Food Research International 172 (2023) 113101

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

The impact of milk storage temperatures on cheese quality and microbial communities at dairy processing plant scale

Lucia Giagnoni [a,b,*], Saptarathi Deb [b], Alessandra Tondello [b], Giulia Zardinoni [b], Michele De Noni [c], Cinzia Franchin [b], Alice Vanzin [b], Giorgio Arrigoni [d], Antonio Masi [b], Piergiorgio Stevanato [b], Alessio Cecchinato [b], Andrea Squartini [b], Carlo Spanu [a]

[a] *Department of Veterinary Medicine, University of Sassari, Via Vienna, 2, 07100 Sassari (SS), Italy*
[b] *Department of Agronomy, Food, Natural Resources, Animals and Environment, DAFNAE, University of Padova, Viale dell'Università 16, 35020 Legnaro, (PD), Italy*
[c] *Latteria Montello S.p.A. Via Fante d'Italia 26, 31040 Giavera del Montello (TV), Italy*
[d] *Department of Biomedical Sciences, University of Padova, Via Ugo Bassi 587b, 35131 Padova (PD), Italy*

ARTICLE INFO

Keywords:
Fresh cheese
Milk refrigeration temperature
Thermal abuse
16S metabarcoding
Dairy processing plant trial

ABSTRACT

Cheese production is an applied biotechnology whose proper outcome relies strictly on the complex interactive dynamics which unfold within defined microbial groups. These may start being active from the collection of milk and continue up to its final stages of maturation. One of the critical parameters playing a major role is the milk refrigeration temperature before pasteurization as it can affect the proportion of psychrotrophic taxa abundance in the total milk bacterial population. While a standard temperature of 4 °C is the common choice, due to its general growth control effect, it does have a potential drawback. This is due to the fact that some cold-tolerant genera present a proteolytic activity with uncompleted proliferation, which could negatively affect curd clotting and regular cheese maturation. Moreover, accidental thermal variations of milk before cheese-making, in a plus or minus direction, can occur both at farm collection sites and during transfer to dairy plant. This present research, directly commissioned by a major fresh cheese-producing company, includes an in-factory trial. In this trial, a gradient of temperatures from 4 °C to 13 °C, which were subsequently reversed, was purposely adopted to: (a) verify sensory alterations in the resulting product at different maturation stages, and, (b) analyze, in parallel, using DNA extraction and 16S-metabarcoding sequencing from the same samples, the presence, abundance and corresponding taxonomical identity of all the bacteria featured in communities found in milk and cheese samples. Overall, 1,714 different variants were detected and sorted into 394 identified taxa. Significant bacterial community shifts in cheese were observed in response to milk refrigeration temperature and subsequently associated with samples having altered scores in sensory panel tests. In particular, proteolytic psychrotrophes were outcompeted by Enterobacteriales and by other taxa at the peak temperature of 13 °C, but aggressively increased in the descent phases, upon the cooling down of milk to values of 7 °C. Relevant clues have been collected for better anticipation of thermal abuse effects or parameter variations allowing for improved handling of technical processing conditions by the cheese manufacturing industry.

1. Introduction

Cheese originated as a component of many regional diets, and is nowadays one of the most frequently consumed foods worldwide, with a steadily increasing market value (FAO, 2022). Cheese texture and organoleptic properties such as flavor and aroma are the result of a complex interaction of factors, including the type and quality of milk, as well as manufacturing and maturing conditions (Mayo et al., 2021). Cheese microbial communities are primarily involved in this process, in particular during the fermentation and ripening stages, contributing to the physicochemical and sensory properties of the final product. In this respect, Bacteria play a decisive role in the accumulation of nutrients, thus impacting on the quality of the product or, on the contrary, potentially causing spoilage and safety issues (Falardeau et al., 2019). The microbiome of cheese varies depending on the cheese type as well as on environmental, ecological, and processing conditions such as milk

* Corresponding author at: Department of Veterinary Medicine, University of Sassari, Via Vienna, 2, 07100 Sassari (SS), Italy.
E-mail address: l.giagnoni@studenti.uniss.it (L. Giagnoni).

https://doi.org/10.1016/j.foodres.2023.113101
Received 14 February 2023; Received in revised form 11 May 2023; Accepted 9 June 2023
Available online 12 June 2023
0963-9969/© 2023 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

species of origin, type of starters, heat treatments (e.g. refrigeration, pasteurization, cooking), and ripening environment (Yeluri Jonnala et al., 2018). A better knowledge of the composition of the cheese microbiome is therefore of crucial importance in understanding and controlling its potential impact on desirable sensory characteristics or, on the contrary, negative product quality (Choi et al., 2020). Although a vast body of knowledge of dairy microbiology has been gathered worldwide over the decades, such studies and their ensuing literature have been mainly cast from an academic perspective, aimed at filling knowledge gaps in terms of taxonomy, diversity, and descriptive aspects of a vast number of different cheeses of local origin (Montel et al., 2014; De Filippis et al., 2018; Tilocca et al., 2020). Most of the studies were purposely designed to capture the actual typical microbiota of each case, either to add value to its distinctiveness for a narrow niche market, or to allow branding protection against imitations for highly popular cheese types. Similar endeavours have seen important developments in the prevention of cheese spoilage and safety issues associated with the presence of foodborne pathogens in dairy products (Rosengren et al., 2010; Choi et al., 2016; Pasquali et al., 2022). Undeniably, a much lesser explored aspect of microbial population dynamics during cheesemaking relates to the effect of different variables, acting as process-ruling parameters, on the quality of the end product. Moreover, this aspect is usually not studied at the actual processing plant stage, by a coordinated approach encompassing academic and industrial expertise. This relates to the immense effort that would be required by companies in agreeing to undertake major explorative process changes, thus diverting from their standard cheese-making procedures. Another reason for cheese manufacturers' reluctance is also due to the need for available dedicated production units, that would be temporarily diverted from benchmark conditions. While that aspect has been for the most part neglected by University-based independent research, it however represents one of the most critical issues to which dairy processing plants seek to achieve genuine answers. The reason it is so critical is linked to the industry's goal of acquiring a deeper understanding of which factors can guarantee reproducibility of the product's desired features as well as facilitating control of processing conditions at all levels.

Indeed, in the present report, the main rationale arose from queries of a private dairy plant located in Northern Italy. The plant's primary interest was focused on how a gradient transect of changes in the temperature of milk storage tanks before cheesemaking would result in a modification of the respective proportions and functional guild dynamics of bacteria that dominate the subsequent process. In particular, data were sought on the conditions governing the relative abundance and prevalence of thermoduric taxa in comparison to psychrotrophic ones, and the containment of the latter to avoid outbreaks of species with proteolytic activity.

The starting milk temperature certainly has a direct influence on consequent cheese biota composition. The refrigeration of raw milk in the dairy industry is aimed at preventing the growth of mesophilic microbiota, including lactose-fermenting species responsible for substrate acidification and having a negative impact on the subsequent cheese-making process. However, the prolonged storage of milk at low temperatures (2 to 6 °C) strongly affects the composition of milk microbiota with a selection of gram-positive and gram-negative psychrotrophic microorganisms, adapted to grow in milk at refrigeration temperatures (De Oliveira et al., 2015; Parente et al., 2020; Paludetti et al., 2020). Psychrotrophs include gram-negative microorganisms such as *Pseudomonas* and *Acinetobacter* (Hahne et al., 2019). Microorganisms belonging to those taxa can produce thermostable extracellular enzymes such as proteases or lipases responsible for off-flavors and other negative effects on aspects of processing, such as cheese yield, stability, as well as nutritional and sensory properties (Lorenzo et al., 2018; Cousin, 1982; Ercolini et al., 2009; Decimo et al., 2014). With the exception of some gram positive genera that are also thermoduric, psychrotrophs are sensitive to pasteurization; however protease and lipase enzymes can remain active even after the heat treatment applied in the dairy industry to eliminate vegetative forms (De Oliveira et al., 2015).

Thermoduric bacteria can survive during the pasteurization of raw milk, leading to a decreased but not complete elimination of the live taxa. As a consequence, the quality of dairy products can be highly influenced by the microbial load allowed to develop in the starting milk. The issue cannot be addressed solely by guaranteeing a low-enough, 'safe', milk storage temperature, such as 4 °C, which is the usual standard adopted. One of the reasons for this is that in the production of cheese, the most frequent negative effects regarding yield and contamination, as mentioned earlier, are linked to enzymes of psychotrophic derivation. Therefore the refrigeration of raw milk, while keeping foodborne pathogens under control, does not ensure ideal conditions for curd clotting, overall cheese formation and its nutritional properties.

Traditionally, in the past the study of cheese microbiota has been carried out using culture-dependent techniques, which providedinsights into its complex microbial composition and contributed to distinguishing and evaluating dairy products. Yet, their limitations regarding the detection threshold, culturability issues and overall quantitative accuracy have fostered culture-independent nucleic acid-based high throughput techniques, now dominant in the contemporary world of microbial community surveys, including those in the dairy context (Almeida et al., 2014; Walsh et al., 2017; De Filippis et al., 2016, Bertuzzi et al., 2018; O'Donnell et al, 2020; Ferrocino et al., 2022).

