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1 **Microbiota of an Italian Grana like cheese during manufacture and ripening unraveled by**
2 **16S rRNA-based approaches**

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20 Running title: 16S rRNA analysis of an Italian hard cheese microbiota

21

22 **ABSTRACT**

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

43

44 **IMPORTANCE**

45 This study highlights the importance of the quality of the raw milk in the production of an hard
46 cheese. Independently from the use of a starter culture, raw milk with low microbiological quality

47 can negatively affect the populations of lactic acid bacteria and as a consequence impact the quality
48 of the final product due to spoilage metabolic processes.

49

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

51 **INTRODUCTION**

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

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

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

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

68 It is well reported that the production is characterized by microbial dynamic changes and bacteria
69 play a primary role in defining its quality (5). Different microbial populations coexist and interact
70 contributing through their metabolism to the development of taste, aroma, texture, shelf-life and
71 safety (6). In particular, the microbial ecology of long ripened cheeses, produced from raw milk and

72 using whey starter cultures, is based on complex interaction among starter lactic acid bacteria
73 (SLAB) and non starter lactic acid bacteria (NSLAB) (7). SLAB give their contribution in the early
74 stages of the cheese making, while NSLAB, which are able to use other carbon sources apart from
75 lactose, become the dominant microbiota of the ripened cheeses (8) and are responsible for the
76 flavour and texture of the cheeses due to their proteolytic and lipolytic activities (7). NSLAB are
77 mainly mesophilic microorganisms originating from raw milk and/or from the environment and
78 their presence may introduce variability into the ripening process (9). For this reason, the
79 development of beneficial NSLAB coming from the natural whey starter, from the raw milk or from
80 the manufacturing environment is crucial to minimize microbial variability during the ripening
81 process and obtain the desired organoleptic characteristics of the cheese. However, several
82 contaminants with spoilage potential may also occur, in most of the cases with negligible effects on
83 the quality of the final product (10).

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

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

97 microbiota, allowing for a greater understanding of microbial community structure and functionality
98 (17, 18).

99

100 **MATERIAL AND METHODS**

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

113 **RT-PCR-DGGE.** Samples preparation and RNA extraction were performed according to the
114 protocol reported by (19) by using the Master-Pure™ Complete DNA and RNA Purification Kit
115 (Epicentre, Madison, WI, USA) following the manufacturer's instructions. Re-suspended RNA was
116 treated with Turbo DNase (Ambion, Italy), in order to eliminate the DNA. Complete DNA
117 digestion was confirmed using 1 µL of extracted RNA in PCR with primers 518r and 338f (21) and,
118 when a PCR product was obtained, the DNase treatment was repeated.

119 Reverse transcription (RT) reactions were performed using the M-MLV reverse transcriptase
120 (Promega, Milan, Italy). Two hundred ng of RNA were mixed with 1 µL of 100 µM of the 518 r
121 primer and the reverse transcription was carried out at 42 °C for 1 h. One µL of the obtained

122 complementary DNA was used as template for the amplification of the bacterial V3 region of the
123 16S rRNA gene and the PCR products were analyzed by means of DGGE, as described by (20).
124 A database of fingerprints was created using the Bionumerics software, version 4.6 (Applied Maths,
125 Sint Marten Latem, Belgium). A combined data matrix that included all the fingerprints from RNA
126 was obtained, and dendrograms of similarity were retrieved using the Dice coefficient and the
127 Unweighted Pair Group Method with the Arithmetic average (UPGMA) clustering algorithm (21).
128 The similarity distance matrix generated through the Bionumerics software was used to build a
129 Partial Least-Squares Discriminant Analysis (PLS-DA) utilizing the “mixOmics” R package
130 (www.r-project.org).
131 Selected DGGE bands were extracted from the gels, using sterile pipette tips, transferred into 50 µL
132 sterile water and incubated overnight at 4 °C. Two µL of the eluted bands were re-amplified using
133 the conditions described above and checked by means of DGGE. The PCR products that gave a
134 single band, co-migrating with the RNA control, were then amplified with the same primers without
135 a GC clamp and sequenced by MWG Biotech (Ebersberg, Germany). The Sanger sequences were
136 aligned in GenBank using the BLAST search program (<http://www.ncbi.nlm.nih.gov/blast/>).
137 **Construction of LAB standard curve for bacterial enumeration.** In order to obtain an
138 enumeration of the active bacterial cells, different standard curves were constructed. Ten ml of
139 milk, whey and ten grams of curd and grated ripened cheese were contaminated with 10 ml of ten-
140 fold serial dilutions of overnight pure cultures mix of *Lactococcus lactis* subsp. *lactis*, *Lactobacillus*
141 *plantarum*, *Lactobacillus pentosus*, *Enterococcus faecium* and *Lb. helveticus* (from the DISAFA
142 collection, all identified by using molecular methods, Bautista Gallego et al. (22) and supplemented
143 with 90 ml of sterile Ringer (Oxoid, Milan, Italy). The samples were homogenized in a Stomacher
144 (Interscience Rockland, MA, USA) for 1 min and 1 ml of each mixture was subjected to RNA
145 extraction as described above. RT was performed using 9 µL of RNA and the resulting cDNA
146 sample was submitted to qPCR. Standard curves were constructed by plotting the threshold cycle
147 (Ct) values against CFU/g or ml on MRS agar. MRS agar plates were incubated at 37°C for 48

