Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.5327/fst.00137



# Effects of cold storage and freezing on sheep's milk

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**Abstract**

Sheep's milk production in Brazil is focused on the industrialization of high added value derivatives. Some factors, such as seasonal production, short lactation period, and microbiological contamination, cause a shortage of this raw material throughout the year. It is well known that low temperatures are used in the conservation of dairy products and the objective of this study was to verify the microbiological and physical-chemical quality in refrigerated and frozen raw sheep's milk. Standard plate counts, psychrotrophics, *Staphylococcus aureus*, total coliforms, and fecal coliforms, as well as titratable acidity, pH, and water activity were analyzed in samples 1 day after refrigeration and 8 and 15 days after freezing temperatures. Overall, the results of refrigerated milk were based on the legislation, but frozen milk showed high values of aerobic plate, *S. aureus*, and acidity, especially after 15 days of storage. Psychrotrophics and coliform counts were also high, indicating a risk for the quality of the raw milk and derivatives, which emphasizes the importance of handler training for good practices in the farm, as well as in the dairy industry. Freezing may be a viable strategy; however, additional studies testing lower temperatures and previous milk pasteurization are necessary to guarantee milk quality and consumer safety.

**Keywords:** cold storage; dairy microbiology; milk quality; ovine milk; preservation.

**Practical Application:** Cold storage of sheep milk can help extend the shelf life of the raw material, resulting in the production of derivatives throughout the year. This monitoring study was carried out and the data obtained can help milk producers and dairy industries to define strategies for preserving the raw material and supplying production in off-season periods.

## 1 INTRODUCTION

In Brazil, sheep's milk production is a relatively recent activity and has shown growth in recent years (Munieweg et al., 2017). Sheep's milk shows differences in composition, such as higher levels of proteins, mainly casein, and fat, compared to cow's milk (Biegalski et al., 2021). Usually, sheep's milk contents vary from 4.7 to 5.4% of proteins, 4.8 to 6.5% of fat, and 4.2 to 4.8% of lactose, with around 0.9% of ash and 16.6% of total solids (Malta et al., 2021; Tribst et al., 2019).

Raising dairy sheep is more common in regions with a temperate climate, as it is in southern Brazil, with mild temperatures and favorable conditions (Rocha Júnior et al., 2022). Sheep milk producers are becoming increasingly aware of the quality control and the importance of adopting good practices in breeding, feeding, and milking (Theodoridis et al., 2022). Proper hygiene, training, and sanitation of the milking equipment and facilities are also crucial to prevent the transmission of pathogens and to maintain milk quality (Taffarel et al., 2015; Theodoridis et al., 2022). Sheep milk production is still relatively small in Brazil compared to other countries; however, there is a potential for growth due to the demand for differentiated products and greater dissemination of the benefits of these products in order to improve human's health (Ferreira et al., 2021; Flis & Molik, 2021; Gavião et al., 2020; Mohapatra et al., 2019; Munieweg et al., 2021).

Low sheep productivity, short lactation period, seasonality, and the susceptibility to microbiological contamination are some reasons for reduced production of cheese and other derivatives in some periods of the year (Biegalski et al., 2021; Park et al., 2006), which requires quality monitoring after milking and before processing (Munieweg et al., 2017; Taffarel et al., 2015). Cold storage of sheep milk may improve shelf life and industrialization time. Therefore, the objective of this study was to evaluate whether sheep's milk refrigeration and freezing temperatures could affect its overall quality.

## 2 MATERIALS AND METHODS

Milk samples from Lacaune ewes were collected in sterile flasks (50 mL) right after milking, from six properties (F1–F6)

*Received 23 Aug., 2023*
*Accepted 24 sept., 2023*
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Funding: National Council for Scientific and Technological Development (CNPq), Research and Innovation Support Foundation of Santa Catarina State (FAPESC), and the Academic Development Program (PDA) of Universidade Federal do Pampa (UNIPAMPA).

located in the states of Rio Grande do Sul and Santa Catarina (southern Brazil) and sent to the laboratory under refrigeration. The flasks were stored at a controlled temperature, under refrigeration (4°C ± 1°C) or freezing (-18°C ± 2°C), during the evaluation period. The analyses were carried out in refrigerated milk 1 day of storage (RE1), and after 8 and 15 days under freezing temperatures (FR8 and FR15, respectively). Three flasks from each property were randomly collected at each analysis time.