In any given sample containing microbes, the microbiome, intended as an indication of presence, abundance and taxonomical lineage can be assessed through DNA extraction, followed by targeted sequencing (amplicon metabarcoding), or shotgun metagenomics and metatranscriptomics. Although methodologies such as the latter shotgun techniques can extract higher information volumes in relation to the amplicon-based approach, their higher cost per sample, the challenges in library construction (e.g. elimination of rRNA in metatranscriptomics) and a heavier demand of computing power for bioinformatics analyses, have limited their routine use in food research. On the other hand, descriptive studies that focus on enriching worldwide databases, e.g. with data that can also be used in *meta*-studies, mainly make use of amplicon metabarcoding. FoodMicrobionet (Parente et al., 2019) is an example of such a database, which, to date, contains data gathered from 120 studies and 5,974 samples. The advantages and limitations of each methodology, including a timeline and milestones achieved in the characterization of microbial communities, are extensively reviewed in the literature (Parente et al., 2019).

The present research aims at assessing the impact made by the refrigeration level of raw milk, purposely stored at different temperatures, on the composition of microbial communities infresh cheese during its ripening/maturation. With this aim in mind, tests were conducted on three different milk storage temperatures before cheesemaking. The 16S-based bacterial metabarcoding was used to investigate the taxonomic identity and relative abundance of microbial communities at three stages of cheese maturation. Particular focus was dedicated to the search for taxa of interest in the dairy process, such as thermoduric and psychrotrophic microorganisms, that could negatively affect milk quality and safety. The experiment was set up at real plant level at the partner dairy company. It involved a step-by-step series of thermal increases to five temperature levels. Although the milk turnover was carried out daily in each case the milk storage tank was allowed to stabilize for 12 days at each chosen temperature before sampling. The set temperatures were increased up to a maximum (13 °C) and then decreased back to the standard one (4 °C); this also allowed verification of the degree needed to reestablish a psychrotrophic milk microbiome after enabling a succession of taxa to exploit the opposite condition.

2. Material and methods

2.1. Sample preparation

Milk and cheese samples used in the present experiment were

provided by the Latteria Montello dairy company, located in Giavera del Montello (province of Treviso), North-Eastern Italy. Raw cows' milk from a partner dairy farm was stored in the usual bulk milk cooling tank, which had a capacity of 4000 L. While the standard bulk tank operating temperature was 4 °C, as a means to simulate thermal abuses in assessing the impact of milk storage temperature on the microbiological community of fresh cheese during ripening, the following temperature shifts were progressively set for the bulk tank: 4 °C, 7 °C, 11 °C, 13 °C and back to 7 °C. Each temperature was maintained for 12 days before sampling, to allow adjustment to the temperature and enable the microbiota to reflect an established equilibrium, rather than representing a sudden change in the bulk tank environment. Bulk tank milk samples were collected each time after the 12 days of adaptation for each of the temperatures. In fact, while milk turnover in and out of the tank took place daily, the cheese-making stage was carried out only with the milk collected on the twelfth day after the start of storage tank conditioning for each of the temperatures tested. Cheese samples from the temperatures that resulted conducive to altered sensory patterns were selected for further investigation by DNA metabarcoding. The graphic abstract shows an outline of the experiment. The milk was within industrial standards, averaging 87.7 % water content, 4.9 % carbohydrates, 3.4 % lipids and 3.3 % proteins. In relation to the routine microbiological cultural analyses the values in the starting milk, for each of the three temperatures of 4 °C, 13 °C, and 7 °C, i.e., the ones selected for metabarcoding based on the altered cheese sensory patterns, were, respectively, the following: Total Bacterial count: 10500, 12,500 12,000 colony forming units (CFU) per ml (Count agar sugar free FIL-IDF; Merck), Coliforms (CFU on Coliform Agar, Biolife, Milan): 200, 1000, 500 CFU per ml).

Milk was picked up, delivered to the dairy plant, and pasteurized (75 °C for 30 s). After pasteurization, aliquots of 50 mL were collected and immediately frozen at −20 °C, to be used later as starting milk controls for metabarcoding analyses. The remaining milk was used to make "caciotta-style" fresh cheese as follows. The pasteurized milk was cooled down to 40 °C, and transferred into a vat where rennet for casein coagulation and an acidifying starter (*Streptococcus thermophilus*) were added. Whey syneresis included a moderate stirring. Once the curd formed, it was sliced with a curd cutter into cubic pieces with 3 cm sides. Whey separation occurred through the reservoir holes and the drained curd was transferred into rectangular molds where it remained until a pH of 55 was reached. This required approximately 2 h, during which molds were inverted twice to favor mass homogeneity. The size of the molds was 10 × 10 × 20 cm. Molds were placed in a 20% NaCl brine solution at 6–7 °C for 30 min and finally moved to the ripening chamber at + 4 °C where they remained for up to 45 days. Subsequently 12 kg blocks of cheese were obtained, featuring: 48–52% water content, 24–26% fat, 22–24 % protein, 0.1–0,5 % lactose, 0,5–0,7% NaCl, 0,98–0,99 water activity Aw.

Three sampling points were analyzed during cheese maturation, namely at time zero (day of production), 15 days and 30 days. For this purpose, two aliquots of 1.5 kg of cheese were collected from each block for the DNA-based analyses and stored at −20 °C for successive analysis.

Customary panel tests were performed in-house by the dairy company to assess which samples would present noticeable altered qualities, thereby selecting those for the bacterial metabarcoding analyses. The sensory parameters taken into consideration included the perception of sharp taste (a ripe taleggio-like piquant sensation) as the main sign of alteration, essentially linked to incipient lipolysis. Such pre-selection led to the choice of samples from milk which had reached a peak temperature of 13 °C (maximum of simulated thermal abuse) and milk that had been returned to 7 °C (descent recovery test towards restoration of pristine communities proportions). As standard control, the cheese from milk maintained at 4 °C was used.

A total of 21 samples were analyzed consisting of 3 samples of milk and 18 samples of cheese, belonging to the 3 temperature regimes (4, 13, 7 °C) tested by 2 replicates at 3 sampling times (0, 15, 30 days of maturation).

2.2. DNA extraction

Samples of 2 mL of thawed pasteurized milk or 1 g of thawed cheese from each of the conditions were transferred into 2 mL eppendorf tubes. Samples were centrifuged at 21,000 g for 10 min. The supernatant fluid was removed and pellets were supplemented with 350 μL of RTL buffer (guanidine thiocyanate 0.12 M, Qiagen, Hilden, Germany) along with a tungsten bead with a diameter of 5 mm (Qiagen, Hilden, Germany). The step of homogenization was carried out in a TissueLyser II (Qiagen, Hilden, Germany) for 5 min at 30 Hertz repeated twice after a 1-minute interval. Samples were centrifuged for 10 min at 21,000 × g to obtain a phase separation to eliminate the floating fat layer, which was removed by pipetting it out from the top. The remaining bottom aqueous phase was subjected to enzymatic treatment by adding 20 μL of a 20 mg/mL lysozyme solution (Thermo Fisher Scientific, Waltham, MA, USA), mixing with a vortex mixer for 3 s, and incubating at 37 °C for 30 min. Subsequently, 20 μL of 20 mg/mL proteinase-K solution (Invitrogen, Carlsbad, CA, USA) was added to the samples which were incubated at 50 °C for 30 min. Samples were finally vortexed for 3 s, centrifuged at 2,500 × g for 6 min, after which the supernatant was collected and DNA was purified using a Biosprint 96 automated workstation (Qiagen, Hilden, Germany) as follows: sample supenatant was transferred in an S-block with 200 μL of isopropanol and 20 μL of MagAttract magnetic beads suspension (Qiagen, Hilden, Germany) and loaded into the instrument. The protocol involved the sequential use of 5 further plates: one S-block with 500 μL of RPW buffer (guanidine hydrochloride, 1.31 M), two plates with 500 μL of 96% ethanol, a subsequent S-block with 500 μL of 0.02% tween solution and a final flat-bottom 96-well plate containing 80 μL of nuclease-free water, where DNA was eluted. The final concentration of DNA was measured with a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using the Qubit™ DNA HS Assay Kit Fluorometer from the same manufacturer.

2.3. Quantitative PCR

The quantitative abundance of bacteria was calculated from the total DNA extracted from cheese samples by Real-Time PCR (qPCR) using the QuantStudio 12 K-Flex (Thermo Fisher Scientific, Waltham, MA, USA) and 16S universal primers (Leigh et al., 2007). The reagent mix was prepared with 2.5 μL PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 0.15 μL each of forward and reverse primer, 0.2 μL nuclease-free-water, and 2 μL target DNA. The thermal cycler was programmed with the following PCR program: pre-incubation at 95 °C for 10 min, 50 cycles of 15 s at 95 °C, and 1 min at 60 °C. All analyses were performed in triplicate. The evaluation of the threshold cycle (Ct) was performed using Past software (Hammer et al., 2001). Depending on data normality and homoskedasticity statuses, Welch ANOVA or Kolmogorov-Smirnov tests were used to detect significant differences (p-value < 0.05) among mean values.

