Microbiota of an Italian Grana-Like Cheese during Manufacture and Ripening Unraveled by 16S rRNA-Based Approaches

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2 **16S rRNA-based approaches**

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

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ABSTRACT

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.

IMPORTANCE

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

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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

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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, beside 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

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97   microbiota, allowing for a greater understanding of microbial community structure and functionality

98   (17, 18).

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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 46ºC, 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 º 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

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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

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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 μl 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 μl 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/) to get

173 the species level as best hit. Statistics and plotting was carried out in R environment (www.r-

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174 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).

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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.*

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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

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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 (g 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
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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.*

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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.

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REFERENCES

1. **Giraffa G**, **Mucchetti G**, **Addeo F**, **Neviani E**. 1997. Evolution of lactic acid microflora during Grana cheesemaking and ripening. Microbiol Alim Nutr **15**:115-122.

2. **Neviani E**, **Bottari B**, **Lazzi C**, **Gatti M**. 2013. New developments in the study of the microbiota of raw-milk, long-ripened cheeses by molecular methods: the case of Grana Padano and Parmigiano Reggiano. Front Microbiol **4**:36.

3. **Lazzi C**, **Rossetti L**, **Zago M**, **Neviani E**, **Giraffa G**. 2004. Evaluation of bacterial communities belonging to natural whey starters for Grana Padano cheese by length heterogeneity-PCR. J Appl Microbiol **96**:481–490.

4. **Rossi F**, **Gatto V**, **Sabattini G**, **Torriani S**. 2012. An assessment of factors characterising the microbiology of Grana Trentino cheese, a Grana-type cheese. Int J Dairy Technol **65**:401–409.

5. **Montel M-C**, **Buchin S**, **Mallet A**, **Delbes-Paus C**, **Vuitton D a**, **Desmasures N**, **Berthier F**. 2014. Traditional cheeses: rich and diverse microbiota with associated benefits. Int J Food Microbiol **177**:136–154.

6. **Holzapfel WH**. 2002. Appropriate starter culture technologies for small-scale fermentation in developing countries. Int J Food Microbiol **75**:197–212.

7. **Gatti M**, **Bottari B**, **Lazzi C**, **Neviani E**, **Mucchetti G**. 2014. Invited review: microbial evolution in raw-milk, long-ripened cheeses produced using undefined natural whey starters. J Dairy Sci **97**:573–91.

8. **Quigley L**, **O'Sullivan O**, **Stanton C**, **Beresford TP**, **Ross RP**, **Fitzgerald GF**, **Cotter PD**. 2013. The complex microbiota of raw milk. FEMS Microbiol Rev **37**:664–98.

9. **Settanni L**, **Moschetti G**. 2010. Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. Food Microbiol **27**:691–7.

10. **Coppola S, Blaiotta G, Ercolini D**. 2007. Dairy products, p 31-90. In Cocolin L, Ercolini D. (ed), Molecular techniques in the microbial ecology of fermented foods. Springer, USA.

13

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.

14

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**

      15

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**, **Coloretti 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 Storia 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 Storia 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

16

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 denaturating 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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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.

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445 **FIG 1**

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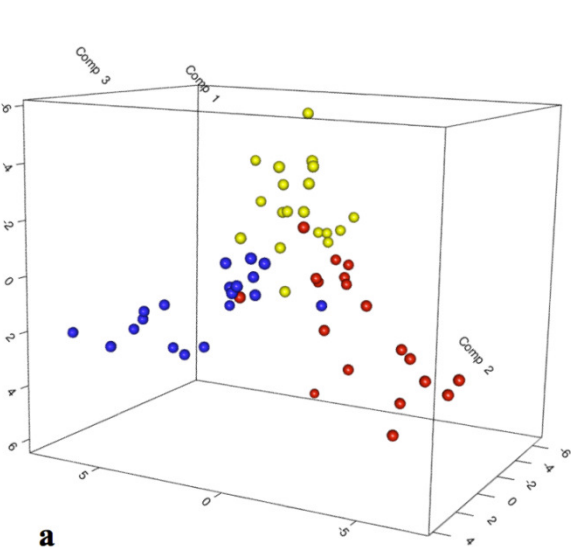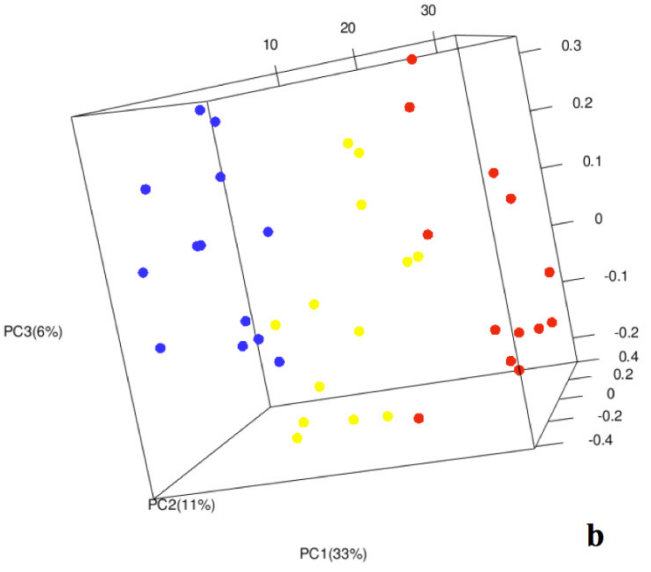
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FIG 2

