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Combining microbiological and technological indicators into a qualitative score for optimizing raw milk refrigeration in cheesemaking

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ABSTRACT

Milk storage plays a crucial role in preserving microbiological and technological qualities required for cheesemaking. This study evaluated the impact of storing raw milk at 4, 6, 8, and 10 °C for 48 h on microbial safety, spoilage dynamics, and cheesemaking suitability. Microbial stability was maintained at ≤8 °C, whereas storage at 10 °C led to significant spoilage development and pathogen proliferation, compromising milk safety. Across all conditions, slower acidification was observed, which may be associated with interactions between starter cultures and the native microflora. Moreover, milk stored at 10 °C showed increased proteolysis and volatile production, due to high levels of presumptive *Pseudomonas* spp. Lower temperatures effectively controlled safety hazards and minimized spoilage but induced increased pH, calcium migration, and casein solubilization, which negatively affect milk cheesemaking properties. By integrating pathogen behavior, psychrotrophic proliferation, and process-relevant indicators, a qualitative decision-support score was developed to guide safe and optimal refrigeration practices. The proposed scoring tool provides an operational framework for risk mitigation and process control in dairy chains, identifying 6 °C/24 h as the safest and most effective compromises, limiting pathogen proliferation while preserving cheesemaking potential. These findings provide evidence-based guidance for dairy chain management, supporting both product safety and cheese quality.

1. Introduction

Raw bovine milk produced on European farms is mainly stored in the farm bulk tank at refrigerated temperatures for periods exceeding 2 days before being delivered to dairy processing plants (O'Connell et al., 2016). Early refrigeration of milk immediately after milking is essential to prevent or slow the growth of the naturally present microflora, which includes indigenous as well as potentially spoilage- or pathogenic microorganisms (Malacarne et al., 2013; Raats et al., 2011). Moreover, extending refrigeration time on the farm could rationalize collection routes, reduce transport costs, and increase flexibility both on the farm and during milk processing (Franciosi et al., 2011). Generally, the time raw milk is stored under refrigerated conditions at the plant before processing varies with milk collection intervals and transport distances. According to European Commission Regulation (EC) No 853/2004, storage limits for raw milk depend on collection frequency: ≤8 °C for daily collection and ≤6 °C for less frequent collection; regarding transport, the milk temperature must not exceed 10 °C upon arrival at the destination facility. At the dairy, milk can be held for up to 48 h, provided the temperature does not exceed 6 °C after 4 h of storage (European Community, 2004).

If refrigeration is a good strategy for preserving product safety and limiting microbial pathogen growth, it may result in unwanted changes in the milk microbiota. As is well known, the microbial diversity of raw milk contributes to the wide variety of sensory characteristics of cheeses, as these communities are primarily involved in fermentation and ripening (Giagnoni et al., 2023; Renoldi et al., 2024). The dominant initial microflora generally includes lactic acid bacteria (LAB), mainly *Lactococcus* and *Lactobacillus* spp., as well as *Leuconostoc*, *Enterococcus*, and *Streptococcus* spp., which grow poorly at low temperatures. Also, the Micrococcaceae family (*Micrococcus* and *Staphylococcus spp.*), *Pseudomonas* spp., and yeasts are present, which can proliferate at low temperatures (Lafarge et al., 2004). As storage time increases and temperature decreases, psychrotrophic microorganisms (*Pseudomonas* spp., *Acinetobacter*) become more dominant in refrigerated raw milk. These microorganisms produce lipolytic and proteolytic enzymes that

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can break down milk fat globules and proteins, resulting in weaker curds, reduced cheese yield, and the release of volatile compounds that might lead to defects in the flavor and texture of cheeses (Decimo et al., 2018; Vithanage et al., 2017; von Neubeck et al., 2015).

In addition to microbiological aspects, refrigeration also affects specific physicochemical characteristics of milk, thereby influencing its cheesemaking properties. These changes depend on temperature and time and, in most cases, are largely reversible with mild heating (around 30 °C) (Maciel et al., 2015; Paggio et al., 2023). Refrigeration primarily causes the dissociation of β- and, to a lesser extent, α-caseins from micelles and the solubilization of calcium phosphate, which can affect the stability and coagulation properties of milk, thus altering its coagulation time and the quality of the curd (Mankai et al., 2012). The release of caseins from the micelle draws some of the calcium phosphate into the soluble phase, increasing the Ca^{2+} concentration. Changes in mineral balance led to higher milk pH, negatively affecting curd properties by diminishing rennet enzyme activity, which works best at a pH of 6.0-6.3 (Malacarne et al., 2013). Casein breakdown due to weakening of hydrophobic interactions induced by low temperatures increases the time required for curd formation, resulting in weak, unstable curds with lower resistance to compression and syneresis (Maciel et al., 2015). Refrigeration also influences some native enzymes in milk. Plasmin is a heat-resistant serine protease that contributes to flavor development and texture formation in hard and semi-hard cheeses by hydrolyzing caseins. However, prolonged milk refrigeration at low temperatures might reduce plasmin activity, potentially slowing cheese ripening and maturation processes (Crudden et al., 2005; Ismail & Nielsen, 2010). The fatty component of milk is also affected by low temperatures. Milk fat exists as globules that solidify at refrigeration temperatures, which can cause fat globule aggregation, affecting the cheesemaking process and the final product's texture. Additionally, refrigeration alters the milk fat globule membrane, releasing phospholipids and glycoproteins into the whey, which may influence cheese yield and quality (Malacarne et al., 2013). Milk changes related to low temperatures are particularly critical in the case of hard and semi-hard cheeses, for which good rennet coagulation capacity is essential, as well as the maturation that should be carried out by an indigenous microflora capable of imparting the desired sensory characteristics to the final product.

The impact of refrigerated storage on milk cheesemaking properties highlights the importance of managing storage temperature and time to control safety hazards and spoilage-related risks while maintaining high milk technological qualities. Psychrotrophic enzymes, pathogen persistence, and milk physicochemical instability can act as critical points along the dairy chain. However, comprehensive tools that integrate microbiological and technological indicators for operational decision-making remain lacking. Therefore, this study not only characterizes microbial and physicochemical dynamics but also proposes a qualitative score designed as a practical decision-support tool for safe milk storage management, in line with industry and regulatory needs.

In this context, this study aimed to evaluate the effects of different refrigeration conditions (4–10 °C for 24–48 h) on the microbiological and physicochemical characteristics of raw milk directly related to cheesemaking performance. By linking storage temperature to both microbial safety and technological suitability, the findings provide practical guidance for the dairy sector to optimize milk-handling strategies. Such knowledge is particularly relevant for ensuring product safety, preserving cheese quality, and supporting more sustainable milk collection and storage practices in modern dairy chains.

2. Materials and methods

2.1. Sampling

Raw cow's milk was supplied by a farm located in the North-East area of Italy. Milk was collected daily, and five aliquots were placed into 250-mL sterile glass bottles. The first aliquot was analyzed immediately upon arrival at the laboratory (control), while the other four were stored at 4, 6, 8, and 10 °C for 48 h. Storage temperatures were selected to represent both regulatory limits (≤6–8 °C) and realistic deviations that may occur in farm tanks or during milk collection, thus enabling a risk-oriented evaluation of microbial behavior. Analyses were carried out after 24 and 48 h. All microbiological, physicochemical, and technological analyses described below were performed on all milk samples across all storage conditions (control, 24 h and 48 h at 4, 6, 8, and 10 °C), unless otherwise specified.

2.2. Microbiological analysis

Milk samples were decimally diluted in Maximum Recovery Diluent (MRD; Oxoid, Milan, Italy), and examined for total mesophilic count onto Gelatin Sugar-Free Agar (Oxoid) plates incubated at 30 °C for 48 h, total and fecal coliforms in ColiID agar (bioMérieux Italia, Florence, Italy) at 37 °C for 24 h, coagulase-positive staphylococci onto Baird Parker agar (Oxoid) at 37 °C for 48 h, presumptive *Pseudomonas* spp. onto *Pseudomonas*-CFC agar (Oxoid) at 30 °C for 24 h, lactobacilli onto MRS agar (Oxoid) pH 5.4 at 37 °C for 48 h under anaerobic conditions in an anaerobic jar using a GasPak system, lactococci onto M17 agar (Oxoid) at 37 °C for 48 h, microstaphylococci on Mannitol Salt agar (Oxoid) at 30 °C for 48 h, and yeasts on Oxytetracycline Glucose Yeast Extract agar at 30 °C for 72 h. For total mesophilic count, coagulase-positive staphylococci, presumptive *Pseudomonas* spp., and microstaphylococci, LOD and LOQ were 10 CFU/mL and 100 CFU/mL, respectively, whereas for total and fecal coliforms, lactobacilli, lactococci, and yeasts, LOD and LOQ were 1 CFU/mL and 10 CFU/mL, respectively.

