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Paola Dolci[1*], Francesca De Filippis[2], Antonietta La Storia[2], Danilo Ercolini[2], Luca Cocolin[1]

[1]Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università degli Studi di Torino, via L. da Vinci 44, 10095 Grugliasco (TO), Italy

[2]Dipartimento di Agraria, Università degli Studi di Napoli Federico II, via Università 100, 80055 Portici, Italy

*corresponding author

Abstract

Fontina Protected Denomination of Origin (PDO) cheese is a full-fat semi-cooked cheese traditionally made in Northwest Italy (Aosta Valley) and manufactured from raw cow's milk. The management of cattle farms in Aosta Valley calls for seasonal migration to high pastures during the summer and the concentration of calving during the autumn and the beginning of the winter. Based on cattle physiology and given to calving seasonality, three cow lactation phases i.e. *post partum*, *oestrus* and early gestation, can be identified and an effect could be hypothesized on average milk composition and on cheese quality.

The aim of the present paper was to investigate the bacterial dynamics during Fontina PDO cheese manufacturing and ripening, in relation to the different lactation stages, in order to evaluate a possible correlation between microbiota and phase of lactation. For this purpose, microbial RNA analysis was carried out by RT-PCR coupled with DGGE and high-throughput sequencing. A good performance of the starter cultures was highlighted throughout Fontina PDO manufacturing and ripening; in fact, the starter prevailed against the autochthonous microbiota. Thus, the microbial activity, which was supposed to affect the final quality of Fontina PDO cheese, appeared to be strictly associated to the presence of the starter, which did not show any difference in its performance according to the different stages of cow lactation. Therefore, the results of this

research highlighted a negligible correlation between the microbiota of raw milk and the organolepitc quality and typicity of Fontina cheese in relation to lactation seasonality.

Keywords

Fontina PDO cheese; lactation; microbiota; RNA; RT-PCR-DGGE; pyrosequencing

1. Introduction

Fontina Protected Denomination of Origin (PDO) is a full-fat semi-cooked washed-rind cheese, that is traditionally made in Aosta Valley (Northwest Italy). Fontina PDO Regulation (revised in 2004) establishes that the cheese has to be manufactured from raw cow's milk produced from the cattle breed "Valdostana", autochthonous of the Aosta Valley region. The management of cattle farms calls for seasonal migration to high pastures, during the summer, to altitude higher than 2000 m in order to follow the vegetative cycle of grassland. Here, cattle is fed on grass, differently from the winter season when the use of hay or concentrated feed is allowed according to the regulation. The mountain pasture custom determines a concentration of calving during the autumn and the beginning of the winter. This organization of animal husbandry, common to many mountain dairy cattle farms, essentially results in three phases of lactation: *post partum* (January to February), *oestrus* (February to March) and early gestation (March to April). It is hypothesized that the different cow physiological states might have an impact on average milk composition and, consequently, on its cheese-making aptitude and on the final quality of the cheese. Remarkably, Fontina cheese manufactured from milk produced during cow *oestrus* stage, is generally known to be of minor quality, in terms of organoleptic characteristics and typicity traits, and this aspect needs appropriate investigation.

The aim of this research was to investigate the dynamics of bacterial populations during Fontina PDO cheese manufacture and ripening, and to evaluate possible correlations between microbiota and different lactation stages. In particular, the performance of the starters used for Fontina PDO production and selected from autochthonous lactic acid bacteria (LAB), was followed together with

the activity of non starter LAB (NSLAB) using a culture-independent approach. Moreover, the role of non dairy (ND) microbiota was considered in order to evaluate, eventually, their interference with starter culture activity.

Ribosomal RNA (rRNA), an already known worthwhile target for a reliable understanding of the food microbial communities (Cocolin et al., 2001; Mills et al., 2002; van Beek et al., 2002), was chosen to follow metabolically active populations from milk to curd and cheese matrices. In particular, both reverse transcription-PCR-denaturing gradient gel electrophoresis (RT-PCR-DGGE) and high-throughput sequencing techniques were performed and the results compared and complemented. RT-PCR-DGGE has been already described as a powerful tool to investigate the microbial biodiversity and activity in different dairy products (Alessandria et al., 2010; Dolci et al., 2010; Dolci et al., 2013; Masoud et al., 2011; Masoud et al., 2012; Randazzo et al., 2002; Rantsiou et al., 2008). At the same time, high-throughput sequencing is emerging as a new culture-independent tool for a quantitative investigation of the structure of microbial communities, beside being much more sensitive to detect sub-dominant populations (Ercolini, 2013). So far this technique was successfully used for an in-depth analysis of the bacterial diversity in a number of dairy foods (Algeria et al., 2012; De Filippis et al., 2014; Ercolini et al., 2012; Masoud et al., 2011; Quigley et al., 2012). In the present study, an estimation of the bacterial abundance was determined to look at the proportions between starter LAB, NSLAB and ND populations during Fontina PDO manufacturing and ripening.

