

Contribution of natural milk culture to microbiota, safety and hygiene of raw milk cheese produced in alpine *malga*

Rosaria Lucchini,¹ Barbara Cardazzo,² Lisa Carraro,² Michele Negrinotti,³ Stefania Balzan,² Enrico Novelli,² Luca Fasolato,² Franco Fasoli,³ Giovanni Farina¹

¹Institute for Experimental Veterinary Medicine of Venezia, Section of Trento;

²Department of Comparative Biomedicine and Food Science, University of Padua, ³Operative Unit of Hygiene and Veterinary Public Health, Agency for Healthcare Services of Autonomous Province of Trento, Italy

Abstract

Processing of alpine milk in *malga* farms is carried out under conditions that can favor contamination by coliforms, coagulase-positive staphylococci, or pathogens such as *Listeria monocytogenes*. With the aim to improve the hygienic characteristics and safety of cheese produced in four *malga* farms the use of lyophilized Natural Milk Culture prepared with selected strains was tested. Two cheesemaking tests were carried out in the same day always starting from the same milk: in the first case following the *malga* recipe that uses either Natural Whey Culture or without the addition of a starter, in the second one using a Natural Milk Culture. Cheesemaking were carried out in four *malga* farms located in the west area of Trentino region within the same week. For hygienic and safety evaluation, aerobic colony count, coagulase-positive staphylococci, *Escherichia coli*, staphylococcal toxins, *Listeria monocytogenes*, and *Salmonella* spp. pH and a_w were determined in raw milk from evening and morning milking, curd in vat, curd after extraction and two months-ripened cheese. Pathogens or toxins, high values of coagulase-positive staphylococci and *E. coli* were not found in cheese samples. However, in the curd coagulase-positive staphylococci reached values almost of 5 Log CFU/g in the two *malga* without starter cultures. The use of Natural Milk Culture reduced *E. coli* counts. In addition, DNA was extracted from cheese samples and from Natural Milk Culture and the composition of the microbial community determined by Next Generation Sequencing method. The determination of cheese microbial communities

demonstrated that the use of Natural Milk Culture exerted different effects in the different *malga*, in any case preserving bacterial biodiversity.

Introduction

In the recent years the interest of consumers toward mountain dairy products has greatly increased and several studies have been carried out on milk and cheese obtained under the unique Alpine conditions. The Alps are a 1300 km mountain chain, located in the north of Italy (Povolo *et al.*, 2013). The ancient tradition of producing raw cow's milk cheese in small dairy farms is common across the Alpine region, including Italian, Austrian, and Swiss Alps. Small farms called *malga* (or *alm* in general) are located between 1000 and 2500 m above sea level and during summer season house the cattle that from the valley are moved to alpine pastures. Both milk collection and cheesemaking are carried out in the *malga* and cheese production follows a procedure that varies for each *malga*, according structural conditions, quality of raw material, hygienic state from milk collection, cheesemaking, to ripening. All such characteristics might either favor or prevent the presence of coliforms, coagulase-positive staphylococci (CPS) and, pathogens such as *L. monocytogenes* (Lm) Contamination can be recurrent, with severe economic losses and risks for food safety. Contamination of dairy products with pathogens can be counteracted by milk pasteurization and/or by using lactic bacterial cultures (LABs) that help curdling and can compete with pathogens preventing their growth (Lucchini 2015, Topisirovic *et al.*, 2006).

Normally, in *malga* farms, commercial starters are not used and microbial fermentation is spontaneous, carried out by the autochthonous microbiota or by adding their own production whey culture (Natural Whey Culture, NWC) (Carafa *et al.*, 2016). While the use of commercial starters may reduce the richness and variability of the cheese microbiota and level off the typical organoleptic characteristics in traditional ripened cheeses, it allows to obtain cheese with desired and predictable properties. In addition, the use of commercial starters improves the standardization of cheesemaking procedures reducing the risk of contamination with pathogens or spoilers (Montel *et al.*, 2014; Mucchetti and Neviani, 2006).

According to the EFSA Report (2014), 5.4% of foodborne outbreaks on 763 occurring in 21 European countries are attributed to the consumption of contaminated

Correspondence: Barbara Cardazzo, Department of Comparative Biomedicine and Food Science, University of Padova, Viale dell'Università, 16, Legnaro, 35020, Padova, Italy.

Tel.: +39.049.8272965 - Fax: +39.049.8272973. E-mail: barbara.cardazzo@unipd.it

Key words: Cheese; Hygiene; Natural Milk Culture; NGS, *malga*.

Contributions: the authors contributed equally.

Conflict of interest: the authors declare no potential conflict of interest.

Funding: none.

Received for publication: 31 July 2017.

Revision received: 10 November 2017.