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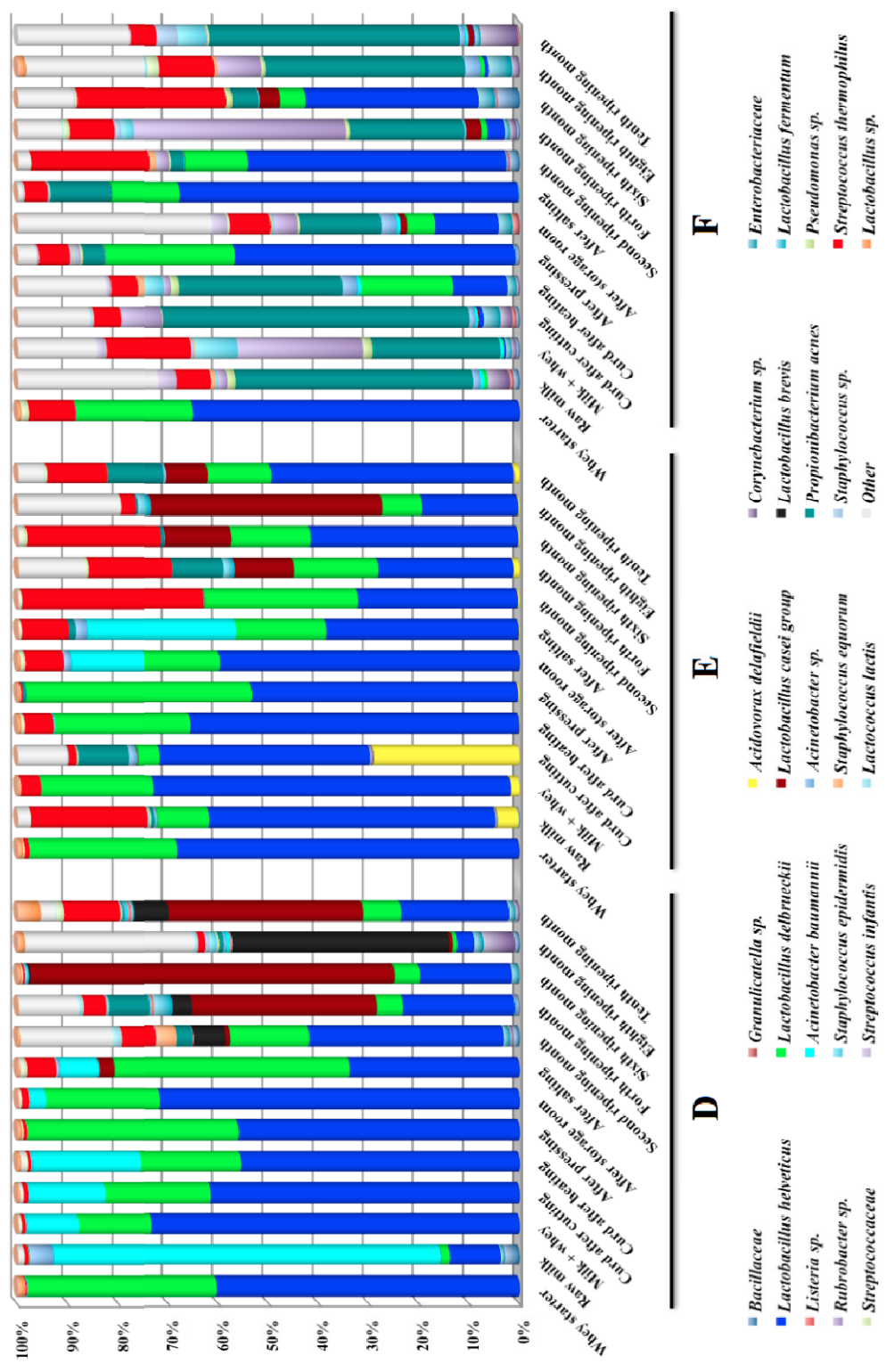
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491   **FIG 3**