Microbiological analyses in all samples followed standardized methodologies and included aerobic plate count (APC), total and thermotolerant coliforms, *Staphylococcus aureus*, and psychrotrophic microorganisms (Brasil, 2022; Silva et al., 2017). The APC was performed by plating on Standard Count Agar (PCA) (Oxoid®), with incubation at 36°C ± 1°C for 24–48 h. For coliforms, the multiple tube technique with inverted Durham was used in a series of three tubes. The presumptive test was performed in tubes containing sodium lauryl sulfate broth (Dinamica®) and the presence of total coliforms was confirmed using bright green broth 2% lactose bile (Himedia®) and incubation at 35°C ± 1°C for 24–48 h, while the thermotolerant coliforms were in EC broth (Himedia®) incubated at 45°C ± 1°C for 24–48 h. The quantification of the most probable number (MPN) was carried out using the Hoskins table.

*Staphylococcus* sp. count was performed using Baird-Parker Agar (Himedia®) medium, enriched with 0.01% potassium tellurite and egg yolk, and incubated at 36°C ± 1°C for 48 h. After counting the plates, characteristic colonies of *S. aureus* (black with halos) were transferred to the Brain Heart Infusion (BHI) (Himedia®) and incubated at 37°C ± 1°C for 24 h, with subsequent tests for catalase and coagulase. The catalase test was performed with a 3% hydrogen peroxide solution and the conjugated coagulase test used rabbit plasma (Probac®). The psychrotrophic counts were done using PCA (Oxoid®) and incubation at 7°C ± 1°C for 10 days. The results were expressed as colony forming unit (CFU)/mL of milk and compared with the limits established by the current Brazilian legislation (Brasil, 2018).

Physicochemical analyses included titratable acidity, water activity (Aw), and pH. Titratable acidity was determined with standardized sodium hydroxide solution and the results were expressed in grams of lactic acid/100 mL of milk (Brasil, 2022). The water activity (Aw) was evaluated using a device model Aqualab 4TE (Decagon®) according to the manufacturer's instructions (MeterGroup, 2023), and the pH was determined using a pH meter model pg1800 after dilution in distilled water (Brasil, 2022).

Microbiological counts were converted into logarithms (log), followed by the calculation of means and standard deviations from the mean. The data were evaluated using the SigmaPlot14.5 program, where analysis of variance was applied followed by the Tukey's test at the 5% level of significance.

## 3 RESULTS

Aerobic plate count, psychrotrophics, and *S. aureus* are shown in Figure 1. The APC values (Figure 1A) observed in refrigerated milk ranged from 4.93 to 6.24 log CFU/mL, and in milk frozen for 8 days the minimum and maximum average values were 5.32 to 6.59 log CFU/mL in Farm 1 and Farm 3, respectively. In milk samples frozen for 15 days, the average values ranged from 5.45 log CFU/mL in F2 to 7.01 log CFU/mL in F3. Interestingly, when comparing the way milk was stored, refrigerated samples from all dairy farms showed significantly lower counts than the respective samples frozen for 15 days. The results showed that the milk with the highest count for this group of indicator microorganisms was the one from the farm 3 (F3), and the milk from the F1 remained with the lowest counts from refrigeration to freezing.

Mean psychrotrophic counts in refrigerated raw milk (Figure 1B) ranged from 4.86 to 6.57 log CFU/mL in farms F6 and F3, respectively. The values reduced significantly in the frozen milk (FR8 and FR15) when compared to the refrigerated milk (RE1). Psychrotrophic counts in sheep milk were high, again with the worst results for F3. The quantification of *S. aureus* (Figure 1C) was gradually higher in samples from properties of F3 and F6 and in frozen milk (FR15) when compared to RE1.