2.3. Growth of microbial pathogens

Milk samples were separately spiked with *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus*. For each species, a pool of three strains (a strain from an international collection and two wild strains isolated in previous studies from dairy sources) was used as follows: *E. coli* DSM 11250, *E. coli* DIAL4315, and *E. coli* DIAL1411, *L. monocytogenes* DSM 20600, *L. monocytogenes* DSA198, and *L. monocytogenes* DSA1195, *S. aureus* DSM 20231, *S. aureus* DIAL411, and *S. aureus* DSA226 (Innocente, Calligaris, et al., 2023; Innocente et al., 2019). The strains were separately grown overnight in BHI at 37 °C, then the cultures of the same species were pooled, and the cells were recovered by centrifugation at 13,000 × *g* for 5 min. Cells were washed twice and resuspended in MRD. The suspension was appropriately diluted and used to inoculate milk (200 mL) at about 10^3 and 10^5 CFU/mL. The milk samples were incubated, and viable counts were determined using (i) Violet Red Bile Lactose agar (Oxoid) incubated at 37 °C for 24 h for *E. coli*, (ii) Palcam agar (Oxoid) incubated at 37 °C for 48 h for *L. monocytogenes*, and (iii) Baird Parker agar incubated at 37 °C for 48 h for *S. aureus*. Results refer to culturable cells recovered on selective media under the applied incubation conditions.

2.4. pH determination

The pH of milk aliquots stored at 4, 6, 8, and 10 °C for 0, 24 and 48 h was measured using a pH meter (Basic 20, Crison Instruments, Spain) equipped with a temperature probe for readings adjustment. Before analysis, the instrument was previously calibrated with standard solutions at pH 4, 7, and 9.

2.5. Starter fermentation kinetics

Fermentation kinetics at 37 °C with a commercial thermophilic starter were determined in control and refrigerated milk samples. 0.25 g of freeze-dried starter culture (MT-2 Montasio, Biochem 2 S. r.l., Treviso, Italy) was resuspended in 50 mL of UHT whole milk at 37 °C for 30 min.

After rehydration, 1 mL of starter culture was used to inoculate 200 mL of milk (final dose: 0.025 g/L). The milk was then incubated at 42 °C for 8 h, and pH was measured every 30 min using a pH meter. The acidification curve was fitted to the Gompertz equation (Zwietering et al., 1990) using the Excel add-in DMFit, which allowed estimation of the lag phase length (Lag, h) and the maximum acidification rate (v_{max}, pH/h). To generate a curve in which the parameter on the y-axis increases over time, the measured pH values were converted into delta pH.

2.6. Calcium content analysis

Calcium content in milk samples was determined by complexometric titration with EDTA (ethylenediaminetetraacetic acid) (Sigma Aldrich, Germany) following the method proposed by Udabage et al. (2000). For the total calcium content, 40 mL of deionized water and 4 mL of 8 M sodium hydroxide solution (Sigma Aldrich, Germany) were added to 10 mL of raw milk. After 10 min, 0.1 g of Patton-Reeder indicator (obtained by mixing 0.5 g of calconcarboxylic acid indicator with 50 g of sodium sulfate, Sigma-Aldrich, Germany) was added to the mixture, which was then titrated with a 0.025 M EDTA solution.

For soluble calcium, 20 mL of milk were centrifuged at 100,000×*g* (36,000 rpm) for 1 h at 4 °C using an ultracentrifuge (Beckman Optima LE-80 K, Beckman Coulter, USA). The obtained whey was then filtered with 0.45 μm polyvinylidene fluoride (PVDF) syringe filters (LLG-Syringe filters, Lab Logistics Group GmbH, Germany) (De La Fuente et al., 1996). Therefore, 10 mL of the filtrate was analyzed using the same procedure as for total calcium. Both total and soluble calcium content were calculated using the following formula:

$$Calcium\ content\ (mg/L) = (\times V_{EDTA} \times AM) / m_{sample} \quad (1)$$

In the formula: C_{EDTA}, is the concentration (mmol/L) of the EDTA solution; V_{EDTA}, is the volume (mL) of EDTA used to titrate; AM, is the atomic mass of calcium; m_{sample}, is the weight (g) of the sample. Colloidal calcium was calculated from the difference between total and soluble calcium.

2.7. Determination of protein content

The content of α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein was determined by HPLC analysis (Bonfatti et al., 2008) on milk and whey obtained after ultracentrifugation (100,000×*g* for 1 h at 4 °C). Briefly, 500 μL of milk or whey were mixed with 500 μL of a solution A (0.1 M Bis-Tris buffer, 6 M guanidine chloride, 5.37 mM sodium citrate, and 19.5 mM dithiothreitol) (Sigma Aldrich, Germany), along with 2 mL of a solution B (acetonitrile, deionized water, and trifluoroacetic acid (TFA) in a ratio of 100:900:1 (v:v), and 4.5 M guanidine chloride) (Sigma Aldrich, Germany). After 5 min, the solution was filtered using 0.45 μm PVDF syringe membrane filters and stored at −18 °C until analysis (Bobe et al., 1998).

Chromatographic analysis was performed using a Varian Pro Star HPLC system (model 230, Varian Inc., USA), equipped with a Rheodyne 7725 injector (IDEX Corporation, USA), a 40 μL loop, and a reversed-phase C8 analytical column (5 μm, 150 × 4.6 mm, 300 Å, PLRP-S Polymer Laboratories, Varian Inc., USA). The gradient elution was carried out using solvent (A) consisting of 0.1% TFA in deionized water, and solvent (B) consisting of 0.1% TFA in acetonitrile. The separation was performed at 1 mL/min and 45 °C for 45 min. Detection was performed at 214 nm using a photodiode array (PDA) detector (Varian, Series Pro Star, mod. 330). Chromatograms were visualized using the System Control-Varian software. For the quantification, calibration curves ($R^2 = 0.999$) were obtained using standard solutions at different concentrations (0.50-7.35 mg/mL) of α-, β-, and κ-casein (Sigma Aldrich, Germany). The instrumental limit of detection (LOD, signal-to-noise ratio of 3:1) was 0.13, 0.12, and 0.30 mg/mL, while the limit of quantification (LOQ, signal-to-noise ratio of 10:1) was 0.42, 0.39, and 0.99 mg/mL for α-, β-, and κ-casein, respectively.

2.8. Determination of proteolysis

Proteolysis in milk samples was determined by HPLC analysis according to Vithanage et al. (2017), with some modifications. Briefly, 40 mL of milk were centrifuged at 16,000×*g* for 5 min at 4 °C (Beckman AVANTI J-25, Beckman Coulter, USA), and the fat was separated using a spatula. Then, 3 mL of supernatant were mixed with 0.36 g of trichloroacetic acid (TCA) (Sigma-Aldrich, Germany). Samples were incubated at 37 °C for 30 min, filtered through 0.45 μm PVDF syringe filters (Millipore, USA), and stored at −18 °C until analysis. Separation of TCA-soluble peptides was carried out adopting the same chromatographic procedure previously described for protein content analysis. Results were expressed as absolute areas of the peaks related to the TCA-soluble peptides.

2.9. Volatile component analysis

Volatile compounds were evaluated using the solid-phase microextraction technique coupled with gas chromatography-mass spectrometry (SPME-GC/MS) following the method described by Innocente, Renoldi, et al. (2023), with minor modifications. For the analysis, 10 mL of milk were placed into 20 mL vials, heated in a water bath at 45 °C, and agitated for 90 min. A 2 cm fused silica fiber, coated with a stationary phase made of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) with a thickness of 50/30 μm (57299-U Supelco, Sigma Aldrich, Germany) was exposed into the headspace of each vial at 45 °C for 45 min. A gas chromatograph (GC Agilent 7890 B, Agilent Technologies, USA) coupled to a mass spectrometer (MS Agilent 5977 A, Agilent Technologies, USA) with an electron impact ionization source and quadrupole analyzer was used. A polar column (Agilent DB-5ms, Agilent Technologies, USA) was employed, with a length of 30 m, an internal diameter of 0.25 mm, and a stationary phase film thickness of 0.5 μm. The fiber was then exposed to the injector at 260 °C for 3 min for desorption. The following operating conditions were set: splitless injection mode; carrier gas (helium) column flow rate at 1 mL/min; column oven temperature program: initial isotherm at 35 °C for 3 min, linear gradient of 3 °C/min up to 240 °C, final isotherm at 240 °C for 12 min; transfer line temperature at 280 °C. For the mass spectrometer, the source and quadrupole temperatures were set to 175 °C and 150 °C, respectively, with a mass range of m/z 40-350. The chromatograms were processed using Agilent MassHunter Qualitative Analysis B.06.00. The identification of the compounds was performed by comparing their mass spectra with those in the database provided with the instrument (NIST14) and their Kovat's retention index (RI) calculated from the retention time of n-alkanes (C7-C30) with those from the literature (https://webbook.nist.gov/chemistry/). Since no internal standards were used for quantification, data were reported as absolute peak areas.

