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This is the author's manuscript

Original Citation:

Availability:
This version is available http://hdl.handle.net/2318/1611504 since 2016-11-14T10:07:37Z

Published version:
DOI:10.1128/AEM.00999-16

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This is the author's final version of the contribution published as:

Alessandria, V; Ferrocino, I; De Filippis, F; Fontana, M; Rantsiou, K; Ercolini, D; Cocolin, L. Microbiota of an Italian Grana-Like Cheese during Manufacture and Ripening, Unraveled by 16S rRNA-Based Approaches. APPLIED AND ENVIRONMENTAL MICROBIOLOGY. 82 (13) pp: 3988-3995. DOI: 10.1128/AEM.00999-16

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Microbiota of an Italian Grana like cheese during manufacture and ripening unraveled by 16S rRNA-based approaches

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Running title: 16S rRNA analysis of an Italian hard cheese microbiota
The microbial ecology of cheese is harbored by a rich and complex interaction between starter lactic acid bacteria (SLAB) and non-starter lactic acid bacteria (NSLAB) mainly originating from raw milk and/or from the environment that can contribute to the final characteristics of cheese. The aim of the present research was the exploration of the active microbiota by RNA-based approaches during the manufacturing and ripening of a Grana like cheese. RT-PCR-DGGE and RNA-based high-throughput sequencing were applied to profile microbial populations, while the enumeration of active bacteria was carried out by using quantitative PCR (qPCR). Three different cheese productions (named D, E and F) collected in the same month from the same dairy plant were analysed. The application of the qPCR protocol revealed the presence of 7 Log CFU/ml of bacterial load in raw milk, while during ripening active bacterial populations ranged from < 4 to 8 Log CFU/ml. The natural whey starters (NWCs) used in the three productions showed the same microbiota composition, characterized by the presence of Lactobacillus helveticus and Lactobacillus delbrueckii. Nevertheless, beta-diversity analysis of the 16S rRNA sequencing data and RT-PCR-DGGE showed a clear clustering of the samples according to the three productions, probably driven by the different milk used. Milk samples were found to be characterized by the presence of several contaminants such as Propionibacterium acnes, Acidovorax, Acinetobacter, Pseudomonas and non-starter lactic acid bacteria (NSLAB). The core genera of the starter tended to limit the development of the spoilage bacteria only in two of the three batches. This study underlines the influence of different factors that can affect the final microbiota composition of the artisanal cheese.

This study highlights the importance of the quality of the raw milk in the production of an hard cheese. Independently from the use of a starter culture, raw milk with low microbiological quality
can negatively affect the populations of lactic acid bacteria and as a consequence impact the quality of the final product due to spoilage metabolic processes.

**Keywords:** raw milk, hard cheese, whey starter, active microbiota

**INTRODUCTION**

Grana like hard cheese is a typical raw cow’s milk cheese produced in the Piedmont region in Northwest of Italy. Curd fermentation is carried out by natural thermophilic whey cultures obtained from the manufacturing of the previous day, after heating at 53–55°C and the ripening is commonly carried out for 12 months.

Natural whey cultures (NWC) are used for several PDO cheeses (Protected Designation of Origin). They are usually dominated by thermophilic lactic acid bacteria (LAB), but sub-dominant species, including mesophilic LAB, may also occur. NWS have a complex microbial association of various species as well as a large number of biotypes (1).

Temperature is the main factors associated with the variability of the microbiota composition of the NWC. Cooking treatment and whey cooling lead to the selection of a characteristic microbiota consisting of thermophilic, aciduric, and moderately heat-resistant LAB (2). Changes of these variable parameters can lead the development of the final cultures.

Previous studies have described in detail the microbial composition of this kind of cheese and showed the predominance of *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus fermentum*, and *Streptococcus thermophilus* as the main species isolated and identified (3, 4).

