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Comparative evaluation of cheese whey microbial composition from four Italian cheese factories by viable counts and 16S rRNA gene amplicon sequencing

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ABSTRACT

The microbiota of cheese whey from four dairy companies located in the North-East of Italy was evaluated during winter and spring using both culture-dependent and -independent methods. Although no significant differences were observed ($p > 0.05$) for any of the six microbial categories analysed by plate counting among the dairy companies, significant differences at the family and genus level were revealed by linear discriminant analysis (LDA). In particular, variations in the abundance of the families *Pseudomonadaceae* and *Enterobacteriaceae*, as well as of the genus *Acinetobacter* were observed and positively correlated with plate count results ($p < 0.05$). The combined use of culture-dependent and -independent approaches gave a better description of the hygienic conditions and food safety aspects and could therefore be suggested as a useful integrated approach.

1. Introduction

Whey is the main by-product from cheese production. Since only a small amount of milk becomes cheese, from approximately 6% (hard cheeses) to 16% (soft cheeses), whey is therefore produced in great quantity, but cannot be easily disposed due to its high organic load, that can reach a chemical oxygen demand (COD) of 100 kg m^{-3} (Prazeres, Carvalho, & Rivas, 2012).

For this reason, historically the whey has been almost entirely used to feed animals, mainly hogs (Schingoethe, 1976). The annual production of whey has been estimated around 190 million tons worldwide (Baldasso, Barros, & Tessaro, 2011) and the EU alone, which is the largest producer of whey globally, accounted for 67 million tons in 2016, according to the European Whey Processors Association (EWPA, 2018). In Italy, whey production is estimated to be between 8 and 9 million tons (Iseppi, Rosa, & Taverna, 2017).

In recent years, an increasing number of published research has allowed a better knowledge of whey composition and this product has hence rapidly gained enormous attention by several industrial sectors for its energetic, pharmaceutical and nutritional potentialities (Escalante, Castro, Amaya, Jaimes, & Jaimes-Estévez, 2018; Lappa et al., 2019; Treu et al., 2019; Zoppellari & Bardi, 2013).

As a matter of fact, whey protein is nowadays considered a universal supplement for its high nutritional value (e.g., essential amino acids and bioactive compounds) (Krissansen, 2007). Whey can also play a significant role on the survival of beneficial or probiotic microbes ingested with foods, as well as to produce organic acids from the fermentation of lactose that is contained in whey at a concentration around 4.5%. On the other side, recycling of this excess production of

62 whey as a fermented medium could be useful not only for the environment but also for a
63 sustainable economy (Jelen, 2003).

64 Bacterial presence in whey is abundant, normally higher than that of milk, since cheese-
65 making procedures favour bacteria growth, even in the case of the use of pasteurised milk, due to
66 the addition of bacterial starter cultures, therefore the microbiological scenario should be
67 carefully evaluated from both a quantitative and qualitative point of view, to make proper use of
68 this material (de Arauz, Jozala, Mazzola, & Penna, 2009; Koller, Hesse, Salerno, Reiterer, &
69 Braunegg, 2011). Whey microbiota is mainly constituted of lactic acid bacteria, mostly
70 belonging to the genera *Lactobacillus* and *Streptococcus*, but other taxa are normally present in
71 lower amounts (e.g., spore-forming and non-spore forming food-borne pathogenic bacteria),
72 whose presence could be considered indicative of some characteristics (e.g., flavours) or
73 problems (e.g., blowing) during whey processing and/or storage (Giraffa & Neviani, 2001;
74 González, Alvarez, Riera, & Alvarez, 2007; Schepers, Thibault, & Lacroix, 2002). Moreover, a
75 preliminary and detailed investigation of specific taxa using High Throughput Sequencing (HTS)
76 can be very useful for downstream processes that utilise cheese whey as source, such as the
77 measurement of fermentative populations in case of energy production (e.g., *Clostridium* and
78 *Lactobacillus*) (Wenzel, Fuentes, Cabezas, & Etchebehere, 2017) and identification of acid-
79 tolerant microbial species for bioproduction of acetic acid and propylene glycol (Veeravalli &
80 Mathews, 2018).