2.10. Qualitative score

To study the overall effect of storage time (*i.e.*, 0, 24, and 48 h) and temperature (*i.e.*, 4, 6, 8, and 10 °C) on the suitability of milk for cheesemaking, microbiological and chemical results were processed to obtain a generic qualitative score (Curic et al., 2008). For all parameters related to physicochemical and microbiological quality indicators (*e.g.*, Lactococci, pH, Ketones), normalization (Y_i) was performed by applying the following Equations:

$$\overline{Y_t} = \frac{Y_0}{Y_i} \quad (2)$$

$$\overline{Y_t} = \frac{Y_i}{Y_0} \quad (3)$$

Where Y_0 is the optimal value corresponding to time zero, and Y_i is a generic measurement. Equation (2) was applied to indicators for which an increase is considered an undesired event, whereas Equation (3) was applied to those where an increase indicates improvement. This transformation forces all indicators to exhibit $\overline{Y_t} \geq 1$ for optimal conditions and $\overline{Y_t} < 1$ for suboptimal ones. For parameters related to the presence of both pathogenic and spoilage microorganisms (*e.g.*, *Listeria monocytogenes*, *Pseudomonas* spp.), a different normalization procedure was adopted, given their critical relevance for food safety (Equation (4)):

$$\overline{Y_t} = \frac{Y_{mo} - Y_i}{Y_{mo} - Y_0} \quad (4)$$

where Y_{mo} is the highest value of the microbial count recorded for specific microorganism, Y_0 is the value corresponding to time zero, and Y_i is the measurement within a certain storage condition (*i.e.*, time and temperature). Finally, for each storage condition, the normalized indicators were combined using the geometric mean according to the formula:

$$Qualitative\ score = \sqrt[N]{\prod_{i=1}^{N} \overline{Y_t}} \cdot 10 \quad (5)$$

where N denotes the number of indicators included (*i.e.*, $N = 16$), the resulting composite Qualitative score ranged from 0 to 10, providing an overall indication of milk suitability for cheesemaking. The data were graphed using OriginPro 2021 (OriginLab, Northampton, MA, USA).

2.11. Statistical analysis

Each analysis was conducted on three milk samples collected from the farm at different times. Moreover, data from physicochemical analysis were expressed as the mean ± standard deviation from at least three analytical determinations on three milk samples. Bartlett's test was conducted to check the homogeneity of variance within all data groups, while the one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test were performed to determine statistically significant differences among means ($p < 0.05$) by using R, version 4.2.3 (The R Foundation for Statistical Computing, Vienna, Austria). For microbiological counts, results below the limit of detection (LOD) were replaced with LOD/2 prior to statistical analysis.

3. Results and discussion

3.1. Effect of storage on the microbiological characteristics of milk

Table 1 displays microbial counts in milk immediately after milking (control) and after 24- and 48-h storage at 4-10 °C. Total and fecal coliforms and coagulase-positive staphylococci were below the limit of detection (LOD) in control milk and across all storage conditions. This indicates good hygienic conditions at milking and low initial contamination, and these indicators were therefore not discussed in terms of growth dynamics. The average total mesophilic bacterial count in the control sample was 4.17 ± 0.11 log CFU/mL, indicating high-quality milk and complying with EU Regulation 853/2004 and literature-reported values (Ercolini et al., 2009; Malacarne et al., 2013; Vithanage et al., 2017). LAB showed a higher prevalence of cocci (3.54 ± 0.17 log CFU/mL) than rods (2.35 ± 0.27 log CFU/mL), which is quite common in raw milk (Carraro et al., 2011; Franciosi et al., 2009). Microstaphylococci and yeasts were present at low levels in control milk, with counts of 3.22 ± 0.46 and 1.80 ± 0.13 log CFU/mL, respectively. These microorganisms can contribute to the sensory characteristics of cheeses through their proteolytic and lipolytic activities and their ability to metabolize lactic acid, free amino acids, and products resulting from protein degradation (Mounier et al., 2006). Presumptive *Pseudomonas* were found at 2.82 ± 0.53 log CFU/mL. They frequently alter cheese, causing discoloration, flavor, and texture defects. Furthermore, they actively contribute to the formation of environmental biofilms, thereby increasing the risk of microbial contamination (Colantuono et al., 2020).

During 48 h of storage, no significant microbial growth was observed at 4 °C and 6 °C. On the other hand, after 24 h at 8 °C, a slight increase (about 1 log CFU/mL) in the lactobacilli count was observed, and their growth continued to increase after 48 h. Lactobacilli originating from milk are often called 'non-starter LAB' (NSLAB). These bacteria grow well in the harsh cheese environment, characterized by low pH, high salt levels, the absence of fermentable carbohydrates, anaerobic conditions, and the presence of bacteriocins produced by starter cultures. They can significantly affect flavor development, texture, and the health benefits associated with cheese consumption (Renoldi et al., 2024; Settanni & Moschetti, 2010). Psychrotrophic lactobacilli, such as *Levilactobacillus brevis* and *Latilactobacillus curvatus*, have been shown to contribute to the sensory characteristics of cheeses through their aminopeptidase and esterolytic activities, as well as their bioactivities (Abarquero et al., 2022; Mushtaq et al., 2021). Lactococci grew at 10 °C; however, they

Table 1
Microbial counts (log CFU/mL, mean ± SD; n = 3) in raw milk samples stored at 4, 6, 8 and 10 °C for 0, 24 and 48 h.

Microbial count	Time (h)	Temperature (°C)			
		4	6	8	10
Total mesophilic bacteria	Control	$4.17^{aA} \pm 0.11$	$4.17^{aA} \pm 0.11$	$4.17^{aA} \pm 0.11$	$4.17^{bA} \pm 0.11$
	24	$4.04^{aA} \pm 0.64$	$3.93^{aA} \pm 0.88$	$4.22^{aA} \pm 0.19$	$4.58^{bA} \pm 1.10$
	48	$3.87^{aB} \pm 0.60$	$4.17^{aAB} \pm 0.73$	$4.35^{aAB} \pm 0.14$	$6.11^{aA} \pm 0.48$
Lactococci	Control	$3.54^{aA} \pm 0.17$	$3.54^{aA} \pm 0.17$	$3.54^{aA} \pm 0.17$	$3.54^{bA} \pm 0.17$
	24	$3.47^{aA} \pm 0.19$	$3.19^{aA} \pm 0.62$	$3.77^{aA} \pm 0.20$	$3.96^{bA} \pm 0.77$
	48	$2.90^{aB} \pm 0.26$	$3.76^{aB} \pm 0.55$	$3.70^{aB} \pm 0.28$	$5.48^{aA} \pm 0.01$
Lactobacilli	Control	$2.35^{aA} \pm 0.27$	$2.35^{aA} \pm 0.27$	$2.35^{bA} \pm 0.27$	$2.35^{cA} \pm 0.27$
	24	$2.64^{aA} \pm 0.38$	$2.56^{aA} \pm 0.43$	$3.36^{aA} \pm 0.26$	$3.75^{bA} \pm 0.58$
	48	$2.35^{aC} \pm 0.53$	$2.93^{aBC} \pm 0.53$	$3.25^{aB} \pm 0.23$	$5.13^{aA} \pm 0.25$
Microstaphylococci	Control	$3.22^{aA} \pm 0.46$	$3.22^{aA} \pm 0.46$	$3.22^{aA} \pm 0.46$	$3.22^{bA} \pm 0.46$
	24	$2.50^{aA} \pm 1.03$	$2.42^{aA} \pm 0.31$	$2.93^{aA} \pm 0.10$	$2.39^{bA} \pm 0.12$
	48	$2.25^{aB} \pm 0.07$	$2.71^{aB} \pm 0.15$	$2.92^{aAB} \pm 0.27$	$4.25^{aA} \pm 0.69$
Yeasts	Control	$1.80^{aA} \pm 0.13$	$1.80^{aA} \pm 0.13$	$1.80^{aA} \pm 0.13$	$1.80^{aA} \pm 0.13$
	24	$1.49^{aA} \pm 0.11$	$1.19^{aA} \pm 0.68$	$1.43^{aA} \pm 0.42$	$1.74^{aA} \pm 0.46$
	48	$1.02^{aA} \pm 0.30$	$1.31^{aA} \pm 1.85$	$1.45^{aA} \pm 0.34$	$2.04^{aA} \pm 0.46$
Pseudomonas spp.	Control	$2.82^{aA} \pm 0.53$	$2.82^{aA} \pm 0.53$	$2.82^{aA} \pm 0.53$	$2.82^{bA} \pm 0.53$
	24	$2.96^{aA} \pm 0.77$	$2.57^{aA} \pm 0.64$	$3.50^{aA} \pm 0.66$	$3.35^{bA} \pm 0.10$
	48	$2.74^{aB} \pm 0.43$	$3.67^{aB} \pm 0.37$	$3.34^{aB} \pm 0.06$	$5.50^{aA} \pm 0.36$

