*Lb. helveticus*
366.0	*Lb. acidophilus*

d = days of ripening

*Values represent the main peaks that identify a strain of a certain species. Size of fragment in basepairs (bp)

Figure 1

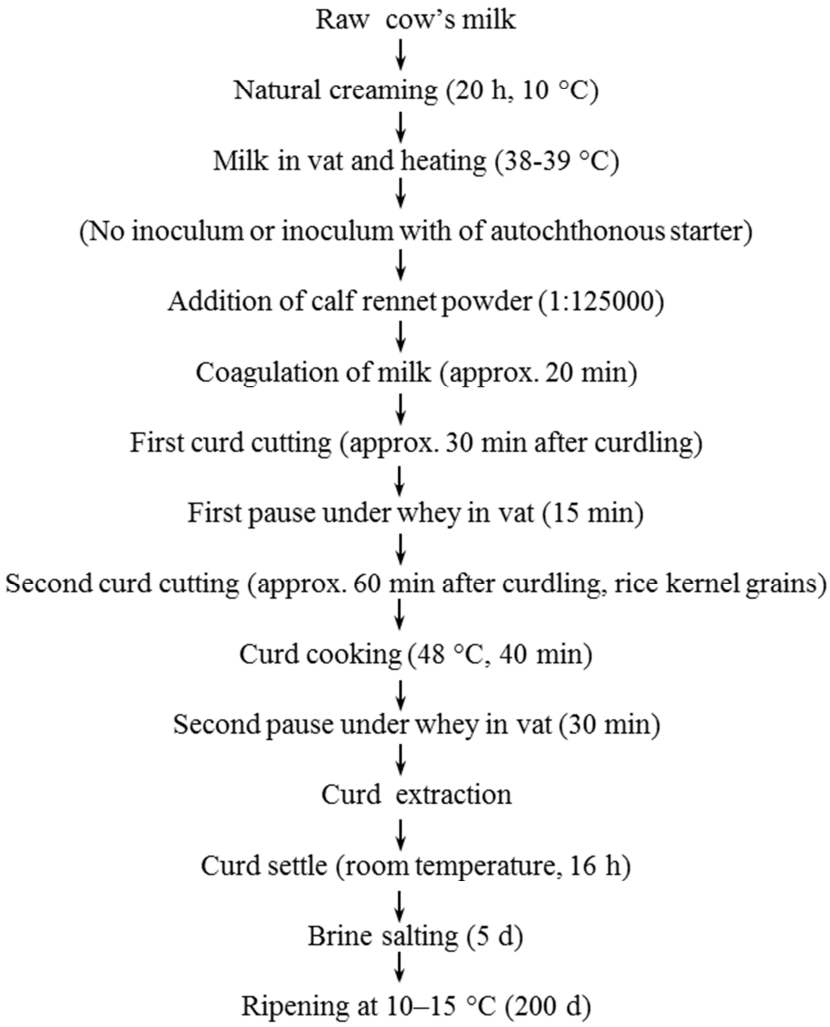
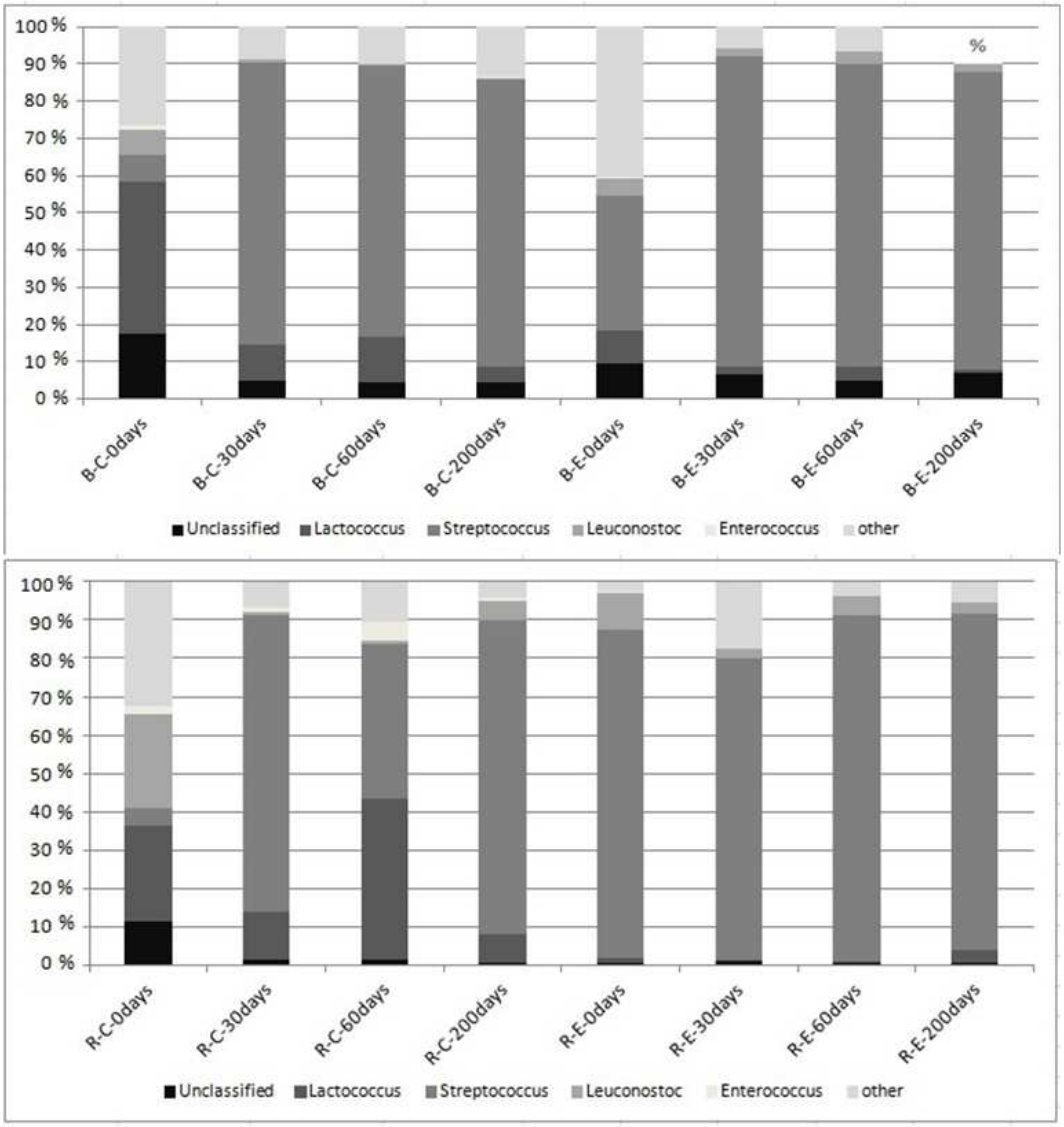

Raw cow's milk
Natural creaming (20 h, 10 °C)
Milk in vat and heating (38-39 °C)
(No inoculum or inoculum with of autochthonous starter)
Addition of calf rennet powder (1:125000)
Coagulation of milk (approx. 20 min)
First curd cutting (approx. 30 min after curdling)
First pause under whey in vat (15 min)
Second curd cutting (approx. 60 min after curdling, rice kernel grains)
Curd cooking (48 °C, 40 min)
Second pause under whey in vat (30 min)
Curd extraction
Curd settle (room temperature, 16 h)
Brine salting (5 d)
Ripening at 10–15 °C (200 d)

Figure 2



Highlights

- The pivotal role of *Streptococcus* and *Lactococcus* genera was highlighted
- Autochthonous starter was found to control undesirable bacteria in curd
- Starter addition determined higher LAB level till 30 days of ripening
- Indigenous NSLAB linked to cheese peculiarity were not affected
- *Enterococcus*, *Lactococcus* and *Lactobacillus* proportion differed among techniques