Total coliforms and fecal coliforms in raw sheep's milk are shown in Table 1. Total coliforms counts were higher in milk collected from farms F1, F3, F4, and F5, while for fecal coliforms only samples from F3 showed high counts.

The pH and water activity (Aw) values in raw sheep's milk are shown in Table 2. The pH values showed statistical differences between the types of storage and time, while the Aw values

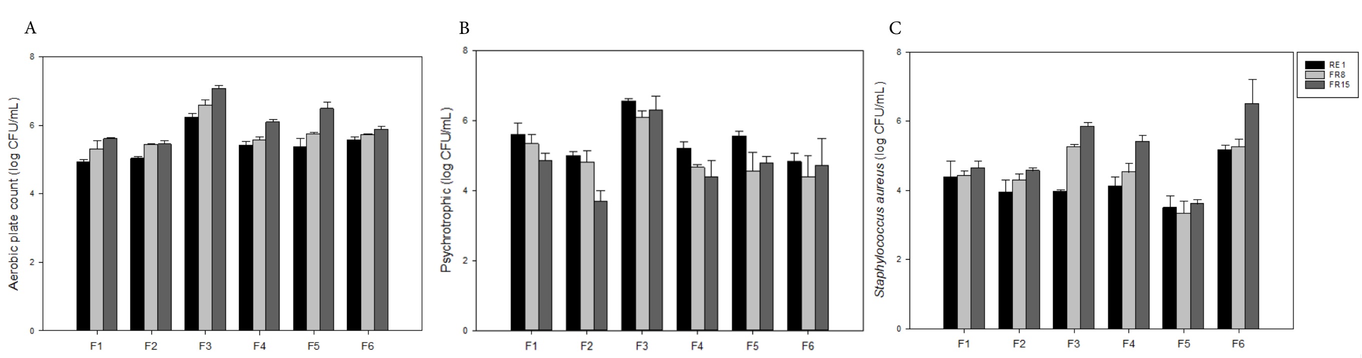


**Figure 1**. (A) Aerobic plate count, (B) psychrotrophic, and (C) *Staphylococcus aureus* in raw sheep milk from six ovine dairy farms of southern Brazil. Milk refrigerated for 1 day (RE1) after milking (black bars); milk frozen for 8 days (FR8) after milking (light gray bar); and milk frozen for 15 days (FR15) after milking (dark gray bar).

did not show significant differences between the RE1 and FR8 groups and both were significantly higher than the FR15 group.

The titratable acidity in raw sheep's milk under refrigeration or freezing is shown in Figure 2. Gradually higher values are verified in sheep milk from each of the properties, as the period of cold storage increases.

## 4 DISCUSSION

The legal limit established for APC for raw milk collected in individual tanks in Brazil is 5.48 log CFU/mL (Brasil, 2018), and the samples from almost all farms (F1, F2, F4, and F5) showed mean values below the legal limit, with only 33.3% (*n* = 2) of improper milk (Figure 1A). Considering the standard for refrigerated raw milk before processing in milk processing industry, the value should not exceed 5.95 log CFU/mL (Brasil, 2018), and therefore only F3 would be above the maximum limit. The FR8 showed an increase in microbial counts when compared to the RE1, with sheep's milk from F1 and F2 within the legal limit. On the contrary, milk from the FR15 group would be within the maximum allowed only in F2, which demonstrates that there is a decrease in the microbiological quality of frozen milk compared to refrigerated samples. When considering the limit of 6.18 log CFU/mL applied by the European Commission for raw milk from species other than cow that will undergo pasteurization (European Commission, 2004), the values would be exceeded by RE1, FR8, and FR15 milk from F3, and FR15 from F5. A previous study on sheep's milk in southern Brazil showed mean counts of 6.06 log CFU/mL on the first day under refrigeration (Munieweg et al., 2017) and, in Greece, counts of 6.20 log CFU/mL were reported in farms with conventional production system (Malissiova et al., 2017).