2. Materials and methods

2.1 Fontina PDO manufacturing and sampling

Fontina PDO cheese production was monitored in three different dairies named, in this paper, A, B and C, and placed at altitudes varying from 600 to 1200 m in Aosta Valley. Cheese-making was followed during three different cow lactation stages, precisely, *post partum* (phase 1), *oestrus* (phase 2) and early gestation (phase 3), and, for each production, two replicates were investigated. The productions were manufactured in the middle period of each phase and the replicates were

carried out with not more of a four day interval. Totally, eighteen production were studied and the following samples were collected: raw milk before addition of starter cultures, curd after 24 hours and cheese at 7, 28, 56 and 84 days of ripening. According to the Fontina PDO Regulation, the addition in the milk of an autochthonous starter cultures is allowed. They are composed of three strains belonging to the species *Streptococcus thermophilus*, *Lactobacillus delbrueckii* and *Lactococcus lactis*, selected and stored at the Institut Agricole Régional of Aosta (Aosta Valley) and added to the milk with an initial load of 10^6 cfu/mL. After the addition of starter cultures, the milk, obtained from a single milking and treated within two hours after milking, is coagulated with calf rennet at 36°C and the curd is cut finely while the temperature is gradually raised to 46–48 °C. The curd, after a brief rest in whey, is collected in molds and pressed to eliminate any residual whey. Rounds are traditionally matured for at least 80 days in natural caves with a temperature that varies from 5 to 12°C.

2.2 Milk pH measurements and chemical determination

The pH measurements were carried out on milk, after the inoculation of the starter cultures, and on 24 h curd samples by using a pH-meter (Sial-micros pH trend 10). All analyses were performed in triplicate. Acidification curves, after starter culture inoculation, were also followed and the time required for the beginning of milk acidification process (D_t) determined as the inflection point of the curves. Milk samples were evaluated for urea content by Milko-Scan FT 6000 (Foss, Hilleroed, Denmark). T-tests were performed to compare data sets of D_t and urea concentration in relation to lactation stage and factory.

2.3 RNA analysis by RT-PCR-DGGE and cluster analysis

Metabolically active populations were followed by direct microbial RNA analysis of milk, curd and cheese samples. Sample preparation and RNA extraction were performed according to the protocol reported by Rantsiou et al. (2008). Three microliters of TURBO - DNase (Ambion, Milan, Italy) was added to digest the DNA in the RNA samples, with an incubation of 3 h at 37 ºC. The presence of residual DNA in the RNA samples was checked by PCR (Cocolin et al., 2001).

RT-PCR was performed with universal primers 338f and 518r (Ampe et al., 1999) annealing to the bacterial V3 region of the 16S rRNA gene, as described by Alessandria et al. (2010). The amplicons obtained were analysed by DGGE with a Dcode universal mutation detection system (BioRad, Milan, Italy) according to the protocol described by Dolci et al. (2008). Selected DGGE bands were extracted from the gels, checked by means of DGGE and sent for sequencing to MWG Biotech (Dolci et al., 2008). Partial 16S rRNA gene sequences were aligned with those in GenBank with the Blast program to determine the closest known relatives of the bands (Altschul et al., 1997). RT-PCR-DGGE profiles were normalized and submitted to Cluster Analysis with the BioNumerics software (Applied Maths, Kortrijk, Belgium). The Pearson product moment correlation coefficient was used to calculate the similarities in DGGE patterns, and dendrograms were obtained by the unweighted pair group method with arithmetic averages.

2.4 RNA analysis by pyrosequencing

The cDNA obtained as previously described (Alessandria et al., 2010) was used to study the microbial diversity by pyrosequencing of the amplified V1-V3 region of the 16S rRNA gene by using primers Gray28f 5'-TTTGATCNTGGCTCAG and Gray519r 5'-GTNTTACNGCGGCKGCTG amplifying a fragment of 520 bp (Ercolini et al., 2012). 454-adaptors were included in the forward primer followed by a 10 bp sample-specific Multiplex Identifier (MID). Each PCR mixture (final volume, 50 μl) contained 50 ng of template cDNA, 0.4 μM of each primer, 0.50 mmol/L of each deoxynucleoside triphosphate, 2.5 mmol/L $MgCl_2$, 5 μl of 10 X PCR buffer and 2.5 U of native *Taq* polymerase (Invitrogen, Milano, Italy). The following PCR conditions were used: 94°C for 2 min, 35 cycles of 95°C for 20 s, 56°C for 45 s and 72°C for 5 min, and a final extension at 72°C for 7 min. After agarose gel electrophoresis, PCR products were purified twice by Agencourt AMPure kit (Beckman Coulter, Milano, Italy), quantified using the QuantiFluorTM (Promega, Milano, Italy) and an equimolar pool was obtained prior to further processing. The amplicon pool was used for pyrosequencing on a GS Junior platform (454 Life Sciences, Roche, Monza, Italy) according to the manufacturer's instructions by using a Titanium chemistry.

2.5 Bioinformatics and data analysis

Raw reads were first filtered according to the 454 processing pipeline. Sequences were then analyzed and further filtered by using QIIME 1.7.0 software (Caporaso et al., 2010). In order to guarantee a higher level of accuracy in terms of Operational Taxonomic Units (OTUs) detection, after the split library script performed by QIIME, the reads were excluded from the analysis if they had an average quality score lower than 25, if they were shorter than 300 bp and if there were ambiguous base calls. Sequences that passed the quality filter were denoised (Reeder and Knight, 2010) and singletons were excluded. OTUs defined by a 97% of similarity were picked using the uclust method (Edgar, 2010) and the representative sequences were submitted to the RDPII classifier (Wang et al., 2007) to obtain the taxonomy assignment and the relative abundance of each OTU using the Greengenes 16S rRNA gene database (McDonald et al., 2012).

Alpha and beta diversity were evaluated through QIIME as recently described (De Filippis et al., 2013). Weighted UniFrac distance matrices (Lozupone et al., 2005) and OTU tables were used to perform Adonis, Anosim, and ANOVA statistical tests through the compare_category.py and the otu_category_significance.py scripts of QIIME, in order to verify the influence of the lactation phase on the microbial population and whether the abundance of any OTUs was significantly associated to a specific lactation phase. An OTU network was generated by QIIME and a bipartite graph was constructed in which each node represented either a sample or a bacterial OTU. Connections were drawn between samples and OTUs, with edge weights defined as the number of sequences from each OTU that occurred in each sample. Networks were visualized using Cytoscape 3.0.2 (Shannon et al., 2003). Sequences are available at the Sequence Read Archive PRJNA230456 of the NCBI.

