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

3

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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](http://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  $\mu$ 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  $\mu$ 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-](http://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](http://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.

301  
302  
303

304 **REFERENCES**

- 305 1. **Giraffa G, Mucchetti G, Addeo F, Neviani E.** 1997. Evolution of lactic acid microflora  
306 during Grana cheesemaking and ripening. *Microbiol Alim Nutr* **15**:115-122.
- 307 2. **Neviani E, Bottari B, Lazzi C, Gatti M.** 2013. New developments in the study of the  
308 microbiota of raw-milk, long-ripened cheeses by molecular methods: the case of Grana  
309 Padano and Parmigiano Reggiano. *Front Microbiol* **4**:36.
- 310 3. **Lazzi C, Rossetti L, Zago M, Neviani E, Giraffa G.** 2004. Evaluation of bacterial  
311 communities belonging to natural whey starters for Grana Padano cheese by length  
312 heterogeneity-PCR. *J Appl Microbiol* **96**:481–490.
- 313 4. **Rossi F, Gatto V, Sabattini G, Torriani S.** 2012. An assessment of factors characterising  
314 the microbiology of Grana Trentino cheese, a Grana-type cheese. *Int J Dairy Technol*  
315 **65**:401–409.
- 316 5. **Montel M-C, Buchin S, Mallet A, Delbes-Paus C, Vuitton D a, Desmasures N, Berthier**  
317 **F.** 2014. Traditional cheeses: rich and diverse microbiota with associated benefits. *Int J Food*  
318 *Microbiol* **177**:136–154.
- 319 6. **Holzappel WH.** 2002. Appropriate starter culture technologies for small-scale fermentation  
320 in developing countries. *Int J Food Microbiol* **75**:197–212.
- 321 7. **Gatti M, Bottari B, Lazzi C, Neviani E, Mucchetti G.** 2014. Invited review: microbial  
322 evolution in raw-milk, long-ripened cheeses produced using undefined natural whey starters.  
323 *J Dairy Sci* **97**:573–91.
- 324 8. **Quigley L, O’Sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD.**  
325 2013. The complex microbiota of raw milk. *FEMS Microbiol Rev* **37**:664–98.
- 326 9. **Settanni L, Moschetti G.** 2010. Non-starter lactic acid bacteria used to improve cheese  
327 quality and provide health benefits. *Food Microbiol* **27**:691–7.
- 328 10. **Coppola S, Blaiotta G, Ercolini D.** 2007. Dairy products, p 31-90. In Cocolin L, Ercolini D.  
329 (ed), *Molecular techniques in the microbial ecology of fermented foods*. Springer, USA.

- 330 11. **Cocolin L, Ercolini D.** 2015. Zooming into food-associated microbial consortia: a “cultural”  
331 evolution. *Curr Opin Food Sci* **2**:43–50.
- 332 12. **Ercolini D.** 2013. High-throughput sequencing and metagenomics: moving forward in the  
333 culture-independent analysis of food microbial ecology. *Appl Environ Microbiol* **79**:3148–  
334 3155.
- 335 13. **Clarke SF, Murphy EF, O’Sullivan O, Ross RP, O’Toole PW, Shanahan F, Cotter PD.**  
336 2013. Targeting the microbiota to address diet-induced obesity: a time dependent challenge.  
337 *PLoS One* **8**:e65790.
- 338 14. **Lessard M-H, Viel C, Boyle B, St-Gelais D, Labrie S.** 2014. Metatranscriptome analysis of  
339 fungal strains *Penicillium camemberti* and *Geotrichum candidum* reveal cheese matrix  
340 breakdown and potential development of sensory properties of ripened Camembert-type  
341 cheese. *BMC Genomics* **15**:235.
- 342 15. **Dugat-Bony E, Straub C, Teissandier A, Onésime D, Loux V, Monnet C, Irlinger F,**  
343 **Landaud S, Leclercq-Perlat M-N, Bento P, Fraud S, Gibrat J-F, Aubert J, Fer F,**  
344 **Guédon E, Pons N, Kennedy S, Beckerich J-M, Swennen D, Bonnarme P.** 2015.  
345 Overview of a surface-ripened cheese community functioning by meta-omics analyses. *PLoS*  
346 *One* **10**:e0124360.
- 347 16. **De Filippis F, Genovese A, Ferranti P, Gilbert JA, Ercolini D.** 2016. Metatranscriptomics  
348 reveals temperature-driven functional changes in microbiome impacting cheese maturation  
349 rate. *Sci Rep* **6**:1–12.
- 350 17. **Dolci P, Zenato S, Pramotton R, Barmaz A, Alessandria V, Rantsiou K, Cocolin L.**  
351 2013. Cheese surface microbiota complexity: RT-PCR-DGGE, a tool for a detailed picture?  
352 *Int J Food Microbiol* **162**:8–12.
- 353 18. **De Angelis M, Montemurno E, Vannini L, Cosola C, Cavallo N, Gozzi G, Maranzano V,**  
354 **Di Cagno R, Gobbetti M, Gesualdo L.** 2015. Effect of whole-grain barley on the human  
355 fecal microbiota and metabolome. *Appl Environ Microbiol* **81**:7945–7956.