It is well reported that the production is characterized by microbial dynamic changes and bacteria play a primary role in defining its quality (5). Different microbial populations coexist and interact contributing through their metabolism to the development of taste, aroma, texture, shelf-life and safety (6). In particular, the microbial ecology of long ripened cheeses, produced from raw milk and
using whey starter cultures, is based on complex interaction among starter lactic acid bacteria (SLAB) and non-starter lactic acid bacteria (NSLAB) (7). SLAB give their contribution in the early stages of the cheese making, while NSLAB, which are able to use other carbon sources apart from lactose, become the dominant microbiota of the ripened cheeses (8) and are responsible for the flavour and texture of the cheeses due to their proteolytic and lipolytic activities (7). NSLAB are mainly mesophilic microorganisms originating from raw milk and/or from the environment and their presence may introduce variability into the ripening process (9). For this reason, the development of beneficial NSLAB coming from the natural whey starter, from the raw milk or from the manufacturing environment is crucial to minimize microbial variability during the ripening process and obtain the desired organoleptic characteristics of the cheese. However, several contaminants with spoilage potential may also occur, in most of the cases with negligible effects on the quality of the final product (10).

The use of 16S analysis through HTS has emerged as a new culture-independent tool and may allow a quantitative investigation of the structure of microbial communities, besides being much more sensitive to detect sub-dominant populations (11, 12). Several studies based on target amplicon sequencing showed that milk source, processing (raw or pasteurized) and addition of various ingredients affect the composition of the microbiota with an impact on the final attributes of the products (13). However, more information is needed regarding the function and the activity of the microbiota during the process. Few studies based on metatranscriptomic are applied on cheese matrix aiming at exploring the function of the cheese associate microbiota (14–16).

The aim of this work was to study the microbial dynamics of the active fraction of the microbiota during the manufacturing and ripening of a raw milk, long-ripened hard cooked Grana type cheese, with particular emphasis on the contribution of milk and whey starter, by coupling RT-PCR-DGGE, qPCR and rRNA-pyrosequencing. The molecular target used in this ecology study was the ribosomal RNA (rRNA), which has been described as an indicator for metabolically active
microbiota, allowing for a greater understanding of microbial community structure and functionality (17, 18).

MATERIAL AND METHODS

Sampling. Three different cheese productions (D, E and F) from the same dairy plant, located in the Piedmont region (North West of Italy), were studied until the tenth month of ripening. The cheese studied is a hard-pressed Grana type cheese. All the productions were carried out in the spring season, in three successive weeks. A full description of the samples is reported in Table 1. The samples were: whey, raw milk (Frisona cow), milk after the addition of whey, curd before and after cutting, after pressing, after storage room at 46°C, after salting, after thermostatic room at 25°C, and from the first to the tenth ripening month. During the first four months of ripening the temperature ranges from 16-17°C with a relative humidity of 80%, while in the following months the temperature reaches 20°C with an humidity of 82%. Samples were transported under refrigeration to the laboratory and were subjected to analysis within 2 h from collection. Sampling on cheese loafs was carried out using a sterile punch, which was inserted perpendicular to the centre of the cheese, and then rotated 360° for digging samples.

RT-PCR-DGGE. Samples preparation and RNA extraction were performed according to the protocol reported by (19) by using the Master-Pure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA) following the manufacturer’s instructions. Re-suspended RNA was treated with Turbo DNase (Ambion, Italy), in order to eliminate the DNA. Complete DNA digestion was confirmed using 1 µL of extracted RNA in PCR with primers 518r and 338f (21) and, when a PCR product was obtained, the DNase treatment was repeated. Reverse transcription (RT) reactions were performed using the M-MLV reverse transcriptase (Promega, Milan, Italy). Two hundred ng of RNA were mixed with 1 µL of 100 µM of the 518 r primer and the reverse transcription was carried out at 42 °C for 1 h. One µL of the obtained
complementary DNA was used as template for the amplification of the bacterial V3 region of the 16S rRNA gene and the PCR products were analyzed by means of DGGE, as described by (20).

A database of fingerprints was created using the Bionumerics software, version 4.6 (Applied Maths, Sint Marten Latem, Belgium). A combined data matrix that included all the fingerprints from RNA was obtained, and dendrograms of similarity were retrieved using the Dice coefficient and the Unweighted Pair Group Method with the Arithmetic average (UPGMA) clustering algorithm (21).

The similarity distance matrix generated through the Bionumerics software was used to build a Partial Least-Squares Discriminant Analysis (PLS-DA) utilizing the “mixOmics” R package (www.r-project.org).

Selected DGGE bands were extracted from the gels, using sterile pipette tips, transferred into 50 µL sterile water and incubated overnight at 4 °C. Two µL of the eluted bands were re-amplified using the conditions described above and checked by means of DGGE. The PCR products that gave a single band, co-migrating with the RNA control, were then amplified with the same primers without a GC clamp and sequenced by MWG Biotech (Ebersberg, Germany). The Sanger sequences were aligned in GenBank using the BLAST search program (http://www.ncbi.nlm.nih.gov/blast/).

