Molecular investigation of bacterial communities during the manufacturing and ripening of semi-hard Iranian Liqvan cheese

Molecular Investigation of Bacterial Communities during the Manufacturing and Ripening of Semi-hard Iranian Liqvan Cheese

M. Ramezani [a], S.M. Hosseini [a], I. Ferrocino [b], M.A. Amoozegar [c, d], L. Cocolin [b, *]

[a] Department of Microbiology, Faculty of Biological Sciences & Technologies, Shahid Beheshti University, Tehran, Iran
[b] University of Torino, Department of Agricultural, Forest and Food Science, Largo Paolo Braccini 2, 10095, Grugliasco, Torino, Italy
[c] Microorganisms Bank, Iranian Biological Resource Centre (IBRC), ACECR, Tehran, Iran
[d] Extremophiles Laboratory, Department of Microbiology, Faculty of Biology and Center of Excellence in Phylogeny of Living Organisms, College of Science, University of Tehran, Tehran, Iran

Abstract

Liqvan (or Lighvan) is a traditional Iranian cheese from the East Azerbaijan province of Iran, which is made of raw ewe´s milk without the addition of a starter. The grazing pastures, environmental conditions and the ancient regional production methods allocate a distinctive microbial ecology to this type of cheese, and these factors are consequently associated with the quality of the product. In this study, the microbiota of the milk, curd and cheese has been investigated using culture independent approaches. Denaturing gradient gel electrophoresis (DGGE) of the bacteria, 16S rRNA based high-throughput sequencing and enumeration of the live bacterial community by means of quantitative PCR (qPCR) have been used for this purpose. The results showed that the main bacterial population in the milk belonged to both microbial contaminants and lactic acid bacteria (LAB). However, both of these populations were totally replaced by LAB during ripening. The present survey contributes by describing the microbiota of this ancient cheese in more detail during fermentation and ripening.

Keywords: Liqvan cheese, Illumina analysis, RNA-based methods

Introduction

Liqvan (or Lighvan) cheese is the most famous traditional, semi-hard, feta like, starter-free, Iranian cheese. It is manufactured from raw ewe's milk in the Liqvan village in the stratovolcano mountainous area of Sahand in the East Azerbaijan province of Iran. The production process is conducted within 2h from milking. Ewe's milk is cooled to 25°C and coagulated with homemade or industrial lamb rennet. After 2 hours of drainage, the coagulum is cut into 15x15 cm cubes and placed in 15-20% saline water, where it is left for 9 to 10 hours. The curd is consequently covered with dry salt and kept in a basin for 2-3 days, during which whey drainage continues. During this period, the cubes are turned (upside down) at least five times. Finally, the resulting curd is placed in 10–12% saline water in metal containers. Ripening takes place in deep natural or manufactured caves over a period of 6 months. This traditional manufacturing process, which has remained unchanged for centuries in this specific area, has attracted particular interest in the last few years (Edalatian et al., 2012; Kafili et al., 2009; Navidghasemizad et al., 2009; Barouei et al., 2008). Typical sensory properties of such traditional cheeses mostly affected by dairy animals' type, breeds and nutrition as well as their indigenous microbiota which drive biochemical process during cheese making. However, this traditional way of production has made also some problems during these days. First of all Standard Organization of Iran recommends strictly the pasteurization of milk to improve the safety of dairies and this process might eliminate the key functional microorganisms involved at cheese ripening and acidification. In spite of food safety, product standardization is necessary

for entrance at international marketing systems so identification and characterization of active starter and non-starter microorganisms during each step of production seems to be important.

Food microbiota is currently monitored by means of a variety of culture dependent and independent techniques. DNA and/or RNA based approaches are considered indispensable tools for the detection, identification and characterization of microorganisms in food, and they have led to a profound change in the microbial ecology study of fermented foods in recent decades. These techniques are faster and more reliable than conventional culturing methods, which fail to reproduce ecological niches and symbiotic relationships (Carraro et al., 2011, Cocolin et al., 2013; Ndoye et al., 2011). Moreover, Next Generation Sequencing (NGS) approaches have been used successfully to monitor microbial communities in foods, especially in milk-based foods lately (Aldrete-Tapia et al., 2014; Bassi et al., 2015; Dugat-Bony et al., 2015; Garofalo et al., 2015; Minervini et al., 2015; O'Sullivan et al., 2015; Alessandria et al., 2016; Dalmasso et al., 2016; De Pasquale et al., 2016; Escobar-Zepeda et al., 2016).

Previous studies on Liqvan diversity, based on culture-dependent and on DNA based DGGE method, led to the identification of some dominant bacteria (Edalatian et al., 2012; Kafili et al., 2009; Navidghasemizad et al., 2009; Barouei et al., 2008). However, to the best of the authors' knowledge, no RNA based studies on this cheese have been carried out so far. Moreover, in order to have a more complete picture of viable microbiota thriving in this cheese during ripening process, we used illumina-sequencing method as a new powerful tool for analyzing of milk-based foods. This thorough analysis of RNA-based microbiota, will help us to follow the trace of possible pathogenic microorganisms and their survival during manufacturing.

Therefore, in this study, the microbial population of the bacterial communities of Liqvan cheese have been studied during its manufacturing through reverse transcriptase PCR (RT-PCR)-DGGE. Furthermore, the total counts and diversity of the viable bacterial populations in the raw milk, curd and ripened cheese have been investigated using RT quantitative PCR (RT-qPCR) and 16S rRNA gene amplicon sequencing, respectively.