Accepted for publication: 11 November 2017.

This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0).

©Copyright R. Lucchini *et al.*, 2018

Licensee PAGEPress, Italy

Italian Journal of Food Safety 2018; 7:6967

doi: 10.4081/ijfs.2018.6967

cheeses. The pathogens most frequently implicated are: Lm, *Staphylococcus aureus*, *Salmonella* spp., Ec O157: H7 and more rarely *Mycobacterium avium* subsp. *paratuberculosis* (Kousta *et al.*, 2010). Many authors describe *S. aureus* as the main cause for food poisoning associated with dairy products, especially for not ripened cheese (Rosengren *et al.*, 2010) and raw milk cheese (Brooks *et al.*, 2012; De Buyser *et al.*, 2001; Duquenne *et al.*, 2010).

Good hygienic practices to reduce risks cannot be always implemented, considering that *malga* facilities are often not sufficiently equipped. Therefore, further options for contamination containment (such as technological practices) should be considered to prevent risks related to the consumption of raw cow's milk cheese.

The purpose of the study was to experiment the use of Natural Milk Culture (NMC) in raw milk processing and to evaluate its effects on microbial composition and on hygiene and safety characteristics of cheese. The obtained results may then be used to identify corrective actions that may be applied by the operators to ensure consumer safety.

Materials and Methods

Cheesemaking

The preparation of experimental NMC was performed by Bioagro Laboratory, Veneto Agricoltura (Thiene, Italy). Three strains were isolated from *malga* milk and curd, tested for technological characteristics and identified by RAPD-PCR methods as two different strains of *Streptococcus thermophilus* and a strain of *Lactobacillus delbrueckii* subsp. *Bulgarius* (Andrighetto *et al.*, 2002). Lyophilized NMC containing the three selected strains cultivated in milk was produced and tested in a previously experimental study (Paternolli *et al.*, 2014).

Traditional mountain *malga* cheese is a semicooked cheese made by mixing raw cow's milk from both morning- and overnight skimmed milk collected the previous evening. Curdling occurs at 35-38°C, milk coagulation is obtained using a commercial rennet, then the curd is cooked at about 43-45°C for at least 30 minutes. The curd is cut into rice-like particles and kept at the same temperature until pouring into molds. After two days of brine salting, wheels of cheese are ripened for two months in a dedicated *malga* room at 12-18°C on wooden shelves, turning them over periodically. Ripening is then completed in a storeroom down in the valley for at least 9-12 months. Such traditional cheese making process was followed in four different *malga*-farms (called M1, M2, M3 and M4), located in the west area of Trentino region. In each farm two cheesemaking procedures were followed: the first one following the *malga* recipe, the second one adding experimental NMC to promote coagulation, together with a commercial rennet. In *malga* M1 and M2 the recipe included the use of NWC, made from *malga* cheesemaker every day and added to drive primary fermentation. In M3 and M4, microbial fermentation was spontaneous, being started and carried on by autochthonous bacteria until the end of the ripening (N).

Sampling

Samples of bulk raw milk, curd from vat, curd after extraction, and two-months ripened cheese were collected. All milk, curd, and cheese samples were analyzed within 24 hours using standard microbiological methods. An aliquot from all cheese samples was frozen and stored at -80°C for NGS analysis. At the same time of cheese sampling, the following cheese quality parameters were evaluated: odor, colour, obvious defects, presence and shape of holes in the cheese.

pH and water activity detection

pH and water activity (a_w) were performed according to ISO 2917:1999 and ISO 21807:2004, respectively. Sample of milk (evening and morning bulk milk, after mixing in vat, before addition of starter cultures, before rennet), samples of curd (curd in vat and after extraction) and two-months ripened cheese were tested for pH. Samples of curd and two-months ripened cheese were analysed for a_w .

Culture-dependent analysis

In order to evaluate cheese for safety and hygienic microbiology criteria, the following analyses were performed. (A) *Salmonella* spp by real time PCR (iQ-check™ *Salmonella* spp II kit Bio Rad, Hercules, CA, USA) with validation certificate AFNOR BRD 07/06-07/04, (B) *Listeria monocytogenes* by real time PCR (iQ-check™ *Listeria monocytogenes* II kitBio Rad, Hercules, CA, USA) with validation certificate No: AFNOR BRD 07/10-04/05, (C) staphylococcal enterotoxins according to the European Union Reference Laboratory for CPS protocol with the VIDAS SET 2 test (bioMerieux, Marcy l'Etoile, France); (D) aerobic colony count (ACC) according to ISO 4833-1:2013, (E) *Escherichia coli* (Ec) according to ISO 16649-2:2001, (F) coagulase-positive staphylococci according to ISO 6888-2:1999/Amd 1 2003.