81 The present study investigated the microbial composition of cheese whey samples
82 collected from four Italian dairy companies located in the North-East of Italy during winter and
83 spring using both culture-dependent and culture-independent approaches, to look for possible

links between technological aspects, environment and microbiota composition, particularly focusing on technological, hygienic and safety aspects.

2. Materials and methods

2.1. Cheese whey sampling

Six samplings of cheese whey were performed, three during March (one sampling per week) and referred to as Winter samples and three in May 2018 (one sampling per week), considered Spring samples, from four different dairy companies (DC) located in the Veneto Region, North-Eastern Italy. Cheese factories were identified by codes, as follows: DC1 (located in the province of Treviso) produces soft (e.g., Casatella, Stracchino) and semi-hard (e.g., Morlacco, Rigatino) cheeses; DC2 (province of Treviso) produces semi-hard cheeses (e.g., Montasio, Moesìn, Agricansiglio); DC3 (province of Treviso) produces soft cheeses (e.g., Casatella DOP, stracchino) and mozzarella; DC4 (province of Vicenza) produces hard and semi-hard cheeses (e.g., Grana). The pH of each cheese whey sample is reported in Supplementary material Table S1.

Fifty millilitre aliquots of whey, collected in the day of production, were cooled on ice and transported under refrigerated conditions to the laboratory where they were immediately subjected to microbiological analyses and in part stored at -20°C for further total bacterial genomic DNA (gDNA) extraction.

2.2. Media and growth conditions

Microbiological analyses of whey samples were performed for the following six bacterial categories, relevant for the dairy sector. Total mesophilic count (TMC) was obtained by the pour plating technique on plate count agar (PCA, Sigma-Aldrich, Milan, Italy) containing 0.1% skim milk (Oxoid, Milan, Italy) incubated at 30 °C for 72 h. *Enterobacteriaceae* were enumerated on violet red bile glucose agar (VRBGA, Sigma-Aldrich) by spread plating after incubation at 37 °C for 24 h. *Pseudomonas* spp. were counted on *Pseudomonas* agar base + CFC supplement (Sigma-Aldrich) containing 10% glycerol by spread plating after incubation at 25 °C for 48 h. Sulphite-reducing clostridia were enumerated on tryptose sulphite cycloserine (TSC) agar (Sigma-Aldrich) containing 5% egg yolk emulsion (Sigma-Aldrich) and 400 mg L⁻¹ cycloserine (Sigma-Aldrich) incubated anaerobically at 37 °C for 48 h. Spores of sulphite-reducing clostridia were enumerated using the same conditions as above but samples were subjected to a heat treatment at 75 °C for 20 min prior to plating, to kill vegetative cells and activate endospores. Aerobic spore former bacteria were plated on PCA after a heat shock treatment at 80 °C for 10 min, and counted after aerobic incubation at 30 °C for 72 h.

2.3. *Whey microbial composition through 16S rRNA gene amplicon sequencing*

Frozen whey samples were thawed on ice, divided into three aliquots of 15 mL each into sterile 50-mL polypropylene Falcon tubes and centrifuged at 4500 × *g* for 15 min at 4 °C. After removal of the supernatant, the pellet was used for total genomic DNA extraction using the DNeasy PowerFood Microbial Kit (Qiagen, Valencia, CA, USA) according to the manufacturer instructions. After reiterated attempts the sample DC4_May1 did not give a sufficient amount of

DNA and was excluded from this study. Total bacterial gDNA was quantified by absorbance measurements at 260 and 280 nm using a Spark 10M reader (Tecan Trading AG, Männedorf, Switzerland), and its integrity was verified by electrophoresis on 1.5% agarose gels.

Total gDNA samples were sent to the Ramaciotti Centre for Genomics (UNSW Sydney, Australia) where amplicon preparation and sequencing were performed. After amplification of the V4 region of the 16S rRNA gene using universal 515f/806r primers, amplicon libraries were generated using the Nextera XT DNA Library Preparation Kit (Illumina Inc, San Diego, CA, USA) and sequenced using the Illumina MiSeq desktop sequencer producing 250 bp paired-end (PE) reads.