Material and methods

Sample collection

Samples of raw milk, curds and cheeses were collected aseptically on-site at five different local authentic production units (A to E) in the Liqvan village, which covers an area of 19.76 square kilometers in the south-western part of Tabriz province. All the milk samples collected at the same day after transferring to the units and cooled at 25 °C. While three days curds gathered after coagulation and drainage. The milk

and curds were transferred directly to the lab, albeit the cheeses were collected after 180 days of ripening from the same source of milk. Three trials as replicates were taken from each dairy for every sampling. The samples were transported at 4-8 °C and kept at -20 °C.

This cheese mainly characterized by a pH value and total fat and protein percentage around 5.02±0.5, 20.3±2.0 and 15.7±1.0, respectively.

Reverse transcription and PCR amplification of the microbial community

Aliquots of 2 milliliters of milk were centrifuged for 10 min at 14,000 × g under refrigerated conditions (4 °C). The samples were placed at -80 °C for 30 min to allow separation of the fat layer, which was then removed by means of a sterile spatula. After thawing, the supernatant was discarded and cell pellets were re-suspended in 1 ml ¼ strength Ringer solution. The mixture was centrifuged at 14,000 × g for 10 min at 4 °C, and the supernatant was again discarded. The pellets were re-suspended in a 50 µl lysozyme solution (50 mg/ml, Sigma, Milan, Italy) and incubated at 37 °C for one hour. The lysate was then subjected to nucleic acid extraction, using the Master-Pure complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA), according to the manufacturer´s instructions.

In the case of the curds and cheeses, five grams of samples was homogenized with 20 ml of ¼ strength Ringer solution. One ml of this solution was transferred to a 1.5 ml micro-tube, and the same procedure as the one described for the milk was followed. The resulting RNA was then treated with 3 µl of TURBO DNase (Ambion, Milan, Italy) and incubated for 3 h at 37 °C in order to eliminate the DNA. Complete DNA digestion was confirmed using 1 µl of extracted RNA in PCR reactions with primers 338f and 518r (Muyzer et al., 1993). If a PCR product was obtained, the DNase treatment was repeated. The resulting RNA was quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Milan, Italy) and standardized at 500 ng/µL.

RT reaction was carried out using M-MLV reverse transcriptase (Promega, Milan, Italy). An aliquot of 500 ng of RNA was mixed with 1 µl of random primer (Promega Milan, Italy) for each sample. A volume of 10 µl was reached by adding DNase- and RNase-free sterile water (Sigma), and the solution was incubated at 75 °C for 10 min. The mixture was then immediately placed on ice for 5 min, and then added to a microtube vial containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 2 mM of each dNTP, 1 µl of 200 U/µl M-MLV and 0.96 U of RNasin ribonuclease inhibitor (Promega). Reverse transcription was carried out at 42 °C for 1 h (Alessandria et al., 2010). The PCR reaction was performed in triplicate in a MyCycler (BioRad, Hercules, CA, USA) on each sample.

The V3 region of the 16S rRNA gene in the bacteria was amplified with primers 338f-GC/518r (Muyzer et al., 1993). PCR amplification was carried out adopting the same procedure used in previous studies on the ripening of hard cheese (Bautista-Gallego et al., 2014).

DGGE analysis and band sequencing

The RT-PCR products were analyzed by means of DGGE, using the DCode system apparatus (BioRad, Hercules, CA, USA). Polyacrylamide gels (8% W/V, Acrylamide-Bisacrylamide 40% solution 37.5:1, 0.8 mm thickness) were prepared using 30-50% urea-formamide denaturing gradients (100% corresponded to 8 M urea and 40% (wt/vol) formamide). The gels were run for 240 min at 130 V, stained with SYBR® Gold Nucleic Acid Gel Stain (Life Technologies, Milano), visualized under UVtransillumination and photographed by using UVIpro Platinum 1.1 Gel Software (Eppendorf).

Identification of the microbial populations was carried out by cutting selected bands from the DGGE gels. DNA was eluted in 50 µl water overnight at 4 °C, and again run in DGGE after re-amplification in order to check the electrophoretic mobility with respect to the excised bands. After the check, DNA was amplified again with non GC-clamp primers and sent for sequencing to GATC-Biotech (Cologne, Germany). A fingerprint database was created using Bionumerics software, version 4.6 (Applied Maths, Sint Marten Latem, Belgium).

Construction of a/the LAB standard curve for viable bacterial enumeration

The viable bacterial populations in the milk, curd and cheese were determined according to Alessandria et al. (2016). Milk, curd and cheese were sterilized. At the same time, overnight pure cultures of *Lactococcus lactis* subsp. *lactis, Lactobacillus plantarum, Lactobacillus pentosus, Enterococcus faecium* and *Lactobacillus helveticus* was prepared. The count of each culture is reached to approximately 10^9 (CFU/g or ml, which checked by culturing methods) and mixed. Serial dilutions of this overnight cultures were prepared using ¼ strength Ringer and 10 ml of each dilution (which contains the final concentrations of 10^8 to 10 CFU/g or ml), inoculated to 10 g or ml of food samples.