Samples of bulk raw milk and samples of curd in vat and after extraction were tested applying A,B,D,E, F analysis. Cheese samples were tested for all of analysis (A-F). The results obtained from the different samples were calculated according to the ISO 7218:2007/Amd 1 2013, corrected version 2014/04/15.

Culture-independent analysis on cheese

The extraction of DNA from 150 mg of cheese and from the pellet obtained from 1mL of NMC was carried out with Invisorb Spin Tissue Mini Kit (Invitex GmbH, Berlin, Germany) following the instructions provided by the kit manufacturer. At the end of the procedure, DNA was eluted in 50 µL of Elution Buffer. The concentration and purity of DNA were analysed using a NanoDrop ND-1000 (Thermo Scientific, Massachusetts, United States). To determine the exact quantity to be used for NGS library construction, an aliquot of diluted (1:200) DNA template was amplified in a final volume of 10 µL containing 5 µL of Platinum SYBR Green qPCR Supermix UDG (Invitrogen, Life Technologies, Monza, Italy), and 0.25 µL of each primer 331F and 797R (10 µM) (Nadkarni *et al.*, 2002). For NGS sequencing, the V3-V4

regions of the 16S rRNA gene were amplified using the primers 331F 5'-TCC-TACGGGAGGCAGCAGT-3' and 797F 5'-GGACTACCAGGGTATCTAATCCTGTT (Nadkarni *et al.*, 2002). The primers were modified with a forward overhang (5'-TCGTCCGGCAGCGTCAGATGTG-TATAAGAGACAG-[locus-specific sequence]-3') and a reverse overhang (5'-GTCTCGTGGGCTCGGAGATGTG-TATAAGAGACAG-[locus-specific sequence]-3'), which was necessary for dual index library preparation. PCR products were purified using AMPure purification kit (Agenour, Beverly, MA). After purification, every sample was quantified using the Qubit® 2.0 fluorometer (Invitrogen). The quality of amplicon libraries was tested using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Libraries were pooled and sequenced by BMR Genomics (Padova, Italy) using a Miseq Illumina 2x300 version 3. Sequence data were deposited in SRA database (accession number SRP109249). Raw sequences data were filtered, trimmed, and merged through with CLC Genomics Workbench. Qiime version 1.9 was used to perform full analysis from operational taxonomic units (OTUs) picking to statistical analysis (Caporaso *et al.*, 2010). The *pick_closed_reference_otus* wrapper (minimum similarity 0.8) was used for OTU picking with Greengenes 13.8 v as reference database. Sequences were then filtered with *filter_samples_from_otu_table.py* to remove any OTU observed in the blank control. OTUs represented by less than 0.005% of total sequences were discarded (Bokulich *et al.*, 2013). To characterize microbial communities and to perform functional analyses, the following wrappers have been employed: *assign_taxonomy* (to assign Greengenes taxonomy to OTUs), *make_otu_table* (to create biom file with OTUs and taxonomy), *compute_core_microbiome* (to calculate the core microbiome), *summarize_taxa_through_plots* (to produce taxonomical files and charts), *alpha_rarefaction* and *beta_diversity_through_plots* (to assess respectively alpha- and beta-rarefaction diversity indices), *principal_coordinates.py* (to compare groups of samples based on phylogenetic distance metrics). ANOVA was performed using CALYPSO version 7.18.

Results and Discussion

pH values

The pH of products obtained by the addition of NMC from different *malga*

farms showed a similar pattern and the curd reached values between 5.2 and 5.4 (as shown in Table 1). The curd obtained with the M1 recipe showed pH values not lower than 6, a circumstance potentially hazardous in presence of pathogens or enterotoxin producer *Staphylococcus aureus* (Schelin *et al.*, 2011). In M2 acidification curves are similar, but it should be pointed out that milk had very low pH values, as can be seen in Figure 1A. In M3 and M4, pH in curd without starter cultures was much higher than that obtained with NMC. Acidification is important for milk coagulation and to counteract the growth of undesirable microorganisms. In fact, low pH values and their rapid decrease, as well as the presence of lactic acid deriving from lactic bacteria metabolism, may inhibit the growth of *S. aureus* (Schelin *et al.*, 2011) and *E. coli* (Presser *et al.*, 1997). Also, the production of enterotoxins is counteracted by low level of pH values and the presence of lactic acid (Schelin *et al.*, 2011).

Culture-dependent analysis

Listeria monocytogenes and *Salmonella* spp were not found in milk, curd and cheese samples. Moreover, staphylococcal enterotoxins were not detectable in two months

ripened cheeses.