Independent 16S rDNA sequences were analysed with the CLC Genomics Workbench software (V.8.0.2, QIAGEN Bioinformatics, Germany) using the Microbial genomics module plugin as described by Treu et al. (2018). Concisely, quality filtering, operational taxonomic unit (OTU) clustering, taxonomical assignment (Greengenes v13_5 database), biodiversity indicators (alpha and beta diversity) calculation were conducted with default parameters. When convenient, OTU consensus sequences of the most interesting taxa were manually verified using MEGABLAST (Database: 16S ribosomal RNA sequences) to improve the taxonomical assignment. Raw reads were deposited in the Sequence Read Archive (SRA) database (<http://www.ncbi.nlm.nih.gov/sra>) under the BioProject PRJNA529382.

Interaction network analysis was constructed to evaluate the association between the most abundant OTUs (relative abundance higher than 0.5%) and microbial load (cfu mL⁻¹) after cheese whey plating on different culture media. Pearson correlation coefficient (r) with a two-sided 95% confidence interval was computed using Graphpad Prism 7 software (Graphpad Prism 7 software, San Diego, CA, USA). Only correlations greater than 0.9 or less than -0.9 were

included in this analysis. Nodes represent microbial information concerning *Enterobacteriaceae*, *Pseudomonas* sp., mesophilic bacteria, sulphite reducing clostridia and total *Clostridium* sp. spores, as well as of the most abundant taxa present in the cheese whey microbiome. The edge weight was calculated by the correlation between the OTUs versus the plate count for each evaluated group, denoting positive or negative significant differences between nodes. Edge-weighted undirected network was visualised and explored using Gephi (Bastian, Heymann, & Jacomy, 2009).

2.4. Statistical analysis

With the exception of alpha diversity comparison conducted with GraphPad Prism 7 (described below), statistical analyses were carried out using MicrobiomeAnalyst (Dhariwal et al., 2017). After data inspection, data filtering (low count and low variance filters) and normalisation (total sum scaling) were conducted with default parameters. When appropriate, data were rarefied to the minimum library size (40,408 sequences). The significant difference of the most abundant OTUs at family and genus level among cheese factories at different times was assessed using the linear discriminant analysis (LDA) effect size (LEfSe) function setting up FDR-adjusted p -value cut-off of 0.5 and log LDA score of 2.0.

Alpha diversity indices and the abundance of specific taxa were compared among dairy companies considering the months of sampling (March and May), as well as between the seasons (winter and spring), using ANOVA with Tukey's multiple comparisons test in GraphPad Prism 7 (GraphPad Software LLC, La Jolla, CA). A significant difference in the bacterial community structure among the four dairy companies during the months of March and May, as well as for

the period of winter and spring, was evaluated by ANOSIM using Bray-Curtis and Jaccard as beta diversity metrics. Principal coordinates analysis (PCoA) was chosen as ordination method and graphs were constructed to explore and to visualise cheese whey microbial dissimilarities among the dairy companies.

3. Results and discussion

3.1. Sampling rationale

The microbiological composition of milk whey has gained great importance in recent years, since this by-product of cheese production is largely used as supplement for the production of many drinks and food products (Zadow, 1992). Four cheese factories located in the Veneto Region (three in the province of Treviso and one in the province of Vicenza) were included in this study. The first sampling was performed at the end of winter, during March, and the second one at the end of spring, in May, to evaluate possible influences of the environmental conditions on the bacterial presence and growth dynamics. Based on pH values (between 6 and 7), all samples were classified as sweet (i.e., not fermented) whey. According to the Agenzia Regionale per la Prevenzione e Protezione Ambientale del Veneto (ARPAV, 2018), the average temperature values recorded in the region during March and May 2018 were 7.1 °C and 20.2 °C, respectively.

3.2. Microbiological analyses

The results of the microbiological analyses on viable bacteria present in whey are shown in Fig. 1, that reports separately the mean values for March and May samplings and the mean of all samples together, separately for each cheese factory.

The mesophilic count is an important parameter to assess the quality and level of hygiene of raw and pasteurised milk. High levels of viable mesophilic microorganisms indicate generally poor conditions in production, storage, and processing of milk, and could also suggest a possible presence of pathogens. Normal values for raw milk range from 10^4 to 10^5 cfu mL⁻¹ (Raats, Offek, Minz, & Halpern, 2011), while in pasteurised milk values are around 10^2 – 10^4 cfu mL⁻¹ (Freitas, Nero, & Carvalho, 2009). Most of the cheeses manufactured in the dairy plants involved in the study are produced from heat-treated milk, and all the products were obtained by the addition of commercial or natural bacterial starter cultures, whose presence is reasonably included in the mesophilic count results, although many technological species are thermophilic and probably not able to develop well at the mesophilic conditions of the analysis (Tarrah et al., 2018). For all cheese factories, the differences between March and May were not statistically significant ($p>0.05$). Although the values of individual samples were highly heterogeneous, ranging from 8.3×10^4 to 2.5×10^8 cfu mL⁻¹, the means of the four dairy factories were not statistically different.