148 hours in micro aerobic conditions. Correlation coefficients (R^2) and efficiency of amplification were
149 calculated as previously described (23).

150 **Detection of total bacteria by qPCR.** Quantitative PCR targeting the V3 region of the 16S rRNA
151 gene were used for the active bacteria quantification. Amplifications were performed in a final
152 volume of 25 μ l in the Chromo 4 Real-Time PCR Detection System (Biorad, Milan, Italy) with the
153 use of SSo Advanced Sybr Green Supermix (Biorad, Italy) and one μ l of cDNA was amplified with
154 338f and 518r primers (20) at a final concentration of 400 nM. Samples were amplified in triplicate
155 using the following conditions: initial denaturation at 95 °C for 5 min and 40 cycles of 95 °C for 15
156 s, 60°C for 30 s.

157

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

167 **Bioinformatics analysis.** 16S rRNA data were analyzed by using QIIME 1.9.0 software (25) and a
168 pipeline previously described (26). OTUs were picked at 99% of similarity by the UCLUST clustering
169 methods (27) and representative sequences of each cluster were used to assign taxonomy using the
170 Greengenes 16S rRNA gene database version 2013 by the RDP classifier (28). When the assigned
171 taxonomy was only at genus level, representative sequences belonging to Lactobacillaceae were
172 checked using the BLAST (BLASTN) search program ([http:// www.ncbi.nlm.nih.gov/blast/](http://www.ncbi.nlm.nih.gov/blast/)) to get
173 the species level as best hit. Statistics and plotting was carried out in R environment (www.r-

174 [project.org](http://www.r-project.org)). Alpha diversity indices were calculated by using the *diversity* function of the vegan
175 package (29). Weighted UniFrac distance matrices obtained through QIIME were imported in R to
176 obtain PCoA (Principal Coordinates Analysis) plots. OTU tables filtered at 0.5% abundance in at
177 least two samples were used for co-occurrence/co-exclusion analysis, carried out by using the psych
178 package of R (www.r-project.org). Correlation matrix was plotted by using the corrplot package of
179 R (30). All the sequencing data were deposited at the Sequence Read Archive of the National
180 Center for Biotechnology Information (SRP044294).

181

182 **RESULTS**

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

195 **RT-PCR-DGGE analysis.** Nineteen samples, from raw milk to cheese ripened for ten months, for
196 each of the three different productions, were analyzed by DGGE. The PLS-DA regression, built
197 using the production batch as discriminatory class (Fig. 1A), showed a certain gradient of separation
198 between the three series of samples. The results of the identification of selected bands are reported
199 in Table 2. NWS used in the three productions showed a similar profile characterized by *Lb.*

200 *helveticus* and, *Lactobacillus delbrueckii* subsp. *bulgaricus*, while *Streptococcus thermophilus* was
201 detected only in the production F. Milks used for productions F and D showed the presence of
202 bands identified as *Lb. helveticus* and, *Lb. delbrueckii* subsp. *bulgaricus* and *Lactobacillus*
203 *acidophilus*, while milk from production E was characterized by *Acinetobacter baumannii*,
204 *Acinetobacter haemolyticus*, *Pseudomonas* sp. and *Streptococcus sanguinis*. Regarding the common
205 species shared among the three batches, *Lb. acidophilus* characterize the ripening time while *S.*
206 *thermophilus* was found during the manufacturing and the early-stage ripening in production E and
207 randomly in F, while it became persistent during the ripening in the production D. *Lactococcus*
208 *lactis* was detected only in few samples during the ripening time only in batch D and E. Regarding
209 the contaminant species, *Bacillus subtilis* was detected mainly in the F samples, while
210 *Acinetobacter baumannii* present randomly from the milk in production D and E, was not found in
211 F samples. *Propionibacterium* sp. was randomly detected in the three productions (Table 2).