The ACC in raw milk were from 4.0 to 6.7 Log CFU/mL, as shown in Table 1. M1 raw milk presented ACC 30°C values less than 5 Log CFU/mL and it complied with the requirements laid down in European Regulation 853/2004, Section IX, Chapter I, point 3 related to raw milk criteria [ACC 30°C <5 Log CFU/mL, rolling geometric average over a two-month period, with at least two samples per month (European Commission, 2004)], assessment of these parameters done before the cattle were moved from the valley to alpine pastures). On the opposite, the other three *malga* farms showed values higher than 5 Log CFU/mL. In M2, ACC values were very high in both evening and morning bulk milk, 6.7 Log CFU/mL and 6.1 Log CFU/mL respectively, while in M3 and M4 only evening bulk milk, (5.8 Log CFU/mL and 6.1 Log CFU/mL respectively). These results likely suggest insufficient hygiene practices either in the milking process and in refrigeration tank sanitization, as well as an insufficient rapid cooling of milk.

CPS contamination in raw milk was similar in all four *malga* farms with mean values of 1.7±0.5 Log CFU/mL in evening

bulk milk and 2.8±0.4 Log CFU/mL in morning bulk milk. CPS were not detectable in any two months-ripened cheese. CPS developed during curdling and after extraction of the curd, not exceeding 6 Log CFU/g in all four *malga* farms, an important threshold for staphylococcal enterotoxins development (Bennett *et al.*, 1983). There was no measurable difference for CPS in cheesemaking process using NMC or *malga* recipe. However in M4, CPS values for curd in vat (3.6 Log CFU/g) and after extraction (3.8 Log CFU/g) made with NMC were 1 log CFU/g lower than CPS values in samples produced with N recipe (4.5 Log CFU/g and 4.6 Log CFU/g respectively). This is in agreement with a role of acidification in reducing CPS counts (Schelin *et al.*, 2011), as in M4 products a lower pH in NMC was observed (Figure 1B). *E. coli* was not detectable in any sample from M1. In M2, *E. coli* reached similar values during cheese production either with NMC or M2 recipe (NWC). In M3, *E. coli* developed with similar values in N_cheesemaking compared to NMC_cheesemaking, although in NMC cheese samples *E. coli* counts were 1.8 Log CFU/g, lower than N_cheese sample (3.3 Log CFU/g). In M4

Table 1. Microbiological counts, pH, water activity and biodiversity of Malga samples.

Samples and analysis	M1_NMC	M1_NWC	M2_NMC	M2_NWC	M3_NMC	M3_N	M4_NMC	M4_N
Evening bulk milk								
ACC	4.0	4.0	6.7	6.7	5.8	5.8	6.1	5.4
CPS	1.6	1.0	1.6	1.6	2.1	2.1	2.3	1.0
Ec	nd	nd	4.4	4.4	1.6	1.6	4.0	3.9
pH	6.7	6.7	5.7	5.7	6.6	6.6	6.5	6.6
Morning bulk milk								
ACC	4.3	4.3	6.1	6.1	4.3	4.3	4.2	4.1
CPS	2.8	2.4	3.4	3.4	2.8	2.8	2.5	2.6
Ec	nd	nd	2.1	2.1	1.6	1.6	1.0	1.6
pH	6.7	6.7	6.6	6.6	6.7	6.7	6.7	6.6
pH Milk in vat	6.5	6.5	6.1	6.2	6.5	6.8	6.9	7.0
pH Milk before starter	6.5	6.5	6.1	6.1	6.5	6.8	6.9	6.9
pH Milk before rennet	6.5	6.3	6.1	6.1	6.5	6.8	6.8	6.9
pH Starting curdling	6.3	6.0	6.0	6.2	6.8	6.9	6.8	7.0
Curd in vat								
CPS	4.1	4.0	3.6	4.0	4.7	4.7	3.6	4.5
Ec	nd	nd	4.8	5.0	3.8	4.0	4.8	6.0
pH	5.3	6.1	5.2	5.5	5.4	6.2	5.4	6.5
Curd after extraction								
CPS	4.1	4.0	3.9	4.0	4.8	4.7	3.8	4.6
Ec	nd	nd	5.0	5.1	4.0	4.1	5.1	6.2
pH	5.3	6.1	5.2	5.3	5.4	6.1	5.3	6.3
Two months ripened cheese								
ACC	7.8	7.4	7.4	7.1	7.4	6.5	7.5	7.0
CPS	nd	nd	nd	nd	nd	nd	nd	nd
<i>E. coli</i>	nd	nd	1.6	1.0	1.8	3.3	1.0	4.5
pH	5.2	5.5	5.5	5.6	5.7	5.7	5.3	5.3
Water activity	0.95	0.95	0.95	0.96	0.96	0.97	0.94	0.94
Biodiversity index (observed species)	88.1	114.9	90.6	114.8	117.8	107	88.5	113.3

nd = not detectable; ACC, CPS and Ec are intended Log CFU/g.

