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## Characterization of microbiota in Plaisentif cheese by high-throughput sequencing

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1 **Characterization of microbiota in Plaisentif cheese by high-throughput sequencing.**

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16 **Key words:** Italian historical cheese, High-throughput sequencing, microbiota

17

18 **Abbreviations:** HTS: high-throughput sequencing; LAB: Lactic Acid Bacteria; NSLAB: non-  
19 starter LAB; ACE: abundance-based coverage estimator; **OTU**: operational taxonomic unit.

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21

22 **Abstract**

23 High-throughput DNA sequencing (HTS) was used in this study to investigate the microbiota of  
24 Plaisentif production, an artisanal antique cheese fabricated in the Italian Alps during the violet's  
25 blooming season. The dynamics of the microbiota was described in four production points for nine  
26 different producers. The bacteria present in all samples correspond to four phyla: Proteobacteria,  
27 Firmicutes, Bacteroidetes, and Acinetobacteria. Of these, Proteobacteria and Firmicutes were the  
28 most abundant in milk and curd whereas Firmicutes dominated in cheese samples. The results  
29 showed a higher bacterial diversity in the initial steps of cheese making (milk, curd), while the final  
30 product presented a lower number of genera mainly represented by lactic acid bacteria. In ripened  
31 cheeses, core bacterial community was composed by the genera Lactococcus, Lactobacillus and  
32 Streptococcus. Although most of the reads from the final ripened cheese correspond to few LAB, it  
33 is still possible to observe some variability between the producers. The HTS **revealed** that some  
34 producers used starters, even if it is not considered by the Plaisentif production's technical policy.  
35 The obtained results highlight the great potential of the HTS methodologies in the dairy industry not  
36 only from the scientific point of view but also from practical approach.

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47 **1. INTRODUCTION**

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49 The ancient Italian dairy tradition is expressed in a wide variety of cheeses strongly related to their  
50 place of origin. Besides the numerous protected designation of origin (PDO) Italian cheeses, there  
51 are also the so-called “historical cheeses”. They all present common features in their fabrication  
52 such as the existence of several small manufacturers in a confined region, a highly variable  
53 production and a limited number of final forms. These characteristics confer the cheeses the role of  
54 niche products.

55 Plaisentif is an Italian historical cheese. It is a hand-made semi-hard cheese, typical from the  
56 Piedmont valleys in the Northwest part of the country. Plaisentif has been produced since the 500’s;  
57 its main particularity is that the milk used to make it is obtained from cows that graze in the  
58 mountains, at an altitude higher than 1800 meters, only during the violet’s blooming period (June -  
59 July). Because of these, Plaisentif is known as the “antique violet cheese”. It is considered as a  
60 niche product, with no PDO or PGI status.

61 Technical policies establish that this cheese should be produced from full-fat raw milk. Fresh  
62 morning milk is mixed with milk of the previous evening (kept at less than 10 °C) and warmed to  
63 33-36°C. Bovine liquid rennet is added maintaining the temperature; the clotting time is one hour.

64 The curd is cut into 5–10 mm particles, collected and placed in molds without being pressed. The  
65 cheese is then salted in brine for approximately 12 hours; dry salting is also done. Finally Plaisentif  
66 is ripened in cellars at 6–10°C with 85% relative humidity for 80 days. At the end of the aging  
67 period the ideal resulting forms are branded in one of the faces.

68 Since the addition of starters is not considered during the Plaisentif manufacturing, the  
69 environmental factors and the milk’s microbial population play a fundamental role in the  
70 characterization of the product. So it becomes interesting to follow the dynamics of bacterial  
71 population from the raw material to the final product.

72 In the past recent years several attempts have been done to characterize the microbial population of  
73 milk and cheese. The identification of microbial species in cheese has been traditionally  
74 determined, as in many other food matrixes, using culture-dependent methods. However, it is well  
75 known that these methodologies are not optimal to survey microbial communities in complex  
76 matrixes, such as cheese and its ripening process.

77 In contrast, culture-independent methods lean on the bacterial genetic material and its analysis.  
78 Since these methods allow a broader examination in short periods of time, they represent ultimate  
79 tools for the detailed study of microbial communities in food matrixes. The PCR-denaturing  
80 gradient gel electrophoresis (PCR-DGGE) (Myers, Maniatis, & Lerman, 1987) and PCR-temporal  
81 temperature gradient gel electrophoresis (PCR-TTGE) (Yoshino, Nishigaki, & Husimi, 1991) are  
82 the most commonly used culture-independent methods to study the microbiota of dairy products  
83 (Alegría et al., 2009; Delgado et al., 2013; Dolci, Alessandria, Rantsiou, Bertolino, & Cocolin,  
84 2010). However there are still some limitations regarding the resolution of these tools, since  
85 different genotypes can derive in similar patterns of migration (Delbes, Ali-Mandjee, & Montel,  
86 2007; El-Baradei, Delacroix-Buchet, & Ogier, 2007; Feurer, Vallaes, Corrieu, & Irlinger, 2004;  
87 Ogier et al., 2004) and are not able to distinguish less-common amplified sequences from the  
88 background noise of the test (Callon, Delbes, Duthoit, & Montel, 2006; Feurer et al., 2004). These  
89 particular problems are enlarged in the analysis of complex matrixes where the diversity of the  
90 microbial communities is considerable.