2.6 Sensory analysis

In order to evaluate the sensory characteristics of the cheeses produced during the experimentation, a panel composed of nine judges trained to evaluate specifically Fontina PDO cheese and with several years of experience in its sensory evaluation was used. The cheese samples analyzed were 1.5 cm thick · 2 cm wide · 8 cm long with rinds cut away. Two portions per

sample were served: one to evaluate odor and flavor and the other to evaluate texture and appearance (Bérodier et al., 1997). Evaluations were carried out in relation to eleven attributes, which were scored on a structured scale from 0 to 10. The sensory attributes were six for odor and flavor (odor quality, odor intensity, sweetness, saltiness, overall taste sensations, aroma typicity), three for texture (elasticity, firmness and overall pleasantness) and two for appearance (color and holes), where aroma typicity and overall pleasantness parameters are considered the more distinctive and significant attributes for the sensory characterization of Fontina.

The differences in the sensory parameters, according to the different phases of lactation, were analyzed by ANOVA and Duncan test by Statistica 7.1 (StatSoft Inc., Tulsa, Oklahoma, USA).

3. Results

3.1 Acidification process and urea concentration

The pH values measured in milk samples (pH_i) before the addition of the starter cultures were ranging from 6.58 to 6.72 and reached values from 5.33 to 5.61 in the curd after 24 hours (pH_{24h}) (Tab. 1). The acidification process showed a different trend related to the different lactation phases. Precisely, the time required for the acidification process to begin (D_t) was, on average, of 444, 456 and 420 minutes referred, respectively, to phase 1 (milk samples A1, B1, C1), phase 2 (milk samples A2, B2, C2) and phase 3 (milk samples A3, B3, C3).

Milk urea concentration, which is an indicator of the health and nutrition status of dairy cows, could influence starter behavior due to the antimicrobial activity of urea molecule (Podhorsky and Cvak, 1989.; Vega-Pérez et al., 2012). The values are shown in Table 1 and were varying from 19.1 mg/dl to 25.2 mg/dl. Some authors showed that an increase in milk urea content influenced milk acidification and led to a decrease of milk clotting ability (Mariani et al., 1992). Actually, in this study, it was not found correlation between these aspects. No significant differences were detected by T-test among acidification and urea concentration according to lactation stage and factory.

3.2 RT-PCR-DGGE data

In Table 2, the main species detected on DGGE gels are summarized according to the different lactation stages and dairy farms. The presence of a species has been considered if the corresponding band occurred at least once in one of the cheese production replicates. On the contrary, the absence of a species is referred to the absence of the corresponding band on both replicates. Direct analysis of bacterial RNA from the milk, curd and cheese matrices highlighted the predominance and persistence of the starter cultures during both cheese-making and ripening in all the productions analyzed. In particular, *Lb. delbrueckii* and *S. thermophilus* were always detected, as metabolically active populations, independently from the variables lactation stage and dairy farm. On the contrary, *L. lactis* showed a minor implantation attitude and it was never found in the cheeses produced in dairy farm A.

Despite the predominance of the starter cultures, in few milk, curd and cheese samples, autochthonous microbiota was found including NSLAB such as *Lactobacillus plantarum* and *Lactobacillus casei* group (Yu et al., 2012). Occasionally, ND species were also detected in the samples analyzed, especially in milk samples. *Staphylococcus aureus*, *Propionibacterium acnes* and *Escherichia fergusonii* were the most recurrent. The psychrophilic species *Marinobacter psychrophilus* and *Psychrobacter maritimus* were also found in milk, curd and cheese samples mostly during the phase 1 (Tab. 2).

DGGE profiles were subjected to cluster analysis and the dendrogram obtained showed that electrophoretic patterns of samples coming from different lactation stages and farms of production tended to group together (Fig. 1). At similarity level of 70%, clusters I, II and IV included samples produced exclusively during the lactation phase 3, while clusters V, VI, VII and VIII grouped samples coming from the phase 1. Clusters IX, X, XI, XII were composed only by samples from phase 2. Moreover, the clustering was somehow dependent on the dairy farm producing the cheeses, underlining the influence of the manufacturing environment and practice on the cheese microbiota (Fig. 1). Milk samples clustered outside the main groups, while cheeses at 28, 56 and 84 days of ripening often showed a high percentage of similarity (clusters II, X and XII, and sub-groups in clusters III, IV, V, VII, VIII and XIII), indicating the establishment of a defined microbiota during late ripening (Fig. 1).

3.3 Pyrosequencing analysis

On the basis of the bacterial dynamics studied by DGGE, milk, curd and cheese samples at the end of ripening were chosen as representative of the different phases of production and also analyzed by pyrosequencing. RNA extracted from the replicates was mixed together before analysis in order to obtain an overall picture of the microbiota for each lactation phase and dairy farm considered. A total of 144,098 raw sequences were obtained and analyzed; 134,171 reads passed the filters applied through the QIIME split_library.py script, with an average value of 4969 reads/sample and an average length of 490bp. The number of OTUs, the Good's estimated sample coverage (ESC), the Chao1 (Chao and Bunge, 2002) and Shannon (Shannon and Weaver, 1949) indices obtained for all the samples are reported in Table 3. The rarefaction analysis and the estimated sample coverage indicated that there was satisfactory coverage for all the samples (ESC > 97%). Interestingly, milk samples from lactation phase 1 always showed a lower level of complexity, compared to those from lactation phases 2 and 3.