Values within the same column followed by different lowercase letters indicate significantly different means ($p < 0.05$), values within the same row followed by different uppercase letters indicate significantly different means ($p < 0.05$).

experienced a longer delay than the lactobacilli. They can also contribute to cheese's final properties, predominantly showing lipolytic or proteolytic activity (Hahne et al., 2019).

After 48 h, an increase of approximately 2 log CFU/mL was observed in the total viable count at 10 °C. *Pseudomonas* likely contributed significantly to this increase (Table 1). In the present study, presumptive *Pseudomonas* spp. remained at relatively low-to-moderate levels under proper refrigeration (≤8 °C), generally around 10^3–10^4 CFU/mL, whereas storage at 10 °C promoted a marked increase, reaching 5.50 ± 0.36 log CFU/mL after 48 h. While this level remains below microbial loads often associated with overt spoilage in fluid milk (frequently reported at around 10^6 CFU/mL), it approaches the range at which the risk of spoilage-related defects becomes more relevant (Su et al., 2025). This is particularly important for psychrotrophic *Pseudomonas*, since strain-dependent production of extracellular thermostable enzymes (*e.g.*, proteases and lipases) during cold storage of raw milk may contribute to quality and technological defects even when cell counts are below classical spoilage thresholds (Maier et al., 2021; Ribeiro Júnior et al., 2018). Given the metabolic activities of this microbial group, storing the milk for cheesemaking at 10 °C for 48 h should be approached with particular caution. Psychrotrophic proliferation at 8–10 °C is a major concern for milk safety and quality control, as lipases and proteases are heat-stable and may survive pasteurization, leading to defects during storage of finished products (Khan et al., 2025).

Raw milk can contain various bacterial pathogens, and their viability may be affected during storage before processing. Most pathogenic species are mesophilic, although some strains can multiply at low temperatures (Kousta et al., 2010; Verraes et al., 2015). Therefore, in this study, pathogens were directly inoculated to test their growth potential under different storage conditions. No pathogens' growth was observed regardless of species, inoculum level, storage time, or temperature below 10 °C (Table 2). As the observations from this study were based on culture-based recovery on selective media, they describe changes in culturable populations and may not have captured cells entering a viable-but-non-culturable (VBNC) state. At 10 °C, *E. coli* and *S. aureus* increased their viability after 48 h at both the inoculation levels tested. *E. coli* is commonly considered an indicator of fecal contamination and lack of sanitation, and it can also reflect inadequate milking practices, including poor operator hygiene and improper cleaning and handling of milking equipment (Cullor, 1997). The presence of *S. aureus* in milk indicates mastitis in dairy herds, although contamination may also occur through human handling and equipment surfaces during milking and subsequent handling (Fagundes et al., 2010). Although strict hygiene protocols during milking and careful monitoring of animal health are commonly practiced and effective at reducing contamination, high counts (*e.g.*, above 10^4 CFU/mL) of these microorganisms in raw milk can still occur (Hill et al., 2012; Öksüz et al., 2004). *L. monocytogenes* showed no growth, even at the highest temperature (Table 2). This last observation is noteworthy because *L. monocytogenes* is a psychrotrophic species that can proliferate even at low temperatures (Lafarge et al., 2004).

The results indicate that refrigerating milk at 8 °C or lower effectively prevented increases in culturable populations of the tested pathogens under experimental conditions. In contrast, storing milk at 10 °C for more than 48 h may pose a slight risk, as both *E. coli* and *S. aureus* can adapt to these conditions. The increase of *S. aureus* at 10 °C is particularly relevant from a food-safety perspective, as temperature abuse in raw milk may allow toxin-producing strains to reach hazardous levels (Lin et al., 2021). Although enterotoxin production was not assessed, the observed microbial dynamics indicate that 10 °C represents a critical deviation to be strictly avoided in dairy food safety management systems. Similarly, the ability of *E. coli* to proliferate at 10 °C underlines that even mild temperature abuses can compromise the microbiological safety margin typically expected before heat treatment. The inhibitory effect of temperature may be amplified by the presence of native flora and the natural antimicrobial systems found in raw milk, such as lactoferrin, lactoperoxidase, and lysozyme. These components help control pathogenic and spoilage organisms, at least during the first few hours after milking (Gay & Amgar, 2005).

In most cheesemaking processes, milk is supplemented with a starter culture, either naturally occurring or specifically selected, before rennet is added. The starter culture initiates the fermentation process, enhancing rennet activity and facilitating the biochemical changes characteristic of ripening (McSweeney, 2004). The growth of starter cultures in milk can be significantly affected by various parameters that may fluctuate during cold storage, such as protein content, total solids, acidity, and microbial flora (Yadav et al., 2018). To assess whether the storage conditions under study influenced the fermentative activity of the lactic flora, milk samples were inoculated with a commercial thermophilic starter, and pH was monitored over time.

Milk acidification by lactic acid bacteria follows a three-phase pattern: a lag phase, a phase of maximum acidification, and, finally, the end of acidification, when a stable pH is reached (Jeanson et al., 2009). The parameter values for lag phase length, maximum rate, and final pH accurately reflect the starter's performance and are influenced by the milk's chemical and physical properties. To evaluate the effect of milk storage conditions on starter acidification kinetics, pH values during incubation were modeled using the triphasic model of Baranyi

Table 2
Viability (log CFU/mL ± SD; n = 3) of pathogens in raw milk stored at 4, 6, 8 and 10 °C for 0, 24 and 48 h.