**Construction of LAB standard curve for bacterial enumeration.** In order to obtain an enumeration of the active bacterial cells, different standard curves were constructed. Ten ml of milk, whey and ten grams of curd and grated ripened cheese were contaminated with 10 ml of ten-fold serial dilutions of overnight pure cultures mix of *Lactococcus lactis* subsp. *lactis*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Enterococcus faecium* and *Lb. helveticus* (from the DISAFA collection, all identified by using molecular methods, Bautista Gallego et al. (22) and supplemented with 90 ml of sterile Ringer (Oxoid, Milan, Italy). The samples were homogenized in a Stomacher (Interscience Rockland, MA, USA) for 1 min and 1 ml of each mixture was subjected to RNA extraction as described above. RT was performed using 9 µL of RNA and the resulting cDNA sample was submitted to qPCR. Standard curves were constructed by plotting the threshold cycle (Ct) values against CFU/g or ml on MRS agar. MRS agar plates were incubated at 37°C for 48 h.
hours in micro aerobic conditions. Correlation coefficients ($R^2$) and efficiency of amplification were calculated as previously described (23).

**Detection of total bacteria by qPCR.** Quantitative PCR targeting the V3 region of the 16S rRNA gene were used for the active bacteria quantification. Amplifications were performed in a final volume of 25 µl in the Chromo 4 Real-Time PCR Detection System (Biorad, Milan, Italy) with the use of SSo Advanced Sybr Green Supermix (Biorad, Italy) and one µl of cDNA was amplified with 338f and 518r primers (20) at a final concentration of 400 nM. Samples were amplified in triplicate using the following conditions: initial denaturation at 95 °C for 5 min and 40 cycles of 95 °C for 15 s, 60°C for 30 s.

**RNA analysis by pyrosequencing.** Samples were carefully selected from the three productions in order to describe the microbial diversity during the whole manufacturing process (Tab. 3). cDNA obtained as explained above was used to study the microbial diversity of the active populations by pyrosequencing of the amplified V1–V3 region of the 16S rRNA gene by using primers and PCR condition previously reported (24). PCR products were purified twice by Agencourt AMPure kit (Beckman Coulter, Milano, Italy), quantified using the QuantiFluor™ (Promega, Milano, Italy) and an equimolar pool was obtained prior to further processing. The amplicon pool was used for pyrosequencing on a GS Junior platform (454 Life Sciences, Roche, Monza, Italy) according to the manufacturer’s instructions by using Titanium chemistry.

**Bioinformatics analysis.** 16S rRNA data were analyzed by using QIIME 1.9.0 software (25) and a pipeline previously described (26). OTUs were picked at 99% of similarity by the UCLUST clustering methods (27) and representative sequences of each cluster were used to assign taxonomy using the Greengenes 16S rRNA gene database version 2013 by the RDP classifier (28). When the assigned taxonomy was only at genus level, representative sequences belonging to Lactobacillaceae were checked using the BLAST (BLASTN) search program (http:// www.ncbi.nlm.nih.gov/blast/) to get the species level as best hit. Statistics and plotting was carried out in R environment (www.r-
Alpha diversity indices were calculated by using the diversity function of the vegan package (29). Weighted UniFrac distance matrices obtained through QIIME were imported in R to obtain PCoA (Principal Coordinates Analysis) plots. OTU tables filtered at 0.5% abundance in at least two samples were used for co-occurrence/co-exclusion analysis, carried out by using the psych package of R (www.r-project.org). Correlation matrix was plotted by using the corrplot package of R (30). All the sequencing data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (SRP044294).

RESULTS

Active bacteria during the production and ripening of the cheeses. Different standard curves were built considering the evolution of the samples during the cheese manufacturing (milk, whey, curd and ripened cheese) in order to obtain a direct quantification of viable bacteria in the samples. For all the samples, the limit of quantification was 4 Log CFU/g or ml. The efficiencies were different based on the matrix and the $R^2$ values was always > 0.922 (data not shown). The results of the quantification, expressed as Log CFU/ml or g, are reported in Table 1. Milk D and E showed microbial loads around 7 Log CFU/ml while 7-8 Log CFU/g were detected in the curds. A slight decrease was observed during the ripening, reaching values ranging 6-7 Log CFU/g, underlining a high amount of active bacteria also at the tenth month of ripening for batch D and E. The application of qPCR highlighted lower counts in most of the samples from the production F, where it was not possible to reach the quantification, since the CT values obtained were out of the linearity range (value < 4 Log CFU/ml or g).

