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Impact of extended refrigerated storage and freezing/thawing storage combination on physicochemical and microstructural characteristics of raw whole and skimmed sheep milk

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ABSTRACT

The impact of freezing (1 month + thawing at 7 or 25 °C) and extended refrigeration (4 days, 7 °C) on physicochemical and microstructural characteristics of raw whole and skimmed sheep milk were assessed. Refrigerated storage resulted in higher sedimentation and creaming (whole milk), possibly due to proteases and agglutinins. Freezing/thawing processes in whole milk increased the particle size and creaming when samples were thawed at 7 °C. Skimmed milk showed an increase in buffering capacity and a reduction in soluble calcium immediately after thawing at 25 °C, suggesting that although the changes in fat are the main alterations caused by slow freezing of sheep milk, minor changes in saline balance can occur. An evaluation of the results showed that frozen and thawed milk in domestic equipment (commonly found in smallholdings) alter the milk microstructure, and it is therefore preferable to use extended refrigeration to accumulate the milk before dairy production.

1. Introduction

Sheep milk is the fourth most consumed milk worldwide (FAOSTAT, 2016); however, favourable commercial use of this milk is a challenge due to the seasonality in milk production, low animal productivity and short periods of lactation (Katsiari, Voutsinas, & Kondyli, 2002). Although these problems could be minimised using reproductive management, this kind of system is hard to be implanted in small or medium-sized farms. Moreover, this kind of farm normally produces a small volume of milk daily, which makes an adequate dairy production scale difficult (Tribst, Ribeiro, Leite Junior, de Oliveira, & Cristianini, 2018).

Frozen milk is commonly used to overcome these limitations, allowing milk storage for days (until reaching a compatible volume with dairy production; Fava, Serpa, Külkamp-Guerreiro, & Pinto, 2013) or months (aiming to solve the problem of seasonality/short lactation; Wendorff, 2001). For small productions, the milk is frozen exclusively to accumulate a sufficient volume for production and is kept frozen for 1 month or less since the high costs incurred by cold equipment and energy consumption for long milk preservation under freezing conditions (>1 month) are not feasible for small producers. To ensure the quality of this milk, it must be frozen in relatively small volumes (avoiding hot spots that favour microbial growth in cooling processes and long thawing times) and preferably cooled at a rapid rate (Haenlein & Wendorff, 2006). Extended cold storage may also be another alternative when milk needs to be stored for only a few days as it has low initial microbial loads (Fonseca et al., 2013), and is refrigerated rapidly and maintained at low temperatures (Yamazi et al., 2013).

Extended cold storage can induce undesirable changes in milk, mainly due to psychrotrophic bacterial growth that produces thermostable lipases and proteases. These enzymes might cause defects in dairy products, especially texture alteration and a bitter or rancid flavour (Fava et al., 2013). On the other hand, changes induced exclusively by cold processes in cow milk (such as solubilisation of calcium phosphate, casein dissociation due to the release of β-casein and a reduction in micelle size) were not observed in sheep milk (Raynal & Remeuf, 2000).

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Freezing processes change sheep milk constituents (Fava, Külkamp-Guerreiro, & Pinto, 2014). Ice crystals (especially at slow freezing rates) entrap the fat globules, causing irreversible damage to the globule structure, with consequent destabilisation of emulsion and coalescence of globules after thawing (Zhang, Mustafa, Ng-Kwai-Hang, & Zhao, 2006). Moreover, intense water crystallisation increases the osmotic pressure of the system (mainly due to high calcium concentration in the reminiscent liquid phase) favouring protein destabilisation and aggregation, which is intensified through milk storage time (Fontecha, Bellanato, & Juarez, 1993; Wendorff, 2001), especially at relatively high temperatures (−18 °C, commonly used in domestic equipment) when compared with lower temperatures (−27 °C) (Wendorff, 2001). The mineral balance can also be affected by freezing, changing the soluble calcium concentration due to forming of a saturated calcium solution during water crystallisation (Kljajevic et al., 2016; Koschak, Fennema, Amundson, & Lee, 1981). Changes in the constituents may modify the physicochemical and microstructural characteristics of sheep milk after thawing; and different levels of changes may occur depending on whether the milk is used immediately after thawing or if it is stored after thawing.

Based on the importance of freezing or extended refrigerated storage for small sheep milk producers, we evaluated the impact of these processes and thawing conditions on the physicochemical and microstructural characteristics of sheep milk. Whole and skimmed milk were intentionally used to differentiate the effect of the studied preservation techniques on milk colloidal suspensions in the presence and absence of lipids, which aimed to better understand the phenomena involving each sheep milk constituent.

2. Materials and methods

2.1. Sheep milk and cold preservation processes

Fresh Lacaune sheep milk (6.1 ± 0.5% fat, 4.9 ± 0.2% protein and 16.8 ± 0.5% of solid content) was obtained in triplicate from the Bela Vista smallholding (Morungaba, Brazil). Each sample was obtained mixing the milk from at least 10 healthy animals. After milking, samples were divided into two parts and one of them was skimmed in a cream milk separator (Separatori D100, Brazil) to 0.3 ± 0.02% fat. The microstructural and physicochemical parameters of whole and skimmed sheep milk were determined using: (i) fresh milk, (ii) milk stored for 4 days in 1 L plastic bags (HDPE) at 7 °C (maximum storage time to total bacteria count $< 1500 \times 10^3$ cfu mL^{-1}; EC, 1994 Directive 94/71/EC), (iii) milk slowly frozen at −18 °C (Brastemp model BVG24H, Brazil) in 1 L plastic bags (HDPE), stored for 1 month (maximum time required for sheep milk storage aiming to accumulate a volume compatible with yogurt/cheese production in smallholdings) and then thawed and evaluated in 4 different conditions, totalling 6 samples for whole sheep milk and 6 samples for skimmed sheep milk: (i) fresh milk; (ii) refrigerated (7 °C/4 days); (iii) frozen + thawed at 7 °C, evaluated immediately after thawing (d_0); (iv) frozen + thawed at 7 °C, evaluated 1 day after thawing, with milk preserved at 7 °C (d_1); (v) frozen + thawed at 25 °C, evaluated immediately after thawing (d_0); (vi) frozen + thawed at 25 °C, evaluated 1 day after thawing, with milk preserved at 7 °C (d_1).

These processes were chosen to simulate the real condition of sheep milk accumulation used in small and medium sized farms.