212 **16S rRNA gene pyrosequencing.** A total of 198,276 raw reads were obtained after the 454
213 processing; 148,944 reads passed the filters applied through QIIME, with an average value of 3,819
214 reads/sample and an average length of 469 bp. The rarefaction analysis and the estimated sample
215 coverage (Table 3) indicated that there was a satisfactory coverage for all the samples (ESC >
216 99%). Whey starters used in the three manufactures showed a similar qualitative and quantitative
217 microbiota composition, characterized by almost the same relative abundance of *Lb. helveticus* and
218 *Lb. delbrueckii* (Fig. 2).

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

222 In Figure 2, only OTUs with a relative abundance of 0.5% in at least two samples are shown. The
223 milk sample from production D was characterized by *Lb. helveticus* (10%) and by the
224 predominance of *Acinetobacter baumannii* (76%), which survived throughout the ripening and
225 appeared in most of the samples of the same batch. *Lb. helveticus* (55%) was the dominant species

226 in milk E, followed by *S. thermophilus* (22%) and *Lb. delbrueckii* (10%). *Propionibacterium acnes*
227 (38%) characterized milk from batch F, while *S. thermophilus* and genera belonging to
228 *Acinetobacter* and *Streptococcaceae* were also present as minor OTUs. The main differences
229 between the three cheese batches were on the presence of several contaminant OTUs, such as *P.*
230 *acnes*, *Acidovorax*, *Acinetobacter* and *Pseudomonas*. Cheese in production D was characterized by
231 the presence of *Lb. casei* group (37%), *Lb. brevis* (6.8%), *Lb. fermentum* (0.6%) and *S.*
232 *thermophilus* (10.8 %), while cheese from batch F showed low levels of NSLAB (< 1%). Moreover,
233 *P. acnes* and *Staphylococcus* were present at 42% and 5% of abundance, respectively. Interestingly,
234 starter species were absent in cheeses from production F during ripening. *S. thermophilus* and *P.*
235 *acnes* were found to be significantly more abundant (g test, $P < 0.001$) in samples from batch F.
236 *Acinetobacter baumannii*, *Lb. delbrueckii* and *Lb. casei* group discriminated samples D, while *Lb.*
237 *helveticus* characterized batch E ($P < 0.001$).

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

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

245

246 **DISCUSSION**

247 Insights into the microbial ecology and active bacterial communities during the manufacturing and
248 ripening of a Piedmont hard cheese were provided in this study. The experimental approach used
249 exploited different culture-independent methods based on RNA. Culture-independent methods have
250 rapidly been recognized as a valuable tool for the study of biodiversity and identification of
251 microbial species in food samples (31). The results of qPCR were able to give more precise

252 information about the total active bacteria counts and showed higher values than those observed by
253 culture-dependent methods on selective media for enumeration of LAB (22), emphasising the
254 presence of other non-LAB populations.

255 By using RT-PCR-DGGE and pyrosequencing, *Lb. rhamnosus* (belonging to *Lb. casei* group) and
256 *Lb. helveticus* were the dominant taxa during the whole manufacturing and ripening process. On the
257 contrary, Bautista-Gallego et al. (23) showed the predominance of *Lb. helveticus* only in the early
258 stages of cheese production, while *Lb. rhamonosus* dominated in the following months. As reported
259 from other studies (32), *Lb. helveticus* can contribute to the ripening process due to its autolytic
260 properties, which could increase the proteolysis in aged cheese and consequently the flavor
261 formation. Moreover, members of the *Lb. casei* group are able to utilize products of *Lb. helveticus*
262 lysis as a unique energy source (33). *L. lactis* was also detected. This evidence is in agreement with
263 recent data concerning the viability of *L. lactis* throughout the manufacturing and ripening of cheese
264 (34, 35).