2.4. Metabarcoding of the bacterial 16S rRNA gene

Library preparation was carried out using a 16S Ion Metagenomics Kit (Thermo Fisher Scientific, Waltham, MA, USA). The protocol involved a first PCR amplification using two different primer sets (V2, V4, V8 and V3, V6-7, V9) for the amplification of hypervariable regions. The PCR program consisted of an initial denaturation of 95 °C for 10 min, followed by 25 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 20 s, and a hold stage of 72 °C for 7 min. Amplicons were quantified and pooled together to obtain a final concentration of 30 ng × μL^{-1}. The subsequent protocol involved the use of an Ion Xpress Plus 9 Fragment Library Kit and an Ion Express Barcode Kit (Thermo Fisher Scientific, Waltham, MA, USA) for barcode ligation. A further amplification step was performed with the following program: 95 °C for 5 min, 7 cycles of

95 °C for 15 s, 58 °C for 15 s, and 70 °C for 1 min, storing the amplified pools at 4 °C. Libraries were quantified using a Qubit 3.0 Fluorometer with a Qubit™ DNA HS Assay Kit, in order to pool libraries at a final concentration of 100 pM. Samples were processed using an Ion 520™ and an Ion 530 ™ Kit – OT2 400 bp (Thermo Fisher Scientific Waltham, MA, USA) following the manufacturer's instructions. Samples were loaded on an Ion 520 chip and the sequencing run was performed in an Ion™ GeneStudio S5 System, both from the same manufacturer.

2.5. Bioinformatics and statistics

Raw reads were processed to trim 20 base pairs on both ends to remove primers using cutadapt v3.4 (Martin, 2011) and analyzed using QIIME2 v2021.4 (Bolyen et al., 2019). High-quality reads were denoised and dereplicated into amplicon sequence variants (ASVs) using the qiime dada2 plugin. To check if sequencing depth had been sufficient, an alfa-rarefaction plot was generated using the "qiime alpha-diversity" plugin. The SILVA SSU v138.1 database was used as a reference for the taxonomic assignment of ASVs (Quast et al., 2012). Chao1, Shannon-Wiener H value, Simpson's 1-D, and Community evenness (e^H/S) were computed from the output data matrix using Past 4.11 software (Hammer et al., 2001). Alpha-diversity and evenness significance of differences were estimated using the MicrobiomeAnalyst online utility (https://www.microbiomeanalyst.ca/) using the Shannon Diversity ecological indicator, and assessing the significance of differences by the nonparametric Kruskal-Wallis statistical test. Before the analysis, the metagenomic data were normalized and filtered excluding reads with an abundance lower than 2 to minimize the effects of sequencing errors. Beta-diversity among samples was visualized using Non Metric Multi-Dimensional Scaling (NMDS) based on Bray-Curtis dissimilarities. The relative abundance % of the major taxa at genus level, and their differential representation analysis in the cheese sample communities were analyzed using the SHAMAN online utility (https://shaman.pasteur.fr). Functional prediction inference from metabarcoding taxonomy was performed using FAPROTAX software (Louca et al., 2016) to predict the main putative metabolic processes of the microbial communities.

3. Results

3.1. Overall diversity and community indexes

In total 6,956,361 paired-end raw reads were produced from the bacterial 16S rRNA gene sequencing. On average, 166,359 reads per sample were obtained, ranging from 10,382 to 1,177,575. Sequences were further classified into 1,714 unique amplified single variants (ASVs), leading to 394 database annotations of different taxonomical subject names.

The global levels of taxa richness, individual abundance, and resulting diversity and evenness indexes for all samples of milk, from each of the three sequentially tested temperatures (4°, 13°, 7 °C), and of each of the deriving cheese products, sampled at the three maturation stages (zero, 15 and 30 days), are presented in Table 1.

In the first instance, the data show bacterial diversity was 4-fold higher in milk compared to cheese in terms of the number of represented taxa; this is also reflected in the values of the two Shannon and Simpson ecological estimators. A further noticeable difference concerns the increasingly higher diversity expressed by these indexes in milk stored at increasing temperatures, and in the corresponding cheese samples. A synopsis of the bacterial diversity unfolding in cheese, and of the community evenness values in relation to both tested variables (milk storage temperature and cheese maturation time) is portrayed in Fig. 1.

While a statistical non-parametric analysis of the data by the Kruskal-Wallis test did not support significance between their differences, the values observed were associated with higher means in the Shannon index for cheese made from milk that had been kept at 13 °C before being processed. On the other hand, community evenness showed less pronounced differences when compared with the two other temperatures and a minimum mean value at the midpoint time of 15 days.

Table 1
Sequencing results in terms of number of different ASV (Amplified Sequence Variants)–defined taxa, sequence reads abundance for each, alpha diversity indexes of Shannon-Wiener H value, Simpson's 1-D, and Community Evenness (e^H/S) for each sample, obtained by the *meta*-barcoding analysis. Prefixes: PM: Pasteurized Milk, C: Cheese. Infixes: Temperature (4°, 13°, or 7 °C)/ maturation time (0, 15, 30 days) for the cheese samples. Suffixes: replicates A or B.

Sample	n. Taxa	Abundance	Shannon	Simpson	Evenness
PM 4°	507	78,190	4.261	0.952	0.140
C 4°/0 A	114	181,251	2.369	0.858	0.094
C4°/0 B	285	190,027	3.578	0.933	0.126
C 4°/15 A	68	45,480	2.437	0.876	0.168
C 4°/15 B	102	233,724	2.286	0.861	0.096
C 4°/30 A	110	85,871	2.532	0.872	0.114
C 4°/30 B	69	51,158	2.390	0.872	0.158
PM 13°	381	110,457	4.499	0.977	0.236
C 13°/0 A	94	7,690	3.493	0.940	0.350
C 13°/0 B	226	90,361	3.750	0.950	0.188
C 13°/15 A	447	871,561	3.520	0.929	0.076
C 13°/15 B	268	311,480	3.270	0.921	0.098
C 13°/30 A	377	320,128	3.661	0.937	0.103
C 13°/30 B	114	12,064	3.417	0.930	0.267
PM 7°	716	151,698	4.576	0.975	0.136
C 7°/0 A	204	178,390	3.071	0.903	0.106
C 7°/0 B	126	123,015	2.719	0.889	0.120
C 7°/15 A	63	20,099	2.368	0.822	0.170
C 7°/15 B	164	154,851	2.930	0.888	0.114
C 7°/30 A	96	58,270	2.565	0.871	0.135
C 7°/30 B	112	119,113	2.550	0.866	0.114

3.2. Dominant taxa in milk and cheese and their major differences in relation to milk storage temperature

Within bacterial communities in milk, at the phylum level, the majority were Proteobacteria with 57.77 % of the sequences, followed by Firmicutes (24.91 %) and Actinobacteriota (17.27 %). In cheese, instead, these values reversed in favor of the Firmicutes (77.27 %), with Proteobacteria at 22.35 % and Actinobacteriota at 0.37 %. The Proteobacteria were mainly of the Gammaproteobacteria class, which encompasses *Pseudomonas*, *Acinetobacter*, *Aeromonas* and various members of the Enterobacteriales order, while the Firmicutes predominantly belonged to the Bacilli class, with the Lactobacillales order and the genera *Streptococcus* and *Lactococcus* dominating the cheese biota. Fig. 2 shows the dominant genera observed in milk or cheese in relation to the milk refrigeration tank temperature.

Milk samples were distinctively characterized by the occurrence of genera from Gammaproteobacteria and by the minimal presence of *Streptococcus* which was instead largely featured in the cheese products, in which it is ascertained to have derived from the starter suspension. Shifts in milk composition appear in passing from the standard cold refrigeration temperature (4 °C) to the peaking value of 13 °C, and through a partial descent to 7 °C. An alteration of the cheese pattern at 13 °C and its partial recovery back towards a *Streptococcus*-dominated assemblage is evident when the temperature is reversed to 7 °C.

3.3. Community shifts as a function of milk storage temperature and cheese maturation stage

To clearly substantiate the actual effects of the experimental rationale, data are presented in greater detail. Focus is on the 25 most abundant genera converting sequence reads abundance counts into percentage values, whose relative differences are emphasized by conditional formatting color shades. Data are shown in Fig. 3. The effect of the temperature rise to 13 °C (middle panel) in fostering the onset of several additional taxa, both in milk and, consecutively, in cheese, is particularly evident. Conversely, the return to an intermediately lower