Psychrotrophic counts were higher in refrigerated milk across all dairy farms (Figure 1B), probably due to the growth of this microbial group being optimized at refrigerated temperatures (Forsythe, 2019). Freezing can contribute to a decrease in microbial metabolism due to the immobilization of free water in the form of ice crystals, which can damage the bacterial cell wall, impairing their viability (Bottiroli et al., 2020; Nespolo et al., 2015). This microbial group in sheep's milk showed no major variations during refrigerated storage, since the average count between producers was 6.02 log CFU/mL (*n* = 12) in the first day and 6.09 log CFU/mL (*n* = 12) in the fourth day under refrigeration (Munieweg et al., 2017). Psychrotrophic group includes potentially proteolytic bacteria that can affect the quality of raw milk and dairy products such as *Pseudomonas*, *Bacillus*, and *Listeria*, as well as lactic acid bacteria such as *Lactococcus*, *Leuconostoc*, and *Lactobacillus* (Forsythe, 2019; Khayrullin & Rebezov, 2023), with spoilage and pathogenic microorganisms.

Brazilian legislation does not set up a limit for *S. aureus* in raw milk, but this microorganism is a well-known cause of clinical and subclinical mastitis in sheep (Bishop & Morris, 2007) and a potentially harmful bacterium (Basanisi et al., 2016), emphasizing the importance of the results observed in the present study (Figure 1C). Mastitis caused by *S. aureus* presents the most severe clinical symptoms and there is also a risk of contamination by its thermostable toxins (Forsythe, 2019).

**Table 1**. Total coliforms and fecal coliforms in raw sheep's milk from dairy farms of southern Brazil.

| Milk sample (farm) | Total coliforms (log MPN/mL) | Fecal coliforms (log MPN/mL) |
|---|---|---|
| RE1 (F1) | > 3.38 | < 0.48 |
| FR8 (F1) | > 3.38 | < 0.48 |
| FR15 (F1) | > 3.38 | < 0.48 |
| RE1 (F2) | 1.45 ± 0.12 | 1.18 ± 0.30 |
| FR8 (F2) | 1.11 ± 0.34 | < 0.48 |
| FR15 (F2) | 1.08 ± 0.16 | < 0.48 |
| RE1 (F3) | > 3.38 | 2.74 ± 0.86 |
| FR8 (F3) | 3.27 ± 0.15 | 2.82 ± 0.29 |
| FR15 (F3) | > 3.38 | > 3.38 |
| RE1 (F4) | > 3.38 | < 0.48 |
| FR8 (F4) | 2.58 ± 0.54 | < 0.48 |
| FR15 (F4) | 2.90 ± 0.32 | < 0.48 |
| RE1 (F5) | > 3.38 | 0.64 ± 0.21 |
| FR8 (F5) | > 3.38 | 0.56 ± 0.06 |
| FR15 (F5) | 3.15 ± 0.15 | < 0.48 |
| RE1 (F6) | 0.86 ± 0.34 | 0.56 ± 0.06 |
| FR8 (F6) | 0.60 ± 0.01 | 0.56 ± 0.06 |
| FR15 (F6) | 1.02 ± 0.23 | < 0.48 |

Note: Values showed as mean ± standard deviation of the mean (*n* = 3); RE1: Milk refrigerated for 1 day after milking; FR8: Milk frozen for 8 days after milking; FR15: Milk frozen for 15 days after milking.

**Table 2**. pH and water activity (Aw) in raw sheep's milk from dairy farms of southern Brazil.

| | RE1 | FR8 | FR15 |
|---|---|---|---|
| Aw | 0.990 ± 0.001 [a] | 0.989 ± 0.003 [a] | 0.977 ± 0.008 [b] |
| pH | 7.15 ± 0.02 [a] | 7.25 ± 0.06 [b] | 6.94 ± 0.07 [c] |

Note: Values showed as mean ± standard deviation of the mean (*n* = 18). Different letters on the same line indicate significant differences ($p < 0.05$); RE1: Refrigerated milk; FR8: Milk frozen for 8 days after milking; FR15: Milk frozen for 15 days after milking.

