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Impact of the preservation methods of sheep milk on the characteristics of Requeijão cremoso processed cheese

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ABSTRACT

Sheep milk refrigerated for up to 10 days and frozen for up to 90 days was evaluated for physicochemical characteristics, proteolytic activity, integrity of protein fractions, and microbiological counts. The impact of refrigeration or freezing on nutritional composition, colour and texture profile of the Requeijão cremoso cheese was also evaluated. Milk refrigerated for up to 6 days or frozen for up to 90 days maintained its physicochemical characteristics, with no measurable changes in protein fractions, and acceptable bacterial counts. Requeijão cremoso made from milk frozen for 30 days was the most similar to that made from fresh milk, followed cheese made from milk refrigerated for 6 days and finally milk frozen for 90 days. Thus, Requeijão cremoso can be produced from milk subjected to refrigerated or frozen storage, with little change in texture and physicochemical characteristics.

1. Introduction

Sheep milk is characterised by a higher concentration of fat, protein, and minerals compared with cow milk, which results in interesting technological, nutritional, and sensory properties (Ranadheera, Naumovski, & Ajlouni, 2018). In addition, α_{S1}- and α_{S2}-casein in sheep milk has 99% similarity with goat milk, being less allergenic than cow milk (Masoodi & Shafi, 2010).

Sheep milk is mainly used to produce cheeses and yoghurts. However, the seasonal reproduction cycle for sheep affects the amount of milk production and thus dairy production chain (Pulina et al., 2018). Moreover, on small farms, the small daily volume of milk produced often requires the milk accumulation for few days until it reaches a volume compatible with minimum processing capacities (Tribst, Ribeiro, Junior, Oliveira, & Cristianini, 2018). Thus, establishing parameters for cold preservation of sheep milk has great importance (Tribst, Falcade, & Oliveira, 2019a; Tribst, Falcade, Ribeiro, Leite Júnior, & Oliveira, 2019b).

Prolonged refrigeration or freezing as alternative preservation approaches for sheep milk as well as its consequences on cheese and yoghurt production have been studied by some authors (Fava, Külkamp-Guerreiro, & Pinto, 2014; Fava, Serpa, Külkamp-Guerreiro, & Pinto, 2013; Tribst et al., 2018, 2019a,b, 2020). Refrigeration temperatures can favour growth of psychrotrophic microorganisms, which produce lipases and heat-resistant proteases (Capodifoglio et al., 2016; Zhang et al., 2015). These enzymes can negatively affect yield and quality of dairy products due to alterations in flavour and aroma and formation of a weak clot during cheese making (Chen, Daniel, & Coolbear, 2003). Freezing can change the structure of milk constituents, leading to an increase in lipid oxidation rate and lipolysis, alteration of soluble and colloidal mineral fractions, and protein destabilisation (Fennema, William, & Elmer, 1973; Kljajevic et al., 2016; Tribst et al., 2019b).

Requeijão cremoso is a type of Brazilian processed cheese that is widely consumed (Van Dender, 2014). It is obtained by mixing

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different types of cheese with emulsifying salts and other dairy ingredients such as butter or cream (Barth, Tormena, & Viotto, 2017; Ferrão et al., 2018). Substitution of calcium by other ions in the protein matrix leads to a loss of matrix hardness and improves spreadability, this is mainly controlled by the protein raw material used (Belsito et al., 2017).

To the best of our knowledge, there are gaps in the literature regarding cold preserved sheep milk, especially: (i) the proteolytic activity and its consequences and (ii) impact of using this milk to produce processed cheeses, aiming to evaluate whether negative effects on hard cheeses would occur in processed cheeses. To fill these gaps and because of the importance of Requeijão cremoso in Brazil (Oliveira et al., 2018; Van Dender, 2014), our objectives were to (i) evaluate the physicochemical properties of sheep milk, presence of proteolytic enzymes and, integrity of the protein fractions during prolonged refrigeration or frozen storage, (ii) determine effects of cold storage on the characteristics of Requeijão cremoso.

2. Materials and methods

2.1. Fresh, refrigerated, and frozen sheep milk

Sheep milk from Lacaune sheep was supplied by a local producer (Três Leites Farm, Lajeado Grande, Brazil). Milk was obtained from 120 healthy animals (at the beginning, middle, and end of lactation) with an average production of 1.6 L per day. Raw milk was stored at 4 °C, and 20 L of milk was obtained 12 h after milking, on two different days. Milk was divided into 36 polypropylene flasks of 100 mL for physicochemical characterisation, and 8 flasks of 1.5 L for production of cheese curd for the manufacture of Requeijão cremoso. The curd and Requeijão cremoso were prepared twice, and assays were carried out in triplicate for each sample.