evening raw milk showed an important contamination from *E. coli* (4.0 Log CFU/mL). In the early stages of curdling, Ec in NMC_curd were 4.8 Log CFU/mL, while in N_curd were 6.0 Log CFU/mL. Values increased in the curd after extraction, respectively 5.1 Log CFU/g and 6.2 Log CFU/g, and decreased in two months-ripened cheese. In N_cheese Ec were 4.5 Log CFU/g, higher than NMC_cheese (1 Log CFU/g, Figure 1C)

Evaluation of cheese quality

After two months of ripening M1 cheeses were similar, but NWC_cheese had an elastic paste, less rich in holes than M1_NMC_cheese. M2_NMC samples showed defects in the cheesemaking as a non-homogeneous color in the center was present with an evident fracture due to an intense fermentation. The external surface of both M2_cheeses had an unpleasant odor probable due to the storage in a poorly ventilated and very humid room. Both M3_cheese revealed numerous small holes typical of the growth of coliforms derived from the starting milk and from poor hygiene during cheesemaking, defects that were much more evident in M3_NWM_cheese. Moreover, the external surface of both M3_cheese smelled of animal and ammonia, due to a poor hygiene of the wooden shelves or in the storage room. M4_NMC_cheese showed a typical aspect of propionic fermentation, while M4_N cheese showed an excessive fermentation not well distributed within the cheese, probable due to sudden temperature changes after curd extraction. Intense fermentation is very likely due to poor hygienic quality of milk. In particular, both M3_ and M4_NMC_cheeses resulted considerably different from N cheeses and the appearance of cheese reflected the load of Ec that suggests fecal contamination during milking and/or cheesemaking (McSweeney PLH, 2007).

Culture-independent analysis on cheese

Microbial community profiles of cheese samples were assessed by 16S amplicon sequencing. A total of 245 OTUs with 97% similarity were obtained after OTU picking and the coverage index is >0.99 for all samples. As expected the phylum Firmicutes dominates the community in all samples (from 64.9 to 99.5%) with the rest composed by Proteobacteria (0.13-32.9%), Actinobacteria (0.11-30.5%) and Bacteroidetes (0.02-22.4%). The most abundant genera belong to endogenous milk microflora such as *Lactobacillus*, *Pediococcus*, *Lactococcus*, and

Enterococcus and contaminants such as *Acinetobacter*, *Chryseobacterium*, and *Staphylococcus*.

The heatmap reported in Figure 2A illustrates the distribution of the most abundant genera in all samples. The clusterization of samples on the top of Figure 2A, based on their bacterial composition, clearly showed the similarity between communities

from the same *malga*. The Principal Coordinates Analysis (PCoA) reported in Figure 2B confirms the similarity in composition of the community in cheese produced in the same *malga*, using the same milk. This result demonstrates that the addition of NMC, while ameliorating hygienic conditions, does not reduce the diversity between cheese communities from different *malga*,

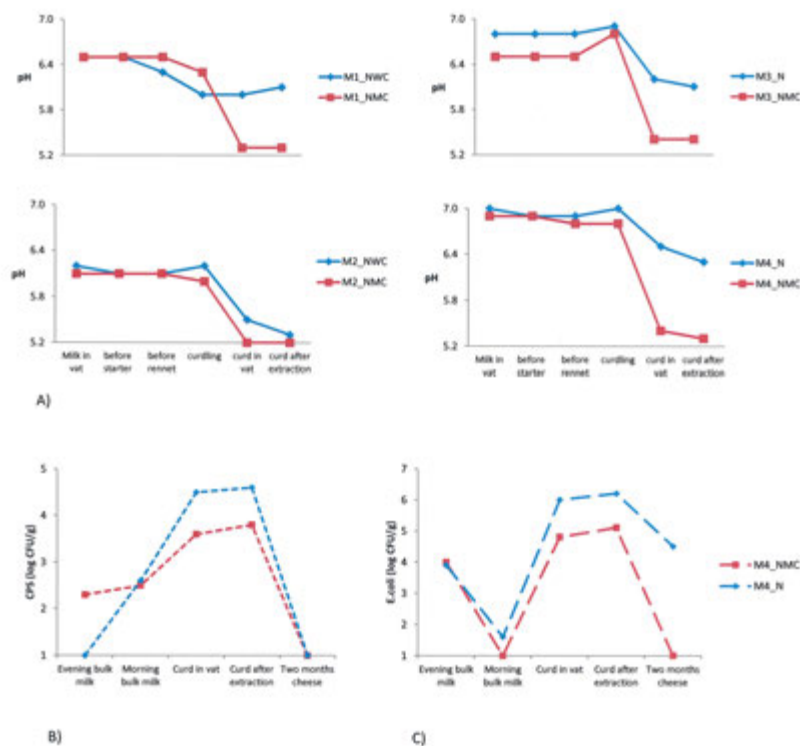


Figure 1. pH values of the two cheesemaking in the four *malga* (A), CPS counts (B) and *E.coli* counts (C) in the two cheesemaking of M4.