In Figure 2, only OTUs with a relative abundance of 0.05% in at least one sample are shown. The milk sampled in the *post-partum* lactation period (phase 1) was unexpectedly characterized by the predominance of a unique species belonging to *Lb. casei* group, which survived throughout the ripening and appeared in most of the cheeses at 84 days. A major biodiversity was highlighted in milk samples during the *oestrus* (phase 2) and early gestation (phase 3), seasons when the dominating microbiota was belonging to species *P. acnes*, to the genera *Staphylococcus* and *Pseudomonas*, and to the family *Enterobacteriaceae*. Microbiota usually colonizing soil and grass habitats such as *Acinetobacter*, *Acidovorax*, *Hymenobacter*, *Brochothrix*, *Actinobacteria* and *Cyanobacteria*, were also found (Fig. 2). Accordingly, Adonis and Anosim statistical tests showed that milk samples were significantly different according to the lactation phase ($P<0.001$). Moreover, *Lactobacillaceae* family abundance, and particularly *Lb. casei* group, was found to be significantly higher in milk samples from the lactation phase 1 by ANOVA ($P<0.001$). On the contrary, no significant difference was found among samples from different dairies ($P>0.05$).

Regardless of the initial quality of the milk, the establishment of two of the starter species, *S. thermophilus* and *Lb. delbrueckii*, was evident in all the curds analyzed after 24 hours from the beginning of the production. *Pseudomonas* and *Enterobacteriaceae* were also strongly present, together with starter cultures, in curd samples produced in dairy farm C during the phases 1 and 3. Moreover, *S. thermophilus* and *Lb. delbrueckii* showed high adaptation throughout the ripening and they were always found in cheeses after 84 days of ripening, except for the cheese manufactured in dairy farm C (phase 2) where the maturing was almost completely carried out by *S. thermophilus* only. On the contrary, the starter species *L. lactis* performed well only in the cheeses produced in dairy farm B during the *post-partum* lactation (phase 1) and early gestation (phase 3) stages (Fig. 2). Despite the prevalence of the starters inoculated, a few autochthonous microorganisms were also found metabolically active, in some cheeses, at the end of ripening. They belonged to *Lb. casei* group and prevailed, particularly, in the products sampled during the phases 1 and 3. Pyrosequencing allowed also the detection of low, but constant incidence of *Pseudomonas*, *Staphylococcus* and *Enterobacteriaceae* in most of the cheeses at 84 days of ripening (Fig. 2). The OTU network in Figure 3 clearly shows a separation between the milk and the curd/cheese samples. A high number of OTUs was shared among curds and cheeses after 84 days of ripening highlighting a core microbiota, while milk samples showed a higher number of unique OTUs. Moreover, the milk samples clustered according to the lactation phases.

3.4 Sensorial evaluation

Results concerning sensory analysis are shown in Table 4. The evaluations given from the nine panelists are reported as mean values and standard deviations from which the final average scores were extrapolated. Generally, most of the sensory attributes of the cheeses, analyzed at the end of ripening, were not markedly affected by the lactation stage of the cows and no significant differences ($P>0.05$) were detected by Duncan test. However, the attributes odor quality and intensity, sweetness, overall taste sensations, hole appearance, aroma typicity and overall pleasantness showed lower scores in cheeses sampled during the lactation phase 2 compared to

the ones coming from the phases 1 and 3. In particular, the cheeses produced during the phase 2 showed an excessive amount of holes.

4. Discussion

Overall, starter cultures were able to outcompete the autochthonous microbiota since the first hours of the fermentation and their performance seemed to be not affected by the different lactation stages. In fact, *S. thermophilus* and *Lb. delbrueckii* were found throughout manufacturing and ripening of Fontina PDO cheese, regardless of farm location and lactation period. Both RT-PCR-DGGE and pyrosequencing were able to highlight the presence and the activity of these two starter species, while they underlined the low ability of *L. lactis* in establishing in Fontina curd and cheese samples. Uniquely, *L. lactis* had a good performance in the cheese manufacturing in the dairy farm B, where it was also detected after three months of ripening. The sensitivity of pyrosequencing allowed to highlight the presence of *L. lactis* also in curds and cheeses produced at dairy farms A and C, but with a very low incidence compared to *S. thermophilus* and *Lb. delbrueckii*. In Fontina cheese making the milk is coagulated at 36 °C and the curd is cut while the temperature is gradually raised to 46–48 °C. The temperature of the process could explain the low frequency of *L. lactis*, whose contribution could be limited only to the first hours of the acidification process. As known, cooking temperature can affect the viability of starter and non starter cultures in hard and semi-hard cheeses (Sheehan et al., 2007). Moreover, Taibi and collegues (2011) identified, in *L. lactis*, a specific core of genes differentially expressed in response to heat stress. These genes are related to the coding of chaperones and proteases and linked to cell division and metabolism. The predominance, in Fontina curd samples, of *S. thermophilus* on *L. lactis* population was already highlighted in previous studies (Giannino et al., 2009; Senini et al., 1997), confirming the different attitudes of the two microorganisms to Fontina cooking temperature.