Milk samples were refrigerated (R) at 4 ± 0.1 °C (Lucadema, LUCA-161/01, São José do Rio Preto, Brazil) for 6 and 10 days. Six days is a common storage condition on small farms, which has been proven to be suitable for preservation of small volumes of sheep milk (Tribst et al., 2019a) and 10 days an abuse condition, to investigate its effect on the milk properties. In addition, milk samples were subjected to frozen storage (F) at −29.8 ± 0.4 °C, in a vertical domestic freezer (Consul, CVU26E, São Paulo, Brazil), and thawed at 4.0 ± 0.1 °C after 15, 30, 45, 60, and 90 days of storage. Fresh milk sample (Fresh), evaluated on the day of milking, was used as a control.

2.2. Cheese curd

Samples of fresh milk, refrigerated milk for 6 days, or frozen milk for 30 and 90 days were pasteurised (1.5 L) at 65 ± 0.1 °C for 30 min, under agitation (300 rpm) in a Thermomix pan (Vorwerk, Cloyes-sur-le-Loir, France) and cooled to 36 °C. After pasteurisation, bacteria were enumerated as described in section 2.6.

Cheese curd was produced as reported by Verruck, Prudêncio, Vieira, Amante, and Amboni (2015) with modifications. For that, 0.40 mL of calcium chloride 50%, 0.02 g of commercial lactic acid culture (Ricaferm MT3) containing *Lactococcus lactis* ssp. *lactis, L. lactis* ssp. *cremosis* and *Streptococccus salivarius* ssp. *thermophilus* (Rica Nata, Piracema, Brazil), and 1.4 mL commercial chymosin rennet (Ha-La) of *Aspergillus niger* var. *awamori* (Christian Hansen, Valinhos, Brazil) were used for 1 L of milk.

The mixture was gently stirred for 1 min in rectangular polypropylene moulds and incubated at 36 ± 0.1 °C for 40 min. After incubation, curd was cut into cubes of approximately 1 cm^3, rested for 5 min, and then stirred (3 min) and at rested (2 min), this was repeated 4 times. Then, curd was separated from the whey using a cylindrical sieve (15 cm in diameter), and pressed (950.2 g) for 20 h at 9 ± 0.1 °C.

2.3. Requeijão cremoso processed cheese

Requeijão cremoso was made with the curds by adding cream (100–124 g) to obtain 14% milk solids-not-fat, 25% fat, and 61% moisture, according to the standard formulation for manufacture of Requeijão cremoso. Then, 3.0% JOHA S9B emulsifying salt (a blend of sodium polyphosphate and tetra sodium pyrophosphate, ICL Food Specialties, Cajati, Brazil) and 0.7% sodium chloride were added. This mixture was melted at 90 °C, under stirring (500 rpm) for 7 min in Thermomix pan (Belsito et al., 2017).

After manufacture, 50 g of Requeijão cremoso were placed in 200 mL polypropylene flasks. The moisture content and pH were determined immediately after production, as described in the 2.4 section. Other assays (instrumental colour, texture profile, and nutritional composition) were performed after 48 h of refrigerated storage (4 °C).

2.4. Physicochemical characterisation

Fresh milk was characterised according to the methodologies of the AOAC (2016): fat (Gerber, method 2000.18), protein (micro-Kjeldahl, method 991.20), ash (method 935.42), total solids (method 990.20), non-fat solids (method 990.21), acidity (method 947.05) and specific minerals (sodium, potassium, calcium, and magnesium) (method 985.35). Lactose was determined by the reducing sugars method (Lane & Eynon, 1923) and pH was measured using a digital pHmeter (Hanna, HI 2221, Amorim, Portugal). Previously stored samples (refrigerated and frozen milk) were monitored for pH and titratable acidity. Both the cheese curd and the Requeijão cremoso cheese were characterised for fat content according to BSI (1989) and protein (method 2001.14 - AOAC, 2016). Other assays (ash, total solids, and solids-not-fat contents) were carried out using the same methods described for fresh milk. All analyses were performed in triplicate.