Regarding the *Enterobacteriaceae* analysis, the reference value for raw milk is 10^2 – 10^4 cfu mL⁻¹ (Tornadijo, García, Fresno, & Carballo, 2001) and less than 10^1 cfu mL⁻¹ for pasteurised milk and whey. This parameter is strictly related to the hygienic conditions of stables and of milking procedures. In the cheese factories studied, values were from 3.7×10^0 to 1.7×10^6 cfu mL⁻¹. Although, with the exception of DC4, values measured in spring appear higher with respect to winter, this difference is not statistically significant. It must be considered that

during cheesemaking procedures and when temperatures are above refrigeration values, these bacteria are able to multiply. Since this analysis does not consider lactic acid bacteria, and therefore starter strains are not counted, this analysis provides a good representation of the overall hygienic conditions.

Pseudomonas is a psychrotrophic bacterial genus, commonly present in milk and in dairy environments, particularly with the non-pathogenic species *P. fluorescens* and *P. putida*, which are non-pathogenic spoiling bacteria frequently responsible for cheese spoilage (Martin, Murphy, Ralyea, Wiedmann, & Boor, 2011). The reference count for milk and cheeses is less than 10^6 cfu mL⁻¹ (Hantsis-Zacharov & Halpern, 2007). Values found in our samples ranged from 1.0×10^1 to 1.5×10^6 cfu mL⁻¹. The distribution among samples was quite uneven, but no significant differences were detected neither between winter and spring samplings nor among the four cheese factories. All mean values related to *Pseudomonas* sp. were below the limit of 10^6 cfu mL⁻¹.

Aerobic sporeformers are a group of bacteria belonging mainly to the genus *Bacillus*, that includes spoiling microbes and also a pathogenic species, namely *B. cereus* (Lücking, Stoeckel, Atamer, Hinrichs, & Ehling-Schulz, 2013). Spores presence in raw milk can range from 10^1 to 10^3 mL⁻¹ (Eijlander et al., 2019). In our study, aerobic sporeformers were from 1.0×10^1 to 2.0×10^4 cfu mL⁻¹, but means were all below 10^3 cfu mL⁻¹.

Regarding *Clostridium*, this genus contains species particularly relevant for the dairy sector because of the ability of these anaerobic microbes to produce considerable amounts of gas during cheese ripening. These bacteria can be present as vegetative forms, detectable by the analysis of sulphite-reducing clostridia, or in the non-viable endospore form and, due to their ubiquitous nature, they are indicative of either environmental or faecal contamination. For many

foods the reference value for sulphite-reducing clostridia is 100 cfu mL⁻¹ and for clostridial endospores is below 1 spore mL⁻¹. Our results (Table 1) revealed that 5 samples out of 24 showed a number of clostridia above the detection limit of the technique, i.e., 1 cfu mL⁻¹, but none exceeded 10 cfu mL⁻¹. Regarding spores, only 3 out of 24 samples scored positive and none exceeded 10 spores mL⁻¹. Company DC4 represents the best situation, since both vegetative cells and endospores were always below the detection limit. This is particularly positive, since this DC produces mainly hard cheeses, which are sensitive to the late blowing defect caused by clostridia.

Considered together, all microbiological analyses gave values within the reference ranges and there was no significant difference detected between winter and spring scores, indicating that environmental conditions, mainly the temperature that in May resulted in 13 °C higher on average with respect to March, did not affect significantly the whey microbial load. Moreover, the mean scores of the four microbial categories tested, were not significantly different among the 4 cheese factories considered, thus indicating that the different types of cheese produced and the technological practices adopted by each company either did not influence microbial populations or such variations were not detectable by canonical microbiological procedures.