In general, the establishment of the starter cultures limited the development of NSLAB, the only exception being the *Lb. casei* group. It was found in almost all the samples of curd and cheese, and, remarkably, was dominant in milk from the first phase of lactation. Thus, this species has to

be considered as significant part of the autochthonous microbiota of Fontina cheese. In some cases, *Lb. casei* was able to compete with the starter cultures, since it was found by pyrosequencing with similar relative abundance. The presence and activity of *Lb. casei* was also highlighted by RT-PCR-DGGE confirming the important presence of this species in Fontina. Regarding NSLAB, the two methods did not always provide the same results, probably due to the different variable regions of ribosomal RNA targeted. The NS *Lb. plantarum*, associated to a variety of environmental and dairy niches (Pisano et al., 2011), and *Lactobacillus diolivorans*, isolated for the first time from maize silage (Krooneman et al., 2002), were only found by RT-PCR-DGGE and the species *Enterococcus faecium* and *E. faecalis*, considered typical in Fontina cheese and related to flavor formation (Giannino et al., 2009; Senini et al., 1997), were detected by RT-PCR-DGGE and pyrosequencing, respectively.

The raw milk analyzed and transformed in the second and third phase of lactation, according to pyrosequencing data, was rich in *P. acnes*, *Pseudomonas*, *Staphylococcus*, *Enterobacteriacee*, generally associated to low quality milk (Quigley et al., 2013), and *Psycrobacter*, *Brochothrix*, *Acinetobacter* and *Cyanobacteria*, already found associated to food and dairy products in other studies (Afzal et al., 2013; Ercolini et al., 2006; Franciosi et al., 2011; Hayes et al. 2002; Meile et al., 2008). As known, raw milk microbiota contributes greatly to the sensory characteristics of raw milk cheeses in terms of the particular flavours and aromas they generate but, at the same time, spoilage flora and potential pathogens can negatively affect the final quality of the product (Mallet et al., 2012). However, in this study, the predominance of the starter cultures over ND microorganisms was already evident in the curd. In fact, starter activity limited the development of these microbial populations throughout Fontina manufacturing and ripening, with the exception of few cheese samples where *Enterobacteriaceae*, *Pseudomonas* and *P. acnes* were detected with moderate incidence. Remarkably, the presence of contaminant microbiota in the milk of the second and third-stage of lactation and, occasionally, in curd and cheese samples of all the lactation phases, cannot explain the minor quality of Fontina, in terms of organolepitc quality and typicity, as detected by sensory analysis. This is probably due to the high performance of the starters, which were dominant in all the productions studied. Accordingly, the OTU network clearly showed

that a high number of OTUs was shared among curds and cheeses after 84 days of ripening without a correlation with the lactation phases.

The cluster analysis of DGGE profiles, obtained by microbial RNA analysis of cheese samples, resulted in a dendrogram showing an evident grouping dependent on the lactation stage, probably due to the occasional presence of dairy and ND species as *Bifidobacterium animalis*, *Citrobacter freundii*, *Corynebacterium variabile*, *Marinobacter psychrophilus*, *Psychrobacter maritimus*, *Enterobacter asburiae*, *Escherichia vulneris*, *Escherichia fergusonii*, in addition to the already cited NSLAB species. In any case, the high incidence of starter cultures, quantified by pyrosequencing, proves that the final quality of Fontina cheese cannot be correlated to autochthonous microbiota.

The metabolic microbial activity, which was supposed to affect the final quality of Fontina PDO cheese, was strictly associated to the presence of the starter, which did not show any difference in its performance according to both lactation stage and dairy farm.

The fact remains that, empirically, in milk from cows in *oestrus* phase of lactation, a slower coagulation process was observed and this can result in a delay of the fermentation process. Actually, in this study, the average D_t calculated for each lactation season was higher in the *oestrus* lactation stage, confirming the difficulty in milk coagulation. Moreover, the sensorial evaluation indicated, generally, a lower quality of the cheeses produced during the *oestrus* lactation phase, especially in terms of appearance, aroma typicity and overall pleasantness.

Nevertheless, the results of this work lead to deny any type of correlation between the microbial dynamics and the quality of Fontina cheese in relation to the different stages of lactation.

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References

Afzal, M.I., Ariceaga, C.C.G., Lhomme, E., Ali, N.K., Payot, S., Burgain, J., Gaiani, C., Borges, F., Revol-Junelles, A.M., Delaunay, S., Cailliez-Grimal, C. 2013. Characterization of *Carnobacterium maltaromaticum* LMA 28 for its positive technological role in soft cheese making. Food Microbiology 36, 223-230.

Alegria, A., Szczesny, P., Mayo, B., Bardowski, J., Kowalczyka, M. 2012. Biodiversity in Oscypek, a Traditional Polish Cheese, Determined by Culture-Dependent and -Independent Approaches. Applied and Environmental Microbiology 78, 1890-1898.

Alessandria, V., Dolci, P., Rantsiou, K., Pattono, D., Dalmasso, A., Civera, T., Cocolin, L. 2010. Microbiota of the Planalto de Bolona: an artisanal cheese produced in uncommon environmental conditions in the Cape Verde Islands. World Journal of Microbiology & Biotechnology 26, 2211-2221.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389-3402.

Ampe, F., Ben Omar, N., Moizan, C., Wacher, C. and Guyot, J.P. 1999. Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. Applied and Environmental Microbiology 65, 5464-5473.

Bérodier, F., Lavanchy, P., Zannoni, M., Casals, J., Herrero, L., Adamo, C. 1997. Guide d'Évaluation Olfacto-Gustative des Fromages à Pâte Dure et Semi-dure. Lwt - Food Science and Technology 30, 653-664.

Caporaso, J.G., Kuczynski J., Stombaugh J., Bittinger K., Bushman F.D. et al. 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7, 335–336.

Chao, A., Bunge, J. 2002. Estimating the number of species in a stochastic abundance model. Biometrics 58, 531-539.

