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Ultrasound processing of fresh and frozen semi-skimmed sheep milk and its effects on microbiological and physical-chemical quality

Celso F. Balthazar[a], Antonella Santillo[b], Jonas T. Guimarães[a], Antonio Bevilacqua[b], Maria Rosaria Corbo[b], Mariangela Caroprese[b], Rosaria Marino[b], Erick A. Esmerino[a], Marcia Cristina Silva[c], Renata S.L. Raices[c], Mônica Q. Freitas[a], Adriano G. Cruz[c], Marzia Albenzio[b,*]

[a] Universidade Federal Fluminense (UFF), Faculdade de Medicina Veterinária, 24230-340 Niterói, Rio de Janeiro, Brazil
[b] University of Foggia (UNIFG), Department of the Science of Agriculture, Food and Environment (SAFE), Via Napoli 25, 71122 Foggia, Italy
[c] Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro (IFRJ), Departamento de Alimentos, 20270-021 Rio de Janeiro, Brazil

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ABSTRACT

The objective of this study was to evaluate the effect of ultrasound treatment on the microbiological quality, protein and free amino acid profile of fresh and frozen stored semi-skimmed sheep milk. Milk was treated as fresh or frozen and stored up to one, three and six months. Output power time and pulse time were the parameters combined to design four different ultrasound (US) treatments: power 78 W and duration 6 min (US1); power 78 W and duration 8 min (US2); power 104 W and duration 4 min (US3) power 104 W and duration 6 min (US4). Pulse duration was of 4 s for each treatment. Sample US1 was discarded due to non effectiveness of US treatment, while other samples showed interesting results. Also, it was verified a frost effect on microorganisms in all samples which were frozen before treatment. No relevant change was reported on amino acid profile. The study showed promising results: the ultrasound treatment inactivated or eliminated the studied contaminant bacteria in semi-skimmed sheep milk, while maintained acceptable amount of lactic bacteria, which could be advantageous for dairy products processing.

1. Introduction

Sheep milk dairy products are considered a delicacy because of their quality and nutritional value; therefore they have gained market size worldwide in the last decade [1,2]. The great amount of fat and protein in sheep milk along with minerals makes the cheese the most important dairy product from sheep milk, due to its high yield and some technological advantage, such as high casein micelles mineralization degree and calcium rennet coagulation being not necessary addition of calcium chloride [3,4].

However, the high content of saturated fat [5] turns this product not ideally for consumption as drinking milk, since it is not seen as much healthy [6], but it is important to the manufacture of fatty dairy products. Moreover, sheep milk are produced by medium and small sheep milk farms, leading producers to freeze their raw milk as a way to store enough amount for further processing into dairy products [1,2]. When sheep milk is frozen below −20 °C, it can preserve protein stability for up to one year of storage [7].

Nowadays, non-thermal approaches are gaining importance in food industry because of the increasing demand for minimally processed, healthy and safe food products, which seem to be the trend of food production for the next years. The non-thermal technologies are known as cleaner processes, efficient in energy expenditure, environmental friendly [8]. Several novel non-thermal process technologies have been developed to help ensure product safety, quality and acceptability, such as: high pressure, pulsed electric field, cold plasma technology, ultrasonication, radiation, ultraviolet and pulsed light. Those have been evaluated in the most diverse productive segments aiming at the establishment of new processes and products that fully meet the wishes of modern consumers [9–11].

Ultrasound (US) processing promises to be a non-thermal method for food preservation, which has the advantage of inactivating microorganisms in food without causing the common side-effects associated with conventional heat treatments, such as high levels of energy and unfavorable impacts on nutritional content, sensory properties and quality of the final product [12]. Food processing using ultrasound

* Corresponding author.
E-mail address: marzia.albenzio@unifg.it (M. Albenzio).