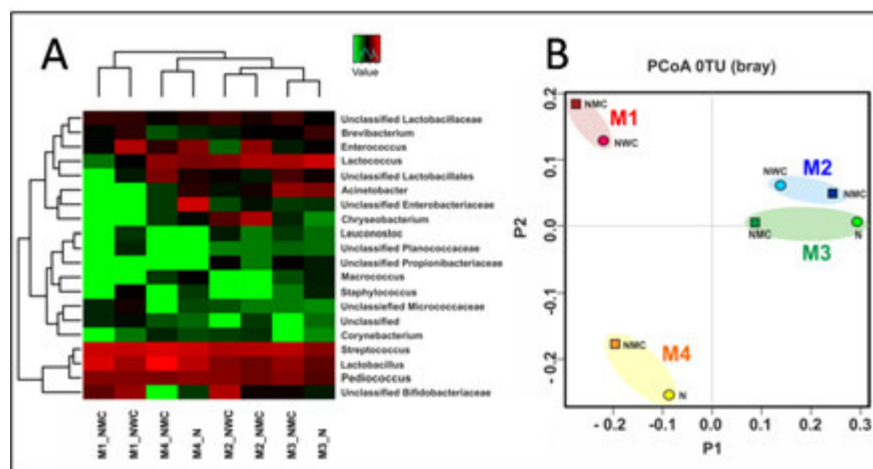


Figure 2. Distribution of the most abundant genera and clusterization of cheese samples based on their bacterial composition (A) and Principal Coordinates Analysis (PCoA) representing the α -diversity among the cheese samples (B).

Table 2 Distribution of the NMC OTUs in Malga samples.

OTU ID	NMC	M1_NMC	M1_NWC	M2_NMC	M2_NWC	M3_NMC	M3_N	M4_NMC	M4_N	taxonomy
816470	50	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae
591907	243	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
760901	459	40	38	27	86	73	19	10	19	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__; s__
308970	1	0	0	0	0	25	0	539	317	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__
4385067	3	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
332486	8	2	2	0	1	0	0	0	1	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__; s__
625693	4	1	0	2	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__; s__
14962	14	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
746246	32	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
805055	649	0	0	0	1	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
327765	1	0	0	0	0	21	0	746	400	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__
573338	12	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
260900	4	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
1147446	3	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
4480185	18	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
1550056	12	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
4429553	8	0	1	0	0	1	0	0	1	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__; s__
554916	9	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
1082539	1	481	622	134	452	533	47	530	550	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus; s__
398350	3	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
355695	6	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
4386115	5	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
4389594	4	1	0	1	1	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__; s__
587574	3	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
139137	4	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
252995	3	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
4333556	10	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
816219	102	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__

Continued on next page.

Table 2. Continued from previous page.

OTU ID	NMC	M1_NMC	M1_NWC	M2_NMC	M2_NWC	M3_NMC	M3_N	M4_NMC	M4_N	taxonomy
366926	3	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
160548	2697	269	285	229	483	441	192	53	96	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Pediococcus; s__
2874742	4	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
218254	29	0	0	0	0	0	0	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
4305372	18	2	6	1	4	5	1	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__; s__
805735	9	1	1	0	1	1	1	0	3	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__; s__
4371645	28	3	3	6	8	10	6	0	0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__; s__

thereby preserving the typicality of products from each *malga*. The α -diversity index (*Observed species* in Table 1) describing the level of microbial biodiversity obtained for each sample confirms such result.

The complete list of the OTU identified in the NMC is reported in Table 2. Summarizing the OTUs in higher taxonomic categories, the NMC contains a predominance of Lactobacillaceae (about the 72% of the population), including the genus *Pediococcus* spp, which represents 60% of the microbial population. Based on results from RAPD analysis performed during strain selection for NMC production (Andrighetto *et al.* 2002; Paternolli *et al.* 2014), a prevalence of the genus *Streptococcus* spp among Lactobacillaceae was expected. NGS-based microbial profiling did not show the presence of *Streptococcus* suggesting that the RAPD approach likely misclassified *Pediococcus* strains as *Streptococcus*. The rest of the bacterial population was composed by Bacillaceae (about 26%), which likely represent a contamination deriving from the milk used to produce NMC. *Pediococcus* has been already reported as the dominant bacteria in *malga* milk, together with *Lactobacillus* (*Pediococcus pentosaceus* and *Lactobacillus paracasei*) (Carafa *et al.*, 2015). Despite its incorrectly defined composition, the NMC demonstrated to have a good performance in cheesemaking. On the other hand, the obtained results highlight that the use of DNA sequencing is essential to improve accuracy and reproducibility in starter production.