3.3. *Metagenomic analysis of whey samples*

Although the use of classical microbiological analyses based on plate count techniques is still widespread for the quality and safety monitoring of food products, such approach does not allow a taxonomical identification of microbes nor it can detect species present in low numbers or in a non-culturable state (Neviani, Bottari, Lazzi, & Gatti, 2013). The advent of DNA-based techniques has allowed a deeper degree of investigation, by eliminating the need to cultivate

bacteria (Pogačić et al., 2010) and, more recently, the next generation sequencing (NGS) technology based on the amplification of the 16S rRNA genes has dramatically increased the potential of the genomic approach and is presently being used routinely for several microbiology investigations in cheese production (Tilocca et al., 2020). Regarding milk whey biodiversity, an increasing interest regarding its bacterial composition is observed over the years (Bertani et al., 2020; Gatti, Bottari, Lazzi, Neviani, & Mucchetti, 2014; Liu et al., 2009; Morandi et al., 2019).

We investigated the microbial composition of 23 cheese whey samples, because one of the samples (DC4_May1) did not give a sufficient amount of DNA after reiterated attempts. Following a deep amplicon sequencing of the hypervariable region 4 (V4) of the 16S rRNA gene, a total of 5,314,530 high-quality sequences with an average length of 255 bp were obtained after the removal of low quality and chimeric sequences. Rarefaction curves evidenced that sequencing depth was enough to cover the microbial diversity of samples (Supplementary material Datasheet 1; Supplementary material Fig. S1). Indeed, Good's coverage index was an average of 99.9% and suggest that the majority of bacterial phylotypes were obtained from all cheese whey samples. The alpha diversity Chao1 and Shannon indices varied significantly ($p < 0.05$) across the samples when values were considered for each DC during a specific month (Fig. 2A,C), but not when samples were grouped by season (Fig. 2B,D). The variations observed in terms of richness and evenness might be a consequence of differences found in the microbial composition of the milk used for cheesemaking, the starter culture of choice and the cheese manufacturing process adopted in each dairy company. Interestingly, the significant ($p = 0.0084$) reduction of Shannon diversity observed on DC2_May when compared with DC2_March is accompanied by an increase of the relative abundance of microorganisms assigned to the taxon *Enterobacteriaceae*.

After the clustering process (97% similarity threshold), a total of 273 OTUs were obtained (Supplementary material Datasheet 1). To improve downstream statistical analyses, 116 OTUs were obtained subsequent to data filtering based on the removal of low count (143 OTUs removed) and low variance (13 OTUs discarded) features. At the phylum level, *Firmicutes* (84.9%) and *Proteobacteria* (15.0%) accounted for 99.9% of the whey bacterial population composition (Fig. 3A), which is in accordance with previous studies on bacterial composition of raw milk (Bonsaglia et al., 2017; Li et al., 2018), milk whey used for ricotta cheese production (Sattin et al., 2016), Poro cheese (Aldrete-Tapia, Escobar-Ramírez, Tamplin, & Hernández-Iturriaga, 2014) and natural whey cultures (NWCs) used in the cheesemaking process of Mozzarella, Grana Padano and Parmigiano Reggiano, three traditional Italian PDO cheeses (De Filippis et al., 2014)

The top ten genera had a relative abundance higher than 1% (Fig. 3B) and, in particular, the four most abundant were *Streptococcus* (64.9%), *Lactobacillus* (18.1%), *Escherichia* (8.5%) and *Acinetobacter* (4.1%), that together accounted for 95.6% of the total bacterial biodiversity, representing the core microbiome in all samples analysed. A significant difference in abundance ($p < 0.05$) was observed for the genera *Streptococcus* and *Lactobacillus* among DCs, which reflects the type of microorganisms used to produce semi-hard and soft cheeses. Except for the DC4, no statistically significant variation during the months of March and May inside the same DC were found considering both genera. Members of the genus *Streptococcus* and *Lactobacillus* are LAB commonly present in dairy products, including strains added as starters during the cheesemaking process, able to develop well at relatively high temperatures and low pH (Rossetti, Carminati, Zago, & Giraffa, 2009). According to Gatti et al. (2014), these two genera form the core microbiome of NWCs used to produce Grana Padano and Parmigiano Reggiano. Although

with significant difference among means (ANOVA, $p = 0.02$), Tukey's multiple comparisons test revealed no significant variation among the DCs with regards to the genus *Acinetobacter*. In this study, *Acinetobacter* was the fourth most abundant genus and it is highly present in cheese whey samples from DC1 in Winter. Despite 16S rRNA analysis used in this study did not reach the species level, the vast majority of microorganisms belonging to the genus *Acinetobacter* include nonpathogenic environmental species able to resist to dry conditions for a long time (Cho et al., 2018) and are commonly found as part of raw milk microbiota as contaminant and spoilage agents in the production of hard cheese (Alessandria et al., 2016; Li et al., 2018).