91 In the last few years the high-throughput DNA sequencing (HTS) technologies and its fast  
92 development have allowed a deeper and precise evaluation of the microbiota of complex matrixes.  
93 With the potential of producing millions of sequence reads in a single run, HTS has revolutionized  
94 the ecological microbial field. It has enabled the accurate identification of microorganisms present  
95 in several contrasting ecosystems (exemplified in Claesson et al., 2009; Roesch et al., 2007; Sogin  
96 et al., 2006) and in food matrixes (as examples see Lusk et al., 2012; Masoud et al., 2011; Roh et

97 al., 2010). This approach has allowed a more detailed perception of the structure and dynamics of  
98 the microbial population in food, overcoming the default limitations of culture-dependent methods.  
99 The main objective of this study was the characterization of the Plaisentif cheese microbiota using a  
100 HTS approach. Since the characteristics of a particular cheese depend mainly on the dynamics of  
101 the microbiota present in it, this study describes the bacteria in cheese as well as in various steps  
102 along its manufacturing and maturation process in order to understand temporal microbiota  
103 changes.

104

## 105 **2. MATERIALS AND METHODS**

106

### 107 **2.1 Sampling and DNA extraction**

108 Samples from nine traditional Plaisentif producers of the Piedmont region were collected. A total of  
109 36 samples, including raw milks (n=9), curds (n=9), 10-day ripened cheeses (n=9) and 80-day  
110 ripened cheeses (n=9) from each producer, were studied.

111 Milk and curd samples were transported to the laboratory immediately after sampling in cooled  
112 conditions, and stored at -20°C until DNA extraction. After 10 and 80 days of ripening, cheese  
113 forms were transported to the laboratory, maintained at 4°C and manipulated in aseptic conditions.  
114 Cheese samples were obtained from the most inner edible part of the forms and stored at -20°C until  
115 DNA extraction.

116 Milk samples (1 ml) were centrifuged at 12,000xg for 30 minutes. The pellets were rinsed in 500 µl  
117 of PBS, centrifuged at 12,000xg for 15 min and finally resuspended in 200 µl of lysis buffer and  
118 proteinase of Dneasy Blood & Tissue kit (Qiagen) (Dalmaso, Civera, La Neve, & Bottero, 2011).  
119 DNA was extracted following the manufacturer's protocol.

120 This same kit was used for the samples of curd, 10-day ripened cheese and 80-day ripened cheese,  
121 but with slight modifications to the provider's protocol: 400 mg of initial sample material and  
122 elution in 50 µl of the corresponding buffer.

123 In order to minimize the bias associated with single extractions, multiple extractions of each of the  
124 36 samples was done and mixed in a final pool.

125 For the lyophilized commercial starters, a total of 0,5 grams were resuspended in 5 ml of sterile BHI  
126 broad culture media (OXOID LTD, Basingstoke, Hampshire, England) and incubated at 37 °C for  
127 24 hours. The DNA of the starters was extracted from one ml of broad, following the Dneasy Blood  
128 & Tissue kit (Qiagen) protocol for Gram positive bacteria.

129 The quantity of DNA extracted was assessed using the Nanodrop 2000 (Thermo Fisher Scientific).

130

## 131 **2.2 High-throughput sequencing and bioinformatic analyses**

132 Illumina libraries were prepared following the method described by Caporaso et al. (2010) using the  
133 NEXTflex 16S V4 Amplicon-Seq Kit (Bioo Scientific, Austin, USA). Briefly, from 50 ng of DNA  
134 template for each sample, the bacterial V4 region of the 16S ribosomal gene was amplified using  
135 the universal primers 515F and 806R tailed with Illumina barcoded adapters (Caporaso et al., 2012)  
136 at the following touchdown PCR conditions: 9 cycles x (15 sec. at 95°C – 15 sec. at 68°C – 30 sec.  
137 at 72°C) and then 23 cycles x (15 sec. at 95°C – 15 sec. at 58°C – 30 sec. at 72°C).