The samples were then homogenized in a Stomacher (Interscience Rockland, MA, USA) for 1 min, and 1 ml of each mixture was subjected to RNA extraction and reverse transcription, as described above. QPCR amplifications, targeting the V3 region of the 16S rRNA gene, were performed in a final volume of 25 µl, using SSo Advanced Sybr Green Supermix (Bio-Rad, Italy). One µl of cDNA was amplified with 338f and 518r primers at a final concentration of 400 nM in a Chromo 4 real-time PCR Detection System (Biorad, Milan, Italy) (Alessandria et al., 2010). In all cases and at each step the bacterial counts was checked by triple plate counting routine methods and Standard curves were constructed by plotting the threshold cycle (C_T) values against log CFU/g or CFU/ml on MRS agar. MRS agar plates were incubated at 37 °C for 48 h in microaerophilic conditions. The correlation coefficients ($R2$) and efficiency of amplification were calculated as described in Higuchi et al. (1993).

Quantification of the viable bacteria in the milk, curd and cheese was carried out by amplifying 1 µl of cDNA, and using the standard curves for the calculation of the counts.

Bacterial RNA analysis by means of Illumina high-throughput sequencing

A high-throughput sequencing approach was applied to a total of 15 samples (5 samples from milk, curd and cheese). cDNA, obtained as explained above, was used to study the microbial diversity of the active populations. The V3–V4 region of the 16S rRNA gene was amplified using the following primers: the 16S Amplicon Forward Primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and the 16S Amplicon Reverse Primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'), as reported in Klindworth et al. (2013). Twenty-five µl PCR reactions were prepared using 12.5 mL of the 2X KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA), 1 µM of each primer, 2.5 µL of cDNA template and PCR grade water. Twenty five cycles of denaturation (95 °C/ 30 sec), primer annealing (55 °C/30 sec) and primer elongation (72 °C/30 sec), followed by a final elongation step (72 °C/ 5 min), were carried out.

The PCR products were purified by means of an Agencourt AMPure kit (Beckman Coulter, Milan, Italy), and the resulting products were tagged by using the Nextera XT Index Kit (Illumina Inc. San Diego, CA), according to the manufacturer's instructions. The sequencing reaction was performed by Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, S.C. Controllo Alimenti e Igiene delle Produzioni (Turin, Italy) with a MiSeq Illumina instrument (Illumina) with V3 chemistry and generated 250 bp paired-end reads according to the manufacturer's instruction. The software used for the base-calling and Illumina barcode demultiplexing processes, were the MiSeq Control Soft. V2.3.0.3, the RTA v1.18.42.0 and the CASAVA v1.8.2.

Bioinformatics analysis

Paired-end reads were first merged using FLASH software (Magoc and Salzberg, 2011) with default parameters. Join reads were further quality filtered (at Phred > Q20) using QIIME 1.9.0 software (Caporaso et al. 2010) through multiple_extract_barcode.py and multiple_split_libraries_fastq.py script. Usearch v8.1 software (Edgar et al., 2011) was adopted for chimera filtering, using the 16S reference databases v9 (RDP classifier training database). OTUs were picked at 99% of similarity by means of UCLUST clustering methods (Edgar, 2010), and representative sequences of each cluster were used to assign taxonomy using the Greengenes 16S rRNA gene database, version 2013, by means of the RDP classifier (Wang et al. 2007). Representative sequences belonging to clusters identified as *Lactobacillus* spp. were double-checked using the BLAST (BLASTN) search program (http:// www.ncbi.nlm.nih.gov/blast/) to get the species level as best hit. Statistics and plotting were carried out in the R environment (www.r-project.org). Alpha diversity indices were calculated using the diversity

function of the vegan package (Dixon, 2003). The Shannon-Wiener diversity index H' was further analyzed using the t-test to assess any differences between the milk, curd and cheese samples. Weighted UniFrac distance matrices and OTU tables were used to perform Adonis and Anosim statistical tests in R environment. A filtered OTU table was generated at 0.5% abundance in at least two samples through QIIME. PICRUSt (Langille et al., 2013) was used to predict the abundance of the gene families, on the basis of 16S rRNA sequence data, as recently described (Ferrocino et al., 2016, Bokulich et al., 2015). Nearest Sequenced Taxon Indexes (NSTI) were calculated in order to evaluate the accuracy of the metagenome predictions (Langille et al., 2013). KEGG orthologs were then collapsed at a hierarchy level of 3, and the table was imported into R. The made4 package was adopted, and hierarchical Ward-linkage clustering, based on the Spearman correlation coefficients of the proportion of the activities belonging to lipid carbohydrates and amino acid metabolism pathways, was used to produce a heatplot.

All the sequencing data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (SRP074051).

Results

Identification and total bacterial counts during ripening

Eleven different bands, corresponding to the bacterial V3 region, were observed in the milk, curd and cheese samples (Tab. 1). *Lactococcus lactis* and *Leuconostoc mesenteroides* were the predominant species that recovered from all of the different types of samples, while *Streptococcus thermophilus* was only found in the curd. On the other hand, bands related to *Enterobacter aerogenes* and *Pseudomonas fluorescens* were mostly abundant in the milk and curd, but there was no trace of them in the cheese samples. Other LAB were randomly distributed between curd and cheese except *Lactococcus raffinolactis* that was not found in cheese.

The results of the quantification RT-PCR showed 8.5± 0.2 log CFU/ml for milk and 5.8± 0.2 and 3.5± 0.1 log CFU/g for curd and cheese, respectively. These data, which all approved by plate counting methods (data was not shown) underline that, the total bacterial populations decreased during the ripening process.