2.5. Protein stability

The protein stability of the milk samples was determined by the ethanol stability test (ES). For this, 2 mL of milk was mixed with 2 mL of ethanol solutions (40–70%, with steps of 2%) in a Petri dish and clot formation was visually evaluated (Lai, Fatimah, Mahyudin, Saari, & Zaman, 2016).

2.6. Microbiological characterisation and proteolytic activity

For microbiological characterisation of milk, total aerobic mesophilic bacteria (TBC) and psychrotrophic aerobic bacteria counts (TPC) (Downes & Ito, 2001) were performed in duplicate.

Proteolytic activity (PA) was determined using azocasein (Sigma Aldrich, Steinheim, Germany) as substrate and measuring the absorbance of the samples in UV–visible spectrophotometer (Biospectro, SP-22 spectrophotometer, San Francisco, CA, USA) at 345 nm (Palomba, Formisano, Arrichiello, Auriemma, & Sarubbi, 2017). The standard curve ($y = -0.09x^2 + 0.32x + 0.01$; R^2 of 1.00) was obtained using liquid protease (0, 0.25, 0.50, 1.00, 2.00 mU mL^{-1}) from *Bacillus* spp. (Sigma Aldrich), with three repetitions. All analyses were carried out in triplicate.

2.7. Polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic profile of the samples was obtained according to Laemmli (1970), with modifications. For sample

preparation, 200 μL aliquots were diluted in water (1:50%, v/v), using the following test conditions: 15% running gel and 3% stacking gel solutions, 20 μL sample, 15 μL standard, which were loaded onto an LCV vertical electrophoresis unit (Loccus Biotecnologia, Cotia, Brazil) and LPS source (Loccus Biotecnologia), at 250 V and 30 mA, for approximately 1 h. At the end of the run, the gel was stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories Inc., Richmond, VA, USA) for 12 h. The gel was unstained in distilled water and subjected to microwave heating to visualise the bands.

2.8. Instrumental colour

Instrumental colour of Requeijão cremoso formulations was determined using a digital colorimeter (HunterLab, MiniScan EZ 4500 L, Reston, VA, USA) with illuminant D65 and 10° observation angle. For the test, ~3 g of sample was placed on an acrylic plate 4 cm in diameter, with a sample thickness of 5 mm. Equation (1) presents the colour variation (ΔE), which was calculated from the L* (luminosity) and b* (chromaticity) values:

$$\Delta E = \left[(L - L_0)^2 + (b - b_0)^2\right]^{\frac{1}{2}} \quad (1)$$

where L_0 and b_0 are parameters from control sample (fresh milk) and L and b are parameters from test sample. Since colour of processed cheese can vary from cream to white (Bosi, 2008), Δ is the difference between each colour parameter of the control sample (fresh milk) and the test sample. The assays were performed 2 days after manufacture, in triplicate.

2.9. Texture profile analysis (TPA)

The texture profile analysis of Requeijão cremoso (hardness, adhesiveness, elasticity, cohesiveness, and gumminess) was performed in a texture analyser (Brookfield, CT3 Texture Analyzer, Middleboro, MA, USA) using an acrylic cylindrical probe of 25.4 mm diameter. The test conditions were load cell of 500 N, compression distance of 20% of the product height, speed of 1.0 mm s^{-1}, and contact time of 5 s, with two penetration cycles (Silva et al., 2012). For tests, 50 g of Requeijão cremoso was placed in a 200 mL polypropylene flask (55 mm top diameter, 50 mm bottom diameter, and 60 mm of height), with a height of 2 ± 0.2 cm (10 ± 0.1 °C). Determinations were carried out in six replicates and 2 days after products manufacture.

2.10. Statistical analysis

All data were analysed by analysis of variance (ANOVA) and Tukey's 5% mean comparison test, using the STATISTICA 13.2 version (StatSoft Inc®, Tulsa, OK, USA). Results were expressed as mean ± standard deviation. Data from milk samples subjected to different storage conditions were evaluated by Principal Component Analysis (PCA) using the software XLSTAT 2015.2.02 (Addinsoft, Paris, France).