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Table 1
Ultrasound processing parameters and samples temperatures after US treatment cycles on fresh and frozen semi-skimmed sheep milk.*

	Power (W)	Time (min)	Pulse (s)	Energy density (J/mL)	Temperature (°C)							
					Day 0		Day 30		Day 90		Day 180	
					1st cycle	2nd cycle	1st cycle	2nd cycle	1st cycle	2nd cycle	1st cycle	2nd cycle
US1	78	6	4	702	40 ± 1^{c}	54 ± 2^{c}	–	–	–	–	–	–
US2	78	8	4	936	53 ± 1^{b}	63 ± 1^{b}	53 ± 2^{b}	65 ± 1^{b}	47 ± 2^{b}	61 ± 1^{b}	40 ± 2^{b}	57 ± 1^{b}
US3	104	4	4	624	52 ± 0^{b}	62 ± 0^{b}	52 ± 0^{b}	63 ± 1^{b}	44 ± 1^{b}	57 ± 1^{c}	41 ± 1^{b}	58 ± 1^{b}
US4	104	6	4	936	59 ± 1^{a}	69 ± 1^{a}	60 ± 0^{a}	69 ± 1^{a}	52 ± 2^{a}	67 ± 1^{a}	50 ± 2^{a}	67 ± 1^{a}

* Different lowercase letters in the same column means significance difference between treatments ($p < 0.05$).

involves mainly the transmission of energy at frequencies higher than 20 kHz, namely high-intensity ultrasound (HIUS), while the low-intensity ultrasound (LIUS) uses very small power levels, normally less than 1 W/cm^2 with frequency range of 5–10 MHz, and causes no physical or chemical alterations in the properties of the treated material [13].

The HIUS is mainly studied for processing liquid foods, like fruit and vegetable juices and dairy products, in which induces mechanical, chemical and biochemical effects via the production and subsequent collapse of cavitation bubbles, generating energy, responsible for the physicochemical and microbiological alterations observed during food processing [14]. The HIUS typically involves shorter processing times, lower water and energy costs, lower production of residual effluents and toxic compounds, in addition to preserving the nutritional characteristics and the sensory aspects of food products [15,16].

Although HIUS treatment has been used in dairy products such as butter [17], cheese [18], chocolate cake, genoise and mousse [19], chocolate milk beverage [20], fermented milk [21], milk [22], prebiotic whey beverage [23], yogurt [24]. Nevertheless, there is no report in the literature about the effect of HIUS processing on sheep milk or frozen stored milk. In this sense, the objective of this study was to evaluate the effect of ultrasound treatment on microbiological quality, protein and free amino acid profiles of fresh and frozen stored semi-skimmed sheep milk.

2. Materials and methods

2.1. Sampling

The experiment was realized with raw sheep milk collected from bulk tanks (6.4 ± 0.5% of fat (v/v) and 11.1 ± 0.4% of non-fat solids (w/v)) from Gentile di Puglia sheep herd located in Foggia, Puglia region, Italy. The raw sheep milk was semi-skimmed up to 1.6 ± 0.2% (w/w) of fat by centrifugation using 4000 rpm for 10 min at 4 °C (Centrifuge 5810 R, Eppendorf AG, Hamburg, Germany). Raw milk proximate composition was determinate as described in Section 2.5.

The semi-skimmed raw milk was divided into four groups: fresh (4 °C) and stored frozen (−20 ± 1 °C) up to one (30 days), three (90 day) and six (180 days) months. Afterwards, each group was divided into six treatments: raw milk (RAW); processed by high-temperature short-time pasteurization (HTST); and high-intensity ultrasound (US1, US2, US3 and US4). All the treatments were performed in duplicate.

The first step of the study was carried out with the processing of the fresh raw semi-skimmed milk by HTST, US1, US2, US3, US4 and RAW. The next step was realized with the processing of the frozen raw semi-skimmed milk with different storage time (30, 90 and 180 days) by HTST, US2, US3, US4 and RAW. The frozen samples were defrosted in refrigerator (4 °C ± 0.5) overnight before processing.

All the microbiological and physico-chemical analysis, from both fresh and frozen milks, were performed right after processing (day 0) and repeated seven days after the processing (day 7) and cold storage (4 ± 0.5 °C) to simulate the shelf life of pasteurized milks in the markets.

2.2. High-temperature short-time (HTST) pasteurization

The HTST pasteurization of fresh or defrosted frozen semi-skimmed raw sheep milk was performed by a heating process in a stainless steel container, double jacket with internal propeller (CASARO, Philips, Netherlands) programmed to achieve 75 °C, maintaining at this temperature for 15 s. The semi-skimmed sheep milk was immediately cooled in ice bath (0 ± 2 °C) and stored in refrigerator (4 ± 0.5 °C) until analysis.