Cocolin, L., Manzano, M., Cantoni, C., Comi, G. 2001. Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the

bacterial population during fermentation of Italian sausages. Applied and Environmental Microbiology 67, 5113-5121.

De Filippis, F., La Storia, A., Villani, F., Ercolini, D. 2013. Exploring the sources of bacterial spoilers in beefsteaks by culture-independent high-throughput sequencing. PLoS One 8, e70222.

De Filippis, F., La Storia, A., Stellato, G., Gatti, M., Ercolini, D. 2014. A selected core microbiome drives the early stages of three popular Italian cheese manufactures. PLoS One 9, e89680.

Dolci, P., Zenato, S., Pramotton, R., Barmaz, A., Alessandria, V., Rantsiou, K., Cocolin, L. 2013. Cheese surface microbiota complexity: RT-PCR-DGGE, a tool for a detailed picture? International Journal of Food Microbiology 162, 8–12.

Dolci, P., Alessandria, V., Rantsiou, K., Bertolino, M., Cocolin, L. 2010. Microbial diversity, dynamics and activity throughout manufacturing and ripening of Castelmagno PDO cheese. International Journal of Food Microbiology 143, 71-75.

Dolci, P., Alessandria, V., Rantsiou, K., Rolle, L., Zeppa, G., Cocolin, L. 2008. Microbial dynamics of Castelmagno PDO, a traditional Italian cheese, with a focus on lactic acid bacteria ecology. International Journal of Food Microbiology 122, 302-311.

Edgar, R.C. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460-2461.

Ercolini, D. 2013. High-throughput sequencing and metagenomics: moving forward in the culture-independent analysis of food microbial ecology. Applied and Environmental Microbiology 79, 3148-3155.

Ercolini, D., De Filippis, F., La Storia, A., Iacono, M. 2012. "Remake" by High-Throughput Sequencing of the Microbiota Involved in the Production of Water Buffalo Mozzarella Cheese. Applied and Environmental Microbiology 78, 8142-8145.

Ercolini, D., Russo, F., Torrieri, E., Masi, P., Villani, F. 2006. Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions. Applied and Environmental Microbiology 72, 4663-4671.

Franciosi, E., De Sabbata, G., Gardini, F., Cavazza, A., Poznanski, E. 2011. Changes in psychrotrophic microbial populations during milk creaming to produce Grana Trentino cheese. Food Microbiology 28, 43-51.

Giannino, M.L., Marzotto, M., Dellaglio, F., Feligini, M. 2009. Study of microbial diversity in raw milk and fresh curd used for Fontina cheese production by culture-independent methods. International Journal of Food Microbiology 130, 188-195.

Hayes, W., White, C.H., Drake, M.A. 2002. Sensory aroma characteristics of milk spoilage by *Pseudomonas* species. Journal of Food Science 67, 448-454.

Krooneman, J., Faber, F., Alderkamp, A.C., Elferink, S.J.H.W.O., Driehuis, F., Cleenwerck, I., Swings, J., Gottschal, J.C., Vancanneyt, M. 2002. *Lactobacillus diolivorans* sp nov., a 1,2-propanediol-degrading bacterium isolated from aerobically stable maize silage. International Journal of Systematic and Evolutionary Microbiology 52, 639-646.

Lozupone, C., Knight, R. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Applied and Environmental Microbiology 71, 8228-8235.

Mallet, A., Guéguen, M., Kauffmann, F., Chesneau, C., Sesboué, A., Desmasures, N. 2012 Quantitative and qualitative microbial analysis of raw milk reveals substantial diversity influenced by herd management practices. International Dairy Journal 27, 13-21.

Mariani, P., Bonatti, P., Sandri, S. 1992. Contenuto di urea, pH, acidità totale e caratteristiche di coagulazione del latte di singoli allevamenti. Industria del latte 28, 3-17.

Masoud, W., Takamiya, M., Vogensen, F.K., Lillevang, S., Abu Al-Soud, W., Sorensen, S.J., Jakobsen, M. 2011. Characterization of bacterial populations in Danish raw milk cheeses made with different starter cultures by denaturating gradient gel electrophoresis and pyrosequencing. International Dairy Journal 21, 142-148.

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

McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., De Santis, T.Z. et al. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archea. ISME Journal 6, 610-618.

Meile, L., Le Blay, G., Thierry, A. 2008. Safety assessment of dairy microorganisms: *Propionibacterium* and *Bifidobacterium*. International Journal of Food Microbiology 126, 316-320.

Mills, D.A., Johannsen, E.A., Cocolin, L. 2002. Yeast diversity and persistence in botrytis-affected wine fermentations. Applied and Environmental Microbiology 68, 4884-4893.

Pisano, M.B., Patrignani, F., Cosentino, S., Guerzoni, M.E., Franz, C.M.A.P., Holzapfel, W.H. 2011. Diversity and functional properties of *Lactobacillus plantarum*-group strains isolated from Italian cheese products. Dairy Science & Technology 91, 65-76.

Podhorsky, M., Cvak Z., 1989. The influence of nonprotein nitrogen on hygiene and processing properties of milk. Prumysl Potravin 40, 83-84

Quigley, L., O'Sullivan, O., Beresford, T.P., Ross, R.P., Fitzgerald, G.F., Cotter, P.D. 2012. High-Throughput Sequencing for Detection of Subpopulations of Bacteria Not Previously Associated with Artisanal Cheeses. Applied and Environmental Microbiology 78, 5717-5723.

Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T.P., Ross, R.P., Fitzgerald, G.F., Cotter, P.D. 2013. The complex microbiota of raw milk. FEMS Microbiological Review 37, 664–698.