16S rRNA gene sequencing

A total of 1,216,136 raw reads (2x250bp) were obtained after sequencing. After joint, a total of 431,012 reads passed the filters applied through QIIME, with an average value of 28,734 reads/sample, and a sequence length of 457 bp. The estimated sample coverage for milk, curd and cheese are 94.69± 1.24, 95.21± 0.77 and 95.43± 1.38, respectively which indicates that there was a satisfactory coverage for all of the samples (ESC > 94%). Moreover, The average OUT numbers between different producers are 2333.8± 515.63 in milk, 1545.6± 489.01 in curd and 1594.4± 298.14 in cheese while Chaol values that

reflect the operational taxonomic unit richness are ranged from 3933.67± 440.16, 2758.24± 904.50 and 2419.38± 306.59 between milk, curd and cheese . In addition, overall sample diversity index (Shannon) are calculated as 6.47± 0.38, 5.17± 0.65 and 5.63±0.42 in milk, curd and cheese, respectively. Besides, the alpha-diversity showed that there was a higher level of complexity ($P < 0.05$) in the milk samples than in the curds and cheeses and between the cheeses and the curds ($P < 0.05$). It was possible to show, through a principal coordinate analysis (PCoA), with a Weighted UniFrac distance matrix as well as from Adonis and Anosim statistical tests, that the samples were grouped together according to their type ($P < 0.001$). In addition, no differences were found between the milk, cheese and curd samples for the different repeats, according to the Adonis and Anosim statistical tests ($P > 0.001$). Adonis and Anosim statistical tests of Weighted UniFrac distance matrix showed. significant difference among sample type ($P < 0.001$). Only OTUs with a relative abundance of 0.5% in at least two samples are shown in Figure 1. The abundance of the OTUs from the milks, curds and cheeses was averaged. The raw milk showed a varied microbiota, characterized by a predominance of *Moraxellaceae* (20% of the relative abundance), *Leuconostoc mesenteroides* (16%), *Staphylococcaceae* (13%), *Lactococcus raffinolactis* (7%), *Pseudomonas* sp. (5%), *Enterobacteriaceae* (6%), and *Lactococcus lactis* (6%).

The curd samples showed a predominance of *Leuconostoc mesenteroides* (43%), *Lactococcus lactis* (11%), *Enterobacteriaceae* (18%) and *Streptococcus* sp. (8%). The cheese samples were characterized by a predominance of *Lactococcus lactis* (30%), *Leuconostoc mesenteroides* (20%), *Lactobacillus fuchuensis* (10%), *Lactobacillus* sp. (10%) and *Pediococcus* sp. (10%).

The main differences between the three types of analyzed samples were due to the presence of several contaminant OTUs, such as *Pseudomonas* and *Moraxellaceae,* which were found to be significantly more abundant (g-test, $P < 0.001$) in the milk samples, while *Enterobacteriaceae* discriminated the curd samples. On the other hand, the cheese samples were characterized by the presence of several lactic acid bacteria (LAB), such as *Lactobacillus curvatus, Lactobacillus zeae*, *Lactobacillus fuchunensis*, *Lactococcus lactis* and *Lactobacillus pentosus* (g-test, $P < 0.001$).

The OTU co-occurrence/exclusion pattern is shown in Figure 2, where only significant correlations are reported (False Discovery Rate - FDR < 0.05). The characteristic OTU of the cheese samples, such as *Lactobacillus curvatus, Lactobacillus zeae*, *Lactobacillus fuchunensis*, *Lactococcus lactis* , *Lactobacillus pentosus* and *Lactobacillus kefiri,* showed the highest number of negative correlations, including a strong exclusion of *Lactococcus* sp., *Moraxellaceae*, *Enterobacteriaceae*, *Enterococcus* sp., *Staphylococcaceae* and other contaminants. *Moraxellaceae* showed a positive correlation with *Pseudomonas* sp., *Rothia* sp. and *Staphylococacceae*.

As far as the predicted metagenomes are concerned, the weighted nearest sequenced taxon index (NSTI) of the samples, expressed as the mean ± SD, was 0.028 ± 0.003. This index is the average branch length

that separates each OTU from a reference bacterial genome, weighted by the abundance of that OTU in the sample. Thus, an NSTI score of 0.028 indicates a satisfactory accuracy for all of the samples (98%). It was possible to differentiate the three different sample types (cheese, curd and milk) on the basis of the predicted gene repertoires, associated with their microbiota (Fig. 3). A sub-cluster was mainly identified for samples derived from the milk samples, and another cluster was identified for most of the cheeses and curd samples. The dominant spoilage-related microbiota from the milk samples could considered, as a microbial proteolitic consortium, due to presence of a presumptive abundance of genes related to lysine, valine and the tryptophan metabolism. Besides, metabolic pathways related to a lipid metabolism, such as a steroid metabolism, and to biosynthesis of the unsaturated fatty acids are also include. The other group (cheese and curd samples) showed a higher presumptive abundance of genes related to a carbohydrate metabolism, such as starch and sucrose metabolisms, amino-sugar and fructose metabolisms as well as amino acid catabolism related genes.