2.2. Physicochemical and microstructural assays

2.2.1. Colour

Milk colour measurement was carried out using an ULTRA PRO colorimeter (HunterLab®, D65, Hunter Associates Laboratory, USA) with an incidence angle of 10° and brightness D65 (Pedras, Tribst, & Cristianini, 2014). An aliquot of 10 mL of milk was placed in glass cuvettes and the colour was measured using the CIELab scale. Lightness was determined as the L* parameter (Pedras et al., 2014) and the total colour difference (ΔE) was calculated considering fresh sheep milk (whole or skimmed) as the initial parameter, following equation (1):

$$\Delta E = \sqrt{(L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2} \quad (1)$$

where L, a and b were the colour parameters of each sample and L_0, a_0 and b_0 were the average colour parameters of fresh whole (for whole samples) or fresh skimmed (for skimmed samples) milk. Each sample was measured 4 times.

2.2.2. Buffering capacity

The buffering capacity of sheep milk samples was evaluated in duplicate according to Huppertz, Grosman, Fox, and Kelly (2004) with few modifications: At 30 s intervals, a 1 mL aliquot of lactic acid (0.18 M) was added to 20 mL of milk samples and then the sample pH was measured. The initial pH of the samples varied from 6.77 to 6.55 (whole milk) and from 6.75 to 6.52 (skimmed milk) and no differences ($P > 0.05$) were observed between any stored sample and control one (data non-shown). The end point of buffering capacity was determined at pH 4.5. A linear regression was adjusted in the linear range of the results (pH 5.8–4.5) to establish the pH rate reduction caused by lactic acid addition (Microsoft Excel, USA), which is directly linked to milk buffering capacity.

2.2.3. Protein aggregate sedimentation

Milk samples were analytically weighed in Eppendorf tubes. Then, tubes were centrifuged (Allegra TM 25R Centrifuge, Beckman Coulter TM, USA) at 3900× g for 30 min at 24 °C (Iordache & Jelen, 2003). The supernatant was drained carefully using a pipette and the residual pellet was dried at 105 °C for 3 h and then it was weighed again. The amount of the sediment formed was recorded as % mass, based on the initial mass of the sample. Each sample was measured 4 times.

2.2.4. Total and soluble calcium

Milk samples were separated by ultracentrifugation at 100,000× g for 1 h at 20 °C and the supernatant (soluble phase − serum milk) was collected. For whole milk, the fat layer was discarded before the serum was collected (Mittal, Ellis, Ye, Das, & Singh, 2015). The soluble calcium was determined in these samples while total calcium was directly determined in whole or skimmed milk. For calcium quantification, 1 g of milk (total calcium) or milk serum (soluble calcium) was digested using concentrated nitric acid at 110 °C, 2 h + 130 °C, 2 h in a block digestion system (Marconi, Model MA 4025). Afterwards, the digested contents were transferred after sufficient rinsing with deionised water into a volumetric flask (25 mL) and filtered using a quantitative filter paper. Then, samples were diluted again up to a calcium concentration ranging between 0.5 and 5% (linear range) and lanthanum (0.5%, w/v) was added to avoid interferences in the sample preparation. Samples were placed into the nebuliser and mixed with an air-acetylene flame (2.5/10 L h^{-1}, around 2000 °C) and the calcium content was analysed in an atomic absorption spectrophotometer (Perkin Elmer Modelo Analyst 200, software Winlab 32) equipped with cathode lamp (FAAS) with emission at 422.67 nm (Theodoropoulos, Turatti, Greiner, Macedo, & Pallone, 2018).

2.2.5. Particle size

The particle size determination of milk samples was carried out using a Mastersize® laser diffraction particle size analyser (Malvern Instruments Ltd, England) equipped with a 300 RF (reverse Fourier) lens and He-Ne laser (λ = 633 nm). Samples were diluted in distilled water until an appropriate obscuration (~14%) was obtained in the diffractometer cell. Particle size distribution analysis was based on a polydisperse model using the following conditions: real refractive index of 1.349, absorption of 0.001 and refractive index of fluid (water) of 1.33 (Fava et al., 2013). The size distribution was characterised by the diameter below which 10 ($d_{0.1}$), 50 ($d_{0.5}$) or 90% ($d_{0.9}$) of the volume of particles were found. These were used to calculate the uniformity distribution (span; equation (2)). The mean particle diameter was evaluated by the particle surface area ($D_{3,2}$; parameter more influenced by smaller particles) and particle volume ($D_{4,3}$; parameter more influenced by larger ones). The specific surface area was also analysed, considering the total area of the particles divided by its total weight (Fava et al., 2013). Each sample was measured at 20 °C in triplicate.

$$Span = \frac{d_{0.9} - d_{0.1}}{d_{0.5}} \quad (2)$$

2.2.6. Near infrared light backscattering

The stability of milk emulsion was monitored using a near-infrared light backscatter at 880 nm (Turbiscan MA2000, Formulaction, France). For this, sheep milk samples were vigorously shaken, 15 mL was placed in borosilicate glass tubes (12 mm inner diameter and 30 mm high) and the experiment's starting time was recorded. The light source scanned the sample at 30 min intervals from bottom to top with an infrared wavelength (880 nm), and measured the percentage of light backscattered during 4 h at 20 °C (Matsumiya, Inoue, Niida, Katagiri, & Nishizu, 2014). The duration of the assays was chosen considering the maximum possible time of keeping the raw milk at room temperature to avoid undesirable changes caused by microbial growth. Each sample was measured in triplicate. For skimmed milk, the backscattered profiles between 1 and 35 mm were collected as raw data. The mean values of the % backscattering (BS) between 5 and 35 mm at times 0 h and 4 h were reported for each sample. The peak areas of delta backscattering (%BS_{4h} − %BS_{0h}) between 1 and 2 mm were determined as sedimentation. For whole milk, the backscattered profiles between 1 and 45 mm were collected as raw data. The creaming phenomenon was characterised by the % of backscattering (BS) at the creaming peak and the thickness of the cream in the tubes.

The creaming peak area corresponds to the sum of the creaming area considering that the beginning of the creaming is the height of the tube where ΔBS (BS_{4h}−BS_{0h}) was ≥0.08 and no further reduction in the BS value was observed until the creaming peak. The end of the creaming was considered as the height of the tube in which the % of BS was the same determined at the beginning of the creaming. The thickness of the cream in the tubes was calculated by the difference in height between the beginning and the end of the creaming in the tube. The data were analysed using the Turbisoft 2.0 software (Zhao et al., 2014).