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

274 PCoA and PLS-DA analyses clearly showed a separation of the samples from the three production
275 batches probably driven by the different milk used. The 16S data highlighted a higher level of
276 complexity in the raw milk used in production F, with the presence of minor OTUs such as
277 *Corynebacterium* sp., *A. baumannii*, *P. acnes*, *Pseudomonas* sp. and *Streptococcaceae*, besides *Lb.*

278 *helveticus* and *S. thermophilus*. The contaminants observed in the raw milk samples were
279 previously detected in milk and cheese and were suggested to cause spoilage (39–41). The
280 differences in microbiota composition of the raw milk used for the three productions can be
281 attributed to the hygiene practices in the farm, including the teat surface hygiene, air, dust, stable
282 conditions, and milking parlor environment, which are responsible for milk contamination (7). It is
283 well demonstrated that the microbiota involved in cheese production are commonly found on the
284 processing surfaces (36) highlighting the importance of appropriate hygienic measures to avoid
285 contamination from the production environment. They are also critical for minimizing
286 contamination and prevent the growth of spoilage microorganism. In addition milk supplied to the
287 farm is not always immediately processed. In particular the storage conditions and storage time
288 before the processing can favor the growth and the development of the minor microbiota which can
289 become dominant, leading to an unacceptable quality of the final dairy product (42, 43).

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

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

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428 **Legends to figures**

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

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

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

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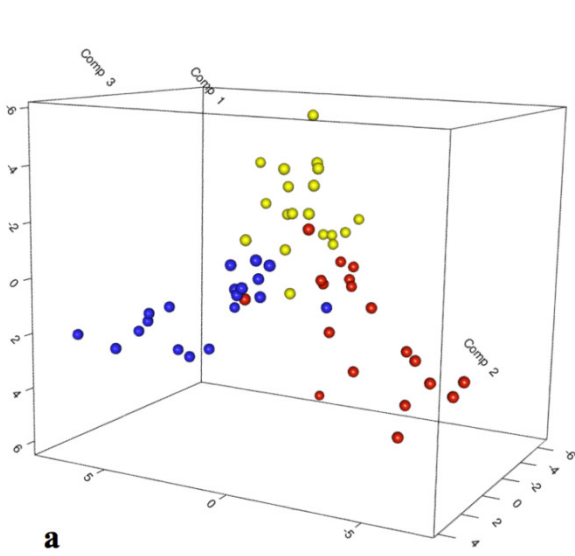
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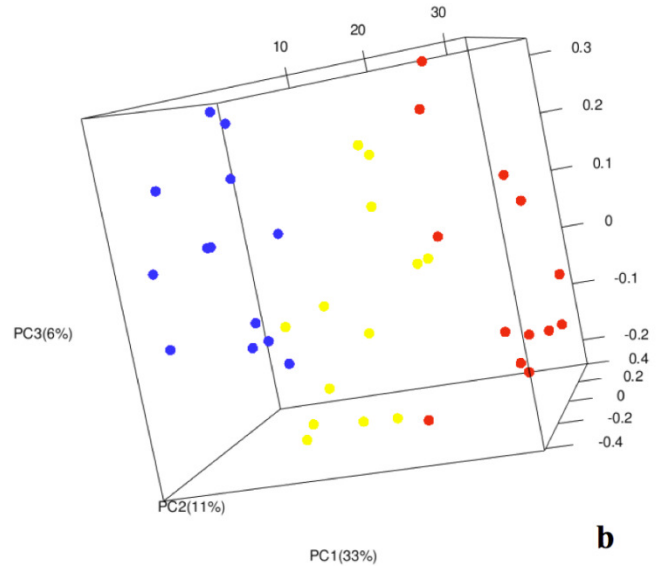
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445 **FIG 1**