Strain	Inoculum (CFU/mL)	Time (h)	4 °C	6 °C	8 °C	10 °C
E. coli	10^3	Control	$3.28^{bA} \pm 0.19$	$3.28^{bA} \pm 0.21$	$3.28^{bA} \pm 0.27$	$3.28^{dA} \pm 0.22$
		24	$3.23^{bA} \pm 1.36$	$3.20^{bA} \pm 0.09$	$3.11^{bA} \pm 0.18$	$3.08^{dA} \pm 0.39$
		48	$3.04^{bB} \pm 0.27$	$3.34^{bB} \pm 0.11$	$3.36^{bB} \pm 0.32$	$4.65^{cA} \pm 0.15$
	10^5	Control	$5.40^{aA} \pm 0.37$	$5.40^{aA} \pm 0.18$	$5.40^{aA} \pm 0.25$	$5.40^{bA} \pm 0.29$
		24	$5.04^{aA} \pm 0.19$	$5.36^{aA} \pm 0.23$	$5.79^{aA} \pm 0.31$	$5.89^{bA} \pm 0.35$
		48	$5.61^{aB} \pm 0.42$	$5.81^{aB} \pm 0.37$	$5.32^{aB} \pm 0.15$	$6.89^{aA} \pm 0.22$
L. monocytogenes	10^3	Control	$3.90^{bA} \pm 0.15$	$3.38^{bA} \pm 0.19$	$3.81^{bA} \pm 0.23$	$3.87^{bA} \pm 0.19$
		24	$3.30^{bA} \pm 0.03$	$3.76^{bA} \pm 0.42$	$3.75^{bA} \pm 0.37$	$3.71^{bA} \pm 0.36$
		48	$3.75^{bA} \pm 0.37$	$3.46^{bA} \pm 0.18$	$3.91^{bA} \pm 0.25$	$3.94^{bA} \pm 0.27$
	10^5	Control	$5.81^{aA} \pm 0.03$	$5.74^{aA} \pm 0.29$	$5.46^{aA} \pm 0.19$	$5.71^{aA} \pm 0.32$
		24	$5.51^{aA} \pm 0.15$	$5.58^{aA} \pm 0.35$	$5.58^{aA} \pm 0.36$	$5.78^{aA} \pm 0.22$
		48	$5.60^{aA} \pm 0.22$	$5.82^{aA} \pm 0.22$	$5.79^{aA} \pm 0.27$	$5.81^{aA} \pm 0.39$
S. aureus	10^3	Control	$3.85^{bA} \pm 0.22$	$3.85^{bA} \pm 0.33$	$3.85^{bA} \pm 0.22$	$3.85^{dA} \pm 0.03$
		24	$3.98^{bA} \pm 0.51$	$3.73^{bA} \pm 0.12$	$3.98^{bA} \pm 039$	$3.94^{dA} \pm 0.42$
		48	$3.77^{bB} \pm 0.08$	$3.85^{bB} \pm 0.32$	$3.99^{bB} \pm 015$	$4.86^{cA} \pm 0.15$
	10^5	Control	$5.80^{aA} \pm 0.37$	$5.80^{aA} \pm 0.18$	$5.80^{aA} \pm 0.25$	$5.80^{bA} \pm 0.31$
		24	$5.89^{aA} \pm 0.19$	$6.20^{aA} \pm 0.23$	$5.92^{aA} \pm 0.18$	$6.08^{bA} \pm 0.25$
		48	$5.91^{aB} \pm 0.42$	$5.94^{aB} \pm 0.37$	$5.83^{aB} \pm 0.06$	$6.49^{aA} \pm 0.14$

Values within the same column followed by different lowercase letters indicate significantly different means ($p < 0.05$), values within the same row followed by different uppercase letters indicate significantly different means ($p < 0.05$).

and Roberts in DMFit, which fits curves characterized by a linear phase preceded and followed by a stationary phase (Pla et al., 2015). As an illustrative example, Fig. 1 presents the fitting of the acidification kinetics of control milk, along with the corresponding R-square values and SE of Fit provided by DMFit, and the estimated kinetic parameters. The complete acidification curves have been added as Supplementary Fig. S1.

The initial pH of the milk was 6.67 ± 0.10 (Table 4), and in milk inoculated without cold storage (control), the pH reached a final value of 4.52 ± 0.10 after a short lag phase (Table 3). The starter was added at a fixed mass-based dose across all trials; however, the viable cell concentration at inoculation (CFU/mL) was not measured. Therefore, fermentation performance is interpreted in terms of acidification kinetics under standardized inoculation conditions rather than absolute initial starter counts. When milk was stored for 24 h, temperature had little impact on fermentation kinetics, except at 8 °C and 10 °C, where the final pH was lower. This pattern may be related to the proteolytic activity of the native milk microflora, which releases amino acids and peptides that stimulate the growth of *S. thermophilus* (Letort et al., 2002; O'Connell et al., 2016). Interestingly, milk held for 48 h exhibited a longer lag phase and a higher final pH than samples incubated for 24 h, regardless of temperature (Table 3). This difference may reflect increased competition between the starter culture and the milk's native microflora, although specific microbial interaction mechanisms were not directly assessed in the present study, and the higher microbial loads in longer-stored samples could have contributed. Moreover, free fatty acids released by lipases and esterases produced by psychrotrophic species can exert antimicrobial activity (Desbois & Smith, 2010), which could also play a role. Surprisingly, as shown in Table 3, milk stored at 10 °C for 48 h had a lower final pH than milk stored at 4-8 °C. The extended incubation at elevated temperatures may have led to the release of amino acids and peptides by the natural milk microflora, thereby potentially stimulating thermophilic starter growth. The optimal fermentative efficiency of the starter culture was achieved when milk was stored briefly, possibly minimizing competition from native microflora. However, at elevated temperatures, the native microflora may proliferate and display proteolytic activity, which may partly explain the observed differences in starter performance.

3.2. Effect of refrigeration on the chemical-physical characteristics of milk

An increase in soluble Ca relative to the control was observed in all samples during the first 24 h of storage, after which values remained stable for the following 24 h (Table 4). At 4 °C, Ca solubilization was markedly greater than in all other samples, reaching 573 mg/L after 24 h. Conversely, milk samples kept at 6 and 8 °C had comparable soluble Ca levels. However, at 10 °C, the soluble Ca concentration was noticeably reduced. Colloidal Ca levels significantly decreased in all samples

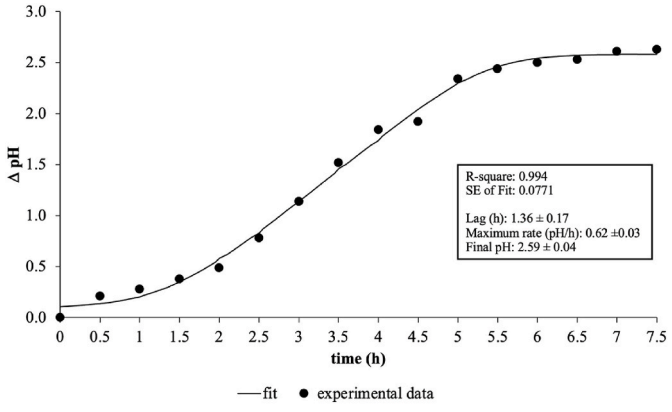

Fig. 1. Acidification curve fitting of control milk.

Table 3
Kinetic parameters of acidification by a thermophilic starter in raw milk stored at 4, 6, 8 and 10 °C for 0, 24 and 48 h.

Temperature	Time (h)	Lag (h)	v_{max} (pH/h)	final pH
Control		$1.25^{c} \pm 0.20$	$0.58^{a} \pm 0.13$	$4.52^{c} \pm 0.10$
4 °C	24	$1.20^{c} \pm 0.12$	$0.52^{a} \pm 0.17$	$4.45^{cd} \pm 0.07$
6 °C	24	$1.27^{c} \pm 0.13$	$0.56^{a} \pm 0.26$	$4.41^{cd} \pm 0.02$
8 °C	24	$1.20^{c} \pm 0.22$	$0.53^{a} \pm 0.04$	$4.33^{d} \pm 0.11$
10 °C	24	$1.23^{c} \pm 0.11$	$0.54^{a} \pm 0.08$	$4.35^{d} \pm 0.03$
4 °C	48	$1.95^{b} \pm 0.14$	$0.55^{a} \pm 0.11$	$4.94^{a} \pm 0.17$
6 °C	48	$1.80^{b} \pm 0.04$	$0.53^{a} \pm 0.10$	$4.90^{a} \pm 0.14$
8 °C	48	$1.98^{b} \pm 0.27$	$0.52^{a} \pm 0.21$	$4.94^{a} \pm 0.09$
10 °C	48	$2.42^{a} \pm 0.08$	$0.57^{a} \pm 0.06$	$4.72^{b} \pm 0.04$

Values within the same column followed by different lowercase letters indicate significantly different means ($p < 0.05$).

stored at varying temperatures over time. This finding suggests that the rise in soluble calcium - more evident at lower storage temperatures - was associated with the release of Ca initially dispersed in colloidal form within the casein micelle. Low refrigeration temperatures cause the release of casein fractions from the micelle, which subsequently draws calcium phosphate molecules into the aqueous phase. Thus, the following chemical dissociation of $Ca_3(PO_4)_2$ separates three calcium ions (Ca^{2+}) and two phosphate ions $(PO_4)^{3-}$, leading to an alteration of the mineral balance in the milk (Mankai et al., 2012; Schroeder et al., 2008).

A slight increase in pH was observed in all samples during the first 24 h of storage (Table 4). This rise, notably greater in milk kept at 4 °C, was probably caused by the solubilization of calcium, shifting from its colloidal state and raising the pH (Malacarne et al., 2013). At 48 h of storage, the pH decreased in all milk samples, likely due to the production of lactic acid by autochthonous lactococci and lactobacilli (McSweeney, 2004). The solubilization of caseins and the release of minerals into the whey, along with an increase in pH, negatively impact curd formation in hard and semi-hard cheesemaking. In fact, casein micelles are stabilized by calcium phosphate bridges. When minerals such as calcium and phosphate dissolve into the whey, these bridges weaken, preventing micelles from aggregating properly, which is essential for curd formation (Post et al., 2012). Moreover, an increase in pH reduces rennet's effectiveness, the enzyme responsible for cleaving κ-casein and allowing micelle aggregation. At higher pH, rennet activity decreases, leading to poor curdling and weak gel formation (Malacarne et al., 2013).