Discussion

Liqvan is a traditional Iranian cheese that has been found to represent an interesting case for the study of fermented food as it originates from a particular region in Iran, and it has long been prepared the same way using an ancient methodology. Moreover, as this cheese is made from raw milk without the addition of starter cultures, it can be speculated that its microbial ecology mainly depends on indigenous microbiota. In this context, the first step towards protecting the microbial diversity of this traditional regional food is to elucidate the evolution of the active microbial populations during its manufacturing and ripening, in order to guarantee both the safety and the quality of the final products (Alegría et al., 2012). Previous studies on Liqvan cheese (Edalatian et al., 2012; Kafili et al., 2009; Navidghasemizad et al., 2009; Barouei et al., 2008), which were mainly based on the DNA PCR-DGGE method, showed that *Lactococcus lactis, Lactococcus raffinolactis, Lactobacillus plantarum, Lactococcus garvieae, Lactobacillus sakei, Lactobacillus casei* and *paracaei, Leuconostoc mesenteroides, Streptococcus* sp. and *Enterobacter* sp. are the predominant groups of bacteria in this cheese.

To the best of our knowledge, no data have been reported on RNA based DGGE or Illumina analysis of the microbiota of this traditional cheese, yet. In our research, the microbial ecology and active bacterial communities of Liqvan cheese have been investigated using different culture-independent methods, at an RNA level, during the ripening process.

Fifteen samples from raw milk, curd and cheese were collected from five different producers in different households in the Liqvan village, East Azerbaijan province of Iran, the Mydanchay district of the central part of the city of Tabriz, and were examined. The microbial investigation focused on bacteria population targeting the V3 region of the 16S rRNA gene.

RNA-seq DGGE analysis of our samples indicates mainly that lactic acid bacteria mostly includes *Lactococcus* sp., *Leuconostoc* sp., *Lactobacillus* sp. and *Streptococcus* sp. are detected in all stages of production while spoilage bacteria like S*taphylococcus* sp., *Enterobacter* sp. and *Pseudomonas* sp. are detected only at milk samples and not in curds and cheese.

Phylogenetic assignment of NGS sequence data analysis revealed that microbial α-diversity was more remarkable at milk level and corresponds to *Firmicutes, Proteobacteria* and *Actinobacteria* however, this diversity of population decreased during ripening dominated by *Firmicutes*.

At the family level, *Moraxcellaceae, Pseudomonadaceae, Corynebacterineae*, *Flavobacteriaceae*, *Comamonadaceae, Staphylococaceae* and *Enterobacteriaceae* were the most abundant contaminant microbiota in the milk, while *Lactobacillaceae*, *Streptococcaceae* and *Leuconostocaceae* were the predominant microbial families in the curds and cheese. There are some number of notable observations among the subdominant populations. Some records from sheep feces, sheep wool, soil and grass contaminants like those that *Brochothrix* sp. and *Rothia* sp. are also detected at milk samples nevertheless there is no trace of them in curd and cheese. Furthermore, *Streptococcus* sp. especially *Streptococcus thermophiles* were detected exclusively in curd samples while its population decreased remarkably in cheese.

The microbial co-occurrence/exclusion patterns also proved these results, as there was a strong co-exclusion effect between *Lactobacillaceae and Enterobacteriaceae,* as well as between *Lactobacillaceae* and *Staphylococcaceae*. *Lactobacillus* species co-occurred each other. These results confirmed that the LAB microbial populations tended to dominate the cheese microbiota and to limit the development of spoilage bacteria, as has recently been demonstrated for other types of cheeses (Alessandria et al., 2016).

This diversity and dynamics of prokaryotes in this study are in agreement with the results that had previously been reported for Liqvan and other semi hard raw milk based cheeses (Bozoudi et al., 2016; Ryssel et al., 2015; Masoud et al., 2011; Rantsiou et al., 2008) although some differences were noted which may related to difference in samples origin and producers.

The use of predicted metagenomes has proved to be useful for the observation of the putative gene repertoires in the analyzed samples. The milk samples were characterized by gene profiles that were related to the aminoacid metabolism, and high proteolitic activity due to the spoilage bacteria, while putative genes characterized the curd and cheese related to carbohydrate depletion, which was presumably associated with the nature of the highly competitive LAB that dominated during the ripening period.

In conclusion, in the present work, the study of microbial population with culture independent techniques has provided a better understanding of the bacterial structure of Liqvan cheese during its processing and ripening.

Acknowledgments

The authors would like to thank the Iranian Biological Resource Center (IBRC), ACECR for their partial financial support. We also dedicate our special thanks to the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, S.C. Controllo Alimenti e Igiene delle Produzioni (Turin, Italy) for performing the 16S rRNA sequencing.

References

Aldrete-Tapia, A., Escobar-Ramírez, M.C., Tamplin, M.L., Hernández-Iturriaga, M., 2014. High-throughput sequencing of microbial communities in Poro cheese, an artisanal Mexican cheese. Food Microbiol. 44, 136–141. doi:10.1016/j.fm.2014.05.022.

Alessandria, V., Ferrocino, I., De Filippis, F., Fontana, M., Rantsiou, K., Ercolini, D., Cocolin, L., 2016. Microbiota of an Italian Grana-Like Cheese during Manufacture and Ripening, Unraveled by 16S rRNA-Based Approaches. Appl. Environ. Microbiol. 82(13), 3988-95.

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. J. Microbiol. Biotechnol. 26(12), 2211-2221.

Alegría, A., Szczesny, P., Mayo, B., Bardowski, J., Kowalczyk, M., 2012. Biodiversity in Oscypek, a Traditional Polish Cheese, Determined by Culture-Dependent and -Independent Approaches. Appl. Environ. Microbiol. 78(6), 1890-1898.