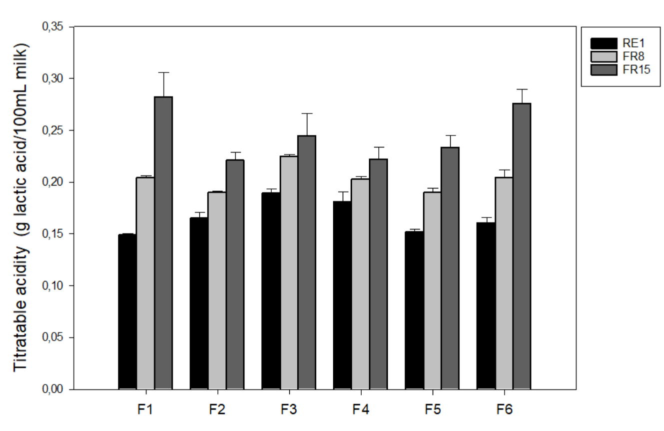


**Figure 2**. Titratable acidity in raw sheep's milk from dairy farms of southern Brazil. Milk refrigerated for 1 day (RE1) after milking (black bars); milk frozen for 8 days (FR8) after milking (light gray bar); and milk frozen for 15 days (FR15) after milking (dark gray bar).

A study evaluating sheep's milk from four farms in the eastern part of Hungary found *S. aureus* counts of 2.6 to 2.8 log CFU/mL in the milk of ewes from two farms and 3.4 log CFU/mL in the milk of the cold storage tank at one of these farms (Tonamo et al., 2020). *S. aureus* counts in raw sheep milk samples in Croatia ranged from 3.0 to 3.8 log CFU/mL, with two-thirds of the samples above the upper limit of 2.0 log CFU/mL (Frece et al., 2016). An evaluation of samples of sheep and goat cheeses, which was carried out in Italy, isolated *S. aureus* in 41.1% ($n$ = 37) (Basanisi et al., 2016), indicating the need to improve herd control in order to reduce milk contamination.

The quantified values for coliforms in the samples of the evaluated dairy farms (Table 1) highlight the importance of proper milking procedures along with the dairy chain. Increased levels of bacteria belonging to the *Enterobacteriaceae* family and coliforms group are considered indicators of poor hygiene and testing in the dairy industry has helped to identify raw milk and dairy products exposed to unsanitary conditions (Martin et al., 2016; Tonamo et al., 2020). The average count of total coliforms in a study carried out in Greece was 3.3 log CFU/mL, both in properties with organic and conventional production (Malissiova et al., 2017). The mean values for *Enterobacteriaceae* in sheep's milk in Croatia averaged between 2.2 and 3.0 log CFU/mL, and 0.7 and 1.1 log CFU/mL for *Escherichia coli* (Frece et al., 2016). Mean for *Enterobacteriaceae* counts in bulk tank milk from three Hungarian dairy sheep farms ranged from 3.9 to 5.6 CFU/mL (Tonamo et al., 2020), demonstrating how difficult it is to control this indicator group. A small fraction of this diverse group of bacteria is of fecal origin, such as *E. coli*, while the majority are from environmental contaminants (Martin et al., 2016). The presence of this group of bulk tank milk bacteria might be related to the growth in milk residues that remain in the equipment (Forsythe, 2019), highlighting the importance of proper sanitation throughout milk processing.

The average water activity was above 0.980 (Table 2), which is compatible with the raw milk (Forsythe, 2019). This parameter represents the water content available for reactions and bacterial growth, and freezing causes the water to remain in the form of ice crystals, making part of the water immobilized, which helps preserve raw milk (Bottiroli et al., 2020; Nespolo et al., 2015). The pH values in the present study varied with the farm and time of storage (Table 2), which was also observed in a study with sheep's milk obtained from dairy farms in southern Brazil kept under refrigeration for 1 to 7 days with pH values 7.04 to 7.35 (Munieweg et al., 2017). One study that evaluated sheep's milk from Poland, which was stored at -24°C for 12 weeks with the mean pH value of 6.65 (Biegalski et al., 2021), demonstrates a decrease in this parameter, possibly due to freezing.