- 356 19. **Rantsiou K, Alessandria V, Urso R, Dolci P, Cocolin L.** 2008. Detection, quantification  
357 and vitality of *Listeria monocytogenes* in food as determined by quantitative PCR. *Int J Food*  
358 *Microbiol* **121**:99–105.
- 359 20. **Alessandria V, Dolci P, Rantsiou K, Pattono D, Dalmasso A, Civera T, Cocolin L.** 2010.  
360 Microbiota of the Planalto de Bolona: an artisanal cheese produced in uncommon  
361 environmental conditions in the Cape Verde Islands. *World J Microbiol Biotechnol* **26**:2211–  
362 2221.
- 363 21. **Vauterin L, Vauterin P.** 1992. Computer-aided objective comparison of electrophoresis  
364 patterns for grouping and identification of microorganisms. *Eur J ClinMicrobiol* **1**:37–41.
- 365 22. **Bautista-Gallego J, Alessandria V, Fontana M, Bisotti S, Taricco S, Dolci P, Cocolin L,**  
366 **Rantsiou K.** 2014. Diversity and functional characterization of *Lactobacillus* spp. isolated  
367 throughout the ripening of a hard cheese. *Int J Food Microbiol* **181**:60–6.
- 368 23. **Higuchi R, Fockler C, Dollinger G, Watson R.** 1993. Kinetic PCR Analysis: real-time  
369 Monitoring of DNA Amplification Reactions. *Bio/Technology* **11**:1026–1030.
- 370 24. **Ercolini D, De Filippis F, La Storia A, Iacono M.** 2012. “Remake” by high-throughput  
371 sequencing of the microbiota involved in the production of water buffalo mozzarella cheese.  
372 *Appl Environ Microbiol* **78**:8142–5.
- 373 25. **Caporaso JG, Lauber CL, Walters WA, Berg-lyons D, Lozupone CA, Turnbaugh PJ,**  
374 **Fierer N, Knight R.** 2011. Global patterns of 16S rRNA diversity at a depth of millions of  
375 sequences per sample. *PNAS* **108**:4516–4522.
- 376 26. **Greppi A, Ferrocino I, La Storia A, Rantsiou K, Ercolini D, Cocolin L.** 2015. Monitoring  
377 of the microbiota of fermented sausages by culture independent rRNA-based approaches. *Int*  
378 *J Food Microbiol* **212**:67–75.
- 379 27. **Edgar RC.** 2010. Search and clustering orders of magnitude faster than BLAST.  
380 *Bioinformatics* **26**:2460–2461.
- 381 28. **McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen**