2.2.7. Confocal microscopy

The sheep milk confocal scanning laser microscopy (CSLM) was based on the method proposed by Oliveira, Augusto, Cruz, and Cristianini (2014). To do this, 1 mL of vigorously stirred sheep milk sample was added to 25 μL fast-green dye FCF (0.1%, w/v, in distilled water) and 40 μL Nile-red (0.234%, w/v, in propane-1,2-diol) (Sigma–Aldrich, Ireland), to observe the protein matrix and fat globules, respectively. A coverglass with 8 chambers was used (Lab-Tek® II Chambered Coverglass, USA) and the confocal imaging was carried out using a Zeiss Upright LSM780-NLO microscope (Carl Zeiss AG, Germany). Representative images of each sample were taken using a 63 × oil immersion objective (numerical aperture = 1.40) at excitation wavelengths of 488 nm to Nile-red (Argon laser) and 633 nm to fast-green (He/Ne laser). The configuration of the images was RGB colour (8 bits) and final resolutions of 0.13 μm pixel^{-1}, 1024 × 1024 pixels in total. This assay was performed only for whole milk samples.

2.3. Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate the effects of frozen and long refrigerated storage milk on physicochemical and microstructural parameters and the Tukey post-hoc test to evaluate the differences between each parameter and also for each sample at 95% of confidence level (XLSTAT software, version 2015.2.02, Microsoft, Inc., USA). Results were expressed as mean ± standard deviation.

3. Results

Table 1 shows the physicochemical parameters of whole and skimmed milk after freezing/thawing and long cold storage. For whole milk, the preservation through the studied processes did not alter the colour parameters (ΔE and L*) and the buffering capacity. Sedimentation analyses showed that extended refrigerated storage significantly increased ($P < 0.05$) mass sedimentation (up to 0.2 g in 100 g, representing 50% increase in sedimentation). In contrast, frozen/thawed samples showed less sedimentation. Soluble calcium reduced ~13% ($P < 0.05$) in milk subjected to freezing (immediately after thawing), while refrigerated storage caused no changes in calcium solubility. For skimmed milk, changes induced by milk preservation methods were more evident than in whole milk. For frozen/thawed samples, the significant changes ($P < 0.05$) were: reversible increase of 2% in L*, slight reduction in sedimentation, 10–20% increase in buffering capacity and for soluble calcium, a reduction in 25% for samples thawed at 25 °C. Although statistically significant, the differences in L* were possibly negligible, since the impact on ΔE was lower than 2; Francis and Clydesdale (1975) reported that only ΔE values higher than 2 are visually perceptible. Moreover, the extended refrigerated milk storage increased 75% of sedimentation (from 0.36 to 0.63%) and 10% of buffering capacity and reduced milk luminosity.

Particle size parameters of sheep milk was characterised as a bimodal (whole) or monomodal distribution (skimmed), with peaks at 0.2 μm (protein) and 1.3 μm (fat) and more than 90% particles lower than 0.30 μm (skimmed) and 2 μm (whole milk), as shown in Table 2. The whole milk particle size was affected when frozen milk was thawed at 7 °C, with a higher volume of larger particles. But their particle characteristics were not affected by long refrigerated storage or for frozen samples thawed at 25 °C. On the other hand, an increase in particle size was observed for sheep milk samples thawed at 7 °C, with an increase in $D_{3,2}$ (~10%), $D_{4,3}$ (~19%) and a reduction in specific surface areas (~8%) and span (~10%). For skimmed milk particle size parameters, the differences observed between the samples were always lower than 2%. Results obtained by Li, Joyner, Carter, and Drake (2018) showed no differences in the sensory perception of viscosity and astringency between cow milk samples with a particle size varying from 0.87 to 0.99 μm (~10% difference in particle sizes). Therefore, although statistically significant, the differences observed in skimmed milk particle size were possibly negligible from a sensory perspective.

Table 1
Physicochemical characteristics of sheep milk (whole and skimmed) after extended refrigerated storage or frozen storage.[a]

Sample	Colour parameters		Sedimentation (%)	Soluble calcium (%)	Buffering capacity (acidification linear slope)
	L*	ΔE			
Whole sheep milk					
Fresh	$70.42 \pm 0.26^{A,B,C,a}$	–	$0.36 \pm 0.1^{B,a}$	$26.5 \pm 1.8^{A,b}$	$-2.24 \pm 0.08^{A,a}$
Refrigerated (7 °C/4 days)	$69.57 \pm 0.83^{B,C,a}$	$0.88 \pm 0.69^{A,a}$	$0.54 \pm 0.1^{A,a}$	$24.8 \pm 0.3^{A,B,C,b}$	$-2.10 \pm 0.03^{B,a}$
Frozen/Thawed at 7 °C (d_0)	$71.02 \pm 0.25^{A,B,a}$	$0.61 \pm 0.23^{A,a}$	$0.19 \pm 0.1^{C,a}$	$22.9 \pm 0.6^{C,b}$	$-2.27 \pm 0.12^{A,B,a}$
Frozen/Thawed at 25 °C (d_0)	$71.80 \pm 0.79^{A,a}$	$1.38 \pm 0.79^{A,a}$	$0.22 \pm 0.1^{C,a}$	$23.5 \pm 1.4^{B,C,a}$	$-2.22 \pm 0.08^{A,B,a}$
Frozen/Thawed at 7 °C ($d_1^{\#}$)	$69.31 \pm 0.80^{C,a}$	$1.11 \pm 0.66^{A,a}$	$0.21 \pm 0.2^{C,a}$	$24.8 \pm 1.7^{A,B,b}$	$-2.26 \pm 0.03^{A,B,a}$
Frozen/Thawed at 25 °C ($d_1^{\#}$)	$69.74 \pm 0.64^{B,C,a}$	$0.68 \pm 0.58^{A,a}$	$0.31 \pm 0.1^{B,a}$	$24.0 \pm 0.7^{B,C,a}$	$-2.15 \pm 0.09^{A,B,a}$
p-value of one-way ANOVA	<0.0001	0.536	<0.0001	<0.0001	0.013
Skimmed sheep milk					
Fresh	$68.06 \pm 0.40^{B,b}$	–	$0.36 \pm 0.1^{B,a}$	$32.5 \pm 1.8^{A,a}$	$-2.22 \pm 0.05^{D,a}$
Refrigerated (7 °C/4 days)	$67.11 \pm 0.09^{C,b}$	$1.01 \pm 0.02^{A,B,a}$	$0.63 \pm 0.1^{A,a}$	$30.4 \pm 0.9^{A,B,a}$	$-2.03 \pm 0.05^{C,a}$
Frozen/Thawed at 7 °C (d_0)	$69.23 \pm 0.44^{A,b}$	$1.17 \pm 0.12^{A,B,a}$	$0.26 \pm 0.2^{B,C,a}$	$26.4 \pm 1.6^{A,B,a}$	$-1.87 \pm 0.1^{A,B,a}$
Frozen/Thawed at 25 °C (d_0)	$69.98 \pm 0.81^{A,b}$	$1.92 \pm 0.35^{A,a}$	$0.18 \pm 0.2^{C,D,a}$	$25.0 \pm 2.7^{B,a}$	$-2.01 \pm 0.06^{B,C,b}$
Frozen/Thawed at 7 °C ($d_1^{\#}$)	$67.52 \pm 0.60^{B,C,b}$	$0.57 \pm 0.06^{B,a}$	$0.11 \pm 0.1^{D,E,a}$	$30.4 \pm 4.8^{A,B,a}$	$-1.82 \pm 0.16^{A,b}$
Frozen/Thawed at 25 °C ($d_1^{\#}$)	$67.18 \pm 0.57^{B,C,b}$	$0.38 \pm 0.13^{B,a}$	$0.07 \pm 0^{E,b}$	$24.7 \pm 0.0^{B,a}$	$-1.87 \pm 0.03^{A,b}$
p-value of one-way ANOVA	<0.0001	0.029	<0.0001	<0.0001	<0.0001