The Brazilian legislation dictates that the titratable acidity in refrigerated raw milk must be between 0.14 and 0.18 g of lactic acid/100 mL of milk (Brasil, 2018) and the average values of the refrigerated samples remained within this limit (Figure 2). However, the same did not occur for samples frozen at 8 and 15 days, in which the maximum limit was exceeded. The tendency of the refrigerated milk to acidify was confirmed in a study with sheep milk refrigerated for 1 to 7 days, in which the values progressively increased over time (Munieweg et al., 2017). Acidification can be caused by microbiological contamination due to the lactic acid production and other organic acids (Forsythe, 2019). The acidification of milk causes the destabilization of the emulsion, leading to economic losses to the producer and to the industry (Fava et al., 2014). Changes in the pH and acidity may affect the coagulation process as well as the water retention capacity of the mass, influencing the size of the formed clot granules, the draining process, the texture, and the microbial growth in cheese (Khayrullin & Rebezov, 2023; Park et al., 2006).

Freezing below -10°C prevents microorganism growth; however, larger ice crystals may be formed, which perforate the fat globules and cause emulsion destabilization (Bottiroli et al., 2020; Nespolo et al., 2015). A study with sheep's milk showed that temperature of -15°C can lead to the formation of larger ice crystals than -27°C, causing damage to fat globules and higher acidity in milk (Park et al., 2006). A study with ultra-high-temperature hydrolyzed lactose milk exposed to slow freezing (-20°C) or fast freezing (-80°C) found that there was no destabilization of the fat or differences in the physical-chemical and sensorial properties of the samples, probably due to prior homogenization to maintain stability upon thawing (Bottiroli et al., 2020). A study carried out in New Zealand showed that rapid freezing produces small ice crystals, allowing sheep's milk to thaw in a matter of minutes instead of hours, and the combination of freezing at -30°C and thawing at 20°C reduces the risk of microbial contamination and deterioration (Morel et al., 2020). In addition, it indicates that freezing at lower temperatures needs to be better studied and that it is important to standardize the pre- and post-freezing steps to maintain the characteristics and quality of sheep´s milk.

The use of frozen milk requires an adequate thawing process at a temperature of 60°C, and partial mixing with fresh milk is recommended before pasteurization and production of cheeses and yogurts (Clark, 2016). The process commonly used in small plants of sheep's milk in Brazil is low-temperature long-time pasteurization, with the aim of eliminating pathogenic microorganisms and reducing deteriorating ones. An alternative could be the freezing of previously pasteurized milk, which would reduce microbiological contamination in the raw milk to be stored under cold conditions.

## 5 CONCLUSION

The evaluation of aerobic plate count and titratable acidity in refrigerated raw sheep's milk showed values within the limits established by law for all properties, except F3. The same did not occur with frozen samples, which reached unreasonable results and could not be manufactured at the dairy industry. Although there is no standardization for the other microbiological and physical-chemical parameters evaluated, the high values observed indicate milk compromised quality.

Freezing is considered efficient in controlling microbial growth, but initial high counts may have caused changes, which could make the use of this raw material unfeasible, possibly affecting the yield and quality of derivatives. The evaluated dairy farms need monitoring and training that mainly focus on good milking practices, temperature control, storage, and overall hygiene. Sheep milk and dairy producers need strategies to preserve the raw material and supply production in between-harvest periods, so additional studies are needed to enable freezing, such as with prior pasteurization, as a way of preserving and maintaining quality, ensuring that consumers have access to high-quality products.

## ACKNOWLEDGMENTS

We are grateful to those responsible for the dairy farms who made it possible to collect samples for analysis.

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