3. Results and discussion

3.1. Characterisation of sheep milk and the impact of refrigerated storage

The sheep milk composition was 6.5 ± 0.3% fat, 5.4 ± 0.1% protein, 4.2 ± 0.0% lactose, 0.89 ± 0.02% ash, and 16.6 ± 0.3% total solids. Mineral content, per 100 g of milk, was 64.0 ± 7.9 mg sodium, 98.5 ± 5.5 mg potassium, 178.5 ± 9.3 mg calcium, and 26.1 ± 4.0 mg magnesium. No significant differences ($P > 0.05$) were observed among samples collected on different days, indicating effective standardisation of the milk.

Table 1 presents results of the microbiological and physicochemical characterisation, protein stability, and proteolytic activity. Mesophilic bacteria (up to 3.70 log cfu g^{-1}) and psychrotrophic microorganisms (up to 4.43 log cfu g^{-1}) counts increased ($P < 0.05$) after refrigerated storage at 4 °C. No differences were observed in bacterial counts between 6 and 10 days of refrigerated storage ($P > 0.05$), possibly due to exponential growth of the microorganisms in the first days of storage, with stationary phase from 6 days to 10 days. These results were expected since refrigeration limits rather than inhibits microbial growth (Tribst et al., 2019a). Other authors have also reported an increase in bacterial counts in refrigerated raw milk (Balthazar et al., 2019; Tribst et al., 2019a), which depends on several factors, including the volume stored, the initial bacterial count, storage temperature, and microbial species present in milk (Tribst et al., 2019a).

In frozen milk, no growth was observed in either mesophilic bacteria or psychrotrophic bacteria under the conditions studied, with results similar to fresh milk ($P > 0.05$). Similar findings were reported by Tribst et al. (2019a), who studied small amounts of milk subjected to freezing and thawing under refrigeration conditions. In turn, Balthazar et al. (2019) reported a reduction in total mesophilic bacteria in sheep milk subjected to frozen storage for 180 days, probably due to exposure of the cells to the osmotic gradient (Soukoulis, Fisk, & Bohn, 2014). Differences between results are due to differences in thawing rate and the diverse microbiota found in the milk of the different studies. Microbiological results allow inferring that freezing is the best method for preservation of sheep milk. However, in this case, it is worth emphasising that milk should be used soon after thawing under refrigeration to prevent microbiological growth (Balthazar et al., 2019; Tribst et al., 2019a).

The acidity of fresh sheep milk (Table 1) was within the acceptable limits reported by Park, Juárez, Ramos, and Haenlein (2007), and the results of pH, titratable acidity, and ethanol stability were similar to those described by other authors for sheep and goat milk (De La Vara et al., 2018; Fava et al., 2014). Among the cold-stored samples, only the milk refrigerated for 10 days showed alterations in physicochemical parameters ($P < 0.05$), with an increase in acidity of 0.1%, pH decreases of 0.3 pH units, and a 15% reduction of ethanol stability. These changes are probably due to the growth of mesophilic aerobic microorganisms during 10 days of storage, with lactic acid production from lactose fermentation (Park et al., 2007; Tribst, Falcade, Leite Júnior, & Oliveira, 2019c).

Accumulation of H^+ ions in milk leads to an increase in acidity, a reduction of pH and protein stability due to the partial dissolution of colloidal calcium phosphate, and reduction of the electrostatic repulsion between protein molecules (De La Vara et al., 2018; Horne, 2016). It is worth noting that although no significant differences were observed between the mesophilic and psychrotrophic bacterial counts for samples subjected to refrigerated storage for 6 and 10 days, longer storage showed significant changes in physicochemical parameters. This result suggests that, despite marginal growth observed between 6 and 10 days of storage, there was an accumulation of H^+ ions in the milk refrigerated for 10 days, which was sufficient to overcome the high buffering capacity of the sheep milk (Tribst et al., 2019c, 2020).

No significant differences were observed for the proteolytic activity (Table 1) of the samples during refrigerated storage ($P > 0.05$), while milk subjected to frozen storage for more than 45 days exhibited a significant increase of this parameter. Even so, the activity was lower (<0.2 mU mL^{-1}) when compared with the findings of Palomba et al. (2017), who reported a proteolytic activity of 1.66 mU mL^{-1}. Higher proteolytic activity was expected in the

Table 1
Evaluation of microbial activity by total bacterial count (TBC) and total psychrotolerant count (TPC), as well as pH, acidity, ethanol stability (ES) and proteolytic activity (PA) for fresh (Fresh), refrigerated (R) and frozen (F) sheep milk over time.[a]