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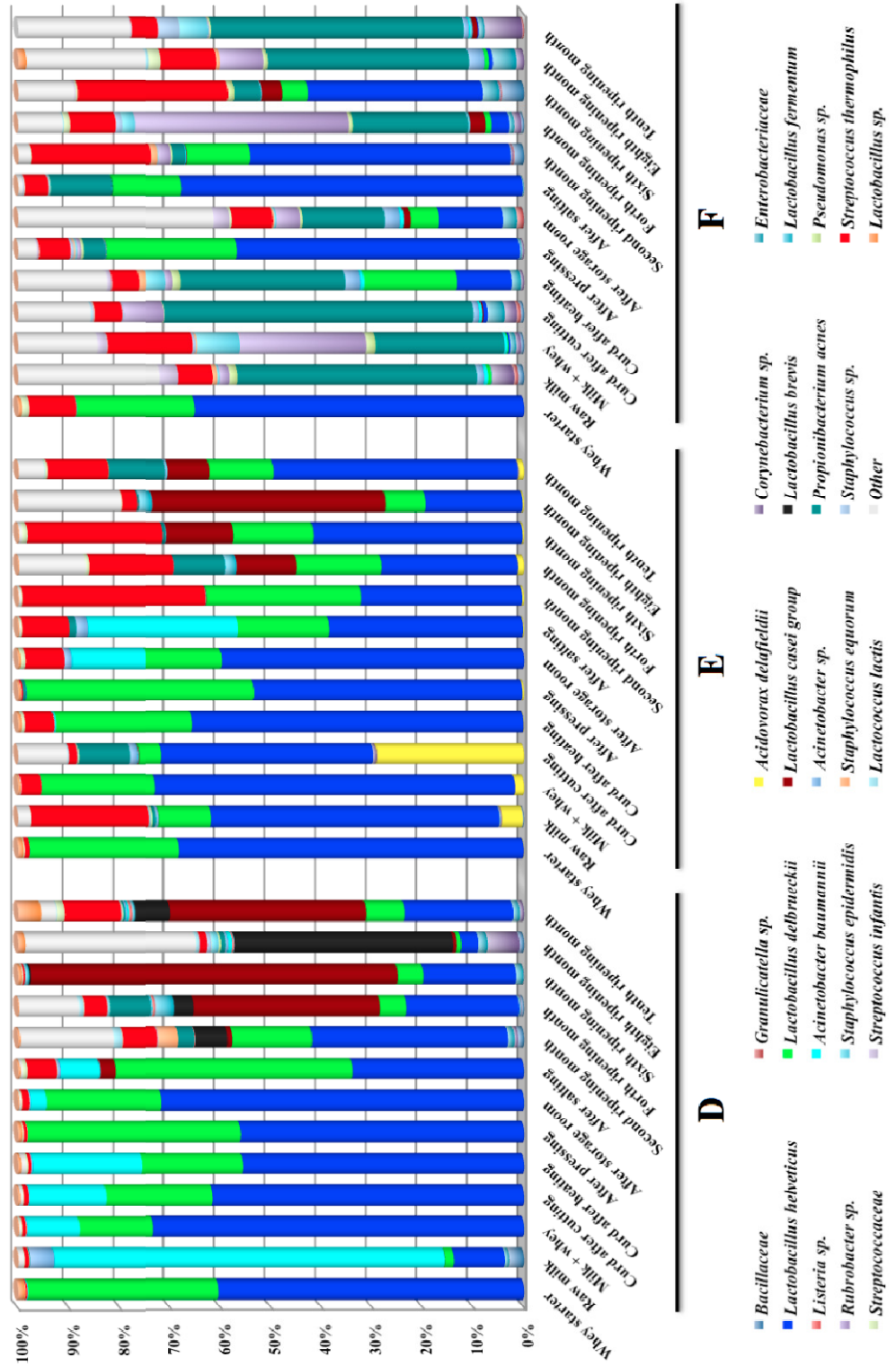
465 **FIG 2**

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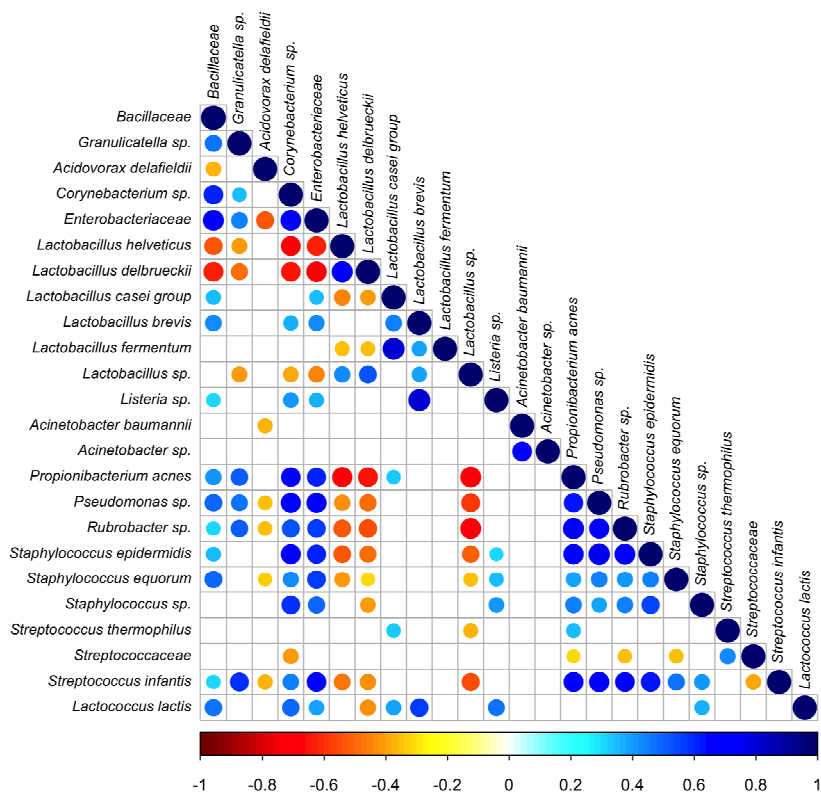
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491 **FIG 3**