[a] Different superscript uppercase letters and lowercase superscript letters indicate a significant difference ($P < 0.05$) between the different cold preservation methods in the same kind of sample and between the different kinds of samples (whole or skimmed) using the same cold preservation method, respectively. Samples designated d_0 were analysed immediately after thawing; those designated d_1 were stored at 7 °C for 1 day after thawing.

Table 2
Particle size parameters of sheep milk (whole and skimmed) after extended refrigerated storage or frozen storage.[a]

Samples	Specific surface area ($m^2\ g^{-1}$)	$D_{3,2}$ (μm)	$D_{4,3}$ (μm)	$d_{0.1}$ (μm)	$d_{0.5}$ (μm)	$d_{0.9}$ (μm)	Span
Whole sheep milk							
Fresh	$21.548 \pm 0.782^{A\dagger}$	0.269 ± 0.009^{B}	0.730 ± 0.050^{B}	0.144 ± 0.002^{B}	0.266 ± 0.011^{B}	$1.828 \pm 0.071^{B,C}$	6.337 ± 0.099^{A}
Refrigerated (7 °C/4 days)	22.093 ± 0.270^{A}	0.262 ± 0.003^{B}	0.704 ± 0.016^{B}	0.143 ± 0.001^{B}	0.257 ± 0.004^{B}	$1.790 \pm 0.029^{C,D}$	6.400 ± 0.101^{A}
Frozen/Thawed at 7 °C (d_0)	19.844 ± 1.746^{B}	0.294 ± 0.025^{A}	0.868 ± 0.137^{A}	0.149 ± 0.005^{A}	0.305 ± 0.038^{A}	$1.875 \pm 0.117^{A,B}$	5.703 ± 0.385^{C}
Frozen/Thawed at 25 °C (d_0)	21.367 ± 0.288^{A}	0.271 ± 0.048^{B}	0.748 ± 0.019^{B}	0.145 ± 0.001^{B}	0.270 ± 0.004^{B}	1.768 ± 0.033^{D}	6.013 ± 0.047^{B}
Frozen/Thawed at 7 °C ($d_1^{\#}$)	19.485 ± 1.817^{B}	0.300 ± 0.028^{A}	0.882 ± 0.130^{A}	0.151 ± 0.006^{A}	0.315 ± 0.047^{A}	1.904 ± 0.112^{A}	5.632 ± 0.507^{C}
Frozen/Thawed at 25 °C ($d_1^{\#}$)	21.352 ± 0.181^{A}	0.271 ± 0.002^{B}	0.752 ± 0.015^{B}	0.145 ± 0.000^{B}	0.269 ± 0.003^{B}	$1.776 \pm 0.022^{C,D}$	6.060 ± 0.042^{B}
p-value of one-way ANOVA	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Skimmed sheep milk							
Fresh	30.507 ± 0.225^{A}	0.190 ± 0.001^{B}	$0.207 \pm 0.003^{B,C}$	0.135 ± 0.001^{A}	$0.196 \pm 0.002^{B,C}$	0.295 ± 0.010^{B}	0.815 ± 0.049^{B}
Refrigerated (7 °C/4 days)	30.696 ± 0.129^{B}	0.189 ± 0.001^{C}	0.206 ± 0.003^{C}	0.134 ± 0.002^{B}	0.195 ± 0.002^{C}	0.296 ± 0.011^{B}	$0.834 \pm 0.057^{A,B}$
Frozen/Thawed at 7 °C (d_0)	30.389 ± 0.047^{A}	$0.191 \pm 0.000^{A,B}$	0.209 ± 0.001^{A}	0.134 ± 0.000^{B}	0.197 ± 0.001^{A}	0.303 ± 0.002^{A}	0.856 ± 0.011^{A}
Frozen/Thawed at 25 °C (d_0)	30.381 ± 0.040^{A}	0.191 ± 0.000^{A}	0.209 ± 0.001^{A}	0.134 ± 0.001^{B}	0.197 ± 0.001^{A}	0.303 ± 0.003^{A}	0.859 ± 0.015^{A}
Frozen/Thawed at 7 °C ($d_1^{\#}$)	30.506 ± 0.054^{A}	0.190 ± 0.000^{B}	$0.208 \pm 0.001^{A,B,C}$	$0.134 \pm 0.001^{A,B}$	$0.196 \pm 0.001^{A,B,C}$	$0.299 \pm 0.004^{A,B}$	$0.839 \pm 0.020^{A,B}$
Frozen/Thawed at 25 °C ($d_1^{\#}$)	30.478 ± 0.059^{A}	$0.190 \pm 0.000^{A,B}$	$0.208 \pm 0.001^{A,B}$	0.134 ± 0.001^{B}	$0.197 \pm 0.001^{A,B}$	$0.300 \pm 0.003^{A,B}$	$0.844 \pm 0.016^{A,B}$
p-value of one-way ANOVA	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.001

[a] Different superscript uppercase letters indicate a significant difference ($P < 0.05$) between the different cold preservation methods in the same kind of sample (whole or skimmed). Samples designated d_0 were analysed immediately after thawing; those designated d_1 were stored at 7 °C for 1 day after thawing.