Sheep milk	TBC (log cfu mL^{-1})	TPC (log cfu mL^{-1})	pH	Acidity (%)	ES (%)	PA (mU mL^{-1})
Fresh	4.19 ± 0.56^{b}	3.85 ± 0.98^{b}	6.56 ± 0.02^{b}	0.21 ± 0.01^{b}	65.00 ± 3.40^{a}	0.02 ± 0.02^{d}
R6	7.06 ± 0.27^{a}	7.70 ± 0.22^{a}	6.49 ± 0.03^{b}	0.24 < 0.01^{b}	63.00 ± 3.40^{a}	0.04 ± 0.02^{cd}
R10	7.89 ± 0.43^{a}	8.28 ± 0.27^{a}	6.26 ± 0.21^{a}	0.31 ± 0.04^{a}	50.00 ± 6.90^{b}	0.06 ± 0.02^{bcd}
F15	6.18 ± 1.71^{b}	4.87 ± 1.47^{b}	6.61 ± 0.05^{b}	0.22 ± 0.02^{b}	64.00 ± 4.60^{a}	0.06 ± 0.02^{bcd}
F30	5.67 ± 1.26^{b}	4.88 ± 1.25^{b}	6.59 ± 0,01^{b}	0.23 ± 0.01^{b}	64.00 ± 4.60^{a}	0.12 ± 0.15^{abcd}
F45	5.64 ± 1.54^{b}	4.54 ± 0.54^{b}	6.56 ± 0.03^{b}	0.23 ± 0.01^{b}	63.00 ± 3.40^{a}	0.30 ± 0.05^{abc}
F60	5.75 ± 1.50^{b}	4.47 ± 0.61^{b}	6.57 ± 0,03^{b}	0.23 ± 0.01^{b}	63.00 ± 3.40^{a}	0.16 ± 0.07^{ab}
F90	5.61 ± 1.59^{b}	4.71 ± 0.50^{b}	6.56 ± 0.04^{b}	0.23 ± 0.01^{b}	63.00 ± 3.40	0.19 ± 0.05^{a}

[a] Data are the means of replicates ± standard deviation; different superscript letters in a column indicate significant differences among samples by Tukey test ($P < 0.05$). The number following the milk description (R or F) indicates time of sample storage (in days).

refrigerated milk, which exhibited high psychrotrophic bacteria counts, this bacterial group is considered the main producer of heat-resistant proteases in milk (Zhang et al., 2015). However, not all species of psychrotrophic bacteria are protease-producers and production rate depends on nutrient availability and storage conditions, including time and temperature (Capodifoglio et al., 2016; Júnior et al., 2018), which may explain the results of the present study.

Fig. 1 shows the electrophoretic profiles of the different milk samples. The electrophoretogram bands were compared with molecular masses of target proteins in the literature to identify the protein fractions (Balthazar et al., 2019; Nguyen, Saeedeh, & Day, 2018). The higher intensity of the casein fractions, when compared with the whey proteins, indicates a higher concentration of casein in sheep milk (Balthazar et al., 2019). Also, as reported by Balthazar et al. (2019), milk samples of the present study exhibited a higher amount of β-lactoglobulin among whey proteins. Concerning casein fractions, no clear link between α and β-casein was observed, possibly due to the high concentration of these proteins in sheep milk (Balthazar et al., 2017).

The cold-stored samples showed electrophoretic profiles similar to fresh milk, except when refrigerated for 10 days, where a reduction of the intensity of the κ-casein fraction was seen, with the emergence of more intense intermediate bands from 10 to 15 kDa, indicating peptide formation (Nguyen, Ong, Lopez, Kentish, & Gras, 2017). Changes were expected due to high counts of psychrotrophic microorganisms, as protease producers (Nörnberg, Tondo, &). However, these results were contradictory to the proteolytic activity and psychrotrophic counts (Table 1). Thus, the present results suggest that proteolysis of κ-casein occurred only in the milk refrigerated for 10 days due to the combined effect of two factors: (i) protease activity, which had more time to act even at low concentrations, and (ii) greater exposure of casein micelles to enzymatic hydrolysis due to destabilisation by pH reduction, with release of colloidal calcium (De La Vara et al., 2018; Horne, 2016).