The protein content of milk, particularly caseins, is an important parameter in cheesemaking, determining milk clotting properties and cheese yield (Wedholm et al., 2006). Table 5 lists the content of individual casein fractions identified and quantified in milk samples immediately after milking. The most abundant fraction was represented by the β-casein, with an average concentration of 13.5 ± 1.3 mg/mL, corresponding to about 44% of total casein, followed by α_{s1}- (30%), κ- (14%), and α_{s2}-casein (12%). These data align with those reported by other authors for raw milk intended for cheesemaking (Guetouache et al., 2014; O'Connell et al., 2017).

To evaluate micellar stability during refrigeration, the caseins released into the whey were quantified after storage at 4, 6, 8, and 10 °C for 0, 24 and 48 h (Table 6).

The κ-casein fraction did not undergo significant changes, and its concentration in the whey remained stable throughout the whole period regardless of storage temperature. α_{s1}-casein, instead, showed an increase in whey during the first 24 h of storage, then reached constant values at longer storage times. This trend is similar to that observed for soluble calcium (Table 4), suggesting a possible relationship between the dissociation of colloidal Ca from the micelle and the release of α_{s1}-casein. The soluble fraction of α_{s2}-casein increased significantly only in milk stored at 4 °C for 48 h. β-casein showed the greatest variation in whey with temperature and storage time. The most notable increase

Table 4
Calcium content and pH of milk samples stored at 4, 6, 8, and 10 °C for 0, 24, and 48 h.

Parameter	Time (h)	4 °C	6 °C	8 °C	10 °C
Soluble calcium (mg/L)	Control	$479^{bA} \pm 3$	$479^{bA} \pm 3$	$479^{bA} \pm 3$	$479^{bA} \pm 3$
	24	$573^{aA} \pm 2$	$555^{aB} \pm 7$	$549^{aB} \pm 4$	$520^{aC} \pm 5$
	48	$568^{aA} \pm 6$	$556^{aA} \pm 5$	$552^{aA} \pm 3$	$518^{aB} \pm 1$
Colloidal calcium (mg/L)	Control	$803^{aA} \pm 3$	$803^{aA} \pm 3$	$803^{aA} \pm 3$	$803^{aA} \pm 3$
	24	$709^{bC} \pm 3$	$726^{bB} \pm 7$	$732^{bB} \pm 5$	$763^{bA} \pm 8$
	48	$713^{bC} \pm 1$	$725^{bB} \pm 4$	$729^{bB} \pm 4$	$761^{bA} \pm 2$
pH	Control	$6.67^{bA} \pm 0.04$	$6.67^{bA} \pm 0.01$	$6.67^{bA} \pm 0.01$	$6.67^{bA} \pm 0.01$
	24	$6.88^{aA} \pm 0.03$	$6.82^{aB} \pm 0.03$	$6.80^{aB} \pm 0.01$	$6.76^{aC} \pm 0.03$
	48	$6.64^{bA} \pm 0.05$	$6.65^{bA} \pm 0.03$	$6.67^{bA} \pm 0.04$	$6.63^{bA} \pm 0.03$

For the same parameter, values within the same column followed by different lowercase letters indicate significantly different means ($p < 0.05$), values within the same row followed by different uppercase letters indicate significantly different means ($p < 0.05$).

Table 5
Concentration (mg/mL mean ± SD; n = 9) and relative percentage of individual casein fractions in control milk.

Fractions	Concentration (mg/mL)	Relative percentage (%)
α_{s1}	9.3 ± 0.3	30
α_{s2}	3.6 ± 0.4	12
β	13.5 ± 1.3	44
κ	4.2 ± 1.1	14

occurred at 4 °C, reaching an average concentration of 6.29 mg/mL in the whey after 48 h. Overall, β-casein solubilization gradually increased during storage in all samples except the milk stored at 10 °C. In this case, it appeared to stabilize after 24 h, showing values significantly lower than those observed in samples at lower temperatures.

Regarding total caseins, an increase in casein content in the whey was observed as a function of storage time and temperature, mainly driven by the β-casein fraction. This phenomenon can be primarily attributed to physicochemical mechanisms. β-Casein is known to exhibit strong temperature-dependent solubility due to its amphiphilic nature and the predominance of hydrophobic interactions in its self-association. At low temperatures, the weakening of hydrophobic interactions promotes the dissociation of β-casein monomers from the casein micelle into the serum phase, particularly in conditions where micellar calcium equilibria are altered (Post et al., 2012). In addition to these physicochemical effects, microbiological factors may also contribute to β-casein solubilization during extended milk storage. The development of psychrotrophic microorganisms can lead to proteolytic activity, further enhancing the release of casein fractions into the whey (Stuknyte et al., 2016). However, this contribution is likely secondary compared to temperature-driven dissociation, especially under conditions of high initial microbiological quality (Table 1). Caseins, particularly α_{s1}-casein and β-casein, are crucial for curd formation in the manufacturing of hard and semi-hard cheeses. Their solubilization at low temperatures led these fractions to be dispersed into the whey rather than forming a solid network, thereby reducing the firmness and cohesiveness of the curd (Mankai et al., 2012).

The peptide content was used to reveal the extent of proteolysis in milk samples stored at different temperatures. Fig. 2 shows the total area values obtained from the sum of the areas of individual peaks identified in milk samples. During the first 24 h, proteolytic activity of milk samples was comparable to control values, with a significant increase only in samples stored at 8 and 10 °C. After 48 h, there were no significant

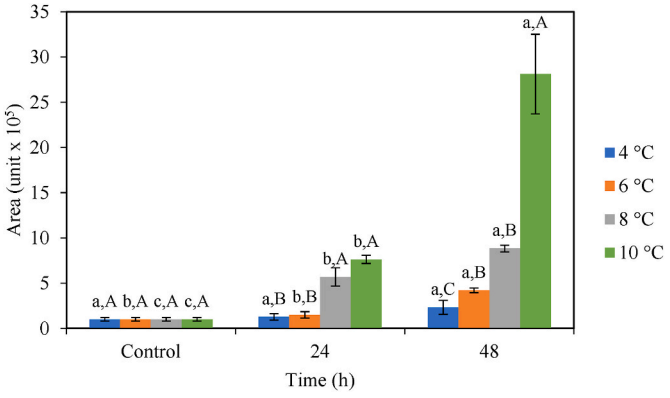

Fig. 2. Evolution of proteolysis in milk stored at 4, 6, 8, and 10 °C for 0, 24, and 48 h. Samples stored at the same temperature and followed by different lowercase letters indicate significantly different means at $p < 0.05$; samples within the same storage time followed by different uppercase letters indicate significantly different means at $p < 0.05$.

Table 6
Concentration (mg/mL mean ± SD; n = 9) of casein fractions in the whey of milk stored at 4, 6, 8, and 10 °C for 0, 24, and 48 h.