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TABLE 1 Results of the total active bacteria counts obtained by qPCR

Sample	Sample description	Production D Log CFU/ml or Log CFU/ g	Production E Log CFU/ml or Log CFU/ g	Production F Log CFU/ml or Log CFU/ g
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1	Whey starter	9,82	6,67	7,9
2	Raw milk	7,52	7,59	*
3	Milk + whey	9,76	7,2	*
4	Curd after cutting	8,22	7,9	*
5	Curd after heating	8,33	7,47	*
6	Curd after pressing	8,43	6,91	*
7	Curd after storage room	7,91	8,16	*
8	Cheese after salting	8,05	7,8	*
9	Cheese after themostatic room	8,35	7,21	*
10	First ripening month	7,77	7,94	*
11	Second ripening month	7,23	8,17	*
12	Third ripening month	8,15	7,71	*
13	Fouth ripening month	7,58	7,67	*
14	Fifth ripening month	8,02	6,23	*
15	Sixth ripening month	7,99	7,98	*
16	Seventh ripening month	7,01	6,3	*
17	Eight ripening month	7	6,73	*
18	Ninth ripening month	6,5	6,97	*
19	Tenth ripening month	5,5	6,5	*

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* Below quantification limit

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521 **TABLE 3** Number of sequences analyzed, observed diversity, and estimated sample coverage for 16S rRNA amplicons from selected samples from
 522 the three productions.

Sample number	Sample Description	Batch														
		D					F					E				
		Reads	OTUs	chao1	Shannon	ESC*	Reads	OTUs	chao1	Shannon	ESC	Reads	OTUs	chao1	Shannon	ESC
1	Whey starter	4570	19	19,17	1,31	1,00	3081	26	32,00	1,55	1,00	2998	21	35,00	1,28	1,00
2	Raw milk	2603	53	72,46	1,46	0,99	7951	103	104,50	4,69	1,00	6337	109	126,65	3,72	1,00
3	Milk + whey	3375	23	35,00	1,33	1,00	4286	97	115,33	4,20	1,00	1843	19	20,50	1,40	1,00
4	Curd after cutting	3272	21	49,00	1,60	1,00	5761	106	109,60	3,63	1,00	3061	85	114,00	3,77	0,99
5	Curd after heating	1990	19	20,50	1,79	1,00	3115	107	111,50	4,30	1,00	3147	29	51,00	1,57	1,00
6	Curd after pressing	3359	17	20,75	1,28	1,00	3785	70	78,25	2,43	1,00	2744	21	30,33	1,34	1,00
7	Curd after storage room	3657	29	35,88	1,32	1,00	3750	151	166,11	5,95	1,00	3191	22	23,20	1,85	1,00
8	Cheese after salting	4348	49	73,00	2,22	1,00	3661	65	72,56	1,89	1,00	8116	52	71,43	2,29	1,00
11	Second ripening month	3111	137	151,50	4,13	0,99	9489	102	108,50	2,54	1,00	2197	35	62,20	1,92	0,99
13	Forth ripening month	4602	108	129,12	3,64	0,99	4722	99	106,50	3,91	1,00	2828	49	119,00	3,22	0,99
15	Sixth ripening month	3883	48	58,11	2,39	1,00	3858	79	85,11	3,76	1,00	2639	36	40,00	2,48	1,00
17	Eighth ripening month	3906	188	201,20	4,52	0,99	5334	41	87,00	0,26	1,00	2495	34	34,43	2,76	1,00
19	Tenth ripening month	3723	140	159,03	4,03	0,99	4891	147	156,00	4,05	1,00	2582	62	72,00	2,95	0,99

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*Estimate sample coverage

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