138 PCR products were purified using the Agencourt XP Ampure Beads (Beckam Coulter). The quality  
139 of the final products was assessed using a Bioanalyzer 2100 (Agilent Technologies). After their  
140 quantification with Qubit (Invitrogen), the samples were pooled in equal proportions and sequenced  
141 paired-end in an Illumina MiSeq with 312 cycles (150 cycles for each paired read and 12 cycles for  
142 the barcode sequence) at the IGA Technology Services (Udine, Italy). To prevent focusing and  
143 phasing problems due to the sequencing of “low diversity” libraries such as 16S amplicons, 30%  
144 PhiX genome was spiked in the pooled library.

145 Raw reads were first filtered with the CLC genomics workbench (Qiagen) for Illumina data sets  
146 with the default parameters. Sequences were then analyzed using QIIME software, version 1.9.0  
147 (Caporaso et al., 2010). OTUs were defined by a 97% of similarity, using the uclust method  
148 (Edgar, 2010). Representative sequences were submitted to the RDPII classifier (Wang, Garrity,

149 Tiedje, & Cole, 2007) to obtain the taxonomy assignment and relative abundance of each OTU  
150 using the Greengenes 16s rDNA database v13.8 (McDonald et al., 2012).

151 Alpha diversity was evaluated through QIIME to generate rarefaction curves, Good's coverage  
152 (Good, 1953), Chao1 (Chao & Bunge, 2002) and ACE (Chao & Lee, 2015), Shannon (Shannon &  
153 Weaver, 1949) and Simpson (Simpson, 1949) diversity indices. Beta diversity was evaluated with  
154 the UniFrac method. Weighted UniFrac distance matrices and OTU tables were used to perform  
155 Adonis and Anosim statistical tests with the compare\_category.py script of QIIME to evaluate  
156 differences between matrixes and producers. Besides, the group\_significance.py script of QIIME  
157 was run to compare the OTUs frequencies across the samples.

158 DNA extracted from the commercial starters provided by the producers was sequenced using the  
159 MicroSeq 500 16S rDNA bacterial sequencing kit (Applied Biosystems). Sequences were aligned  
160 with the NCBI database.

161

### 162 **3. RESULTS and DISCUSSION**

163

164 The quality and safety of cheeses made from raw milk can be derived from the comprehension of  
165 their microbial composition. A wide extent of molecular methodologies, apart from culturing, has  
166 been used to describe the microbial diversity and its dynamics all along the cheese manufacturing  
167 and ripening process (Jany & Barbier, 2008).

168 Several studies have reported the structure and transformation of the microbiota of PDO cheeses  
169 with a high commercial interest using HTS methodologies. Traditional dairy products (Alegría,  
170 Szczesny, Mayo, Bardowski, & Kowalczyk, 2012; Ercolini, De Filippis, La Stora, & Iacono, 2012;  
171 Quigley et al., 2012), industrial cheese's manufacture (Masoud et al., 2012) kefir grains and  
172 beverages (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011; Leite et al., 2012; Nalbantoglu et al.,  
173 2014) have been analyzed under this approach.



174 The Mozzarella, Grana Padano and Fontina cheeses are among the Italian products that have  
175 already been surveyed with HTS (De Filippis, La Stora, Stellato, Gatti, & Ercolini, 2014; Dolci, De  
176 Filippis, La Stora, Ercolini, & Cocolin, 2014; Ercolini et al., 2012). However there are no previous  
177 descriptions for historical Italian cheeses. For this reason the present study characterized the  
178 microbial communities present in the manufacturing process of Plaisentif, using the HTS approach.

179

### 180 **3.1 Characteristics of sequencing data**

181 We recovered a total of 10,453,450 high-quality 16S rDNA gene sequences with an average  
182 sequence length of 252 bp. The numbers of reads for each matrix were 2,285,535 for milk samples;  
183 2,376,825 for curd; 2,971,829 for 10-days ripened cheese and 2,819,261 for 80-days ripened cheese  
184 samples (Table 1). Sampling completeness assessed by Good's coverage estimator returned values  
185 above 99% in all cases (Table 1). Rarefaction curve analysis showed a trend to level off strongly  
186 suggesting a sufficient sampling of the microbial communities. However milk samples showed a  
187 higher number of observed OTUs with an ampler range, compared to the rest of the matrixes  
188 (Figure 1). Simpson and Shannon indices revealed a higher diversity in milk. Richness estimators  
189 (Chao1 and ACE) showed a decreased tendency at the end of ripening period (Table 1).

190

### 191 **3.2 Variability of the microbial composition, from milk to the final product**

192 The microorganisms present in all the samples correspond to four phyla: *Proteobacteria*,  
193 *Firmicutes*, *Bacteroidetes*, and *Acinetobacteria* (Table S1 in the supplementary material). These  
194 results are consistent with the phyla present in milk (Quigley, O'Sullivan, et al., 2013), Danish raw  
195 milk cheese (Masoud et al., 2011), short-timed ripened cheese, and other artisanal products (Alegría  
196 et al., 2012; Fuka et al., 2013; Quigley et al., 2012; Riquelme et al., 2015).