To identify bacterial families and genera whose relative abundances differed significantly among the four DCs enrolled in this study, LDA scores were computed. In total, a higher and significant abundance difference was observed for 13 families and 17 genera (Fig. 3C,D). The families *Streptococcaceae* and *Lactobacillaceae* displayed the highest abundance (LDA score > 6) in DC1 and DC2, respectively. With regard to the families *Pseudomonadaceae* and *Enterobacteriaceae*, a significant bacterial difference was observed, respectively, in DC2_March (LDA score > 5) and DC4 (LDA score > 6), while increased relative abundance for the family *Lachnospiraceae* was observed for both dairy companies when compared with DC1 and DC3 (Fig. 3A). Members of the family *Enterobacteriaceae*, such as *Escherichia*, *Erwinia*, *Klebsiella* are commonly found in less industrialised dairy products, for instance in mozzarella samples, as sub-dominant taxa (De Filippis et al., 2014). Moreover, this taxon can negatively affect sensory properties of cheese due to proteolytic and lipolytic activities or gas production (early blowing) (Mladenović, Muruzović, Žugić Petrović, Stefanović, & Čomić, 2018), while members of the family *Pseudomonadaceae*, such as *Pseudomonas putida* and *P. fluorescens*, are well-known food spoilers determining changes in colour and aroma, particularly during prolonged storage at

refrigeration temperatures (Champagne et al., 1994). Although described as part of the normal microbiota in milk samples obtained from healthy cows (Ganda et al., 2017), the family *Lachnospiraceae* (order *Clostridiales*) arises as a non-conventional taxon with higher relative abundance mainly in whey sampled from DC2 (LDA score > 5) with respect to all other dairy companies. Abundantly present in the digestive tract of mammals, members of this family are chemoorganotrophic, strictly anaerobic, fermentative and able to convert complex carbohydrates to short-chain fatty acids (SCFA) by fermentation (Meehan & Beiko, 2014).

Multivariate analyses using Bray-Curtis and Jaccard as distance metrics indicate moderate microbial dissimilarity (ANOSIM, $p < 0.001$, $R=0.59904$) among the groups and suggest that cheese whey microbiome profiles differed according to the month of sampling and dairy company. As observed in Fig. 3A,C, two separate clusters are distinguishable for companies DC2 and DC4, which can be explained by their differences in terms of genera composition and relative abundance during the period analysed. On the contrary, cheese whey microbial composition did not differ between the samplings occurred in Winter and Spring (ANOSIM, $p < 0.023$, $R=0.047255$) (Fig. 3B,D). The percentage of variation explained by the plotted PCoA1 and PCoA2 accounted for 89.7% and 72.2% considering, respectively, Bray-Curtis and Jaccard as distance metrics.

We investigated the relationships between the bacterial community composition considering the three samples per period (Winter and Spring) of each dairy company and the numbers (cfu mL⁻¹ of culturable *Enterobacteriaceae*, *Pseudomonas* sp., mesophilic bacteria and aerobic spore-formers using Pearson's correlation coefficient (r) (Fig. 4). Notwithstanding the use of network analysis is still relevant to explore the correlation between OTUs and variables (Barberán, Bates, Casamayor, & Fierer, 2012), it is worth mentioning that the use of the current