Comparing the NMC composition at OTU level (each OTU represents a strain) with the OTU distribution in cheese samples demonstrates that none of the strains deriving from NMC became predominant in

cheese (see Table 2) and the genus *Pediococcus* is present in all the cheese samples (with or without the addition of NMC). As a matter of fact, the addition of NMC has different effects in each *malga* (the α -diversity index in M1 and M4 slightly decreases and in M2 and M3 slightly increases) suggesting that the starter microflora does not affect the composition of the cheese bacterial community, which largely depends on the endogenous microflora of milk.

Conclusions

Alpine dairy products are generally manufactured from not pasteurized raw milk, in working environments that often do not ensure good hygiene practices. As a consequence, more frequently than other productions, such products might represent a risk for public health because they could be contaminated with food-borne pathogens. While it might not be always possible to increase the level of hygienic practices, simple modifications of the technological process such as the use of NWC or selected NMC might help reducing the risks associated to the consumption of raw cow's milk cheese. In fact, the present study shows that the addition of NMC, prepared using endogenous technologically active strains in raw milk cheesemaking promotes the acidification process and drives the primary fermentation. To this end, *malga* cheesemakers need guidelines to produce NWM, otherwise it can happen as in M1_NWM where pH did not decrease below 6. The results obtained here confirm that quality of raw milk and hygiene of work areas remain critical to determine

cheese quality and safety. It was shown that adequate growth of lactic bacteria during dairy farming contributes to the control of the multiplication of potentially pathogenic bacteria, with particular reference to enterotoxin-producing staphylococci, and can help in the production of safer cheese (Bennett *et al.*, 1983; Cretenet *et al.*, 2011, Schelin *et al.*, 2011). Moreover, even when starting from milk of poor hygienic quality, as in the case of M3 and M4, the addition of NMC led to substantial reduction of *E. coli* microbial load in two-months ripened cheese. The study also demonstrates that addition of NMC produced with selected indigenous strains does not significantly affect the cheese microflora, and NMC may be used without losing the typicality of cheese from individual *malga* farms.

References

- Andrighetto C, Borney F, Barmaz A, Stefanon B, Lombardi A, 2002. Genetic diversity among *Streptococcus thermophilus* isolated from Italian traditional cheese. *Int Dairy J* 12:141-4.
- Bennett, RW, Amos, WT. 1983. *Staphylococcus aureus* growth and toxin production in imitation cheese. *J Food Sci* 48:1670-3.
- Bokulich, NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA, Caporaso JG, 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods* 10:57-59.
- Brooks JC., Martinez B, Stratton J, Bianchini A, Krokstrom R, Hutkins R, 2012. Survey of raw milk cheeses for microbiological quality and prevalence of foodborne pathogens. *Food*