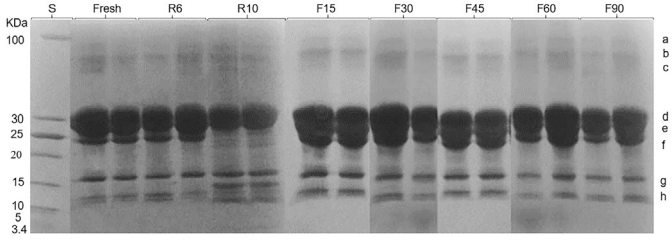

Fig. 1. Representation of the protein profile in SDS-PAGE electrophoresis gel from fresh and cold preserved sheep milk. S, standard; Fresh, fresh milk; R, refrigerated milk; F, frozen milk. The number following the milk description (R or F) indicates the time of sample storage (in days); a, immunoglobulin; b, lactoferrin; c, serum albumin; d, α-casein; e, β-casein; f, κ-casein; g, β-lactoglobulin; h, α-lactalbumin.

Absence of visible protein degradation of samples qualitatively estimated by SDS-PAGE confirmed that the cold storage conditions (refrigeration for 6 days or freezing for up to 90 days) did not affect extensively protein integrity due to proteolysis. It was not possible to guarantee that the initial hydrolysis did not occur, since separation of similar molecular masses of proteins (i.e., fractions slightly hydrolysed) is difficult by the SDS-PAGE method (Sharma et al., 2021).

The principal component analysis (PCA) of the physicochemical and microbiological data indicated in a simple manner the impact of each type of storage condition had on parameters studied (Fig. 2). Prolonged refrigeration led to bacterial growth with consequent acidification, pH reduction, and lower ethanol stability. Freezing for up to 90 days ensured microbiological and physicochemical stability, except for proteolytic activity, which increased with freezing time. However, this increase was not capable of increasing proteolysis in milk (Fig. 1), which was also reported by Palomba et al. (2017).

3.2. Influence of preservation methods of sheep milk on the manufacture of Requeijão cremoso

The physicochemical characteristics and structural properties of the casein micelles, including the size, proportion of casein fractions, colloidal calcium phosphate concentration, and genetic variants may influence the rheological properties of the curd and, consequently, its ability to retain milk constituents in cheese, mainly water, casein, and fat (Bittante, Penasa, & Cecchinato, 2012). In the present study, there was no difference in milk

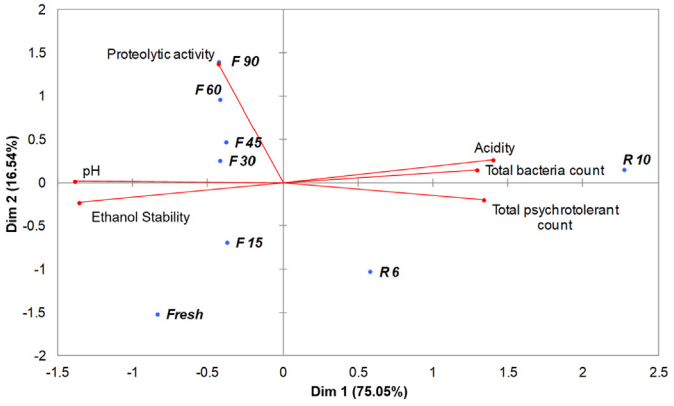

Fig. 2. Principal component analysis of fresh and cold preserved sheep milk. Fresh, fresh milk; R, refrigerated milk; F, frozen milk. The number following the milk description (R or F) indicates the time of sample storage (in days).

Table 2
Physicochemical and composition of cheese curd and Requeijão cremoso processed cheese manufactured with fresh and cold preserved sheep milk.[a]