Casein fraction	Time (h)	4 °C	6 °C	8 °C	10 °C
α_{s1}	Control	$0.38^{bA} \pm 0.01$	$0.38^{bA} \pm 0.01$	$0.38^{bA} \pm 0.01$	$0.38^{bA} \pm 0.01$
	24	$0.66^{aA} \pm 0.11$	$0.63^{aA} \pm 0.03$	$0.72^{aA} \pm 0.04$	$0.70^{aA} \pm 0.03$
	48	$0.76^{aA} \pm 0.04$	$0.71^{aA} \pm 0.09$	$0.67^{aA} \pm 0.07$	$0.71^{aA} \pm 0.05$
α_{s2}	Control	$1.69^{bA} \pm 0.11$	$1.69^{aA} \pm 0.11$	$1.69^{aA} \pm 0.11$	$1.69^{aA} \pm 0.11$
	24	$1.67^{bA} \pm 0.21$	$1.50^{aA} \pm 0.03$	$1.39^{aA} \pm 0.23$	$1.57^{aA} \pm 0.01$
	48	$2.02^{aA} \pm 0.07$	$1.79^{aAB} \pm 0.26$	$1.56^{aAB} \pm 0.09$	$1.43^{aB} \pm 0.24$
β	Control	$1.21^{cA} \pm 0.10$	$1.21^{bA} \pm 0.10$	$1.21^{bA} \pm 0.10$	$1.21^{bA} \pm 0.10$
	24	$4.05^{bA} \pm 0.88$	$3.55^{aA} \pm 0.13$	$4.48^{aA} \pm 0.12$	$2.73^{aB} \pm 0.21$
	48	$6.29^{aA} \pm 0.25$	$4.50^{aB} \pm 0.91$	$4.96^{aB} \pm 0.22$	$2.76^{aC} \pm 0.22$
κ	Control	$1.36^{aA} \pm 0.11$	$1.36^{aA} \pm 0.11$	$1.36^{aA} \pm 0.11$	$1.36^{aA} \pm 0.11$
	24	$1.23^{aA} \pm 0.09$	$1.21^{aA} \pm 0.03$	$1.17^{aA} \pm 0.04$	$1.19^{aA} \pm 0.05$
	48	$1.28^{aA} \pm 0.04$	$1.09^{aA} \pm 0.37$	$1.18^{aA} \pm 0.05$	$1.13^{aA} \pm 0.12$
Total	Control	$4.34^{cA} \pm 0.10$	$4.34^{bA} \pm 0.10$	$4.34^{bA} \pm 0.10$	$4.34^{bA} \pm 0.10$
	24	$7.31^{bA} \pm 1.38$	$6.58^{aA} \pm 0.20$	$7.04^{aA} \pm 0.74$	$5.97^{aA} \pm 0.13$
	48	$10.06^{aA} \pm 0.40$	$7.79^{aB} \pm 1.62$	$7.96^{aB} \pm 0.43$	$5.59^{aC} \pm 0.02$

For the same casein fraction, values in the same column followed by different lowercase letters indicate significantly different means at $p < 0.05$, values in the same row followed by different uppercase letters indicate significantly different means at $p < 0.05$.

changes in proteolytic activity in samples stored at 4 °C, while a significant increase in activity was observed in milk samples stored at 6, 8, and 10 °C. This increase is likely due to increased load and metabolic activity of psychrotrophic microorganisms, especially *Pseudomonas*, which is well known for its proteolytic activity (Ertan et al., 2015). However, proteolysis was observed only after 48 h of storage, likely because psychrotrophic microorganisms initially adapt to the temperature and show reduced metabolic activity, releasing extracellular enzymes only in the later stages of growth (Izidoro et al., 2013).

It should be highlighted that the milk sample stored at 10 °C, in which presumptive *Pseudomonas* proliferated after 48 h (Table 1), had the lowest release of calcium and soluble caseins in the whey during storage. Therefore, it can be hypothesized that the limited solubilization of β-casein observed in this sample may be due to microbiological activity rather than solely to a physicochemical mechanism, which is unlikely to be triggered at this temperature. On the other hand, the solubilization of β-casein and calcium observed in samples stored at lower temperatures might be primarily attributed to a physicochemical phenomenon rather than a microbiological one. The cheese yield depends on numerous factors, including processing parameters, but primarily on the milk's casein content. In fact, the loss of proteins caused by proteolytic activities during the storing of milk negatively affects curd stability, syneresis rate, water retention capacity, and consequently, the quality and yield of the cheese (Mankai et al., 2012).

3.3. Effect of refrigeration on the volatile profile of milk

A total of 25 different volatile compounds were identified in the headspace of milk samples (Table S2), mostly short- and medium-chain fatty acids, carbonyl compounds (aldehydes and ketones), and alcohols. In Fig. 3, the PCA biplot and heatmap related to the distribution of the main classes and volatile compounds detected in the headspace of samples stored at different temperatures over time are reported. The first principal component (PC1) accounted for 78.9% of the variability, while the second principal component (PC2) accounted for 15.4%, explaining 94.3% of the total variance (Fig. 3A). In general, PC1 could be interpreted as a temperature-driven gradient of microbial metabolic intensity, separating mildly active refrigerated samples from highly metabolically active milk stored at 10 °C, while PC2 could reflect qualitative differences in dominant metabolic pathways, distinguishing ketone-associated profiles from acid- and aldehyde-rich profiles. Milk samples stored at 4 °C and 6 °C for 24 and 48 h were clustered with the control milk and were characterized by a balanced volatile profile and limited compound production (Fig. 3A), consistent with mild metabolic activity of the microflora (Table 1). In contrast, milk stored at 8 °C was associated with ketones and compounds such as dimethyl sulfide, dimethyl ether, and ethyl acetate. Among ketones, 2,3-butanedione and acetoin were predominant after 24 and 48 h, respectively (Fig. 3B). These compounds, with buttery and milky notes, can derive both from LAB citrate metabolism and from microstaphylococci or enterococci activity (Chammas et al., 2006; McSweeney, 2004).

Samples stored at 10 °C showed the most altered profile, with a marked accumulation of acids, alcohols, and aldehydes (Fig. 3A). High levels of 3-methylbutanoic acid (isovaleric acid) were detected after 48 h, together with other acids (butanoic, 2-methylbutanoic, pentanoic, hexanoic, octanoic) (Fig. 3B) commonly linked to *Pseudomonas* metabolism but also produced by the lipid metabolism of certain LAB and microstaphylococci (Bekker et al., 2016; Decimo et al., 2018; Morales et al., 2005). These acids are associated with rancid, pungent, or fruity notes (Bekker et al., 2016; Decimo et al., 2018). Acetic acid was also abundant in both samples stored at 10 °C (Fig. 3B), likely originating from heterofermentative LAB (Smit et al., 2005). Regarding alcohols, 2-methyl-1-butanol and 2,6-dimethyl-4-heptanol were distinctive at 10 °C, contributing fruity and yeasty aromas (Decimo et al., 2018; Lee et al., 2013). Finally, the aldehydes 2-methylbutanal and 3-methylbutanal, both linked to amino acid catabolism, were detected at higher levels in milk samples stored at 10 °C, conferring bitter-almond and malt-like notes (Smit et al., 2005).

In milk, the development of aromatic compounds is mainly attributed to the activity of the microflora, including psychrotrophic species such as *Pseudomonas* spp., micrococci, and staphylococci, whose concentrations increase at higher storage temperatures (Samaržija et al., 2012). However, a possible role of lactococci and lactobacilli, which were found in concentrations approximately 2 log higher in milk stored at 10 °C compared to those stored at lower temperatures, should not be excluded since these are important dairy microorganisms that can play a pivotal role during cheese maturation as well (McSweeney, 2004; Smit et al., 2005).

Overall, both storage time and temperature markedly influenced the volatile profile of raw milk, reflecting the impact of storage conditions on microbial communities and their metabolism. Refrigeration at ≤6 °C preserved a balanced profile; storage at 8 °C induced an intermediate shift characterized by increased ketones; and storage at 10 °C for 48 h promoted compounds typically associated with spoilage and flavor defects.

Balancing milk cheese-making potential and microbiological quality is crucial in dairy processing, especially in refrigerated milk storage intended for cheese production. These two aspects are interconnected but often at odds over extended storage at varying temperatures. A qualitative score, based on chemical, biochemical, and microbiological results related to milk cheesemaking potential and quality, was used to summarize the effect of refrigerated storage on milk performance. The developed qualitative tool was particularly designed to be sensitive to critical safety events (*e.g.*, microbiological spoilage) while limiting the influence of a single exceptionally good parameter from disproportionately inflating the final score. Fig. 4 shows the evolution of the computed qualitative score as a function of storage time and temperature. Milk stored at 4 and 6 °C for 24 h achieved higher qualitative scores, with 6 °C for 24 h representing the most favorable compromise between microbial stability and cheesemaking potential. However, at 48 h, scores declined due to casein and calcium loss and mild proteolysis. Milk stored at 8 °C showed stable bacterial levels but reduced cheesemaking efficiency. Extended storage and temperatures above 8 °C significantly reduce the microbiological quality, potentially increasing spoilage and pathogenic risks and negatively affecting cheese yield due to intense proteolysis and production of undesired volatile compounds. Since storage at 10 °C for 48 h enabled the growth of pathogenic microorganisms, these conditions must be strictly avoided a priori, regardless of the technological parameters considered, in order to preserve the microbiological safety of raw milk prior to processing. Finally, the best compromise was achieved at 6 °C for 24 h, confirming that both temperature and time are decisive for the suitability of raw milk for cheesemaking. It should be noted that these findings were obtained using milk with high initial microbiological quality collected under good hygienic conditions. Therefore, the identification of 6 °C for 24 h as the most favorable compromise should be interpreted within this context, as different initial milk quality levels may require more conservative storage conditions to ensure microbiological safety and technological performance. From a risk-management perspective, these results highlight a dual challenge: ensuring microbiological safety while simultaneously preventing spoilage-related quality defects and preserving adequate cheesemaking functionality. The qualitative score developed in this study integrates these competing dimensions, providing dairy operators with a practical tool to quantitatively assess how deviations in milk storage conditions increase both microbial hazards and technological risks. Although the qualitative score integrates multiple analytical dimensions, its routine calculation may represent a practical challenge for dairy plants, due to analytical costs, the need for specialized expertise, and the time required to obtain comprehensive microbiological and chemical results. For this reason, the score is not intended to be calculated autonomously by each dairy plant for every milk batch. Rather, it should be regarded as a decision-support framework, developed and validated on representative