RT-PCR-DGGE analysis. Nineteen samples, from raw milk to cheese ripened for ten months, for each of the three different productions, were analyzed by DGGE. The PLS-DA regression, built using the production batch as discriminatory class (Fig. 1A), showed a certain gradient of separation between the three series of samples. The results of the identification of selected bands are reported in Table 2. NWS used in the three productions showed a similar profile characterized by *Lb.*
helveticus and, Lactobacillus delbrueckii subsp. bulgaricus, while Streptococcus thermophilus was detected only in the production F. Milks used for productions F and D showed the presence of bands identified as Lb. helveticus and, Lb. delbrueckii subsp. bulgaricus and Lactobacillus acidophilus, while milk from production E was characterized by Acinetobacter baumanni, Acinetobacter haemolyticus, Pseudomonas sp. and Streptococcus sanguinis. Regarding the common species shared among the three batches, Lb. acidophilus characterize the ripening time while S. thermophilus was found during the manufacturing and the early-stage ripening in production E and randomly in F, while it became persistent during the ripening in the production D. Lactococcus lactis was detected only in few samples during the ripening time only in batch D and E. Regarding the contaminant species, Bacillus subtilis was detected mainly in the F samples, while Acinetobacter baumannii present randomly from the milk in production D and E, was not found in F samples. Propionibacterium sp. was randomly detected in the three productions (Table 2).

**16S rRNA gene pyrosequencing.** A total of 198,276 raw reads were obtained after the 454 processing; 148,944 reads passed the filters applied through QIIME, with an average value of 3,819 reads/sample and an average length of 469 bp. The rarefaction analysis and the estimated sample coverage (Table 3) indicated that there was a satisfactory coverage for all the samples (ESC > 99%). Whey starters used in the three manufactures showed a similar qualitative and quantitative microbiota composition, characterized by almost the same relative abundance of Lb. helveticus and Lb. delbrueckii (Fig. 2).

Alpha-diversity indices (Table 3) showed that raw milk samples from batches F and E had a generally higher level of complexity compared to D. On the contrary, cheese from batch D showed a higher level of complexity during the ripening.

In Figure 2, only OTUs with a relative abundance of 0.5% in at least two samples are shown. The milk sample from production D was characterized by Lb. helveticus (10%) and by the predominance of Acinetobacter baumannii (76%), which survived throughout the ripening and appeared in most of the samples of the same batch. Lb. helveticus (55%) was the dominant species
in milk E, followed by *S. thermophilus* (22%) and *Lb. delbrueckii* (10%). *Propionibacterium acnes* (38%) characterized milk from batch F, while *S. thermophilus* and genera belonging to *Acinetobacter* and *Streptococcaceae* were also present as minor OTUs. The main differences between the three cheese batches were on the presence of several contaminant OTUs, such as *P. acnes, Acidovorax, Acinetobacter* and *Pseudomonas*. Cheese in production D was characterized by the presence of *Lb. casei* group (37%), *Lb. brevis* (6.8%), *Lb. fermentum* (0.6%) and *S. thermophilus* (10.8 %), while cheese from batch F showed low levels of NSLAB (< 1%). Moreover, *P. acnes* and *Staphylococcus* were present at 42% and 5% of abundance, respectively. Interestingly, starter species were absent in cheeses from production F during ripening. *S. thermophilus* and *P. acnes* were found to be significantly more abundant (t test, P < 0.001) in samples from batch F. *Acinetobacter baumannii, Lb. delbrueckii* and *Lb. casei* group discriminated samples D, while *Lb. helveticus* characterized batch E (P < 0.001).

Nevertheless, OTUs characteristic of each production drove the sample clustering according to the batch (Fig. 1B) and sample differentiation according to the batch was supported by Adonis and Anosim statistical tests (P < 0.001).

The OTU co-occurrence/exclusion pattern is shown in Figure 3, where only significant correlations are reported (at False Discovery Rate - FDR < 0.05). *Lb. delbrueckii, Lb. helveticus* and *Lb. casei* group showed the highest number of negative correlations, including a strong exclusion with *Pseudomonas* and other contaminants.