Fig. 1 shows the results of near infrared light backscattering of whole and skimmed sheep milk samples after immediate vigorous shaking and after 4 h of rest. Immediately after shaking, only whole refrigerated samples showed a visible cream formation, while other whole samples showed different cream formations only after 4 h of rest at 20 °C. For skimmed milk, cream formation was not relevant but a peak observed due to sample behaviour in the base of the tube (ΔBS) revealed a sedimentation process. The cream formation parameters (% BS in the cream peak and area of creaming) showed no differences ($P > 0.05$) between control and stored samples (Table 3). On the other hand, cream thickness was ~30% higher for samples thawed at 7 °C, indicating a higher occurrence of cream in these samples.

Parameters obtained from near infrared light backscattering of skimmed milk (Table 4) showed ~3% increase in % BS (compared with control) for all frozen/thawed samples immediately after they were shaken ($\%BS_{0h}$). However, at the end of the test (4 h later), only the sample thawed at 7 °C was different from the control ($\%BS_{4h}$). No significant differences ($P > 0.05$) in the ΔBS and area of sedimentation values were observed for almost all the samples compared with control, indicating that the formation or suspension of sediments in frozen/thawed processes was insignificant. This is corroborated by the results of milk sediments (Table 1), which showed that the sedimentation occurrence was very small in the samples (<0.36%).

The confocal microscopy of the whole milk samples (Fig. 2) showed that long refrigerated storage and freezing/thawing processes increase the fat globule size and no changes were observed in the protein network.

The most important results were evaluated using principal component analysis (Fig. 3). For both samples (whole and skimmed), fresh and refrigerated milk showed similar behaviour. Among the frozen/thawed samples, milk thawed at 25 °C + 1 day of storage at 7 °C was the most similar to fresh milk, while thawed samples evaluated immediately after thawing were the most different. For whole sheep milk, the parameters related to fat particle size were the main differential factors in the samples, while skimmed milk was mainly differentiated by physicochemical parameters such as soluble calcium, buffering capacity and luminosity (L*).

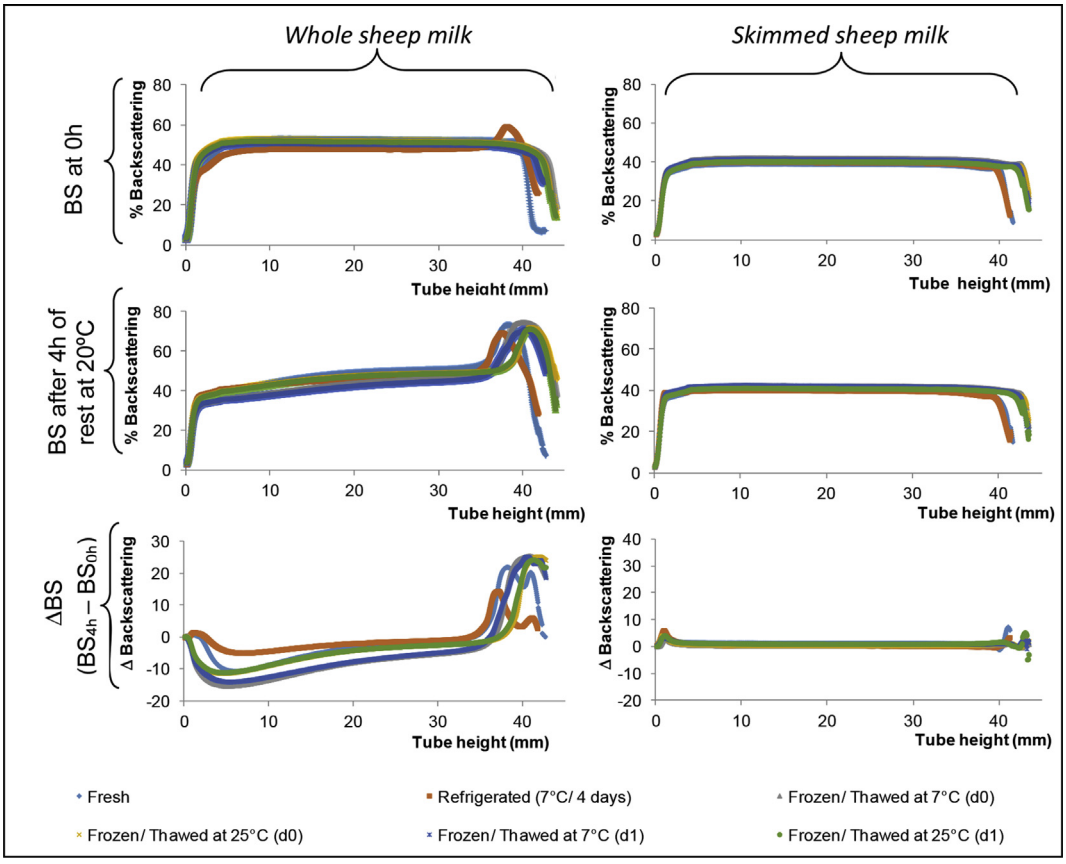

Fig. 1. NIR-backscattering milk tube scans of whole or skimmed sheep milk after extended refrigerated storage (4 days) or frozen/thawed storage combination (d_0 and d_1 at 7 °C, and d_0 and d_1 at 25 °C).

Table 3
Parameters obtained from the NIR light backscattering scan (0 and 4 h of samples maintained at 20 °C) of whole sheep milk after extended refrigerated storage or frozen storage.[a]

Samples	% BS creaming peak	Area of creaming (mm %BS)	Thickness of creaming (mm)
Fresh	$73.4 \pm 0.4^{A,B\dagger}$	60.6 ± 7.6^{A}	5.3 ± 0.4^{C}
Refrigerated (7 °C/4 days)	$73.8 \pm 0.7^{A,B}$	73.2 ± 5.8^{A}	$5.9 \pm 0.6^{B,C}$
Frozen/Thawed at 7 °C (d_0)	$73.9 \pm 1.5^{A,B}$	95.3 ± 20.2^{A}	$6.9 \pm 0.4^{A,B}$
Frozen/Thawed at 25 °C (d_0)	72.2 ± 0.4^{B}	66.3 ± 1.4^{A}	5.7 ± 0.3^{C}
Frozen/Thawed at 7 °C ($d_1^{\#}$)	75.6 ± 1.9^{A}	91.0 ± 25.4^{A}	7.1 ± 0.7^{A}
Frozen/Thawed at 25 °C ($d_1^{\#}$)	71.8 ± 0.4^{B}	64.6 ± 3.5^{A}	5.5 ± 0.1^{C}
p-value of one-way ANOVA	0.02	0.06	<0.0001

[a] Different superscript uppercase letters indicate a significant difference ($P < 0.05$) between the different cold preservation methods. Samples designated d_0 were analysed immediately after thawing; those designated d_1 were stored at 7 °C for 1 day after thawing.