197 Of these, *Proteobacteria* and *Firmicutes* were abundantly present in both milk and curd samples;  
198 whereas in cheese samples *Firmicutes* were mainly observed (Table S1 in the supplementary

199 material). The statistical analyses, Adonis and Anosim, showed that the samples varied significantly  
200 ( $P < 0.001$ ) from one matrix to another.

201 In this study the use of the V4 region of the 16S rDNA allowed the bacterial identification at the  
202 genus level. This taxonomical resolution might be insufficient for those genera that comprise  
203 pathogenic species (*Staphylococcus*, *Enterococcus*, *Streptococcus*, *Acinetobacter*). This argument is  
204 also valid for the lactic acid bacteria (LAB) genera where some species are well-known starters  
205 strains and others participate on the flavour and organoleptic characteristics of the final product.  
206 Still the identification at genus level provides a general and informative insight into the bacterial  
207 population present in the studied matrixes.

208 A total of 6 genera (*Acinetobacter*, *Chryseobacterium*, *Enhydrobacter*, *Lactococcus*, *Streptococcus*,  
209 and *Sphingomonas*) were found to constitute the largest group present in milk samples.

210 *Lactococcus* spp. and *Streptococcus* spp. comprise some LAB species commonly present in dairy  
211 products (*Lactococcus lactis*, *Streptococcus thermophilus* respectively). Some *Acinetobacter* spp.  
212 and *Chryseobacterium* spp. have also been found in bovine raw milk (Quigley, O'Sullivan, et al.,  
213 2013). The rest of the genera are frequently associated to environmental microbial sources specially  
214 water and dairy equipment (Quigley, O'Sullivan, et al., 2013).

215 In addition, *Pseudomonas*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Psychrobacter* and  
216 *Staphylococcus* genera were also identified in milk (Table S1 in the supplementary material). Some  
217 species of the LAB genera *Enterococcus*, *Leuconostoc* and *Lactobacillus* are implicated in the  
218 maturation and flavor development of dairy products. Some species of the *Staphylococcus* genus  
219 even if it is not a LAB, also participates in these traits. Figure 2a shows the relative abundance of  
220 the most prevalent genera in the analyzed milk samples.

221 In the curd the percentages of *Lactococcus* spp. and *Streptococcus* spp. increase, although the  
222 diversity observed in milk was still visible (Figure 2b). However this diversity began to flatten out  
223 after 10 days of ripening. It is possible to observe in Figure 2c how *Lactococcus* spp. and  
224 *Streptococcus* spp. predominated among the others.

225 In the final product, after 80 days of ripening, almost all of the reads correspond to LAB. The  
226 genera *Acinetobacter* and *Enhydrobacter* present in milk and curd were significantly reduced  
227 (Figure 2d). They are commonly recognized as environmental contaminants and cause of milk  
228 spoilage due to their proteolytic and lipolytic activities (Hantsis-Zacharov & Halpern, 2007; Montel  
229 et al., 2014). Some members of the *Acinetobacter* genus, including *A. baumannii*, are considered  
230 opportunistic pathogens. Since the genus *Enhydrobacter* includes until now only one species *E.*  
231 *aerosaccus* we can intuit that the reads correspond to this bacteria.

### 232

### 233 3.3 Identification of core bacterial community members in Plaisentif

234 The microbial composition of Plaisentif – the final product – was dominated by LAB, as expected  
235 to be for a raw milk ripened cheese. According to the abundance of reads it was possible to  
236 differentiate the microbial population in three main categories. *Lactococcus*, *Lactobacillus* and  
237 *Streptococcus* compose the dominant genera group. It is interesting to note that in all samples the  
238 relative abundance of at least one of these genera is present between 40% and more than 90% of the  
239 respective total reads (Fig. 2d, Table S1 in the supplementary material). Some species of these three  
240 genera contribute to the acidification of the curd and casein proteolysis. The metabolism of amino  
241 acids and fatty acids by these LAB are major contributions for the flavor development  
242 (McSweeney, 2004; Randazzo, Vaughan, & Caggia, 2006; Skelin et al., 2012). Plaisentif's  
243 dominant genera exactly correspond to those found previously in raw milk ripened cheeses  
244 (Masoud et al., 2011). While some differences are present compared with other studies. These  
245 dissimilarities might be due to the different types of milk (Fuka et al., 2013) and to the shorter  
246 ripening period of the cheeses (Quigley et al., 2012; Riquelme et al., 2015).