Randazzo, C.L., Torriani, S., Akkermans, A.D.L., de Vos, W.M., Vaughan, E.E. 2002. Diversity, dynamics, and activity of bacterial communities during production of an artisanal sicilian cheese as evaluated by 16S rRNA analysis. Applied and Environmental Microbiology 68, 1882-1892.

Rantsiou, K., Urso, R., Dolci, P., Comi, G., Cocolin, L. 2008. Microflora of Feta cheese from four Greek manufacturers. International Journal of Food Microbiology 126, 36-42.

Reeder, J., Knight, R. 2010. Rapidly denoising pyrosequencing amplicon reads by exploiting rank-abundance distributions. Nature Methods 7, 668-669.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., et al. 2003 TM4: a free, open-source system for microarray data management and analysis. Biotechniques 34, 374-378.

Senini, L., Cappa, F., Cocconcelli, P.S., 1997. Use of rRNA-targeted oligonucleotide probes for the characterization of the microflora from fermentation of Fontina cheese. Food Microbiology 14, 469–476.

Shannon, C.E., Weaver, W. 1949. The mathematical theory of communication. Urbana: University of Illinois Press. 125 p.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., et al. 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Research 13, 2498–2504.

Sheehan, J.J., Fenelon, M.A., Wilkinson, M.G., McSweeney, P.L.H. 2007. Effect of cook temperature on starter and non-starter lactic acid bacteria viability, cheese composition and ripening indices of a semi-hard cheese manufactured using thermophilic cultures. International Dairy Journal 17, 704–716.

Taïbi, A., Dabour, N., Lamoureux, M., Roy, D., LaPointe, G. 2011. Comparative transcriptome analysis of *Lactococcus lactis* subsp. *cremoris* strains under conditions simulating Cheddar cheese manufacture. International Journal of Food Microbiology 146, 263–75.

van Beek, S., Priest, F.G. 2002. Evolution of the lactic acid bacterial community during malt whisky fermentation: A polyphasic study. Applied and Environmental Microbiology 68, 297-305.

Vega-Pérez, J.M., Periñán, I., Argandoña, M., Vega-Holm, M., Palo-Nieto, C., Burgos-Morón, E., López-Lázaro, M., Vargas, C., Nieto, J.J., Iglesias-Guerra F. 2012. Isoprenyl-thiourea and urea derivatives as new farnesyl diphosphate analogues: synthesis and in vitro antimicrobial and cytotoxic activities. European Journal of Medicinal Chemistry 58, 591-612

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R. 2007. Naïve Bayesan classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental Microbiology 73, 5261-5267.

Yu, J., Sun, Z.H., Liu, W.J., Bao, Q.H., Zhang, J.C., Zhang, H.P. 2012. Phylogenetic study of *Lactobacillus acidophilus* group, *L. casei* group and *L. plantarum* group based on partial hsp60, pheS and tuf gene sequences. European Food Research and Technology 234, 927-934.

Figure 1. Dendogram obtained from the cluster analysis of RT-PCR-DGGE profiles of bacterial microbiota detected in milk, curd and Fontina samples produced in three dairy farms during different cow lactation stages.

Figure 2. Incidence of the major taxonomic groups detected by pyrosequencing throughout manufacturing and ripening of Fontina PDO cheese (see Figure 1 for sample abbreviation). Only OTUs with an incidence above 0.05% in at least one sample are shown.

Dairy farm and	pH_i	pH_{24h}	D_t (min)	Urea (mg/dl)

Figure 3. Simplified illustration of possible cheese - microbe networks. Network diagrams are color- and symbol-coded by sample type and lactation phase.

	Mean	SD	Mean	SD	Mean	SD	Mean	SD
A1	6.61	0.04	5.60	0.19	402	21	23.4	1.6
A2	6.66	0.03	5.41	0.06	443	69	22.2	1.4
A3	6.68	0.02	5.36	0.02	395	34	19.1	1.7
B1	6.62	0.01	5.61	0.28	402	29	21.1	2.8
B2	6.65	0.01	5.35	0.11	451	54	21.3	1.8
B3	6.72	0.05	5.45	0.05	400	38	22.4	3.2
C1	6.58	0.03	5.44	0.07	528	35	21.6	1.1
C2	6.62	0.02	5.33	0.09	474	65	25.2	0.8
C3	6.69	0.03	5.41	0.03	465	36	20.2	2.9

Table 1. Analysis of the milk used for the production of Fontina PDO cheese in the dairy farms A, B and C during the lactation phases 1, 2 and 3: pH measurements of milk (pH_i) and after 24 hours (pH_{24h}), time required for the beginning of the acidification process (D_t) and milk urea concentration.

*A, B and C are referred to the three dairy farms and 1, 2 and 3 to the three different lactation phases. SD, standard deviation

Table 2. Occurrence of bacterial species in milk, curd and Fontina PDO cheese samples by RT-PCR-DGGE according to the different lactation phase and dairy farm.