Barouei, J., Karbassi, A., B. Ghoddusi, H., Mortazavi, A., 2008. Lactic microflora present in Liqvan ewes' milk cheese. Int. J. Food. Prop. 11(2), 407-414.

Bassi, D., Puglisi, E., Cocconcelli,P.S., 2015. Understanding the bacterial communities of hard cheese with blowing defect. Food. Microbiol. 52, 106-118.

Bautista-Gallego, J., Alessandria, A., Fontana, M., Bisotti, S., Taricco, S., Dolci, P., Cocolin, L., Rantsiou, K., 2014. Diversity and functional characterization of *Lactobacillus* spp. isolated throughout the ripening of a hard cheese. Int. J. Food. Microbiol. 181, 60-66.

Bokulich, N., Amiranashvili, L., Chitchyan, K., Ghazanchyan, N., Darbinyan, K., Gagelidze, N., Sadunishvili, T., Goginyan, V., Kvesitadze, G., Torok, T., A. Mills, D., 2015. Microbial biogeography of the transnational fermented milk matsoni. Food. Microbiol. 50, 12-19.

Bozoudi, D., Torriani, S., Zdragas, A., Litopoulou-Tzanetaki, E., 2016. Assessment of microbial diversity of the dominant microbiota in fresh and mature PDO Feta cheese made at three mountainous areas of Greece. LWT-food. Sci. Technol. 72, 525-533.

Carraro, L., Maifreni, M., Bartolomeoli, I., Martino, M. E., Novelli, E., Frigo, F., Marino, M., Cardazzo, B., 2011. Comparison of culture-dependent and –independent methods for bacterial community monitoring during Montasio cheese manufacturing. Res. Microbiol. 162, 231-239.

Cocolin, L., Alessandria, L., Dolci, P., Gorra, R., Rantsiou, K., 2013. Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. Int. J. Food. Microbiol. 167, 29-43.

Dalmasso, A., Dolores Soto del Rio, M.D.L., Civera, T., Pattono, D., Cardazzo, B., Bottero, M.T., 2016. Characterization of microbiota in Plaisentif cheese by high-throughput sequencing. LWT – Food. Sci. Technol. 69, 490-496.

De Pasquale, I., Di Cagno, R., Buchin, S., De Angelis, M., Gobbetti, M., 2016. Spatial distribution of the metabolically active microbiota within Italian PDO ewes' milk cheeses. PLoS One 11, 1–23. doi:10.1371/journal.pone.0153213.

Dixon, P., 2003. VEGAN, a package of R functions for community ecology. J. Veg. Sci. 14, 927–930.

Dugat-Bony, E., Straub, C., Teissandier, A., Onésime, D., Loux, V., Monnet, C., Irlinger, F., Landaud, S., Leclercq-Perlat, M.-N., Bento, P., Fraud, S., Gibrat, J.-F., Aubert, J., Fer, F., Guédon, E., Pons, N., Kennedy, S., Beckerich, J.-M., Swennen, D., Bonnarme, P., 2015. Overview of a surface-ripened cheese community functioning by meta-omics analyses. PLoS One 10, e0124360. doi:10.1371/journal.pone.0124360.

Edalatian, M.R., Habibi Najafi, M.B., Mortazavi, A., Mayo, B., 2012. The biodiversity and evolution of lactic flora during ripening of the Iranian semisoft Lighvan cheese. Int. J. Dairy. Technol. 65(1), 81-89.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 27(16), 2194-2200. doi: 10.1093/bioinformatics/btr381 [PMID 21700674].

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

Escobar-Zepeda, A., Sanchez-Flores, A., Baruch, M.Q., 2016. Metagenomic analysis of a Mexican ripened cheese reveals a unique complex microbiota. Food. Microbiol. 57, 116-127.

Ferrocino, I., Greppi, A., Storia, A.L., Rantsiou, K., Ercolini, D., Cocolin, L., 2016. Impact of nisin-activated packaging on microbiota of beef burgers during storage. Appl. Environ. Microbiol. 82(2), 549-559.

Garofalo, C., Osimani, A., Milanović, V., Aquilanti, L., De Filippis, F., Stellato, G., Di Mauro, S., Turchetti, B., Buzzini, P., Ercolini, D., Clementi, F., 2015. Bacteria and yeast microbiota in milk kefir grains from different Italian regions. Food. Microbiol. 49, 123-33.

Higuchi, R., Fockler, C., Dollinger, G., Watson, R., 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Nat. Biotechnol. 11, 1026-1030.

Kafili, T., Razavi, S.H., Emam Djomeh, Z., Naghavi, M.R., Álvarez-Martín, P., Mayo, B., 2009. Microbial characterization of Iranian traditional Lighvan cheese over manufacturing and ripening via culturing and PCR-DGGE analysis: identification and typing of dominant lactobacilli. Eur. Food. Res. Technol. 229, 83–92.

Klindworth, A., Pruesse, E., Schweer, T., Peplles, J., Quast, C., Horn, M., Oliver Glöckner, F., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic. Acids. Res. 41(1).

Langille, M. G.I., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J., Clemente, J.C., Burkepile, D.E., Vega Thurber, R.L., Knight, R., Beiko, R.G., Huttenhower, C., 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 8, 1-10.

Magoc, T., Salzberg, S., 2011. FLASH: Fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 27(21), 2957-2963.

Masoud, W., Takamiya, M., K. Vogensen, F., Lillevang, S., Al-Soud, W.A., Sørensen, 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. Int. Dairy. J. 21, 142-148.