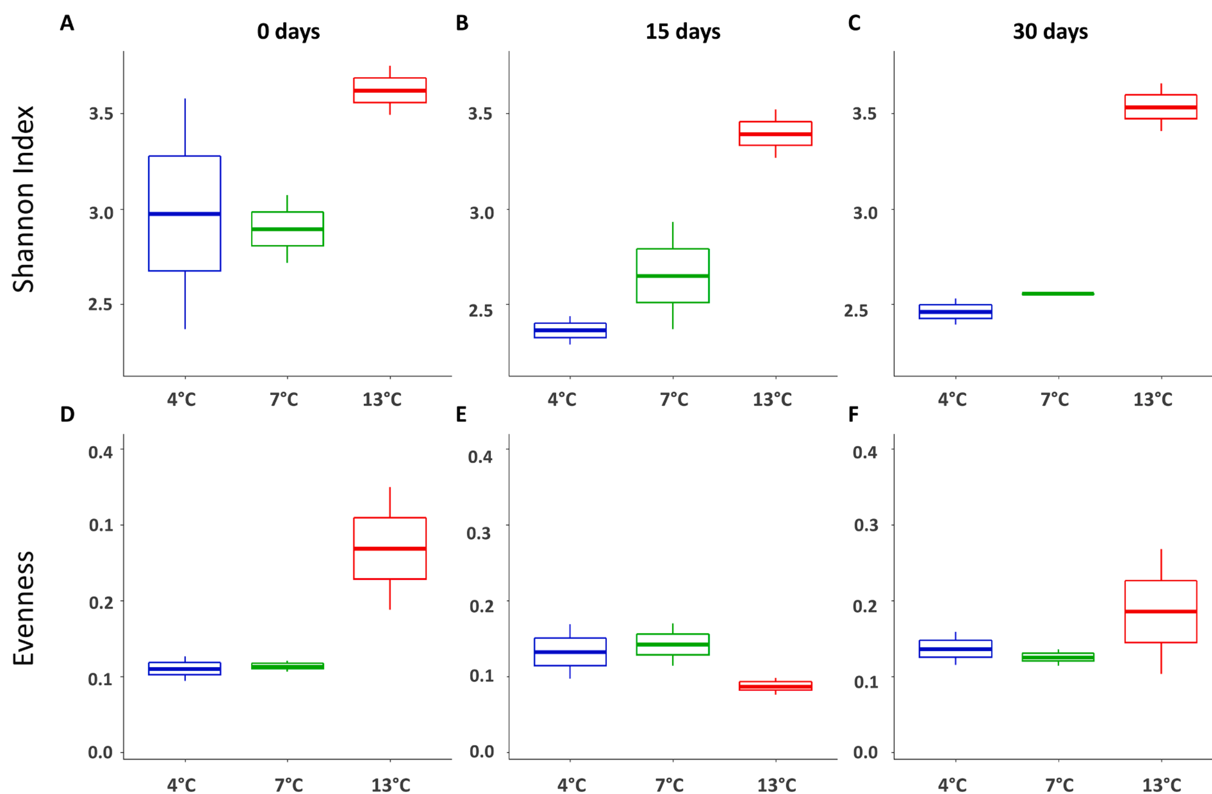

Fig. 1. Boxplots depicting Shannon alpha-diversity (A-C), and community evenness (D-F) as a function of milk storage temperature prior to pasteurization and processing, or of cheese maturity stage (Time zero: A, D; 15 days: B, E; 30 days: C, F).

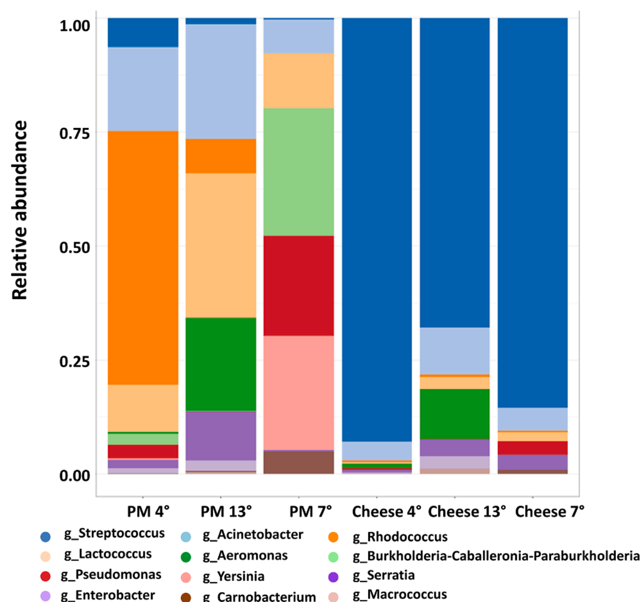

Fig. 2. Relative abundance % of the major taxa at genus level occurring in pasteurized milk (PM) stored at the three different temperatures and in the three corresponding cheese preparations originating from each. For the cheese samples the three maturation stages data are averaged together within the same histogram bar.

temperature value (7 °C) shows an ongoing recovery towards the pre-warming tank conditions of the starting control temperature.

Milk samples at the initial temperature featured fewer dominant members, among which were *Rhodococcus* (phylum Actinobacteriota) and *Acinetobacter* (Proteobacteria). In all cheese samples, the higher temperature storage step brought about a relative decrease in abundance of the otherwise dominant *Streptococcus*, whose content dropped from an average of 94.4 % to values between 51.2 % and 67.4 %. The drop is parallel to the rise of several other genera such as *Aeromonas*, *Enterobacter*, *Sphingomonas*, *Carnobacterium*, *Macrococcus*, *Citrobacter*, *Methylobacterium*, *Salmonella*, *Hafnia*, *Staphylococcus*, *Klebsiella* that were either rarely occurring, absent or below detection limits in cheese produced from milk stored at the 4 °C standard refrigeration conditions.

3.4. Multivariate ordination of samples and associations with temperature levels or key taxa

The grouping of samples based on the difference in taxonomy of each community was assessed through the Non-Metric Multidimensional Scaling (NMDS) ordination method, using Bray-Curtis dissimilarity values as distance units. A PERMANOVA statistical analysis indicated a significant p-value < 0.001 for separation under a very low stress factor value (0.072), indicative of a good representation of the samples clustering in the reduced dimensions. The NMDS plot of Fig. 4 shows the relationship between the independent variable of milk temperature and three major representative taxa.

The analysis revealed that sample clusterization in three well-resolved clouds was almost completely influenced by the temperature of milk storage prior to cheese-making. Cheese maturation days, on the contrary, appeared to have a minimal effect in separating their communities with respect to initial milk temperature. Samples at 13 °C also had the most variable and least clustered ordination in comparison to both of the two lower temperatures. In selecting three species extremely relevant to the cheese formation process, in this image we highlight their opposite behavior using the color-coded abundance gradients. In Fig. 4 b, the decrease of *Streptococcus thermophilus* is seen as inversely proportional to the variation in temperature. On the other hand, a different dynamic is displayed by the psychrotrophic *Pseudomonas* sp. (Fig. 4c) whose maxima of occurrence are less extreme and are associated with

	PM 4°	C4°/0A	C4°/0B	C4°/15A	C4°/15B	C4°/30A	C4°/30B	PM 13°	C13°/0A	C13°/0B	C13°/15A	C13°/15B	C13°/30A	C13°/30B	PM 7°	C7°/0A	C7°/0B	C7°/15A	C7°/15B	C7°/30A	C7°/30B
Streptococcus	5.18	94.60	67.23	93.23	98.14	90.86	95.28	1.14	51.22	51.66	63.59	62.77	67.46	59.92	0.27	69.33	87.27	89.37	75.08	88.33	88.40
Acinetobacter	14.77	4.31	7.42	5.63	1.16	6.76	3.75	20.52	13.07	13.69	9.73	8.27	6.36	8.80	6.09	9.64	3.06	2.99	7.78	3.10	2.80
Aeromonas	0.42	0.00	8.08	0.00	0.00	0.00	0.00	16.80	13.84	11.59	8.74	11.42	7.41	9.95	0.01	0.05	0.03	0.00	0.05	0.00	0.01
Lactococcus	8.20	0.01	3.29	0.02	0.01	0.05	0.10	25.86	2.26	2.36	2.43	2.33	3.06	2.52	9.81	2.47	1.85	1.38	2.78	1.32	1.72
Rhodococcus	44.77	0.33	0.51	0.25	0.31	0.25	0.16	6.18	0.42	1.13	0.18	0.26	0.87	0.67	0.00	0.18	0.29	0.02	0.65	0.23	0.19
Serratia	1.46	0.06	3.84	0.06	0.01	0.07	0.07	8.64	5.05	4.25	3.11	2.25	3.60	4.77	0.21	4.76	1.81	2.03	6.64	2.09	1.85
Pseudomonas	2.35	0.34	0.13	0.47	0.19	1.31	0.36	0.13	0.08	0.08	0.10	0.09	0.18	0.23	17.97	5.24	2.62	2.37	2.79	2.07	1.83
Burkholderia	1.93	0.00	0.01	0.00	0.00	0.00	0.00	0.07	0.00	0.02	0.01	0.01	0.03	0.00	22.80	0.00	0.00	0.00	0.01	0.01	0.00
Yersinia	0.36	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	20.38	0.00	0.00	0.00	0.00	0.00	0.00
Enterobacter	0.90	0.00	1.70	0.00	0.00	0.00	0.00	1.91	3.19	2.39	2.17	3.06	1.67	3.19	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Sphingomonas	7.63	0.02	0.06	0.02	0.02	0.08	0.00	0.57	0.03	0.12	0.05	0.04	0.19	0.14	3.05	0.15	0.01	0.00	0.06	0.09	0.05
Carnobacterium	0.17	0.00	0.06	0.00	0.00	0.00	0.00	0.14	0.00	0.04	0.05	0.01	0.06	0.00	4.05	0.92	1.34	0.48	0.99	1.01	1.01
Macrococcus	0.00	0.00	1.43	0.00	0.00	0.00	0.00	0.40	1.35	1.45	1.13	0.38	1.57	0.91	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Citrobacter	0.00	0.00	0.45	0.00	0.00	0.00	0.00	0.95	0.53	0.87	0.70	0.59	0.99	0.71	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Vibrio	0.60	0.08	0.00	0.00	0.00	0.00	0.00	2.07	0.00	0.00	0.00	0.00	0.01	0.00	1.46	0.01	0.00	0.00	0.00	0.00	0.00
Methylobacterium	1.79	0.00	0.00	0.00	0.01	0.02	0.02	0.13	0.03	0.08	0.04	0.01	0.13	0.14	0.70	0.27	0.00	0.01	0.03	0.02	0.02
Salmonella	1.24	0.00	0.35	0.00	0.00	0.00	0.00	0.27	0.00	0.29	0.26	0.03	0.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Hafnia	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.56	0.46	0.32	0.19	0.14	0.15	0.12	0.02	0.00	0.00	0.00	0.00	0.00	0.00
Lactobacillus	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.01	0.00	0.01	0.00	0.00	1.19	0.09	0.15	0.00	0.13	0.11	0.09
Mucilaginibacter	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.52	0.00	0.00	0.00	0.00	0.00	0.00
Staphylococcus	0.03	0.00	0.31	0.00	0.00	0.00	0.01	0.11	0.00	0.14	0.22	0.09	0.40	0.22	0.07	0.00	0.00	0.00	0.00	0.00	0.00
Herbaspirillum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.27	0.00	0.00	0.00	0.00	0.00	0.00
Cutibacterium	0.26	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.72	0.00	0.00	0.04	0.00	0.00	0.00
Microbacterium	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.73	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00
Klebsiella	0.10	0.00	0.05	0.00	0.00	0.00	0.00	0.14	0.00	0.13	0.07	0.11	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Fig. 3. Top 25 most abundant taxa at genus level, ranked in decreasing order of their mean percent abundance in all samples of milk or cheese. The three sections of the image correspond to the three temperatures of milk collection; left: 4 °C, center: 13 °C, right: 7 °C. Milk values are shown as single separate columns left of their derived cheese products, which are in turn ordered by cheese maturation time, in each of the three panels. Shades from green to red of the Microsoft Office Excel conditional formatting tool evidence abundance changes and, in particular, the appearance of additional taxa at the mid-trial peak temperature of 13 °C. Sample names in the headline follow the same legend used in Tab. 1. Note: *Burkholderia* also includes *Caballeronia, Paraburkholderia*; *Methylobacterium* includes also *Methylorubrum*; *Hafnia* also includes *Obesumbacterium.* (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the cloud of samples that in panel 4a correspond to a milk temperature of 7 °C. Finally, the full opposition of outcome is the one exemplified by *Lactococcus lactis*, which was most scarce at the coldest temperature of 4 °C and highest at the warmest one of 13 °C (Fig. 4d).