Table 4
Parameters obtained from the NIR light backscattering scan (0 and 4 h of samples maintained at 20 °C) of skimmed sheep milk after extended refrigerated storage or frozen storage.[a]

Samples	%Backscattering (0 h)	%Backscattering (4 h)	Δ BS (4 h- 0 h)	Area sedimentation peak (1–2 mm)
Fresh	$38.8 \pm 0.2^{E\dagger}$	40.2 ± 0.1^{B}	1.4 ± 0.1^{A}	$2.46 \pm 0.20^{A,B}$
Refrigerated (7 °C/4 days)	$39.7 \pm 0.5^{D,E}$	40.0 ± 0.1^{B}	$0.5 \pm 0.3^{B,C}$	3.56 ± 1.01^{A}
Frozen/Thawed at 7 °C (d_0)	41.9 ± 0.4^{A}	42.3 ± 0.9^{A}	$0.9 \pm 0.7^{A,B,C}$	2.11 ± 0.45^{B}
Frozen/Thawed at 25 °C (d_0)	$40.6 \pm 0.1^{B,C}$	$41.1 \pm 0.1^{A,B}$	0.4 ± 0.1^{C}	$2.52 \pm 0.38^{A,B}$
Frozen/Thawed at 7 °C ($d_1^{\#}$)	$41.3 \pm 0.6^{A,B}$	42.3 ± 0.9^{A}	$1.0 \pm 0.3^{A,B}$	$2.71 \pm 0.22^{A,B}$
Frozen/Thawed at 25 °C ($d_1^{\#}$)	$40.0 \pm 0.1^{C,D}$	$41.0 \pm 0.2^{A,B}$	1.1 ± 0.2^{A}	$3.01 \pm 0.16^{A,B}$
p-value of one-way ANOVA	<0.0001	<0.0001	0.005	0.028

[a] Different superscript uppercase letters indicate a significant difference ($P < 0.05$) between the different cold preservation methods. Samples designated d_0 were analysed immediately after thawing; those designated d_1 were stored at 7 °C for 1 day after thawing.

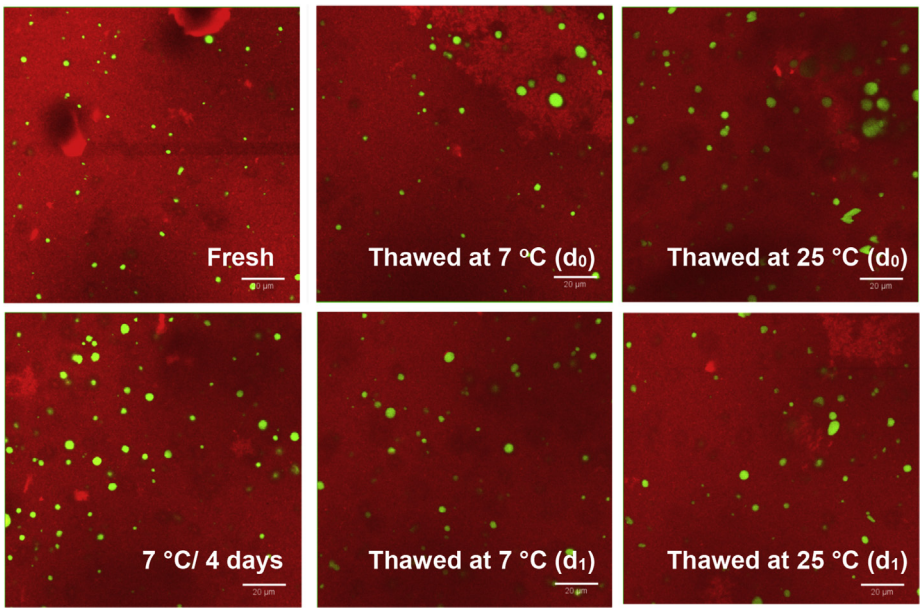

Fig. 2. Images obtained by confocal microscopy for the of whole sheep milk after extended refrigerated storage (4 day) or frozen/thawed storage combination (d_0 and d_1 at 7 °C, and d_0 and d_1 at 25 °C). The green colour represents the fat globules; the red colour represents the protein network. White bar size indicates 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