247 The second group contained sub-dominant genera corresponding to frequently encountered ones  
248 (1%-0.01% of the total reads of each samples): *Leuconostoc*, *Enterococcus*, *Acinetobacter*,  
249 *Chryseobacterium*, *Staphylococcus*, *Enhydrobacter*, *Sphingomonas*, *Bacillus*, *Corynebacterium*,

250 *Pseudomonas*. From these, members of the *Leuconostoc* spp. and *Enterococcus* spp. are known for  
251 their role in the flavor and texture development of cheese (Montel et al., 2014).

252 The third group consisted of rare sequences which were detected occasionally (comprising 0.01% -  
253 0.0001% of the total reads of each samples): *Granulicatella*, *Brevibacterium*, *Salinicoccus*,  
254 *Vagococcus*, *Anaerobacillus*, *Sphingobacterium*, *Klebsiella*, *Carnobacterium*, *Pediococcus*,  
255 *Brachy bacterium*, *Morganella* *Erwinia*, *Psychrobacter*, *Ralstonia*, *Veillonella*, *Cloacibacterium*,  
256 *Actinomyces*, *Flavobacterium*, *Capnocytophaga* genera. Some authors suggest that the rare  
257 biosphere can importantly influence the organoleptic characteristics of traditional products (Pedrós-  
258 Alió, 2007; Sogin et al., 2006). It is interesting to note that the rare biosphere of Plaisentif  
259 comprises also non-starter LAB (NSLAB) (*Carnobacterium* spp., *Pediococcus* spp., *Vagococcus*  
260 spp.) that encompass some species that are often abundant in almost all cheese varieties, whether  
261 traditional or not (Delcenserie et al., 2014; Montel et al., 2014).

262 Members of the Enterobacteriaceae family (*Klebsiella* spp., *Morganella* spp., *Erwinia* spp.) were  
263 also present as it was previously observed in other raw milk artisanal cheeses. In general, their  
264 presence indicates poor hygienic conditions during the manufacture process. However the number  
265 of reads present in all the Plaisentif samples are scarce. Sequences for major foodborne pathogens  
266 were not found. These observations strongly suggest microbial safety of the final products were  
267 satisfactory despite their precarious production conditions in the mountains.

268

### 269 3.4 Comparison between cheese producers

270 Figure 3 shows the most abundant genus for each of the nine Plaisentif makers in the different  
271 stages of production. It can be observed that five producers (A, B, C, D, and E) presented a high  
272 number of *Streptococcus* spp. reads and lower percentage for other LAB (*Lactococcus* spp. and  
273 *Lactobacillus* spp.).

274 Looking more into detail the evolution of the microbial communities of each producer, it can be  
275 observed that 4 of them presented a significant increase in the number of *Streptococcus* spp. reads

276 from milk to curd, remaining high until the end of the ripening period (producers A, B, C, and D)  
277 (Figure 3). This crest profile could only be explained with the fact that **commercial starters** were  
278 added in order to standardize the production, even though it is not contemplated in the Plaisentif  
279 technical production policy. It was only after this scrutiny that the producers confirmed a recent use  
280 of the supplementation. **We asked for a sample of the starters that they used and sequenced them**  
281 **with the Sanger method, allowing the identification at the species level. The results indicated that**  
282 **the added species was *Streptococcus thermophilus* (data not shown). This result corroborates the**  
283 **NGS observed profiles for these producers.**

284 On the other hand, **producers** E and I showed a constant microbial profile since the beginning of the  
285 process mainly composed of LAB (*Streptococcus* spp. and *Lactococcus* spp. respectively). **It is**  
286 **important to mention that these two producers denied the use of starters. So this observation might**  
287 **have different explanations. There are** reports where a considerable abundance of these two genera  
288 was already present in raw milk (Quigley, McCarthy, et al., 2013; Quigley, O'Sullivan, et al.,  
289 2013). Another possible **reason**, considering the results of the previous producers, could be the use  
290 of starters as a remote habitual practice, masking the crest profile. The colonization of the working  
291 environment, surfaces and dairy equipments by LAB facility resident strains could also explain this  
292 observation (Bokulich & Mills, 2013; Montel et al., 2014).

293 Producers F, G and H presented a higher **diversity** of the microbial communities in all the analyzed  
294 matrixes, probably as a consequence of traditional dairy practices, following the technical  
295 production policy of Plaisentif. It can be presumed that the organoleptic properties present in the  
296 cheese of these producers are the result of the microbial population present in milk and the  
297 environmental factors.

298 It is also interesting to note that the curds of producers G and H had an increase in the genus  
299 *Acinetobacter* compared to the number of reads present in the other matrixes. Since this genus is  
300 commonly found in soil and water (Quigley, O'Sullivan, et al., 2013), the raise in the number of

301 reads could be associated with contaminants of the boiler or tools used in the early stages of  
302 processing (knife, saber, harp and molds).