3.5. Significant trade-offs in differentially represented taxa between pairs of temperatures

A differential analysis of the cheese sample communities was also run in order to extract all cases of taxa having statistically significant differences of representation in relation to chosen variables, and to rank them in order of fold change extent. The analysis included both comparisons between timewise consecutively adjacent temperature pairs (from 4 °C to 13° and from 13 °C to 7 °C) and the non-adjacent case (4 °C-7 °C).

The two temporally closest pairwise comparisons were the ones showing the highest number of changes. Increasing from 4° to 13 °C brought a significant increase for11 taxa and a significant decrease for three. After the temperature peak, lowering it back to 7 °C was even more effective, as it caused a downshifting for 13 taxa and an increase for 4. On the other hand, comparison of the non-adjacent indirectly connected temperatures of 4 °C and 7 °C, in which temperature changes were achieved in the opposite (decreasing) direction, showed the lowest extent of shifts (5 and 3 respectively).

3.6. Quantitative assessment of total bacteria in cheese by 16S gene qPCR amplification

In order to inspect possible differences related to milk storage temperature or cheese maturation stage in terms of total bacterial density within the cheese samples, a quantitative approach by RealTime Polymerase Chain Reaction was used. This targeted the general bacterial gene encoding the small subunit ribosomal RNA (16S) PCR by universal eubacterial primers. Results are shown in Table 2, in which data are comparatively arranged in different sets to highlight either milk storage temperature-related differences across different times of maturation, or time-related differences in samples coming from the same milk storage temperature.

All data ranged across a maximum span of three Ct values, encompassing density values within an 8-fold scale of density differences. Significance was observed in relation to maturation time, with a consistent increase of bacterial density at the intermediate stage of 15 days, which continued through to the final 30 days stage in the standard 4 °C preparation, but reversed in both higher milk temperature experiments.

The across-temperature comparisons showed significant differences in most cases, with the exception of 4° and 13° at time zero, and a consistently lower density of bacteria in the cheese samples deriving from milk that had been stored at the peak temperature of 13 °C.

3.7. Functional phenotypes prediction of the microbial communities

The predictive inference of community functions in relation to the known metabolic and ecological properties obtained by the FAPROTAX analysis, indicated as dominant functional groups chemoheterotrophy, fermentation, aerobic chemoheterotrophy, aromatic compound degradation, hydrocarbon degradation, aromatic hydrocarbon degradation and aliphatic non methane hydrocarbon degradation. The functional groups showed no statistical differences between samples (T-test, p-value ≤ 0.05), in line with a tightly controlled man-managed technology process of a given kind.

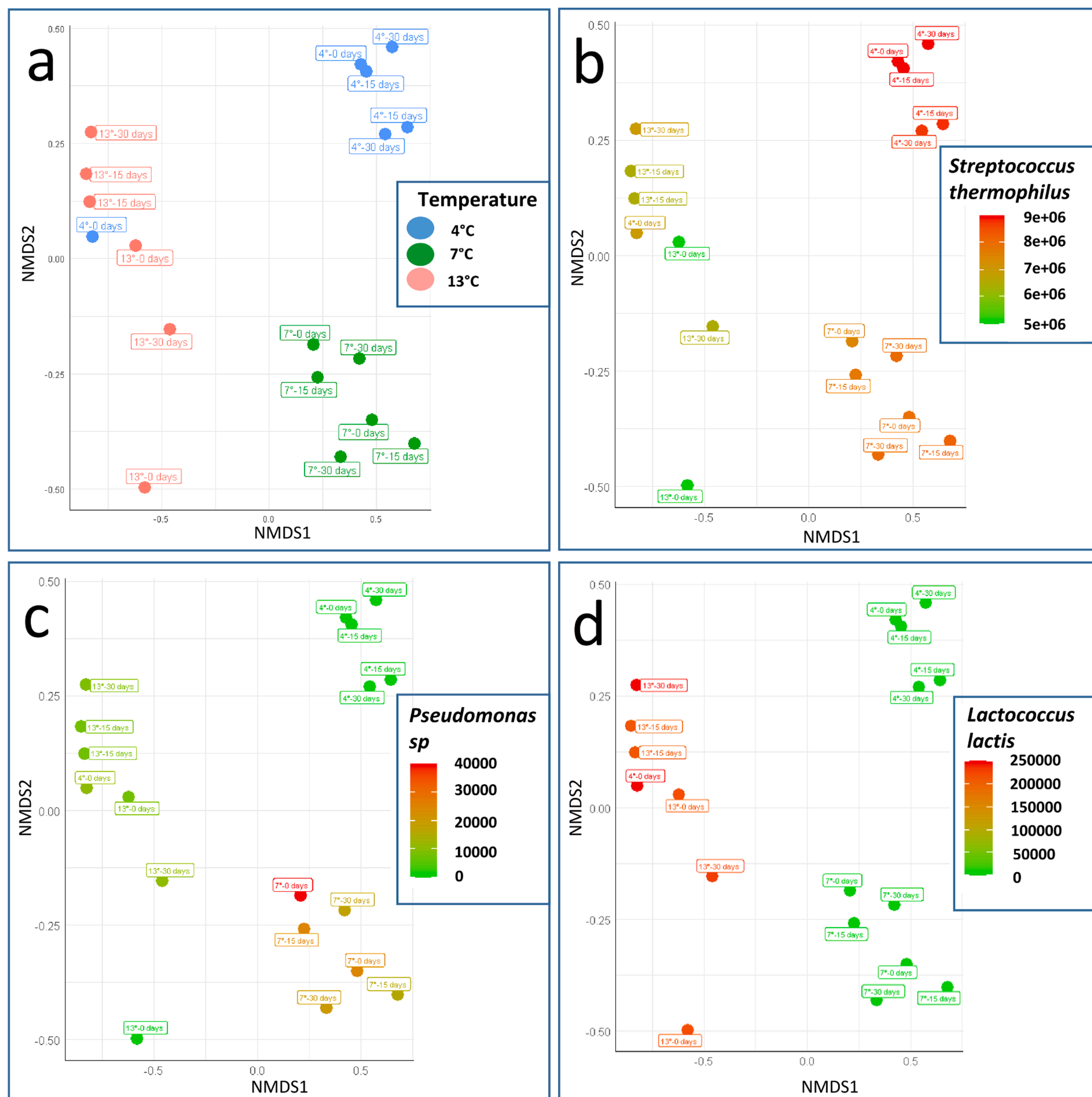

Fig. 4. Non-Metric Multidimensional Scaling (NMDS) of the sequencing data matrix for the cheese samples, showing the resulting spatial ordination of each bacterial community and their correspondence to: originating milk storage temperatures (a), quantitative abundance of *Streptococcus thermophilus* (b), or *Pseudomonas* sp. (c); or *Lactococcus lactis*.

4. Discussion

The experimental goal of the present investigation involved a deliberate simulation of thermal abuse and of its retrogression, within an actual dairy processing supply chain. The consequences in terms of altered proportions of bacterial functional guilds were unraveled by culture independent community DNA *meta*-barcoding. A question of major interest to the partner company hosting the trial was the assessment of the reciprocal dominance of bacterial groups with psychrotrophic activity relating to plain thermoduric taxa upon possible temperature changes. Such thermal alterations were purposely driven and pushed to their limits in order to assess the full potential extent of the phenomena and the timing needed for their full recovery. The reason for such practical interest from the cheese manufacturing industry was the fact that accidental low range shifts in temperature, possibly occurring either at farm level or during milk transportation to dairy plants, are technically likely to occur in routine production cycles. However, it was still unclear as to whether these shifts contribute towards any irregularities in the quality outcome of the product. Therefore, the purpose of this study was to acquire and interpret information relating to the effects of a tightly manipulated variable, on the downstream cascade of biotic interactions unfolding in the maturing cheese product.