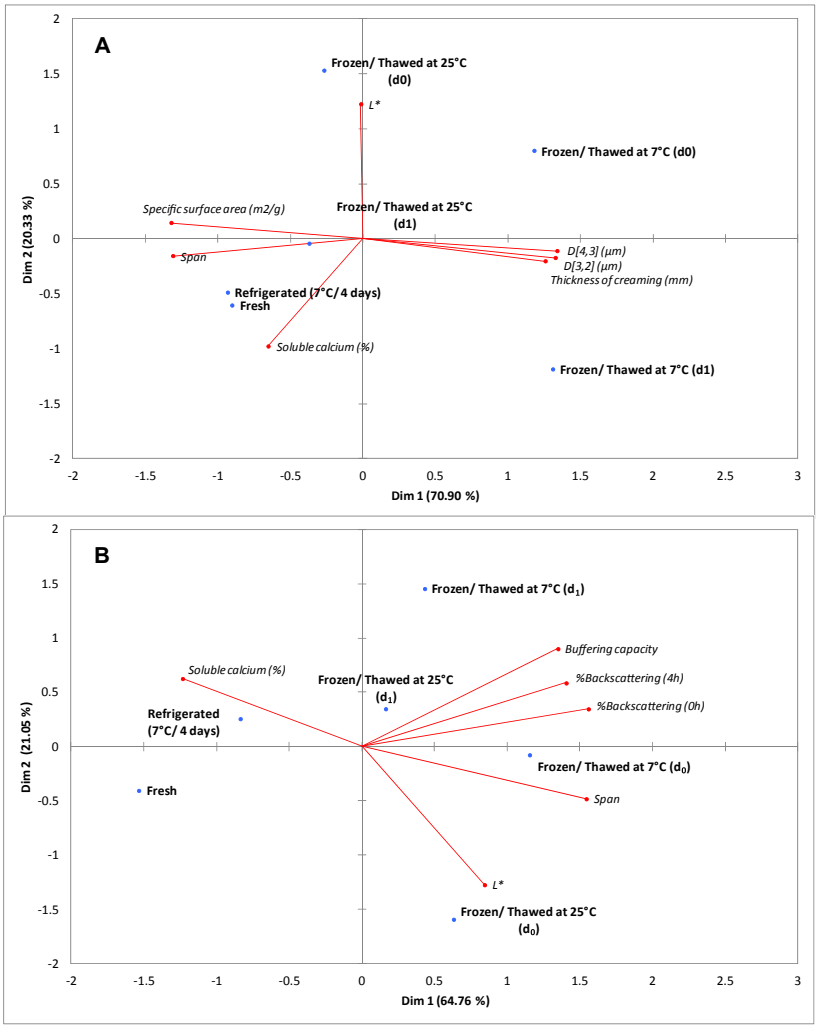

Fig. 3. Principal component analysis of (A) whole or (B) skimmed sheep milk after extended refrigerated storage (4 day) or frozen/thawed storage combination (d_0 and d_1 at 7 °C, and d_0 and d_1 at 25 °C). Only parameters for which at least 10% difference in the samples results were observed were included in the PCA.

4. Discussion

Storing raw sheep milk at smallholdings is a challenge because the required accumulation time exceeds the 48 h limit (time established by regulatory law of milk production/storage as safe from a microbiological point of view). To solve this problem, small-scale sheep milk producers keep the milk refrigerated for a longer time (4 days) than is acceptable or freeze the milk (if more than 4 days is necessary) until enough volume is accumulated for production. However, they report a depreciation in the sensorial characteristics of milk (especially the texture) and defects in the products obtained from these milks.

The differences in the physicochemical and microstructural properties among the milk of different species after freezing and thawing are related to the differences in structure and size of casein micelle, proportion of salts at colloidal and aqueous phases, protein interactions, and the size and concentration of fat globules (Reynal-Ljutovac, Park, Gaucheron, & Bouhallab, 2007; Wendorff, 2001).

Among the impacts of freezing and thawing sheep milk, the main expected problems are the drilling of fat globules, creaming and coalescence acceleration, changes in salt balance, modification of protein hydration layer and protein destabilisation (Haenlein & Wendorff, 2006; Walstra, Wouters, & Geurts, 2006; Zhang et al., 2006). These changes are due to the partial freezing of water and the formation of a supersaturated salt solution during the freezing process (Haenlein & Wendorff, 2006; Walstra et al., 2006; Zhang et al., 2006). Although impacted by the freezing rate (previously studied by Wendorff, 2001), we believe that the final consequences in milk constituents and physicochemical characteristics are also dependent on the rate of thawing (linked to the temperature used) and the possibility to recover, at least partially, its salt balance after a resting time subsequent to thawing. Therefore, we chose the thawing (7 or 25 °C) and resting time (zero or 1 day) conditions aiming to find a way of minimising changes caused by the freezing process when it is mandatory. Moreover, we also examined the impact of extended refrigeration storage (4 days) to determine the less damaging form of milk conservation when only 3 or 4 days are needed to accumulate sufficient milk volume for processing. The storage period of refrigerated samples was limited to 4 days considering that longer storage would lead to the growth of psychrotrophic microorganisms above an adequate limit ($>5 \times 10^6$ cfu mL^{-1}, according to Pinho, 2006). This, in turn, would produce undesirable proteases and lipases that can cause defects in dairy products (Garnica, Santos, & Gonzalo, 2011).

The physicochemical parameters and their microstructure are highly important for dairy products such as fermented milk, cheese and dried concentrated dairy products (Haenlein & Wendorff, 2006; Pavic, Antunac, Mioc, Ivankovic, & Havranek, 2002; Ramos & Juarez, 2011). Alterations in these parameters can modify processing time [fermentation (Haenlein & Wendorff, 2006; Tribst et al., 2018), coagulation (Haenlein & Wendorff, 2006; Pazzola et al., 2013; Ramos & Juarez, 2011), concentration or drying (Kelly & Fox, 2016)], yield (Haenlein & Wendorff, 2006; Pazzola et al., 2013; Zhang et al., 2006) and can cause undesirable changes in products, such as higher acidity and syneresis in fermented products (Tribst et al., 2018), excessive elasticity or hardness of cheese (Guinee, 2016), as well as alterations in powdered product characteristics (Kelly & Fox, 2016).

The main effects on sheep milk stored under refrigeration at 7 °C for 4 days were higher sedimentation, an increase in the buffering capacity (skimmed milk), fast creaming after stirring and enlarged fat globules. The fast creaming can be explained by agglutinins, which are complexes of cryoglobulins (mainly immunoglobulin M) and lipoproteins that induce flocculation of the fat globules. These have higher activity at low temperatures (Walstra et al., 2006) and higher molecular mobility under cooling temperatures rather than freezing conditions, facilitating the creaming process due to factors described in Stokes' law (Walstra et al., 2006). The higher sedimentation probably occurred due to favoured growth of psychrotrophic microorganisms at low temperatures, as the proteases produced by these microorganisms are able to induce the formation of protein aggregates (Garnica et al., 2011; Oliveira, Leite Júnior, Tribst, & Cristianini, 2018). Additionally, the microbial growth in these samples (total bacterial count and total psychrotrophic counts were around 5×10^5 cfu mL^{-1}, with consequent acidity increase of 0.03%) possibly changed the milk buffering capacity. Despite these modifications, extended refrigerated storage was less negative than the freezing/thawing processes. Therefore, amongst the methods studied for milk preservation, producers should preferably choose extended refrigeration if the milk accumulated over 4 days guarantees an adequate volume of milk for dairy manufacturing. Moreover, the results corroborated that common phenomena observed in cow milk preservation at low temperatures such as solubilisation of calcium colloidal phosphate and reduction of protein particle size due to β-casein dissociation do not occur in sheep milk (Haenlein & Wendorff, 2006; Raynal & Remeuf, 2000; Walstra et al., 2006). On the other hand, the other observed phenomena, such as agglutinin activity and the production of undesirable enzymes by psychrotrophs also occur in cow milk, but the agglutinin impact on cow milk tends to be lower due to the lower content of fat compared with sheep milk (Walstra et al., 2006).