- 382 **GL, Knight R, Hugenholtz P.** 2012. An improved Greengenes taxonomy with explicit ranks  
383 for ecological and evolutionary analyses of bacteria and archaea. *ISME J* **6**:610–8.
- 384 29. **Dixon P.** 2003. VEGAN, a package of R functions for community ecology. *J Veg Sci*  
385 **14**:927–930.
- 386 30. **Friendly M.** 2002. Corrgrams. *Am Stat* **56**:316–324.
- 387 31. **Ercolini D, Moschetti G, Blaiotta G, Coppola S.** 2001. The potential of a polyphasic PCR-  
388 DGGE approach in evaluating microbial diversity of natural whey cultures for water-buffalo  
389 Mozzarella cheese production: bias of culture-dependent and culture-independent analyses.  
390 *Syst Appl Microbiol* **24**:610–7.
- 391 32. **Zambonelli C, Chiavari C, Benevelli M, Coloretto F.** 2002. Effects of lactic acid bacteria  
392 autolysis on sensorial characteristics of fermented foods. *Food Technol Biotechnol* **40**:347–  
393 351.
- 394 33. **Sgarbi E, Bottari B, Gatti M, Neviani E.** 2014. Investigation of the ability of dairy  
395 nonstarter lactic acid bacteria to grow using cell lysates of other lactic acid bacteria as the  
396 exclusive source of nutrients. *Int J Dairy Technol* **67**:342–347.
- 397 34. **Dolci P, De Filippis F, La Stora A, Ercolini D, Cocolin L.** 2014. rRNA-based monitoring  
398 of the microbiota involved in Fontina PDO cheese production in relation to different stages  
399 of cow lactation. *Int J Food Microbiol* **185**:127–35.
- 400 35. **Ruggirello M, Dolci P, Cocolin L.** 2014. Detection and viability of *Lactococcus lactis*  
401 throughout cheese ripening. *PLoS One* **9**:e114280.
- 402 36. **Stellato G, De Filippis F, La Stora A, Ercolini D.** 2015. Coexistence of lactic acid bacteria  
403 and potential spoilage microbiota in a dairy-processing environment. *Appl Environ*  
404 *Microbiol* AEM.02294–15.
- 405 37. **Meile L, Le Blay G, Thierry A.** 2008. Safety assessment of dairy microorganisms:  
406 *Propionibacterium* and *Bifidobacterium*. *Int J Food Microbiol* **126**:316–20.
- 407 38. **Thierry A, Maillard M-B, Bonnarme P, Roussel E.** 2005. The addition of

- 408 *Propionibacterium freudenreichii* to Raclette cheese induces biochemical changes and  
409 enhances flavor development. J Agric Food Chem **53**:4157–65.
- 410 39. **Ercolini D, Russo F, Ferrocino I, Villani F.** 2009. Molecular identification of mesophilic  
411 and psychrotrophic bacteria from raw cow's milk. Food Microbiol **26**:228–231.
- 412 40. **Giannino ML, Aliprandi M, Feligini M, Vanoni L, Brasca M, Fracchetti F.** 2009. A  
413 DNA array based assay for the characterization of microbial community in raw milk. J  
414 Microbiol Methods **78**:181–188.
- 415 41. **Masoud W, Takamiya M, Vogensen FK, Lillevang S, Al-Soud WA, Sørensen SJ,**  
416 **Jakobsen M.** 2011. Characterization of bacterial populations in Danish raw milk cheeses  
417 made with different starter cultures by denaturing gradient gel electrophoresis and  
418 pyrosequencing. Int Dairy J **21**:142–148.
- 419 42. **O'Sullivan DJ, Cotter PD, O'Sullivan O, Giblin L, McSweeney PLH, Sheehan JJ.** 2015.  
420 Temporal and spatial differences in microbial composition during the manufacture of a  
421 continental-type cheese. Appl Environ Microbiol **81**:2525–2533.
- 422 43. **Gargouri A, Hamed H, Elfeki A.** 2013. Analysis of raw milk quality at reception and  
423 during cold storage: combined effects of somatic cell counts and psychrotrophic bacteria on  
424 lipolysis. J Food Sci **78**:M1405–11.