Particular care was taken in the setup to ensure that each of the targeted thermal conditions would be attained as a result of a slow, gradual increase, through steps. Once attained, the temperature would be kept at that level for 12 days before sampling of the daily milk load was carried out. It is worth clarifying that the 12 day lapse was not the time that a single batch of milk was kept in the tank. In fact, it was the time during which each given temperature was maintained in the tank, where the milk was regularly refreshed on a daily basis, as is standard practice for a dairy production chain. The participating dairy plant decided it was important to allow 12 days of operation (with daily collecting and refilling of milk but without sampling analyses) as

Table 2
Means of the qPCR Cycle Threshold (Ct) values observed for the different samples. Each Ct value indicates at which of the repeated doubling cycles of the chain reaction amplification run, the first positive signal was detected. Lower numbers correspond therefore to higher target DNA density within the sample, and each integer number increment equates a target concentration of half with respect to the prior cycle. Top panels: same temperature/different times comparisons; bottom panels: same time/different temperature comparisons. Within each triad, values sharing the same letter are not significantly different ($p < 0.05$).

Sample	Ct	Sample	Ct	Sample	Ct
C 4°/0	13.86±0.76 a	C 13°/0	14.23 ±0.23a	C 7°/0	12.61±0.83 a
C 4°/15	12.42 ±0.21b	C 13°/15	13.39 ±0.16b	C 7°/15	11.67±0.85 a
C 4°/30	12.09±0.23c	C 13°/30	14.61 ±0.29c	C 7°/30	12.92±0.48 a
Sample	Ct	Sample	Ct	Sample	Ct
C 4°/0	13.86±0.76 a	C 4°/15	12.42 ±0.21a	C 4°/30	12.09±0.23 a
C 13°/0	14.23±0.23 a	C 13°/15	13.39 ±0.16b	C 13°/30	14.61 ±0.29b
C 7°/0	12.61 ±0.83b	C 7°/15	11.67 ±0.85a	C 7°/30	12.92±0.48c

acclimatization of the tank would start to reflect a given microbial community legacy only after a period of sufficient conditioning. Therefore, this approach to the gradient end was meant to make the tank environment an established equilibrium rather than that of a sudden change. To guarantee smooth progression, the steps also involved two further temperature levels in the uprising phase before the peak (7 °C and 11 °C ascending), which accounted for a total conditioning time that spanned a two-month period, from start to finish. Another important aspect is that the trial has been serial and not parallel. This means that the same tank was conditioned in time to the sequentially changing temperatures, implying also a series of dependent data with an environmental legacy exerted from the prior communities. This aspect was deemed important as it ruled out a stochasticity and possible randomness of variability in the founding starting points that would have occurred in a parallel setup. The preference for sample-dependence is due to the fact that in real industrial circumstances, a thermal abuse event occurs to a chain line which was, up to that point, operated as standard, bringing about a possible change. The concept also involves a truly connected successional series of perturbations occurring within the same enviroment, allowing more grounded interpretations to be drawn from the standpoint of applied ecology.

Interestingly, the taxonomic diversity of pasteurized milk stored at higher-than-standard temperatures was only moderately higher (Tab.1), while that of the respective cheese samples showed a much more evident increase as early as at 0 days, and, moreover, without ulterior gains over the following month of maturation (Tab.1, Fig. 1). It is important to also bear in mind that, given the DNA-based experimental methodology adopted, which is based on a target gene amplification following a thermal denaturation at 95 °C, milk pasteurization (at 75 °C) is not bound to negatively affect the results. This is because DNA from both live and dead bacterial cells would be equally processed, denatured, and their DNA equally sequenced. In fact, in this kind of study, any live cell in the environment under study is killed by the DNA extraction protocol. In the present study, results regarding milk are therefore meant to be interpreted as also revealing the load of bacteria from raw milk. This is irrespective of pasteurization after which the milk samples, destined to metabarcoding, had been immediately frozen, while awaiting the thawing and DNA extraction steps.

However, the ecological parameter that showed a more noticeable difference in milk at the peak temperature of 13 °C was community evenness (Tab. 1), reflecting the higher abundance value for several of the taxa that associated with the more mesophilic storage temperature, indirectly leading to a lower dominance of the few that characterize colder temperatures.

Compositionally, the standard 4 °C milk from the farm involved in this trial was high in *Rhodococcus*.(Fig. 2) This is a typical occurrence, reportedly more associated to the milk of cows having lower estimated breeding values, in respect to their resilience to mastitis (Tarrah et al., 2022). The higher temperatures brought about visibly irregular successional instances of dominance in milk, with the peak temperature community also showing its role in challenging the starter-driven *Streptococcus* prevalence in the corresponding cheese (Fig. 2). This figure shows how bacterial community composition in milk differs greatly across the three temperatures and that cheese made from milk stored at 13 °C shows an even lower distribution of *Streptococcus*, in spite of its inoculation as a cheese starter.

The clearest view of the global effect of the present experimental approach is offered by the sequential left-to-right observation of the three portions in Fig. 3. In essence, the color coding demonstrates that attaining a storage temperature which is over fourfold higher had a boosting effect on the relative abundance of a pre-existing minority of taxa, as well as an advent of additional ones. These were interpreted as either newcomers or as previously cryptic cells reaching detectable thresholds of abundance.

This phenomenon is paralleled by the percentage lowering of the otherwise cheese-dominant taxon *Streptococcus thermophilus*, as can be visually appreciated by the paler shade of green of the first row in the middle panel part of the figure. Equally informative is the view of the right 1/3 of the image, in which one can notice that the picture is being reset back to a level which starts to mirror the initial array of cheese data (the left 1/3 of the same image). This indicates that reversing the direction of the milk tank temperature change by refrigeration guides communities back towards the pristine reference point. This is a relevant piece of knowledge as it shows a reversibility of the phenomenon and how a single manipulated variable of temperature is technically sufficient to achieve system resilience in spite of possible community legacy interference. It can be seen that the community at the initial standard milk T of 4 °C is similarly mirroring the one restored by the lowering of the temperature to 7 °C, after having reached the peaks of complexity, with the rise of additional taxa occurring at the central panel of 13 °C. Consequently, the technical view is that a thermal abuse occurring at a higher temperature does not lead to a drift towards an unknown outcome, as pristine communities can be restored by temperature lowering. For a dairy factory, this evidence can be of great relevance in deciding under which circumstances they must disassemble and clean segments of their production chain, or when counting on their resilience to act on process ruling parameters.

Regarding the microbial diversity assessment potential of the method employed, it is also worth commenting that the percentage data shown in Fig. 3 are the consolidated sums of different rows pointing to each given genus. For example, in the original full sequence matrix of the results, the top scoring *Streptococcus* is represented by 110 different amplified sequence variants, most of which are annotated under the *S. thermophilus* species and others under the *Streptococcus* sp. denomination. Considering also that data are denoised and filtered for possible sequencing errors, by excluding reads occurring less than twice, and that 97 out of those 110 unique *Streptococcus* sequences are found more than 10 times in the project, one can appreciate the actual extent of microbiodiversity today featured today by this long-ago man-selected microbiological type of environment.

To have a broader appreciation of the level of hidden diversity, in spite of the known nomenclatural taxonomy, one can consider that, with 1,714 being the unique amplified single variants found in the whole analysis dataset, and the taxonomical database annotations being only 394, the ratio of actual variants per known name is over 4.3. We have covered the issue of potentially overlooked diversity in environmental metabarcoding studies in a prior report (Fasolo et al., 2020).

As stated, *S. thermophilus* was identified as the most abundant species in cheese with a mean of 89.8% in cheese deriving from milk stored at 4 °C, dropping to a relative abundance average of 59.4% in cheese from milk stored at 13 °C and restoring back to 82.9% in cheese from milk stored at 7 °C. Even if we disregard the present experimentation, its presence in cheese is in line with common aspects of dairy technology, with *L. lactis* and *S. salivarius* subsp. *thermophilus* both being major players in cheese-making as well as widely used as starter cultures, (Panagou et al., 2013; Shani et al., 2021) the present case being no exception.