- Microbiol 31:154-8.
- Caporaso JG, Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R, 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7:335-6.
- Carafa I, Nardin T, Larcher R, Viola R, Tuohy K, Franciosi E, 2015. Identification and characterization of wild lactobacilli and pediococci from spontaneously fermented Mountain cheese. *Food Microbiol* 48:123-32.
- Carafa I, Clementi F, Tuohy K, Franciosi E, 2016. Microbial evolution of traditional mountain cheese and characterization of early fermentation cocci for selection of autochthonous dairy starter strains. *Food Microbiol* 53:94-103.
- Cretenet M, Nouaille S, Thouin J, Rault L, Stenz L, Francois P, Hennekinne JA, Piot M, Maillard MB, Fauquant J, Loubière P, Le Loir Y, Even S, 2011. *Staphylococcus aureus* virulence and metabolism are dramatically affected by *Lactococcus lactis* in cheese matrix. *Environ Microbiol Rep* 3:340-51.
- De Buyser ML, Dufour B, Maire M, Lafarge V, 2001. Implication of milk and milk products in foodborne diseases in France and in different industrialized countries. *Int J Food Microbiol* 67:1-17.
- Duquenne M, Fleuret I, Aigle M, darrigo C, borezée-Durant E, Derzelle S, Bouix M, Deperrois-Lafarge V, Delacroix-Buchet A, 2010. Tool for Quantification of Staphylococcal Enterotoxin Gene in Cheese. *App Environ Microbiol* 76:1367-74.
- European Commission, 2004. Regulation of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for the hygiene foodstuffs, 853/2004/CE. In: *Official Journal*, L 139/55, 30/04/2004
- European Commission, 2005. Commission Regulation (EC) of 15 November 2005 on microbiological criteria for foodstuffs. 2073/2005/CE. In: *Official Journal*. L 338/1. 22/12/2005.
- European Food Safety Authority (EFSA), 2014. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Foodborne Outbreaks in 2012. *EFSA J*, 12(2), 3547.
- European Screening Method of the European Union-Reference Laboratory for Coagulase Positive Staphylococci, including *Staphylococcus aureus*, 5th ed. 2010. Ostyn A, Pruffer AL, Papinaud J, Hennekinne J-A, Assere A, Lombard b. Detection of staphylococcal enterotoxins types SEA to SEE in all types of food matrices. EU-RL CPS; ANSE; Maisons-Alfort, France (1-12).
- ISO, 1999. Meat and meat products – measurements of pH – reference method. ISO Norm 2917:1999(E). International Standardization Organization ed., Geneva, Switzerland.
- ISO, 2001. Microbiology of food and animal feeding stuffs – horizontal method for the enumeration of beta-glucuronidase-positive *Ec* – part 2: colony-count technique at 44°C using 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide. ISO Norm 16649-2:2001. International Standardization Organization ed., Geneva, Switzerland.
- ISO, 2003. Microbiology of food and animal feeding stuffs – horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species). Part 2: technique using rabbit plasma fibrinogen agar medium ISO Norm 6888-2:1999/Amd.1:2003. International Standardization Organization ed., Geneva, Switzerland.
- ISO, 2014. Microbiology of food and animal feeding stuffs – determination of water activity. ISO Norm 21807:2004(E). International Standardization Organization ed., Geneva, Switzerland.
- ISO, 2013. Microbiology of the food chain – Horizontal method for the enumeration of microorganism – part 1: colony count at 30 degrees C by the pour plate technique. ISO Norm 4833-1:2013. International Standardization Organization ed., Geneva, Switzerland.
- ISO, 2013. Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations. Amendment 1. ISO Norm 7218:2007/Amd.1:2013(E). International Standardization Organization ed., Geneva, Switzerland.
- Kousta M, Mataragas M, Skandamis P, Drosinos EH, 2010. Prevalence and sources of cheese contamination with pathogens at farm and processing levels. *Food Control* 21:805-15.
- Lucchini R, 2015. Quanto sono sicuri i formaggi delle nostre malghe. Esperienze pratiche e indicazioni per la produzione. *L'allevatore Trentino* 3:20-5
- McSweeney PLH (Ed), 2009. Cheese problems solved. Cambridge: Woodhead publishing limited, pp 119-123, 131-132, 140-144.
- Montel MC, Buchin S, Mallet A, Delbes-Paus C, Vuitton DA, Desmasures N, Berthier F, 2014. Traditional cheeses: rich and diverse microbiota with associated benefits. *Int J Food Microbiol*. 177:136-54.
- Mucchetti G, Neviani E 2006. Microbiologia e tecnologia lattierocasearia. Qualità e sicurezza. Ed. Tecniche Nuove, Milano, 400.
- Nadkarni, M A, Martin, F E, Jacques, N A, Hunter, N, 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* 148:257-66.
- Paternolli S, Pedrolli I, Paolazzi G, Andrighetto C, Lombardi A, Lucchini R, 2014. Sviluppo di colture batteriche autoctone con attitudine casearia e competitiva nei confronti i patogeni per la valorizzazione dei prodotti caseari d'alpeggio: applicazione in campo. *Atti XXIV Convegno AIVI Bologna* 10-12 settembre 2014 pp 21-22
- Povolo M, Pelizzola V, Passolungo L, Biazzi E, Tava A, Contarini G, 2013. Characterization of two *Agrostis-Festuca* alpine pastures and their influence on cheese composition. *J Agric Food Chem* 61:447-55.
- Presser KA, Ratkowsky PA, Ross T 1997. Modelling the growth rate of *E coli* as a function of pH and lactate acid concentration. *Appl Environ Microbiol* 63:2355-60.
- Rosengren A, Fabricius A, Guss B, Sylven S, Lindqvist R, 2010. Occurrence of foodborne pathogens and characterization of *Staphylococcus aureus* in cheese produced on farm-dairies *Int J Food Microbiol* 144:263-9.
- Schelin J, Wallin-Carlquist N, Cohn MT, Lindqvist R, Barker GC, Radstrom P, 2011. The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment *Virulence* 2:580-92.
- Topisirovic L, Kojic M, Fira D, Golic N, Strahinic I, Lozo J, 2006. Potential of lactic acid bacteria isolated from specific natural niches in food production and preservation. *Int J Food Microbiol* 127:230-5.