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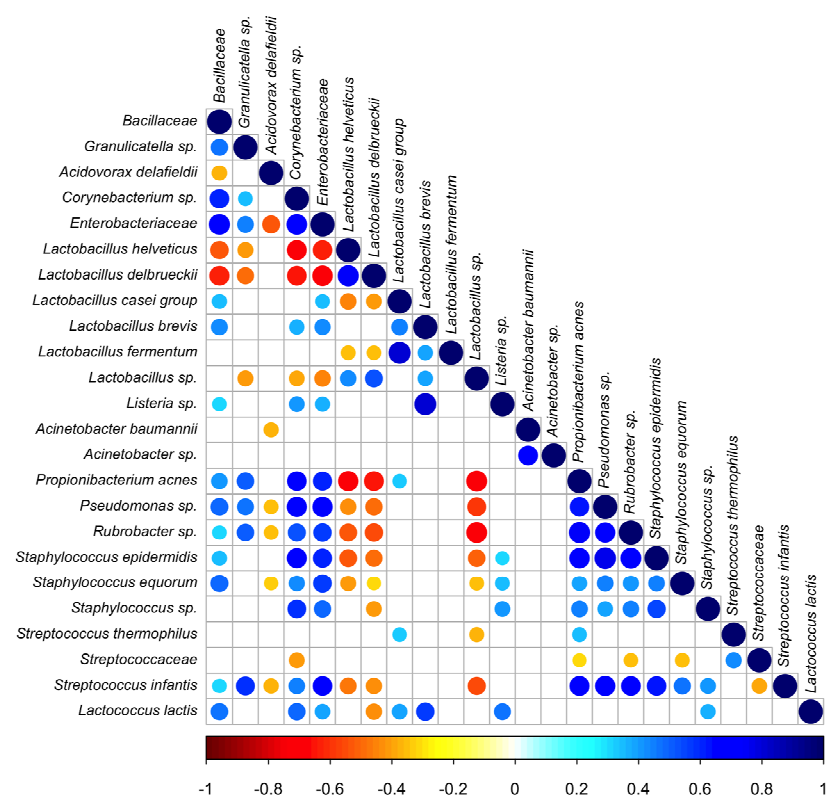
TABLE 1 Results of the total active bacteria counts obtained by qPCR

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

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 fter 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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510   * Below quantification limit

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512 **TABLE 2** Results of the identification of selected RT-PCR-DGGE band sequencing.

			Production D*	Production E	Production F
		Sampling points	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Closest relative §	% identity	Accession number			
Lactobacillus helveticus, Lactobacillus crispatus	100%	CP002427			
Lactobacillus delbrueckii subsp. *bulgaricus*	98%	HG423863			
Streptococcus thermophilus	100%	CP000419			
Bacillus subtilis	100%	KF303131			
Lactobacillus acidophilus	99%	NR_075048			
Acinetobacter baumannii	99%	KC253268			
Lactobacillus rhamnosus	100%	NR_102778			
Klebsiella sp.	100%	AB267070			
Acinetobacter haemolyticus	100%	KC178880			
Uncultured *Propionibacterium*	99%	JQ962690			
Lactococcus lactis	98%	HF677502			
Paracoccus aminophilus	100%	NC_022042			
Lactobacillus casei	99%	AY773945			
Pseudomonas sp.	100%	KF511637			
Methylobacterium radiotolerans	100%	NR_074244			
Streptococcus sanguinis	99%	KC628757			

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514 § 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
515 the DGGE profiles. The sequences obtained were aligned with those in GenBank with Blast program

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517 *Sample descriptions are reported in Table 1

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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.

		Batch														
		D					F					E				
Sample number	Sample Description	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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524 *Estimate sample coverage

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