Minervini, F., Lattanzi, A., Angelis, M.D., Celano, G., Gobbetti, M., 2015. House microbiotas as sources of lactic acid bacteria and yeasts in traditional Italian sourdoughs. Food. Microbiol. 52, 66-76.

Muyzer, G., De Waal, EC., Uitterlinden, AG., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59:695–700.

Navidghasemizad, S., Hesari, J., Saris, P., Nahaei, M.R., 2009. Isolation of lactic acid bacteria from Lighvan cheese, a semihard cheese made from raw sheep milk in Iran. Int. J. Dairy. Technol. 62(2), 260-264.

Ndoye, B., Rasolofo, E.A., LaPointe, G., Roy, D., 2011. A review of the molecular approaches to investigate the diversity and activity of cheese microbiota. Dairy. Sci. Technol. 91, 495–524.

O'Sullivan, D.J., Cotter, P.D., O'Sullivan, O., Giblin, L., McSweeney, P.L.H., Sheehan, J.J., 2015. Temporal and Spatial Differences in Microbial Composition during the Manufacture of a Continental-Type Cheese. Appl. Environ. Microbiol. 81, 2525–2533.

Rantsiou, K., Urso, R., Dolci, P., Comi, G., Cocolin, L., 2008. Microflora of Feta cheese from four Greek manufacturers. Int. J. Food. Microbiol. 126 (1-2), 36-42.

Ryssel, M., Johansena, P., Al-Soud, W.A., Sørensen, S., Arneborg, N., Jespersen, L., 2015. Microbial diversity and dynamics throughout manufacturing and ripening of surface ripened semi-hard Danish Danbo cheeses investigated by culture-independent techniques. Int. J. Food. Microbiol. 215, 124-130.

Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microb 73(16): 5261-5267.

462 **Table 1:** Identification of the selected bands from bacterial DGGE gels and their occurence in the samples
463 considered in this study

Bacterial species	**Stage of manufacturing**		
	Milk	Curd	Cheese
Lactococcus lactis ssp. *lactis*	+	+	+
Lactococcus lactis ssp. *cremoris*	-	+	+
Leuconostoc mesenteroides	+	+	+
Lactococcus raffinolactis	+	+	-
Lactobacillus pentosus	-	-	+
Lactococcus garvieae	-	+	+
Enterobacter aerogenes	+	+	_
Pseudomonas fluorescens	+	-	-
Lactobacillus fuchuensis	-	-	+
Staphylococcus sp.	+	-	-
Streptococcus thermophilus	-	+	-

464
465
466
467
468
469
470
471
472
473
474
475
476
477

FIG 1: Relative abundance of the major taxonomic groups detected by means of 16S sequencing. Only OTUs with an incidence above 0.5% in at least two samples are shown. The abundance of OTUs from the milks, curds and cheeses was averaged.