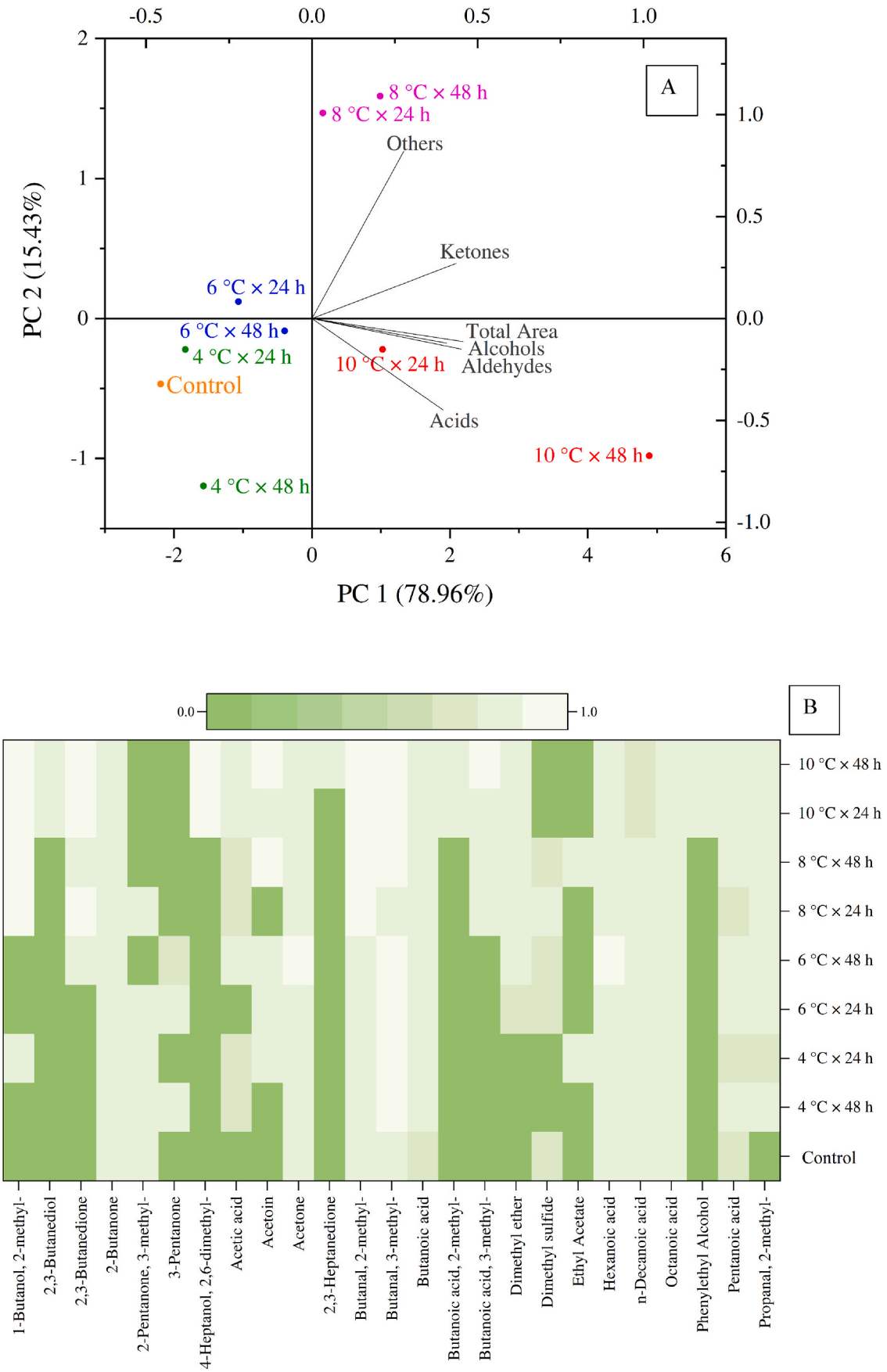

Fig. 3. PCA biplot (A) and heatmap representation (B) of volatile compounds generated in milk samples stored at 4, 6, 8, and 10 °C for 0, 24, and 48 h.

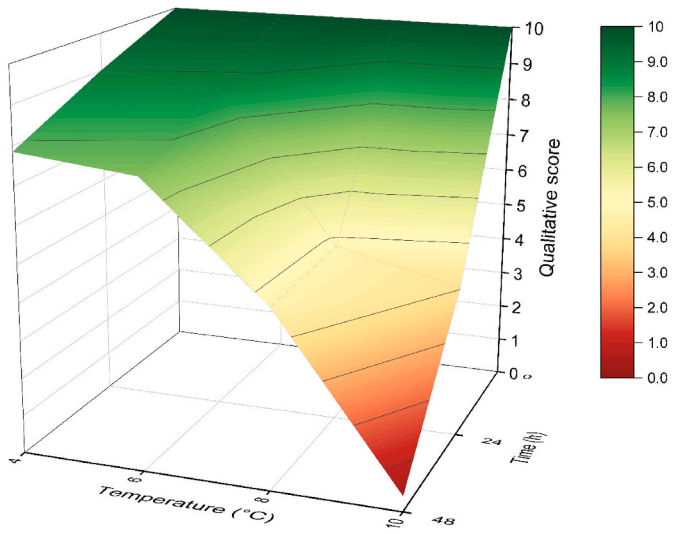

Fig. 4. Representation of qualitative score related to the milk cheese-making potential and quality as a function of storage times (24-48 h) and temperatures (4, 6, 8, 10 °C).

production scenarios, that can provide science-based guidance for defining acceptable combinations of storage time and temperature. Once established, such an integrated score can support risk-based management strategies by helping dairies set preventive refrigeration guidelines that balance microbiological safety, spoilage control, and cheesemaking performance, without requiring the full analytical workload on a routine basis.

4. Conclusions

The storage temperature of raw milk during the pre-processing phase plays a critical role in determining its microbiological stability and chemical-physical integrity, both of which are essential for its technological suitability, particularly in cheesemaking. Storage at 4 °C proves highly effective in limiting microbial growth, including spoilage organisms such as presumptive *Pseudomonas* spp. and pathogens like *E. coli*, *L. monocytogenes*, and *S. aureus*. However, it also accelerates the dissociation of calcium and casein fractions (especially β- and αs-caseins) from the micellar structure, which may adversely affect milk's coagulation properties. Conversely, while storage at 10 °C better preserves micellar stability, it also permits the proliferation of spoilage and pathogenic microorganisms, leading to increased proteolytic activity and the development of volatile compounds. The observed delay in acidification after starter culture inoculation in milk stored for 48 h, regardless of temperature, further suggests competitive interactions between the native microflora and the added starter cultures, potentially impacting fermentation performance. By combining these different aspects into a qualitative score, this study provides a comprehensive evaluation of storage conditions, showing that 6 °C represents the most effective compromise. Importantly, time also emerged as a critical factor: the highest qualitative score was obtained at 4 and 6 °C for 24 h, while extended storage (48 h) led to a decline in milk performance even under optimal temperature. These findings highlight the importance of optimizing milk refrigeration protocols not only to ensure safety but also to preserve its functional properties in dairy applications. Overall, the findings provide operational evidence to support the dairy industry in refining milk refrigeration protocols, ensuring product safety, improving cheese quality, and promoting more sustainable practices along the dairy chain.

CRediT authorship contribution statement

Niccolò Renoldi: Writing – original draft, Visualization, Methodology, Investigation. **Marilena Marino:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation. **Marco Lopriore:** Writing – review & editing, Formal analysis. **Anna Rossi:** Writing – review & editing, Investigation. **Giulia Di Filippo:** Writing – review & editing, Investigation. **Nadia Innocente:** Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2026.112155.

Data availability

Data will be made available on request.

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