Item	Fresh	R6	F30	F90
Cheese curd				
pH	6.14 ± 0.33[a]	6.00 ± 0.51[a]	5.91 ± 0.52[a]	6.05 ± 0.44[a]
Fat (%)	21.00 ± 1.78[a]	20.67 ± 2.38[a]	20.70 ± 1.79[a]	20.42 ± 0.86[a]
Protein (%)	15.44 ± 1.43[a]	16.24 ± 0.05[a]	15.88 ± 1.37[a]	15.38 ± 0.11[a]
TS (%)	39.43 ± 0.88[a]	39.76 ± 0.33[a]	40.20 ± 2.12[a]	40.11 ± 0.90[a]
NFS (%)	18.43 ± 0.88[a]	19.09 ± 0.33[a]	19.50 ± 2.12[a]	19.69 ± 0.90[a]
Ash (%)	1.89 ± 0.08[a]	1.79 ± 0.16[a]	1.96 ± 0.04[a]	1.82 ± 0.02[a]
Requeijão cremoso				
pH	6.68 ± 0.48[a]	6.61 ± 0.41[a]	6.74 ± 0.43[a]	6.52 ± 0.47[a]
Fat (%)	20.50 ± 3.00[a]	20.50 ± 1.97[a]	19.25 ± 0.96[a]	19.33 ± 0.52[a]
Protein (%)	8.81 ± 0.51[a]	9.07 ± 0.20[a]	9.05 ± 0.60[a]	9.33 ± 0.17[a]
TS (%)	35.17 ± 0.06[a]	36.35 ± 1.59[a]	36.61 ± 1.52[a]	36.82 ± 1.39[a]
NFS (%)	14.67 ± 0.06[b]	15.85 ± 1.59[ab]	17.36 ± 1.52[a]	17.49 ± 1.39[a]
Ash (%)	3.74 ± 0.08[a]	3.70 ± 0.29[a]	3.85 ± 0.12[a]	3.73 ± 0.02[a]
FDM (%)	58.29 ± 0.10[a]	56.50 ± 2.51[a]	52.66 ± 2.23[b]	52.57 ± 2.00[b]

[a] Abbreviations are: fresh, fresh milk; R, refrigerated milk; F, frozen milk; TS, total solids; NFS, not-fat solids; FDM, fat in dry matter. Data are means of replicates ± standard deviation; different superscript letters in a column indicate significant differences among different samples by Tukey test ($P < 0.05$). The number following the milk description (R or F) indicates the time of sample storage (in days).

composition, and preservation processes (6 days of refrigerated storage and 30 or 90 days of frozen storage) thus, there were no significant ($P > 0.05$) changes in the physicochemical parameters (Table 2).

The Requeijão cremoso from curd made with fresh milk and previously frozen milk showed an average pH of 6.14 ($P > 0.05$). The difference in pH between Requeijão cremoso and cheese curd is due to addition of emulsifying salts (tetrasodium pyrophosphate and sodium polyphosphate) and cream to the formulations, which modified the buffering capacity. The pH values of the Requeijão cremoso in this study were higher than the values recommended by Van Dender (2014) for Requeijão made with cow milk (5.4–6.2).

Fat, protein, total solids, and ash contents of Requeijão cremoso ($P > 0.05$) had no differences among the formulations produced with fresh and cold-preserved milk. The not-fat solid (NFS) and fat dry matter (FDM) showed differences ($P < 0.05$) between the samples of Requeijão cremoso made from frozen and fresh milk, with higher NFS and consequently lower FDM in the Requeijão cremoso from frozen milk. These differences were small and probably inherent to the adapted lab-scale production of Requeijão cremoso (small differences in the cream added for each formulation/batch to reach the target final composition, described in item 2.3).

The Requeijão cremoso cheeses and the control made from fresh milk were subjected to instrumental colour measurements to determine the ΔE parameter. A colour difference noticeable to the human eye occurs at ΔE > 2 (Francis & Clydesdale, 1975), here the Requeijão cremoso formulations did not differ from the control for the colour parameters, all formulations had ΔE < 1.53 (data not shown).

Texture profile analysis (TPA) revealed differences between samples produced with fresh and preserved milk. Refrigerated milk showed ~20% higher hardness, adhesiveness, and gumminess ($P < 0.05$). Considering the similar composition of samples from fresh and refrigerated milk (Table 2), differences observed may be related to the greater emulsification capacity of the caseins from refrigerated milk. Greater emulsification can probably be explained by slight proteolysis of casein fractions, especially κ-casein and β-casein, caused by microbial and endogenous proteases (Zhang et al., 2015). Although no visible changes were observed in the electrophoretic profile of refrigerated milk for 6 days, it is known that SDS-PAGE is only a qualitative measure of protein hydrolysis and is not able to distinguish low hydrolysis levels, especially when the concentration of protein is high in the sample (Sharma et al., 2021). This probably explains why changes in κ-casein (visible formation of peptides of 10–15 kDa from κ-casein – Fig. 1) were only observed after 10 days of refrigerated storage and no changes were observed in β-casein bands (50% of total sheep milk casein; Tavaria, Reis, & Malcata, 2006), even though this fraction is most susceptible to proteolysis under refrigeration, due to its partial solubilisation (Manfredini & Massari, 1989).