**DISCUSSION**

Insights into the microbial ecology and active bacterial communities during the manufacturing and ripening of a Piedmont hard cheese were provided in this study. The experimental approach used exploited different culture-independent methods based on RNA. Culture-independent methods have rapidly been recognized as a valuable tool for the study of biodiversity and identification of microbial species in food samples (31). The results of qPCR were able to give more precise
information about the total active bacteria counts and showed higher values than those observed by culture-dependent methods on selective media for enumeration of LAB (22), emphasising the presence of other non-LAB populations.

By using RT-PCR-DGGE and pyrosequencing, *Lb. rhamnosus* (belonging to *Lb. casei* group) and *Lb. helveticus* were the dominant taxa during the whole manufacturing and ripening process. On the contrary, Bautista-Gallego et al. (23) showed the predominance of *Lb. helveticus* only in the early stages of cheese production, while *Lb. rhamnosus* dominated in the following months. As reported from other studies (32), *Lb. helveticus* can contribute to the ripening process due to its autolytic properties, which could increase the proteolysis in aged cheese and consequently the flavor formation. Moreover, members of the *Lb. casei* group are able to utilize products of *Lb. helveticus* lysis as a unique energy source (33). *L. lactis* was also detected. This evidence is in agreement with recent data concerning the viability of *L. lactis* throughout the manufacturing and ripening of cheese (34, 35).

The microbial co-occurrence/exclusion patterns suggested that the presence of *Lb. helveticus* and *Lb. delbrueckii* co-excluded the presence of other populations. Those results confirmed that the core microbial genera of the starter tend to dominate the cheeses microbiota and to limit the development of spoilage bacteria and contaminants (36). On the other hand, the 16S data revealed that *P. acnes*, present as main contaminant in milk from production F, showed a co-occurrence pattern with other contaminant taxa. Moreover, it remained metabolically active until the end of the ripening, confirming the impact of raw milk quality in the development of the microbiota during ripening. As previously demonstrated *Propionibacterium* is normally isolated from milk and cheese and may contribute to the formation of distinctive flavors arising from its metabolism (37, 38).

PCoA and PLS-DA analyses clearly showed a separation of the samples from the three production batches probably driven by the different milk used. The 16S data highlighted a higher level of complexity in the raw milk used in production F, with the presence of minor OTUs such as *Corynebacterium* sp., *A. baumannii*, *P. acnes*, *Pseudomonas* sp. and *Streptococcaceae*, besides *Lb.*
*helveticus* and *S. thermophilus*. The contaminants observed in the raw milk samples were previously detected in milk and cheese and were suggested to cause spoilage (39–41). The differences in microbiota composition of the raw milk used for the three productions can be attributed to the hygiene practices in the farm, including the teat surface hygiene, air, dust, stable conditions, and milking parlor environment, which are responsible for milk contamination (7). It is well demonstrated that the microbiota involved in cheese production are commonly found on the processing surfaces (36) highlighting the importance of appropriate hygienic measures to avoid contamination from the production environment. They are also critical for minimizing contamination and prevent the growth of spoilage microorganism. In addition milk supplied to the farm is not always immediately processed. In particular the storage conditions and storage time before the processing can favor the growth and the development of the minor microbiota which can become dominant, leading to an unacceptable quality of the final dairy product (42, 43).

This study highlights and confirms that the microbial quality of raw milk has an important effect on the development of the contaminant microbiota during ripening of a Grana-like cheese. The results showed that the core genera of the starter tended to limit the development of the spoilage bacteria. On the other hand in the case of the batch F the whey starter was not competitive enough against the contaminant species, which impacted the microbial composition of the final products. For this production it was also observed a low amount of active cells in the milk and during manufacturing and ripening.

This study underlines the influence of different factors that can affect the final microbiota composition. Further studies based on metatranscriptomics and metabolomics are needed for verifying the effect of the subdominant microbiota of the raw milk on the organoleptic and sensorial properties of the cheeses.
REFERENCES


12. Ercolini D. 2013. High-throughput sequencing and metagenomics: moving forward in the
3155.

13. Clarke SF, Murphy EF, O’Sullivan O, Ross RP, O’Toole PW, Shanahan F, Cotter PD.
2013. Targeting the microbiota to address diet-induced obesity: a time dependent challenge.
PLoS One 8:e65790.

fungal strains Penicillium camemberti and Geotrichum candidum reveal cheese matrix
breakdown and potential development of sensory properties of ripened Camembert-type