303 In conclusion HTS technology has allowed the characterization of the microbiota present in  
304 Plaisentif cheese, as an alternative approach to traditional culture-independent methods. It also  
305 provided several snapshots of the intermediate steps during the cheese production, enabling to track  
306 and follow the progress of the bacterial communities from raw milk to the final ripened cheese. The  
307 composition of Plaisentif's core confirmed the scarce standardization of niche products, a sign of  
308 artisanal production.

309 Lastly, the study of the dynamics of bacteria present in different cheese-manufacturing steps  
310 allowed not only the surveillance of the process but also revealed unexpectedly practices that were  
311 not considered in the production's policy, such as the starter addition.

312 The obtained results underline the considerable potential of HTS in the dairy industry not only from  
313 the scientific approach but also as a potential tool for the surveillance of good practices in the  
314 production of cheese.

315

316

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325

326

327 **References**

- 328 Alegría, Á., Álvarez-Martín, P., Sacristán, N., Fernández, E., Delgado, S., & Mayo, B. (2009).  
329 Diversity and evolution of the microbial populations during manufacture and ripening of  
330 Casín, a traditional Spanish, starter-free cheese made from cow's milk. *International*  
331 *Journal of Food Microbiology*, 136, 44–51.
- 332 Alegría, Á., Szczesny, P., Mayo, B., Bardowski, J., & Kowalczyk, M. (2012). Biodiversity in  
333 Oscypek, a traditional Polish cheese, determined by culture-dependent and-independent  
334 approaches. *Applied and Environmental Microbiology*, 78, 1890–1898.
- 335 Bokulich, N. A., & Mills, D. A. (2013). Facility-specific “house” microbiome drives microbial  
336 landscapes of artisan cheesemaking plants. *Applied and Environmental Microbiology*, 79,  
337 5214–5223.
- 338 Callon, C., Delbes, C., Duthoit, F., & Montel, M. C. (2006). Application of SSCP-PCR  
339 fingerprinting to profile the yeast community in raw milk Salers cheeses. *Systematic and*  
340 *Applied Microbiology*, 29, 172–180.
- 341 Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al.  
342 (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature*  
343 *Methods*, 7, 335–336.
- 344 Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012).  
345 Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq  
346 platforms. *ISME Journal*, 6, 1621–1624.
- 347 Chao, A., & Bunge, J. (2002). Estimating the number of species in a stochastic abundance model.  
348 *Biometrics*, 58, 531–539.
- 349 Chao, A., & Lee, S.M. (2015). Estimating the number of classes via sample coverage. *Journal of*  
350 *the American Statistical Association*, 87, 210–217.

351 Claesson, M. J., O’Sullivan, O., Wang, Q., Nikkila, J., Marchesi, J. R., Smidt, H., et al. (2009).  
352 Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring  
353 microbial community structures in the human distal intestine. *PLoS ONE*, *4*, e6669.

354 Dalmaso, A., Civera, T., La Neve, F., & Bottero, M. T. (2011). Simultaneous detection of cow and  
355 buffalo milk in mozzarella cheese by Real-Time PCR assay. *Food Chemistry*, *124*, 362–366.

356 De Filippis, F., La Stora, A., Stellato, G., Gatti, M., & Ercolini, D. (2014). A selected core  
357 microbiome drives the early stages of three popular Italian cheese manufactures. *PLoS ONE*,  
358 *9*, e89860.

359 Delbes, C., Ali-Mandjee, L., & Montel, M. C. (2007). Monitoring bacterial communities in raw  
360 milk and cheese by culture-dependent and -independent 16S rRNA gene-based analyses.  
361 *Applied and Environmental Microbiology*, *73*, 1882–1891.

362 Delcenserie, V., Taminiau, B., Delhalle, L., Nezer, C., Doyen, P., Crevecoeur, S., et al. (2014).  
363 Microbiota characterization of a Belgian protected designation of origin cheese, Herve  
364 cheese, using metagenomic analysis. *Journal of Dairy Science*, *97*, 6046–6056.

365 Delgado, S., Rachid, C. T. C. C., Fernández, E., Rychlik, T., Alegría, Á., Peixoto, R. S., et al.  
366 (2013). Diversity of thermophilic bacteria in raw, pasteurized and selectively-cultured milk,  
367 as assessed by culturing, PCR-DGGE and pyrosequencing. *Food Microbiology*, *36*, 103–  
368 111.

369 Dobson, A., O’Sullivan, O., Cotter, P. D., Ross, P., & Hill, C. (2011). High-throughput sequence-  
370 based analysis of the bacterial composition of kefir and an associated kefir grain. *FEMS*  
371 *Microbiology Letters*, *320*, 56–62.