Texture of Requeijão cremoso produced with frozen milk had minor differences (around 10% in hardness and gumminess, close to the expected deviation in TPA analysis of food) between formulations produced with fresh milk and milk frozen for 30 days. However, Requeijão cremoso produced with milk frozen for 90 days was different from the control sample for most texture parameters ($P < 0.05$), with increases ranging from 24% to 57% in hardness, adhesiveness, elasticity, and gumminess. Comparing cheese from frozen for 30 and 90 days (Table 1) and composition of the Requeijão cremoso produced with these milk samples (Table 2), differences observed in the Requeijão cremoso texture might be attributed to the effects of protein destabilisation caused by long freezing, which is associated with high calcium content and lactose crystallisation (Wendorff, 2001) and/or protein rearrangement due to protein–protein interaction (Fava et al., 2013). Casein is the main structural component of the curd and its network stiffness depends on the degree of openness, the amount of bounded water in the protein matrix, and content of fat and free water (Prentice, Langley, & Marshall, 1993). Thus, changes induced by prolonged freezing probably affected functional properties of the proteins, such as water holding capacity and emulsification capacity, thus modifying curd rigidity and, consequently, texture.

In general, processed cheese made from cows' milk at pH > 6.2 has low hardness due to the weak protein–protein interactions. However, results of the texture profile analysis of the Requeijão cremoso formulations showed no consistency defects (Table 3), probably due to buffering capacity and high protein and mineral levels of sheep milk, which allowed for better consistency of the Requeijão cremoso at a higher pH. As reported by Van Dender

Table 3
Influence of refrigerated (R) and frozen (F) sheep milk preservation methods on the texture profile of Requeijão cremoso compared with that obtained with fresh sheep milk.[a]

Item	Fresh	R6	F30	F90
Hardness (N)	0.97 ± 0.03[c]	1.19 ± 0.04[b]	0.88 ± 0.04[d]	1.35 ± 0.03[a]
Adhesiveness (mJ)	5.26 ± 0.34[c]	6.30 ± 0.35[b]	4.85 ± 0.24[c]	8.28 ± 0.47[a]
Cohesiveness	0.69 ± 0.04[a]	0.71 ± 0.04[a]	0.68 ± 0.04[a]	0.74 ± 0.03[a]
Elasticity (mm)	10.39 ± 0.38[b]	10.96 ± 0.26[b]	10.70 ± 0.87[b]	12.89 ± 0.21[a]
Gumminess (N)	0.68 ± 0.04[c]	0.82 ± 0.06[b]	0.59 ± 0.02[d]	0.98 ± 0.04[a]

[a] Data are means of replicates ± standard deviation; different superscript letters in a row indicate significant differences among different samples by Tukey test ($P < 0.05$). The number following the milk description (R or F) indicates the time of sample storage (in days).

(2014), the texture parameters of Requeijão can be affected by its solids-not-fat content.

Studies have shown that freezing causes changes in fat globules and saline balance, and the negative impact of refrigerated storage (7 °C) for 4 days is lower when compared with frozen storage (−18 °C) for 30 days (Tribst et al., 2019b). Results of the present study differed from the literature in some aspects, possibly due to several factors, including the freezing, refrigeration, and thawing conditions, as well as storage temperatures and composition of the raw material.

4. Conclusion

Sheep milk subjected to refrigerated storage at 4 °C for 6 days, and frozen storage at −29.8 °C for 90 days maintained its physicochemical and microbiological quality, with no measurable impact on the protein fractions. However, refrigerated storage for 10 days affected the pH, acidity, and ethanol stability, with an increase in mesophilic and psychrotrophic bacteria counts, resulting in protein degradation, evidenced by appearance of intermediate bands in the range of 10−15 kDa, indicating formation of peptides.

Cold storage of milk minimally affected the characteristics of Requeijão cremoso cheese produced, except for the texture of samples. Regarding texture, samples manufactured with fresh milk was more similar to that made with 30 days frozen milk, followed 6 days refrigerated milk and finally 90 days frozen milk. Therefore, overall evaluation of the results suggests that refrigeration of sheep milk for 6 days, or freezing for up to 90 days, were effective for storage of milk for the manufacture of Requeijão cremoso.

Credit author statement

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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.

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