approach has limitations since no adjustments for multiple comparisons were made and additional studies using larger cohorts must be conducted to verify this correlation. Once anaerobic spore-formers (sulphite reducing *Clostridium* and total *Clostridium* spores) were present below 20 cfu mL⁻¹, they were excluded from further analyses. A positive and significant correlation was observed considering cheese whey samples collected in May (Supplementary material Datasheet 1, Tables 1–3). The genus *Klebsiella* was positively correlated with *Enterobacteriaceae* and mesophilic bacteria plate counting ($p < 0.05$) only for DC1, whereas the genus *Acinetobacter* was associated with mesophilic bacterial load ($p < 0.01$) in DC2. Interestingly, the genus *Pseudomonas* was positively correlated with high levels of culturable bacteria when DC1, DC2 and DC3 were considered, mainly with regards DC3 ($p < 0.001$), but not in DC4. The dairy company 4 produces semi-hard and hard cheeses (e.g. Grana Padano PDO) and the high temperatures (± 55 °C) used during the cheese-making process can determine a low presence of the psychrotrophic *Pseudomonas* in the whey. Finally, the presence of aerobic spore-formers was positively correlated with *Geobacillus* sp. ($p < 0.01$), present in samples obtained from DC3 in March, a thermophilic strain associated to biofilm formation in the manufacturing plants (Scott, Brooks, Rakonjac, Walker, & Flint, 2007).

4. Conclusions

In view of the continuously growing interest in the use of whey and whey-based products for human nutrition, it becomes increasingly important to gain detailed information on its microbial composition in relation to hygiene and food safety. The combined use of culture-independent approaches and microbial profiling using next-generation sequencing can strengthen

conventional microbiological analyses in dairy companies to achieve better food safety and hygienic levels. The possibility to describe the whey microbiota and in particular to look for the presence of potentially dangerous microbial taxa and its relationship with farming or industrial procedures and techniques could be of help in better dealing with this product and to protect consumers regarding safety aspects. In the present work, we have shown that no significant differences were detected when only bacterial plate counting was used; however, the NGS approach highlighted that microbial profiles significantly varied among the four dairy companies examined, concerning mainly the families *Pseudomonadaceae* and *Enterobacteriaceae*.

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Figure legends

Fig. 1. Viable bacteria (■, enterobacteriaceae; ■, pseudomonas; ■, aerobic sporeformers; ■, mesophilic) of cheese whey samples determined by plate count analyses related to each dairy company (DC 1–4). Each bar under “March” and “May” represents the mean of the three samples collected during the month. Each “mean” bar includes all 6 samples obtained from each dairy company.

Fig. 2. Alpha diversity indices Shannon and Chao1 used to compare cheese whey samples from different dairy companies (DC) considering the factors “month” (A and C) and “season” (B and D). Asterisks represent statistically significant differences among groups (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

Fig. 3. Bacterial profile at the phylum (A) and genus (B) levels. Dot plot of the LDA scores computed for family and genus with differential abundance in cheese whey sampled from four different dairy companies in the North-East of Italy.

Fig. 4. Principal coordinate analysis (PCoA) based on Bray-Curtis (A and B) and Jaccard (C and D) distance metrics. Samples are coloured according to the month (A and C) and according to the season (B and D). Standard error ellipses show 95% confidence areas.

Fig. 5. Correlation network analysis between bacterial taxa obtained by 16S rRNA gene amplicon sequencing and bacterial plate counting (*Enterobacteriaceae*, *Pseudomonas* sp., mesophilic bacteria and aerobic spore-formers). Nodes are represented either by OTUs

(relative abundance higher than 0.5%) or culturable category. The solid line (edge) between nodes denotes the positive ($r > 0.9$; $p < 0.05$), or negative ($r < -0.9$; $p < 0.05$) correlation.

Table 1

Number of sulphite reducing clostridia and clostridial spores (cfu mL⁻¹) present in the 24 whey samples. ^a

Dairy company	Sulphite reducing clostridia						Clostridial spores					
	Winter1	Winter2	Winter3	Spring1	Spring2	Spring3	Winter1	Winter2	Winter3	Spring1	Spring2	Spring3
DC1	0	0	0	0	< 10	0	0	0	0	< 10	< 10	0
DC2	0	< 10	0	0	< 10	< 10	0	0	0	0	< 10	0
DC3	0	0	< 10	0	0	0	0	0	0	0	0	0
DC4	0	0	0	0	0	0	0	0	0	0	0	0

^a “Zero” means that the number of bacteria was below the detection limit of the technique, i.e. 1 cfu mL⁻¹. When 1 to 10 colonies developed on the plate, the term “<10” is used since few per plate cfu (below 20) are not considered sufficient to calculate a reliable concentration of the sample.

A **B** **C** **D**

A **B** **C** **D**