The NMDS multivariate ordination panels compared in Fig. 4 extract the hierarchical ranking of the milk temperature variable as the main driver of community composition in comparison to cheese maturation times. As Fig. 4a shows, the three clustering clouds of communities rather strongly match the three milk temperature values, with the highest one (13 °C) being the least concentrated, in agreement with its higher variability and lower dominance of communities. The relative positions of the samples from different maturation times, that can be tracked by the sample labels shown near each point in the same figure, are clustered in suborder within each of the three temperatures data clouds. The other three panels have been chosen as examples of alternative selection responses by three representative and physiologically-contrasting taxa. *S. termophilus* has the highest abundance score (red data points) in cheese from milk stored at the coldest (4 °C) temperature, and encounters its minima (green data points) at the opposite warmest one of 13 °C (Fig. 4b). The psychrotroph *Pseudomonas* sp., instead expresses its maximum abundance in the communities corresponding to a temperature of 7 °C. Lastly, a partially complementary behavior is

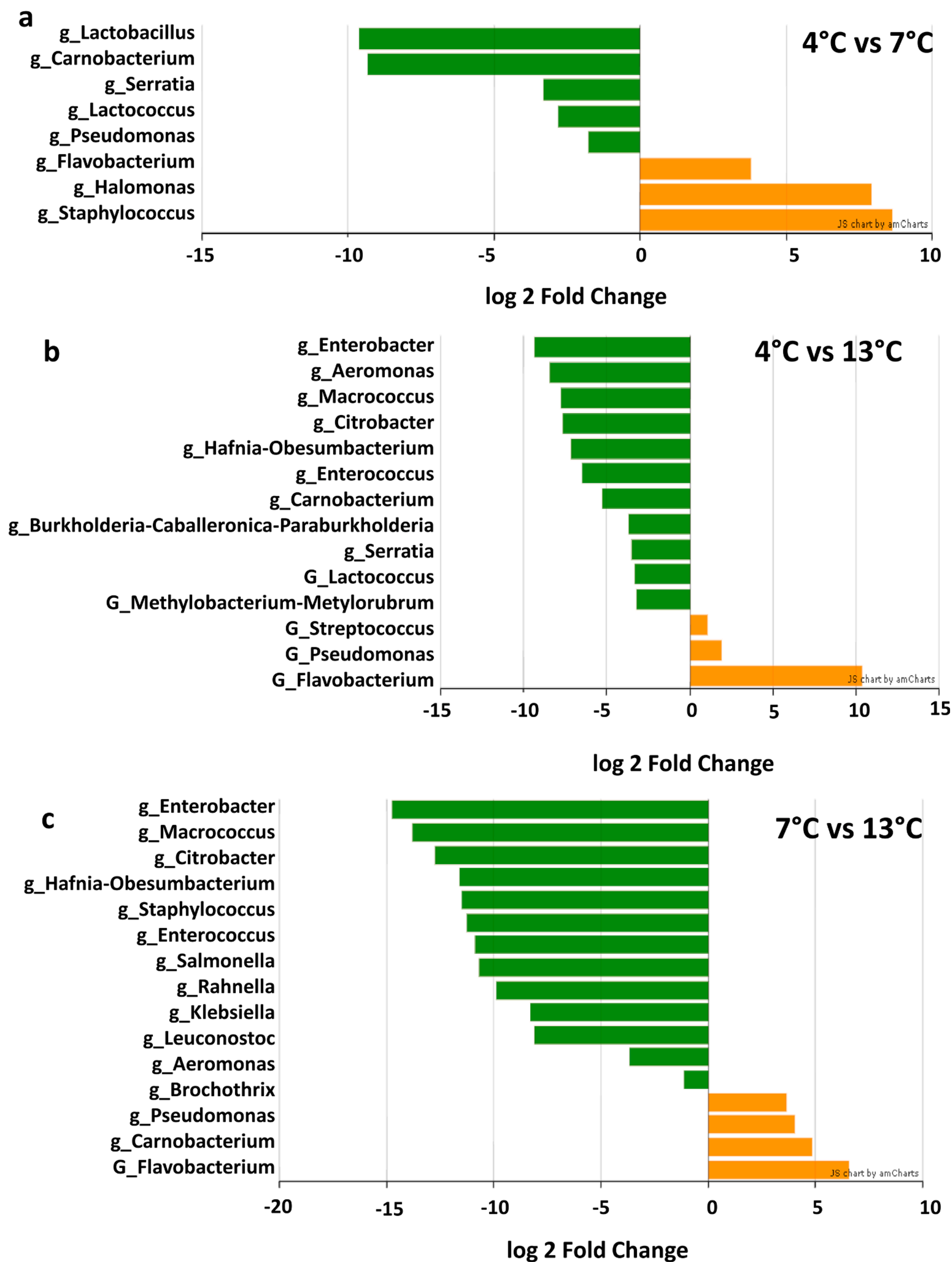

Fig. 5. Genera displaying significantly different representation ($p < 0.05$) in the sample communities upon comparisons between pairs of temperatures. a: 4 °C vs 7 °C, b: 4 °C vs. 13 °C; c: 7 °C vs 13 °C. The log 2 fold change extent is plotted on the horizontal scale. Up-shifted taxa are indicatd by rightwards orange bars and down-shifted ones by leftward green bars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

displayed by *Lactococcus lactis* (Fig. 4d), which fills the opposite temperature niche when compared to *Streptococcus* (Fig. 4b), being mostly featured at 13 °C and minimally present at 4 °C and 7 °C.

The information conveyed in Fig. 3 and Fig. 4 is complemented by a list of all those taxa whose reciprocal fluctuations are also statistically supported in the differentially represented genera analysis reported in the three pairwise temperature comparisons in Fig. 5. Here also less abundant but significantly shifting taxa are listed and the log2 fold change allows to assess the extent of variation. The invasion of several milk-opportunistic Gammaproteobacteria is well evidenced in the contrast between 4 °C and 13 °C, and equally clear is their retreat when the temperature of the same milk tank is reversed from 13 °C back to 7 °C.

The functional inference of metabolic profiles in this survey was run essentially as a check for any unexpected contamination by microorganisms with phenotypes not related to the usual dairy guilds. The FAPROTAX method was in fact primarily developed to explore environmental sites in which various unpredicted metabolisms could occur. Therefore, when dealing with man-managed technical contexts such as this, the technique essentially has the role of ruling out or spotting trends of aberrant metabolic drift. In this case, the predicted phenotypes and their proportions were in line with the known food microbial community framework.

In terms of bacterial density within the maturing cheese, as shown in Tab. 2, the situations in which milk had been stored at higher temperatures (13 °C) also revealed the dynamics in which the total bacterial concentration, in a statistically significant manner, resulted two- to four-fold lower than in the standard control cheese. Apart from showing taxonomical differences, this evidence casts a light on another interesting and overlooked aspect of food microbiology, namely that dairy process efficiency, its timing, and the organoleptic properties of the final product, are in large part direct functions of the kinetics, enzymology, and concentration of the biotic microbial agents engaged in the transformation.

With regard to the relevant technical questions which the dairy company sought to answer in this trial, the tests allowed us to determine to what extent an irregular temperature ramping in the milk collection and transfer stage would carry over its effect on cheese production via microbial community metabolism. In this respect it is worth recalling that the two temperatures, besides the standard one, corresponding to samples selected for metabarcoding, were those, among a wider series, that generated products with properties found deviant from standards in the company's test panel judgement.

In terms of addressing critical factors among the psychrotrophs, a main concern in dairy processes is a possible rise of *Pseudomonas*, a major agent of proteolysis, and that its dynamics in relation to temperature are not univocally predictable. Through this analysis it has been possible to observe that a rise to 13 °C of the milk storage temperature caused a reduction of its relative abundance to an extent of 2 log2 fold change (Fig. 5 b), but a lowering of the temperature to 7 °C, i.e., still higher than standard, had formerly caused its increase by as much as 4 log2 fold change (Fig. 5c). The same can be seen in the NMDS of Fig. 4c). Its individual dynamics in each different sample can be further inspected in the 9th row of Fig. 3, observing its very low percentages in all 4 °C samples. There is a further reduction occurring at 13 °C, and a dramatic increase unfolding at 7 °C with peaks as high as 5%. With milk at 13 °C the numerical competition with Enterobacteriales, among which *Serratia, Klebsiella, Vibrio*, and others, including coliforms such as *Citrobacter*, outcompeted *Pseudomonas*. The condition recreated in the present trial by incomplete milk refrigeration (13 °C) has caused a consortium whose proportions show that they have indeed downshifted *Pseudomonas* by the rise of several Enterobacteriales and similar taxa. Among psychrotrophs, the behavior of *Pseudomonas* was moreover different from that of other taxa such as *Serratia*, which was equally featured across cheese from milk kept at 13 °C or 7 °C, or that of saccharolytic ones, such as *Aeromonas*, which bloomed only in cheese from milk stored at 13 °C.

In conclusion, the data fulfilled the needs of the industrial partner by providing a series of clues to anticipate the outcome of product quality properties in relation to thermal abuses and their reversion. It also linked perceivable olfactory and taste aspects during customary in-house panel tests to defined patterns of microbial accumulation proportions.

The simulations enabled us to measure and disengage the relative importance of variables, linked to the phases of transporting milk from the farm, from those pertaining to subsequent processing at the cheese production plant. All these pieces of acquired information correspond to an advancement of knowledge in the field from a scientific point of view, as well as enabling the implementation of relevant controls that can be applied with enhanced technical awareness by the food industry.