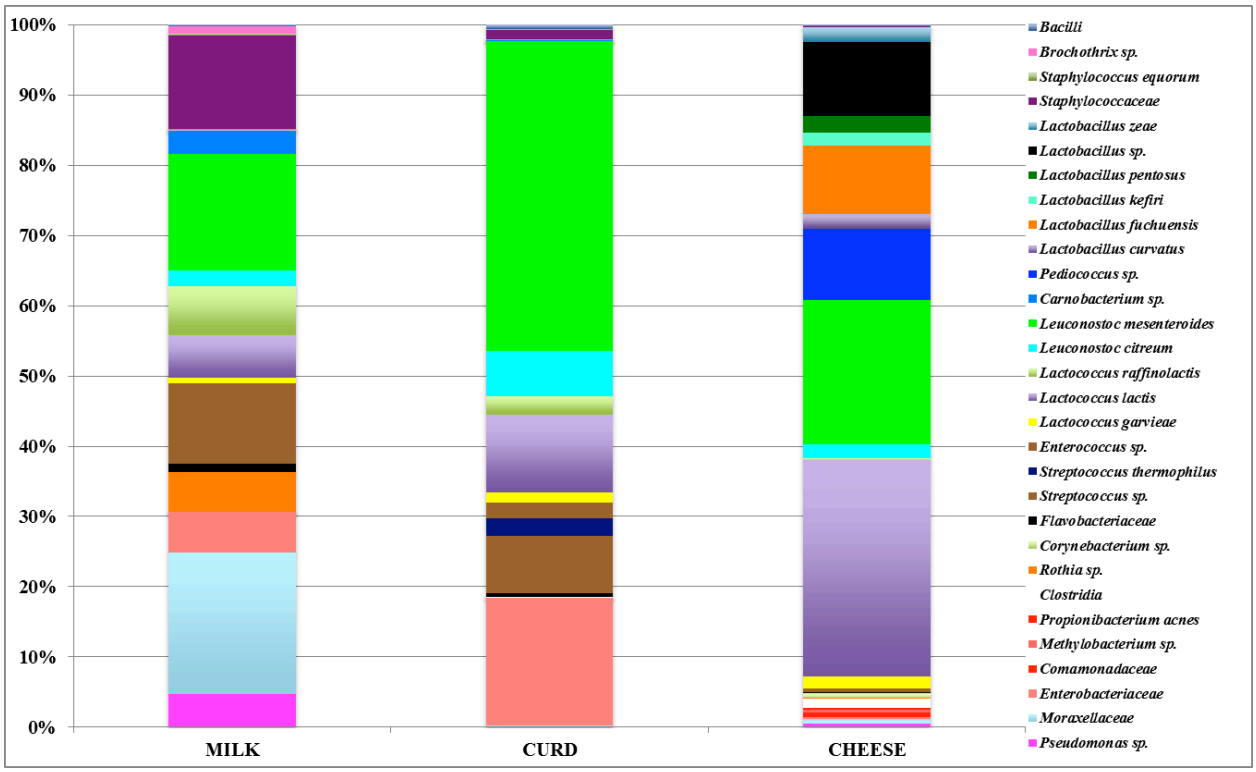

FIG 2: Significant co-occurrence and co-exclusion relationships between bacterial OTUs. Spearman's rank correlation matrix of OTUs with > 0.5% abundance in at least 2 samples. Strong correlations are indicated with large squares, whereas weak correlations are indicated with small squares. The color of the scale bar denotes the nature of the correlation, with 1 indicating a perfectly positive correlation (dark blue) and -1 indicating a perfectly negative correlation (dark red). Only significant correlations (FDR < 0.05) are shown.

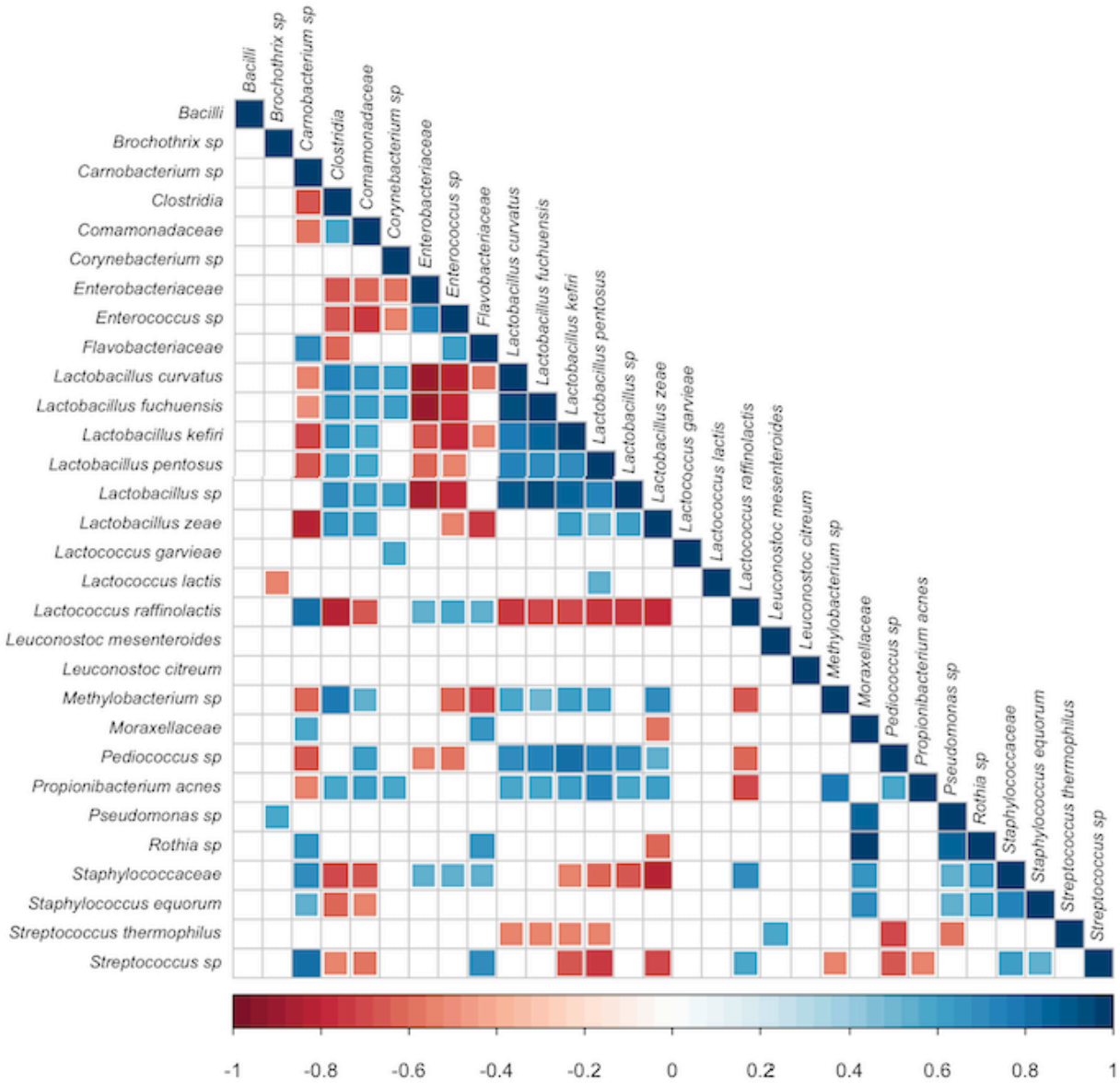

FIG 3: Heat plot of the abundances of genes presumptively belonging to carbohydrate (blue squares), amino acid (red squares) and lipid (green squares) metabolism pathways in the milk (upper blue bar), curd (upper green bar) and cheese (upper red bar). Rows and columns are clustered by means of Ward linkage hierarchical clustering. The intensity of the colors represents the degree of correlation between the samples and KO as measured by Spearman's correlations.