2.3. High-intensity ultrasound processing (HIUS)

The HIUS processing of the semi-skimmed sheep milk was adapted from the procedure reported by Bevilacqua et al. [25] to inactivate food born bacteria in skim milk. The processing parameters like power, duration, temperature and energy density are presented in Table 1. The energy density (ED) applied to the samples was calculated according to the Eq. (1).

$$ED(J/mL) = \frac{Nominal\ power\ (W) \times Process\ time(s)}{Sample\ volume\ (mL)} \quad (1)$$

Briefly, aliquots of 40 mL of fresh or defrosted semi-skimmed raw sheep milk were submitted to ultrasound treatment with a VC Vibra Cell Ultrasound (US) equipment, model VC 130 (Sonics and Materials Inc., Newtown, CT, USA); the equipment works at 20 kHz (frequency) and 130 W (maximum power). The probe (5 × 60 mm; diameter × the active component of horn) was put 2–3 cm below the surface of milk. The processing parameters varied in power level (78 and 104 W) and duration of the treatment (4, 6 and 8 min), and the duration of pulse were kept constant (4 s); the treatment was performed as a two cycle processing, that is each US combination was done twice. Before processing, each sample was maintained refrigerated (4 ± 2 °C) and the milk temperature was monitored after each US-cycle (Table 1), and left at room temperature (22 ± 2 °C) for 10 min between each US-cycle. Before each US-cycle, the probe was washed with sterile distilled water. After the second cycle of US processing the samples were stored at refrigerator (4 ± 0.5 °C) until further analysis.

2.4. Microbiological analyses

For microbiological analysis, serial dilutions (1:10) of processed semi-skimmed sheep milk were performed in sterile 0.9% NaCl water (w/v) (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). The range of serial dilutions was 10^{-1} to 10^{-5} decided based on somatic cell counts per milliliters (mL) results of fresh milk and European legislation [26]. Total aerobic mesophilic bacteria (TAMB) was counted using Plate Count Agar (CM0325, Oxoid, Hampshire, England) by pour plate technique and aerobic condition, incubated for 24–48 h at 36 ± 1 °C. Total coliform count (TCC) was determined in Violet Red Bile Glucose

Agar (CM0485, Oxoid, Hampshire, England) using spread plate method and anaerobic or microaerophilic conditions incubated for 24–48 h at 36 ± 1 °C. *Staphylococcus* spp. coagulase positive was isolated and counted on Baird Parker Agar (CM0275, Oxoid, Hampshire, England) supplemented with egg yolk tellurite (SR0054C, Oxoid, Hampshire, England) using spread plate method and aerobic condition, incubated for 24–48 h at 36 ± 1 °C. For lactic *streptococci* and *lactobacilli*, there were used M17 Agar (CM0785, CM0275, Oxoid, Hampshire, England) supplemented with 10% lactose (LP0070, Oxoid, Hants, UK) and M.R.S. Broth (CM0359, Oxoid, Hampshire, England) supplemented with 1.5% Agar Bacteriological (LP0011, CM0275, Oxoid, Hampshire, England), respectively, using pour plate technique, both in anaerobic or microaerophilic conditions, incubated for 48–72 h at 36 ± 1 °C. The bacterial counts were expressed as colonies forming units (CFU) per mL of sample. The microbial analyses were performed in triplicate.

2.5. Physical-chemical analyses

The raw semi-skimmed sheep milk proximate composition and somatic cell count were performed in triplicate using MilkoScanTM FT 120 (FOSS, Denmark) and Fossomatic TM Minor (FOSS, Denmark) according to the FIL-International Dairy Federation 148 A: 95 norm [27], respectively, before processing. The pH were determined according to the International Dairy Federation standard [28] after processing (0 d) and at day 7 (7 d) in fresh and defrosted (30, 90, 180 days) samples. The analysis was conducted in triplicate.

The protein profiles were acquired by SDS-PAGE according to Tidona et al. [29] and was evaluated only in RAW and processed samples (US and HTST) right after processing (day 0) of the fresh and defrosted milks (30, 90 and 180 days). Briefly, aliquots (0.1 mL) of milk samples was mixed with 0.9 mL buffer solution (0.125 M Tris HCl, 4% SDS, 2% glycerol, 2% 2-mercaptoethanol, 0.03 mM bromophenol blue, pH 6.8) and heated at 100 °C for 5 min. The protein profiles were identified on SDS-PAGE gels, using 15% acrylamide for the resolving gel and 4% for the stacking gel. As molecular ladder, a low molecular weight standard kit (LMW Calibration kit, Amersham, GE Healthcare UK limited, Little Chalfont, Buckinghamshire, UK) was used and lactic proteins were identified according to Grappin et al. [30] based on molecular weight; 4 mL of standard and 5 mL of samples were loaded into the gels. Electrophoretic gels were acquired using the analysis software Quantity One® (Bio-Rad Laboratories, Inc, USA) for image analysis. The analysis was carried out in triplicate.

