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

Abstract

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

1. INTRODUCTION

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

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

Technical policies establish that this cheese should be produced from full-fat raw milk. Fresh morning milk is mixed with milk of the previous evening (kept at less than 10 ºC) and warmed to 33-36ºC. Bovine liquid rennet is added maintaining the temperature; the clotting time is one hour. The curd is cut into 5–10 mm particles, collected and placed in molds without being pressed. The cheese is then salted in brine for approximately 12 hours; dry salting is also done. Finally Plaisentif is ripened in cellars at 6–10°C with 85% relative humidity for 80 days. At the end of the aging period the ideal resulting forms are branded in one of the faces.

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

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

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

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

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

2. MATERIALS AND METHODS

2.1 Sampling and DNA extraction

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

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

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

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

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

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

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

2.2 High-throughput sequencing and bioinformatic analyses

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

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

Raw reads were first filtered with the CLC genomics workbench (Qiagen) for Illumina data sets with the default parameters. Sequences were then analyzed using QIIME software, version 1.9.0 (Caporaso et al., 2010). OTUs were defined by a 97% of similarity, using the uclust method (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 Storia, & 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 Storia, Stellato, Gatti, & Ercolini, 2014; Dolci, De
176 Filippis, La Storia, 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.

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

3.3 Identification of core bacterial community members in Plaisentif

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

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

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

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

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

3.4 Comparison between cheese producers

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

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

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

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

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

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

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

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

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

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

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

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

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

Tables caption

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

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