The freezing/thawing processes altered (reversibly or not) whole and skimmed raw sheep milk. For whole milk, impacts on fat are more significant than those on other constituents, which was evidenced by particle size increase, surface area reduction, higher cream thickness formed by the natural skimming process (%BS after 4 h) and the images via confocal microscopy. These effects were expected as slow freezing induces big crystal ice formation (Pazzolla et al., 2013) that entraps the membrane of fat globules (Zhang et al., 2006) reducing the stability of the natural milk emulsion after thawing.

Interestingly, these alterations were more relevant for samples thawed at 7 than at 25 °C. This difference might be linked to the time required for complete thawing at different temperatures (at 7 °C and 25 °C with thawing processes of 14 h and 8 h, respectively) or due to the difference in the fat crystallinity of both samples. Therefore, the slow thawing at 7 °C probably favoured slow and continuous partial coalescence (clumping) of the sheep milk fat (Walstra et al., 2006) or favoured the agglutinin activity (Walstra et al., 2006). Moreover, sheep milk has a high concentration of saturated fatty acids, which has high melting temperature (Haenlein & Wendorff, 2006). Therefore, at 7 °C, the sheep milk fat was mostly in crystal form, favouring fat coalescence.

Comparing the expected impact of freezing on sheep and cow milk fat, a similar clumping intensity might occur. Although cow milk commonly has a lower content of fat, the size of cow milk fat globules is higher than those in sheep milk (~4.5 μm compared with ~2 μm; Haenlein & Wendorff, 2006), and is therefore highly impacted by large ice crystals. In relation to protein stability, the aforementioned authors reported that cow milk can be affected by freezing processes, explained by the phenomena of salting out and deposition of calcium phosphate in the casein micelles, which is reversible depending on the temperature and time of storage (Walstra et al., 2006). This phenomenon also could occur in sheep milk when subjected to an extended frozen preservation (>3 months; Wendorff, 2001). In general, sheep milk is characterised by a lower colloidal stability than cow milk (Reynal-Ljutovac et al., 2007).

Overall, the evaluation of whole milk results suggests that milk thawing at room temperature was preferable to minimise changes

in the microstructure of milk. However, it is extremely important to be careful about microbiological hazards. Ideally, milk should be used immediately after thawing to prevent long exposures of the milk to temperatures that favour microbial growth. It is important to note that previous studies have shown that the growth of mesophilic and psychotrophic microorganisms were <1 log cycle after freezing sheep milk in 1 L bags followed by thawing at 7 or 25 °C (data not shown). Therefore, good quality milk will remain suitable to be used after freezing/thawing in the cited conditions.

In the absence of fat, the subtler consequences of freezing/thawing processes can be observed, mainly a reduction in soluble calcium concentration (samples thawed at 25 °C) and an increase in the buffering capacity for all evaluated conditions. At a slow freezing rate (common for domestic equipment), soluble calcium is allowed to precipitate as colloidal calcium phosphate in the micelles (Koschak et al., 1981), since the ionic strength increase (expected due to the continuous increase in salt concentrations in the remaining non-frozen solution during the freezing process; Walstra et al., 2006) occurs slowly (Koschak et al., 1981).

Therefore, a reduction in soluble calcium after thawing milk at 25 °C can possibly be explained by the relative fast thawing process, with no sufficient time to recover the calcium distribution equilibrium. Additionally, our results indicate that this ionic strength change might be responsible for the increase in the buffering capacity of skimmed sheep milk. We believe that this fact is mainly related to saline equilibrium than to changes in protein due to the complementary results (absence of sedimentation and changes in particle size in skimmed milk), to the stabilisation effect on protein caused by colloidal calcium phosphate (CCP) precipitation (Koschak et al., 1981) and also due to the relative short storage period at −18 °C (1 month). Previously published results suggest that significant changes in sheep milk proteins only occurs after 3 months of storage at temperatures higher than −18 °C (Haenlein & Wendorff, 2006; Wendorff, 2001), which do not meet the real needs of sheep milk producers.

Moreover, the higher buffering capacity explains the longer time requirement for producing yoghurt from frozen milk, as well as its higher acidity compared with yoghurt made from fresh sheep milk (Tribst et al., 2018). In addition to yoghurt manufacturing, the higher buffering capacity of frozen/thawed milk can affect the pH reduction in ripened cheese, changing its elasticity and hardness, salt penetration and cheese rind formation (Guinee, 2016).

Previous results showed few or none alterations in sheep milk physicochemical characteristics and microstructural after freezing (Fava et al., 2014, 2013; Katsiari et al., 2002; Voutsinas, Katsiari, Pappas, & Mallatou, 1996). However, others showed significant changes in pH (Kljajevic et al., 2016), emulsion stability and flavour of cow milk (Katsiari et al., 2002). The differences amongst the results of our research and those published in previous articles might be explained by the different forms of freezing [volume, rate (fast or slow), and pre-treatment (heating, concentration), thawing (fast or slow), temperature (under refrigeration or not) and shaking)] and also by the analytical refinement used to evaluate the changes caused by freezing since the use of low discriminative analysis tends to minimise differences between samples evaluated in previous studies (Fava et al., 2014, 2013; Katsiari et al., 2002). Therefore, our work elucidates that freezing causes changes in fat globules and possibly in the saline balance of the sheep milk. These alterations are more relevant for fat globules and calcium equilibrium if milk is thawed at 7 and 25 °C, respectively.

5. Conclusions

We concluded that freezing sheep milk at −18 °C and thawing it at 7 °C results in an increase of fat globule size and higher creaming separation compared with fresh milk, whereas fast thawing (at 25 °C) resulted in higher concentrations of colloidal calcium. Moreover, skimmed milk frozen/thawed at different conditions showed an increase in the buffering capacity, which might have been associated to changes in the milk saline equilibrium. Additionally, we concluded that although extended refrigeration also changes sheep milk physicochemical characteristics (mainly fat agglomeration and sedimentation), the impact of this preservation technique is less undesirable than freezing/thawing.

Therefore, evaluating all the results we observed that up to 4 days of milk accumulation in refrigerated storage is a better option compared with the freezing strategy. For longer preservation time, when freezing is mandatory, milk thawing at room temperature (25 °C) was preferable to minimise changes in the microstructure of whole milk. In addition, no significant benefits on milk physicochemical characteristics or microstructure were observed after one day of resting subsequent to milk thawing. Therefore, milk must be preferentially used immediately after complete thawing for dairy product manufacturing. The knowledge gained from these alterations can be used to create strategies to minimise the undesirable impact of using cold preserved sheep milk for dairy manufacturing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2019.02.013.

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