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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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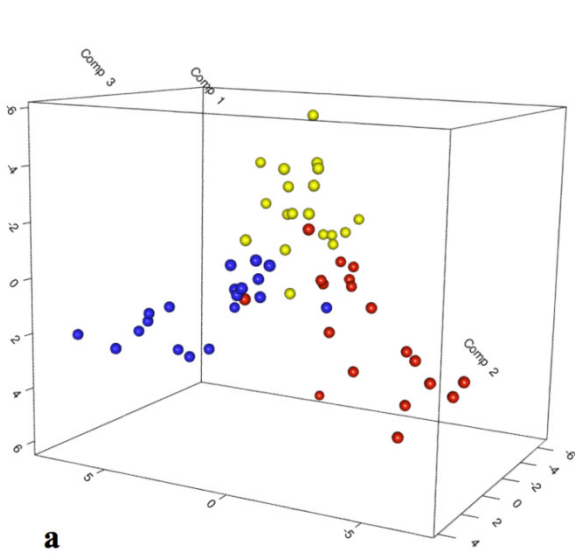
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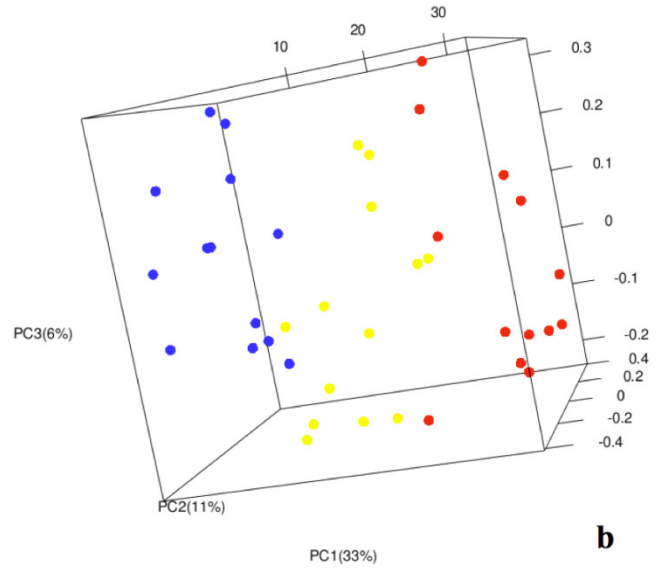
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445 **FIG 1**

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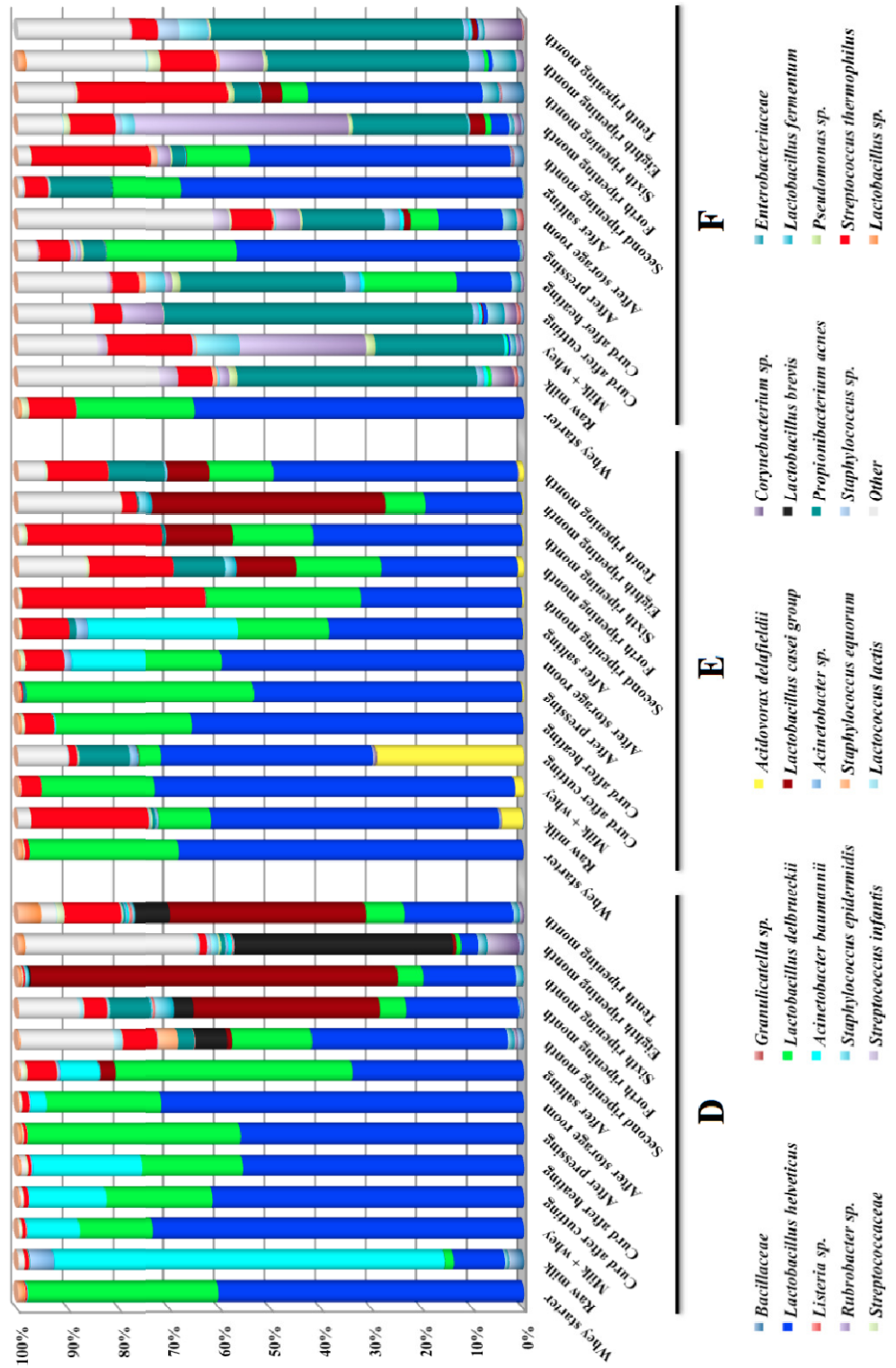
465 **FIG 2**