15. Dugat-Bony E, Straub C, Teissandier A, Onésime D, Loux V, Monnet C, Irlinger F,
Landaud S, Leclercq-Perlat M-N, Bento P, Fraud S, Gibrat J-F, Aubert J, Fer F,
Overview of a surface-ripened cheese community functioning by meta-omics analyses. PLoS
One 10:e0124360.

reveals temperature-driven functional changes in microbiome impacting cheese maturation

2013. Cheese surface microbiota complexity: RT-PCR-DGGE, a tool for a detailed picture?
Int J Food Microbiol 162:8–12.


28. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen


38. Thierry A, Maillard M-B, Bonnarme P, Roussel E. 2005. The addition of


Legends to figures

**FIG 1** Plot A - Partial Least-Squares Discriminant Analysis (PLS-DA) model built on the similarity distance matrix based on RT-PCR-DGGE fingerprint profiles. Plot B - Principal Coordinates Analysis (PCoA) of weighted UniFrac distances for 16S rRNA (cDNA) gene sequencing data. Samples are color coded as a function of the batch: D (blue), F (red) and E (yellow).

**FIG 2** Abundance of the major taxonomic groups detected by pyrosequencing. Only OTUs with an incidence above 0.5% in at least two samples are shown. Samples are grouped according to batch.

**FIG 3** Significant co-occurrence and co-exclusion relationships between bacterial OTUs. Spearman’s rank correlation matrix of OTUs with > 0.5% abundance in at least 2 samples. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The color of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (dark blue) and -1 indicating a perfectly negative correlation (dark red). Only significant correlations (FDR < 0.05) are shown.
TABLE 1 Results of the total active bacteria counts obtained by qPCR

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample description</th>
<th>Production D</th>
<th>Production E</th>
<th>Production F</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Log CFU/ml or Log CFU/ g</td>
<td>Log CFU/ml or Log CFU/ g</td>
<td>Log CFU/ml or Log CFU/ g</td>
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</tr>
<tr>
<td>1</td>
<td>Whey starter</td>
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<td>6.67</td>
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<td>Raw milk</td>
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<tr>
<td>3</td>
<td>Milk + whey</td>
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<td>4</td>
<td>Curd after cutting</td>
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<td>7.9</td>
<td>*</td>
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<td>Curd after heating</td>
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<td>6</td>
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<td>Curd after storage room</td>
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<td>8</td>
<td>Cheese after salting</td>
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<td>9</td>
<td>Cheese after themostatic room</td>
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<td>7.21</td>
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<td>5.5</td>
<td>6.5</td>
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* Below quantification limit
TABLE 2 Results of the identification of selected RT-PCR-DGGE band sequencing.

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<th>Closest relative</th>
<th>% identity</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
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<td>Lactobacillus helveticus, Lactobacillus crispatus</td>
<td>100%</td>
<td>CP002427</td>
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<tr>
<td>Lactobacillus delbrueckii subsp. bulgaricus</td>
<td>98%</td>
<td>HG423863</td>
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<td>Bacillus subtilis</td>
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<tr>
<td>Lactobacillus acidophilus</td>
<td>99%</td>
<td>NR_075048</td>
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<tr>
<td>Acinetobacter baumannii</td>
<td>99%</td>
<td>KC253268</td>
</tr>
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<td>Bacillus coagulans</td>
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<td>NR_051758</td>
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<td>Propionibacterium acnes</td>
<td>99%</td>
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<td>Acinetobacter haemolyticus</td>
<td>99%</td>
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<td>JQ406046</td>
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<td>Lactobacillus casei</td>
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<td>Pseudomonas sp.</td>
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<td>KF511637</td>
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<td>NR_074244</td>
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<td>Streptococcus sanguinis</td>
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<td>KC628757</td>
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</table>

Species identified from sequencing of bands in the profiles of each sample. The presence of the black box indicates the presence of the bands in the DGGE profiles. The sequences obtained were aligned with those in GenBank with Blast program.

Sample descriptions are reported in Table 1.
TABLE 3 Number of sequences analyzed, observed diversity, and estimated sample coverage for 16S rRNA amplicons from selected samples from the three productions.

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<thead>
<tr>
<th>Sample number</th>
<th>Sample Description</th>
<th>D</th>
<th>Batch</th>
<th>E</th>
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<td>Shannon</td>
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</table>

*Estimate sample coverage