372 Dolci, P., Alessandria, V., Rantsiou, K., Bertolino, M., & Cocolin, L. (2010). Microbial diversity,  
373 dynamics and activity throughout manufacturing and ripening of Castelmagno PDO cheese.  
374 *International Journal of Food Microbiology*, *143*, 71–75.

375 Dolci, P., De Filippis, F., La Stora, A., Ercolini, D., & Cocolin, L. (2014). RRNA-based  
376 monitoring of the microbiota involved in Fontina PDO cheese production in relation to



377 different stages of cow lactation. *International Journal of Food Microbiology*, 185, 127–  
378 135.

379 Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*,  
380 26, 2460–2461.

381 El-Baradei, G., Delacroix-Buchet, A., & Ogier, J. C. (2007). Biodiversity of bacterial ecosystems in  
382 traditional Egyptian Domiati cheese. *Applied and Environmental Microbiology*, 73, 1248–  
383 1255.

384 Ercolini, D., De Filippis, F., La Stora, A., & Iacono, M. (2012). “Remake” by high-throughput  
385 sequencing of the microbiota involved in the production of water buffalo mozzarella cheese.  
386 *Applied and Environmental Microbiology*, 78, 8142–8145.

387 Feurer, C., Vallaes, T., Corrieu, G., & Irlinger, F. (2004). Does smearing inoculum reflect the  
388 bacterial composition of the smear at the end of the ripening of a French soft, red-smear  
389 cheese? *Journal of Dairy Science*, 87, 3189–3197.

390 Fuka, M. M., Wallisch, S., Engel, M., Welzl, G., Havranek, J., & Schloter, M. (2013). Dynamics of  
391 bacterial communities during the ripening process of different Croatian cheese types derived  
392 from raw ewe’s milk cheeses. *PLoS ONE*, 8, e80734.

393 Good, I. J. (1953). The population frequencies of species and the estimation of population  
394 parameters. *Biometrika*, 40, 237–264.

395 Hantsis-Zacharov, E., & Halpern, M. (2007). Culturable psychrotrophic bacterial communities in  
396 raw milk and their proteolytic and lipolytic traits. *Applied and Environmental Microbiology*,  
397 73, 7162–7168.

398 Jany, J. L., & Barbier, G. (2008). Culture-independent methods for identifying microbial  
399 communities in cheese. *Food Microbiology*, 25, 839–848.

400 Leite, A., Mayo, B., Rachid, C., Peixoto, R., Silva, J., Paschoalin, V., et al. (2012). Assessment of  
401 the microbial diversity of Brazilian kefir grains by PCR-DGGE and pyrosequencing  
402 analysis. *Food Microbiology*, 31, 215–221.

403 Lusk, T. S., Ottesen, A. R., White, J. R., Allard, M. W., Brown, E. W., & Kase, J. A. (2012).  
404 Characterization of microflora in Latin-style cheeses by next-generation sequencing  
405 technology. *BMC Microbiology*, *12*, 254.

406 Masoud, W., Takamiya, M., Vogensen, F. K., Lillevang, S., Al-Soud, W. A., Sørensen, S. J., et al.  
407 (2011). Characterization of bacterial populations in Danish raw milk cheeses made with  
408 different starter cultures by denaturing gradient gel electrophoresis and pyrosequencing.  
409 *International Dairy Journal*, *21*, 142–148.

410 Masoud, W., Vogensen, F. K., Lillevang, S., Abu Al-Soud, W., Sørensen, S. J., & Jakobsen, M.  
411 (2012). The fate of indigenous microbiota, starter cultures, *Escherichia coli*, *Listeria innocua*  
412 and *Staphylococcus aureus* in Danish raw milk and cheeses determined by pyrosequencing  
413 and quantitative real time (qRT)-PCR. *International Journal of Food Microbiology*, *153*,  
414 192–202.

415 McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., Desantis, T. Z., Probst, A., et al. (2012).  
416 An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary  
417 analyses of bacteria and archaea. *ISME Journal*, *6*, 610–618.

418 McSweeney, P. L. H. (2004). Biochemistry of cheese ripening: Introduction and overview. *Cheese:*  
419 *Chemistry, Physics and Microbiology*, *1*, 347–360.

420 Montel, M.C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D. A., Desmasures, N., et al.  
421 (2014). Traditional cheeses: Rich and diverse microbiota with associated benefits.  
422 *International Journal of Food Microbiology*, *177*, 136–154.

423 Myers, R. M., Maniatis, T., & Lerman, L. S. (1987). Detection and localization of single base  
424 changes by denaturing gradient gel electrophoresis. *Methods in Enzymology*, *155*, 501–527.