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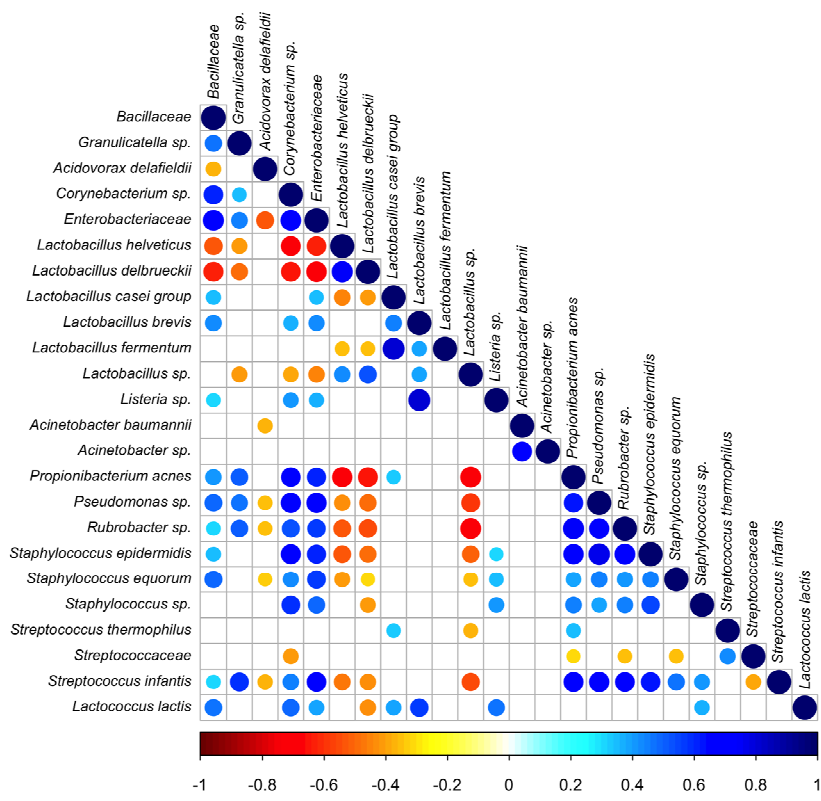
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491 **FIG 3**

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