[a]Species detected on DGGE gels	RNA analysis								
	Dairy farm A			Dairy farm B			Dairy farm C		
	Phase 1	Phase 2	Phase 3	Phase 1	Phase 2	Phase 3	Phase 1	Phase 2	Phase 3
Streptococcus thermophilus	$+_{Cu,Ch}$	$+_{Cu,Ch}$	$+_{Cu,Ch}$	$+_{Cu,Ch}$	$+_{Ch}$	$+_{Cu,Ch}$	$+_{Cu,Ch}$	$+_{Cu,Ch}$	$+_{M,Cu,Ch}$
Lactococcus lactis	-	-	-	$+_{M,Cu,Ch}$	-	$+_{Cu,Ch}$	-	$+_{M,Cu,Ch}$	$+_{Cu,Ch}$
Lactobacillus delbrueckii	$+_{Cu,Ch}$	$+_{Cu,Ch}$	$+_{Cu,Ch}$	$+_{Cu,Ch}$	$+_{Cu,Ch}$	$+_{Cu,Ch}$	$+_{Cu,Ch}$	$+_{M,Cu,Ch}$	$+_{Cu,Ch}$
Lactobacillus casei group	$+_{M}$	-	-	$+_{M,Cu}$	-	$+_{M}$	$+_{M,Cu,Ch}$	$+_{Ch}$	$+_{M,Ch}$
Lactobacillus plantarum	-	$+_{M}$	$+_{M,Cu}$	-	$+_{Ch}$	$+_{M}$	-	$+_{M,Ch}$	$+_{M,Ch}$
Lactobacillus diolivorans	-	-	-	-	-	$+_{Ch}$	-	-	-
Enterococcus faecium	-	-	-	-	-	-	-	$+_{Ch}$	-
Marinobacter psychrophilus	$+_{M}$	-	-	$+_{M}$	-	-	$+_{M,Ch}$	-	-
Psychrobacter maritimus	$+_{Cu}$	-	-	-	$+_{Ch}$	-	$+_{Ch}$	-	-
Enterobacter asburiae	-	-	-	-	-	-	$+_{Cu,Ch}$	-	-
Escherichia vulneris	-	-	-	-	-	-	-	$+_{Ch}$	-
Escherichia fergusonii	-	$+_{Cu}$	-	-	-	$+_{M}$	-	-	$+_{Ch}$
Staphylococcus aureus	-	$+_{M}$	$+_{M}$	-	-	-	-	-	-
Propionibacterium acnes	-	-	$+_{M}$	-	$+_{M}$	-	$+_{M}$	$+_{M}$	$+_{M}$
Corynebacterium variabile	-	-	-	-	$+_{Ch}$	-	-	-	-
Bifidobacterium animalis	-	-	-	-	-	-	$+_{Ch}$	-	-
Citrobacter freundii	-	-	-	-	-	-	-	$+_{Ch}$	-
Bradyrhizobium sp.	-	-	-	-	-	-	-	$+_{M}$	$+_{M}$

[a] Plus and minus signs mean, respectively, presence and absence on RT-PCR-DGGE gels of the band referred to the species indicated
Subscripts M, Cu and Ch are referred to milk, curd and Fontina cheese samples where the species indicated were found

Table 3. Observed diversity and estimated sample coverage for 16S rRNA amplicons analyzed in this study.

Sample	OTUs	Chao1	Shannon	ESC
1A_milk	25	47,75	0,94	97,37
1A_cu24h	25	29,67	1,35	99,32
1A_ch84d	69	74,53	2,54	99,51
2A_milk	290	295,88	4,55	99,83
2A_cu24h	72	120,75	1,67	98,45
2A_ch84d	54	75,08	1,52	99,21
3A_milk	240	251,89	3,38	99,73
3A_cu24h	28	61,00	1,26	99,75
3A_ch84d	72	112,63	2,39	99,16
1B_milk	23	42,50	0,16	99,80
1B_cu24h	40	65,67	1,51	99,34
1B_ch84d	96	135,38	3,09	99,16
2B_milk	283	293,16	5,22	99,59
2B_cu24h	23	30,86	1,21	99,63
2B_ch84d	61	73,67	2,21	99,37
3B_milk	228	250,52	3,50	98,99
3B_cu24h	33	55,67	1,64	99,43
3B_ch84d	98	133,77	3,70	98,77
1C_milk	30	30,55	0,23	99,93
1C_cu24h	78	133,50	3,04	98,27
1C_ch84d	41	71,60	1,61	99,40
2C_milk	105	110,28	2,63	99,49
2C_cu24h	64	73,55	2,59	99,46
2C_ch84d	181	201,07	1,26	99,80
3C_milk	125	132,89	5,49	98,11
3C_cu24h	56	71,33	2,30	98,96
3C_ch84d	70	93,00	2,86	99,30

Abbreviations: OTU, operational taxonomic unit; ESC, estimated sample coverage. Chao1, Shannon and ESC were calculated with Qiime at the 3% distance level.

Table 4. Comparison of the sensorial characteristics of Fontina PDO cheeses, at the end of ripening, produced in the three different lactation stages.

		Sensory attributes of the cheeses										
Lactation phase		odour quality	odour intensity	sweetness	saltiness	overall taste sensations	aroma typicity	elasticity	firmness	overall pleasantness	colour	holes
1	MEAN*	**6.2**	**6.2**	**6.2**	**6.1**	**5.9**	**5.9**	**5.9**	**6.0**	**6.0**	**6.3**	**6.0**
	SD	0.2	0.3	0.3	0.3	0.4	0.5	0.6	0.7	0.4	0.6	1.0
2	MEAN*	**5.7**	**5.8**	**6.0**	**6.1**	**5.7**	**5.6**	**6.3**	**6.2**	**5.8**	**6.6**	**6.8**
	SD	0.3	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.3	0.1	0.2
3	MEAN*	**6.0**	**5.9**	**6.1**	**6.1**	**5.9**	**5.9**	**6.2**	**6.3**	**5.9**	**6.6**	**6.6**
	SD	0.4	0.3	0.5	0.5	0.5	0.5	0.3	0.5	0.5	0.4	0.3

*Mean values are not significantly different ($P<0.05$) according to Duncan test