425 Nalbantoglu, U., Cakar, A., Dogan, H., Abaci, N., Ustek, D., Sayood, K., et al. (2014).  
426 Metagenomic analysis of the microbial community in kefir grains. *Food Microbiology*, *41*,  
427 42–51.

428 Ogier, J. C., Lafarge, V., Girard, V., Rault, A., Maladen, V., Gruss, A., et al. (2004). Molecular  
429 fingerprinting of dairy microbial ecosystems by use of temporal temperature and denaturing  
430 gradient gel electrophoresis. *Applied and Environmental Microbiology*, *70*, 5628–5643.

431 Pedrós-Alió, C. (2007). Dipping into the rare biosphere. *Science*, *315*, 192–193.

432 Quigley, L., McCarthy, R., O’Sullivan, O., Beresford, T. P., Fitzgerald, G. F., Ross, R. P., et al.  
433 (2013). The microbial content of raw and pasteurized cow milk as determined by molecular  
434 approaches. *Journal of Dairy Science*, *96*, 4928–4937.

435 Quigley, L., O’Sullivan, O., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2012).  
436 High-throughput sequencing for detection of subpopulations of bacteria not previously  
437 associated with artisanal cheeses. *Applied and Environmental Microbiology*, *78*, 5717–5723.

438 Quigley, L., O’Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P.  
439 D. (2013). The complex microbiota of raw milk. *FEMS Microbiology Reviews*, *37*, 664–698.

440 Randazzo, C. L., Vaughan, E. E., & Caggia, C. (2006). Artisanal and experimental Pecorino  
441 Siciliano cheese: Microbial dynamics during manufacture assessed by culturing and PCR-  
442 DGGE analyses. *International Journal of Food Microbiology*, *109*, 1–8.

443 Riquelme, C., Câmara, S., Enes Dapkevicius, M. L. N., Vinuesa, P., da Silva, C. C. G., Malcata, F.  
444 X., & Rego, O. A. (2015). Characterization of the bacterial biodiversity in Pico cheese (an  
445 artisanal Azorean food). *International Journal of Food Microbiology*, *192*, 86–94.

446 Roesch, L. F., Fulthorpe, R. R., Riva, A., Casella, G., Hadwin, A. K., Kent, A. D., et al. (2007).  
447 Pyrosequencing enumerates and contrasts soil microbial diversity. *ISME Journal*, *1*, 283–  
448 290.

449 Roh, S. W., Kim, K. H., Nam, Y. D., Chang, H. W., Park, E. J., & Bae, J. W. (2010). Investigation  
450 of archaeal and bacterial diversity in fermented seafood using barcoded pyrosequencing.  
451 *ISME Journal*, *4*, 1–16.

452 Shannon, C. E. & Weaver, W. (1949). A mathematical theory of communication, *AT&T Bell*  
453 *Laboratories Technical Journal*, *27*, 359–423.

454 Simpson, E. H. (1949). Measurement of diversity. *Nature*, *163*, 688.

455 Skelin, A., Fuka, M. M., Majhenič, A. Č., Redžepović, S., Samaržija, D., & Matijašić, B. B. (2012).  
456 Phenotypic and genotypic characterization of indigenous *Lactobacillus* community from  
457 traditional Istrian Ewe's cheese. *Food Technology and Biotechnology*, *50*, 362–370.

458 Sogin, M. L., Morrison, H. G., Huber, J. A., Welch, D. M., Huse, S. M., Neal, P. R., et al. (2006).  
459 Microbial diversity in the deep sea and the underexplored “rare biosphere.” *Proceedings of*  
460 *the National Academy of Sciences*, *103*, 12115–12120.

461 Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naïve Bayesian classifier for rapid  
462 assignment of rRNA sequences into the new bacterial taxonomy. *Applied and*  
463 *Environmental Microbiology*, *73*, 5261–5267.

464 Yoshino, K., Nishigaki, K., & Husimi, Y. (1991). Temperature sweep gel electrophoresis: a simple  
465 method to detect point mutations. *Nucleic Acids Research*, *19*, 3153.

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469 **Figure 1** - Rarefaction curves of microbial population from the studied matrixes.

470 **Figure 2** - Relative abundance (%) of sequences assigned to genus level from a matrix point of  
471 view: a) milk, b) curd, c) cheese 10 days and d) cheese 80 days.

472 **Figure 3** - Relative abundance (%) of sequences assigned to genus level from the producers  
473 perspective.

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476 **Tables caption**

477 **Table 1** – Numbers of sequence tags, OTUs observed, coverage and richness estimators for all  
478 studied samples

479 **Table S1** - Percentages of the most abundant taxonomical groups for nine producers in each matrix.

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