CRediT authorship contribution statement

Lucia Giagnoni: Conceptualization, Formal analysis, Validation, Methodology, Investigation, Writing – original draft. **Saptarathi Deb:** Data curation, Formal analysis, Methodology. **Alessandra Tondello:** Formal analysis, Investigation, Validation, Writing – review & editing. **Giulia Zardinoni:** Writing – review & editing. **Michele De Noni:** Methodology, Investigation. **Cinzia Franchin:** Validation. **Alice Vanzin:** Data curation. **Giorgio Arrigoni:** Funding acquisition. **Antonio Masi:** Funding acquisition. **Piergiorgio Stevanato:** Resources. **Alessio Cecchinato:** Project administration, Funding acquisition, Writing – review & editing. **Andrea Squartini:** Conceptualization, Resources, Funding acquisition, Supervision, Writing – review & editing. **Carlo Spanu:** Conceptualization, Resources, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was made possible in part by a research grant from Latteria Montello S.p.A., to the University of Padova, Contract "*Analisi microbiologiche e proteomiche su latte e prodotti della sua lavorazione*"

References

Almeida, M., Hébert, A., Abraham, A. L., Rasmussen, S., Monnet, C., Pons, N., & Renault, P. (2014). Construction of a dairy microbial genome catalog opens new perspectives for the metagenomic analysis of dairy fermented products. *BMC Genomics, 15*, 1–16. https://doi.org/10.1186/1471-2164-15-1101

Bertuzzi, A. S., Walsh, A. M., Sheehan, J. J., Cotter, P. D., Crispie, F., McSweeney, P. L., & Rea, M. C. (2018). Omics-based insights into flavor development and microbial succession within surface-ripened cheese. *MSystems, 3*(1), e00211–e217. https://doi.org/10.1128/mSystems.00211-17

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., & Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *NatureBiotechnology, 37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Choi, K. H., Lee, H., Lee, S., Kim, S., & Yoon, Y. (2016). Cheese microbial risk assessments-a review. *Asian-Australasian Journal of Animal Sciences, 29*(3), 307–314. https://doi.org/10.5713/ajas.15.0332

Choi, J., Lee, S., Rackerby, B., Goddik, L., Frojen, R., Ha, S., Kim, J. H., & Park, S. H. (2020). Microbial communities of a variety of cheeses and comparison between core and rind region of cheeses. *Journal of Dairy Science*. https://doi.org/10.3168/jds.2019-17455

Cousin, M. A. (1982). Presence and activity of psychrotrophic microorganisms in milk and dairy products: A review. *Journal of Food Protection, 45*(2), 172–207. https://doi.org/10.4315/0362-028X-45.2.172

Decimo, M., Morandi, S., Silvetti, T., & Brasca, M. (2014). Characterization of gram-negative psychrotrophic bacteria isolated from Italian bulk tank milk. *Journal of Food Science, 79*(10), M2081–M2090. https://doi.org/10.1111/1750-3841.12645

De Filippis, F., Genovese, A., Ferranti, P., Gilbert, J. A., & Ercolini, D. (2016). Metatranscriptomics reveals temperature-driven functional changes in microbiome impacting cheese maturation rate. *Scientific Reports, 6*(1), 21871. https://doi.org/10.1038/srep21871

De Filippis, F., Parente, E., & Ercolini, D. (2018). Recent past, present, and future of the food microbiome. *Annual Review of Food Science and Technology, 9*, 589–608. https://doi.org/10.1146/annurev-food-030117-012312

Oliveira, G. B. D., Favarin, L., Luchese, R. H., & McIntosh, D. (2015). Psychrotrophic bacteria in milk: How much do we really know? *Brazilian Journal of Microbiology, 46*, 313–321. https://doi.org/10.1590/S1517-838246220130963

Ercolini, D., Russo, F., Ferrocino, I., & Villani, F. (2009). Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk. *Food Microbiology, 26*(2), 228–231. https://doi.org/10.1016/j.fm.2008.09.005

Falardeau, J., Keeney, K., Trmčić, A., Kitts, D., & Wang, S. (2019). Farm-to-fork profiling of bacterial communities associated with an artisan cheese production facility. *Food Microbiology, 83*, 48–58. https://doi.org/10.1016/j.fm.2019.04.002

FAO. 2022. Dairy Market Review: Emerging trends and outlook 2022. Rome. Available at: https://www.fao.org/3/cc3418en/cc3418en.pdf Accessed January 26, 2023.

Fasolo, A., Treu, L., Stevanato, P., Concheri, G., Campanaro, S., & Squartini, A. (2020). The hidden layers of microbial community structure: Extracting the concealed diversity dimensions from our sequencing data. *FEMS Microbiology Letters, 367*(11), fnaa086. https://doi.org/10.1093/femsle/fnaa086

Ferrocino, I., Rantsiou, K., & Cocolin, L. (2022). Investigating dairy microbiome: An opportunity to ensure quality, safety and typicity. *Current Opinion in Biotechnology, 73*, 164–170. https://doi.org/10.1016/j.copbio.2021.08.009

Hahne, J., Isele, D., Berning, J., & Lipski, A. (2019). The contribution of fast growing, psychrotrophic microorganisms on biodiversity of refrigerated raw cow's milk with high bacterial counts and their food spoilage potential. *Food Microbiology, 79*, 11–19. https://doi.org/10.1016/j.fm.2018.10.019

Hammer, Ø., Harper, D. A., & Ryan, P. D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia electronica, 4*(1), 9. http://palaeo-electronica.org/2001_1/past/issue1_01.htm.

Leigh, M. B., Pellizari, V. H., Uhlík, O., Sutka, R., Rodrigues, J., Ostrom, N. E., … Tiedje, J. M. (2007). Biphenyl-utilizing bacteria and their functional genes in a pine root zone contaminated with polychlorinated biphenyls (PCBs). *The ISME Journal, 1*(2), 134–148. https://doi.org/10.1038/ismej.2007.26

Lorenzo, J. M., Munekata, P. E., Dominguez, R., Pateiro, M., Saraiva, J. A., & Franco, D. (2018). In *Main groups of microorganisms of relevance for food safety and stability: general aspects and overall description* (pp. 53–107). Academic Press. https://doi.org/10.1016/B978-0-12-811031-7.00003-0.

Louca, S., Parfrey, L. W., & Doebeli, M. (2016). Decoupling function and taxonomy in the global ocean microbiome. *Science, 353*(6305), 1272–1277. https://doi.org/10.1126/science.aaf4507

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet Journal, 17*(1), 10–12. https://doi.org/10.14806/ej.17.1.200

Mayo, B., Rodríguez, J., Vázquez, L., & Flórez, A. B. (2021). Microbial interactions within the cheese ecosystem and their application to improve quality and safety. *Foods, 10*(3), 602. https://doi.org/10.3390/foods10030602

Montel, M. C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D. A., Desmasures, N., & Berthier, F. (2014). Traditional cheeses: Rich and diverse microbiota with associated benefits. *International Journal of Food Microbiology, 177*, 136–154. https://doi.org/10.1016/j.ijfoodmicro.2014.02.019

O'Donnell, S. T., Ross, R. P., & Stanton, C. (2020). The progress of multi-omics technologies: Determining function in lactic acid bacteria using a systems level approach. *Frontiers in Microbiology, 10*, 3084. https://doi.org/10.3389/fmicb.2019.03084

Paludetti, L. F., Kelly, A. L., & Gleeson, D. (2020). Effect of thermoresistant protease of Pseudomonas fluorescens on rennet coagulation properties and proteolysis of milk. *Journal of Dairy Science, 103*(5), 4043–4055. https://doi.org/10.3168/jds.2019-17771

Panagou, E. Z., Nychas, G.-J.-E., & Sofos, J. N. (2013). Types of traditional Greek foods and their safety. *Food Control, 29*, 32–41. https://doi.org/10.1016/j.foodcont.2012.05.050

Parente, E., De Filippis, F., Ercolini, D., Ricciardi, A., & Zotta, T. (2019). Advancing integration of data on food microbiome studies: FoodMicrobionet 3.1, a major upgrade of the FoodMicrobionet database. *International Journal of Food Microbiology, 305*, Article 108249. https://doi.org/10.1016/j.ijfoodmicro.2019.108249

Parente, E., Ricciardi, A., & Zotta, T. (2020). The microbiota of dairy milk: A review. *International Dairy Journal, 107*, Article 104714. https://doi.org/10.1016/j.idairyj.2020.104714

Pasquali, F., Valero, A., Possas, A., Lucchi, A., Crippa, C., Gambi, L., Manfreda, G., & De Cesare, A. (2022). Occurrence of foodborne pathogens in Italian soft artisanal cheeses displaying different intra-and inter-batch variability of physicochemical and microbiological parameters. *Frontiers in Microbiology, 13*. https://doi.org/10.3389/fmicb.2022.959648

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., … Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research, 41*(D1), D590–D596. https://doi.org/10.1093/nar/gks1219

Rosengren, Å., Fabricius, A., Guss, B., Sylvén, S., & Lindqvist, R. (2010). Occurrence of foodborne pathogens and characterization of Staphylococcus aureus in cheese produced on farm-dairies. *International Journal of Food Microbiology, 144*(2), 263–269. https://doi.org/10.1016/j.ijfoodmicro.2010.10.004

Shani, N., Isolini, D., Marzohl, D., & Berthoud, H. (2021). Evaluation of a new culture medium for the enumeration and isolation of Streptococcus salivarius subsp. thermophilus from cheese. *Food Microbiology, 95*, Article 103672. https://doi.org/10.1016/j.fm.2020.103672

Tarrah, A., Callegaro, S., Pakroo, S., Finocchiaro, R., Giacomini, A., Corich, V., & Cassandro, M. (2022). New insights into the raw milk microbiota diversity from animals with a different genetic predisposition for feed efficiency and resilience to mastitis. *Scientific Reports, 12*(1), 13498. https://doi.org/10.1038/s41598-022-17418-2

Tilocca, B., Costanzo, N., Morittu, V. M., Spina, A. A., Soggiu, A., Britti, D., & Piras, C. (2020). Milk microbiota: Characterization methods and role in cheese production. *Journal of Proteomics, 210*, Article 103534. https://doi.org/10.1016/j.jprot.2019.103534

Walsh, A. M., Crispie, F., Claesson, M. J., & Cotter, P. D. (2017). Translating omics to food microbiology. *Annual Review of Food Science and Technology, 8*, 113–134. https://doi.org/10.1146/annurev-food-030216-025729

Yeluri Jonnala, B. R., McSweeney, P. L., Sheehan, J. J., & Cotter, P. D. (2018). Sequencing of the cheese microbiome and its relevance to industry. *Frontiers in Microbiology, 9*, 1020. https://doi.org/10.3389/fmicb.2018.01020