Free amino acids (FAA) were determined by High-Performance Liquid Chromatography (HPLC) according to the method of Marino et al. [31] and carried out only in RAW and processed samples (US and HTST) right after processing (day 0) of the fresh and defrosted milks (30, 90 and 180 days). The HPLC system (1260 Infinity series, Agilent Technologies, Waldbronn, Germany) was composed of a binary pump equipped with a micro vacuum degasser, thermostat-controlled auto sampler, column compartment, a fluorescence detector (model G1321A), and a diode array detector (model G1315A). The analysis were performed using a Zorbax Eclipse AAA column (150 × 4.6 mm i.d., prepacked with 3.5 µm particles; Palo Alto, CA); the column temperature was set at 40 °C. The mobile phase comprised a 40 mM $NaH_2PO_4{\cdot}H_2O$ solution (phase A) and a mixture of water, methanol, and acetonitrile (10:45:45 v/v/v; phase B). Individual amino acid peaks (Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val) were identified by comparing their retention times with specific standards (Sigma-Aldrich, St. Louis, MO).

2.6. Statistical analyses

All the variables were tested for normal distribution and transformed in logarithm form to normalize their frequency distribution, when necessary. Data were processed by ANOVA using the GLM procedure for repeated measure of SAS [32]. Principal component analysis (PCA) was conducted using the XLSTAT software version 2018.4 (Adinsoft, Paris, France). PCA data were auto scaled before the analysis, being the matrix data set composed of 16 rows (semi-skimmed sheep milk samples at times fresh, 90 and 180 days) and 10 columns (free amino acids).

3. Results and discussion

3.1. Microbiological evaluation after HTST and US processing

The effectiveness of the US treatment depends on the food composition, among other parameters [33]. The solid compounds in sheep milk are higher than in cow milk [3]; thus, the ultrasound parameters were adapted to semi-skimmed sheep milk. Previously, there was tested one cycle US processing, which was not efficient to assure the microbiological quality in skimmed sheep milk, due to bacterial results of US processed samples were similar ($p > 0.05$) to raw skimmed sheep milk bacterial counts (data not shown). Therefore, two sonication cycles were performed. The temperatures reached by the milks during the process ranged from 40 to 69 °C in fresh or frozen stored semi-skimmed sheep milk samples after different US treatments (Table 1).

The raw sheep milk collected from the bulk tanks showed satisfactory sanitary parameters with 347,000 somatic cells (data not shown) and total bacterial count of 4.23 log cfu/mL, being in accordance to Directive 853/2004 [34]. There could be verified diminish of bacteria counts after milk processing by high-intensity ultrasound (HIUS) and pasteurization (HTST) in fresh skimmed milk and in frozen samples stored for different periods (30, 90, 180 days at −20 °C ± 1) before processing. Pasteurization aims to decrease the contaminant and pathogenic bacteria to ensure the products safety [26]. As well, US processing targets to inactivate enough spoilage microorganisms to be known as a potential alternative to this technology [35]. The mechanism of HIUS to inactivate microorganisms lies on acoustic cavitation, generated by the application of low frequencies, with the instantaneous formation of micro-bubbles, followed by an immediate collapse, resulting in great micro shearing rates in the milk, resulting result in the breaking and shearing of microorganism cell walls [36].

3.1.1. Fresh semi-skimmed sheep milk processing

The first step of microbiological evaluation was carried out after processing of fresh semi-skimmed sheep milk samples by HTST and HIUS. The bacterial counts were realized immediately after processing (day 0) and after 7 days of cold storage at 4 °C (± 0.5, shelf life; Fig. 1).

US1 presented significantly higher counts in all tested media than HTST or the other HIUS (US2, US3 or US4) in the study ($p < 0.05$), meaning that US1 parameters were inefficient to inactivate the milk bacteria. In addition, bacteria continued growing significantly on US1 during shelf life (4 °C ± 0.5; $p < 0.05$), as found in RAW sample. For those reasons, the US1 was excluded from the second step of the study with frozen stored milk.

The ultrasound processing of fresh milk, except the treatment US1, significantly decreased the bacterial contamination, in comparison to the RAW milk sample, for every bacterial group tested ($p < 0.05$), as well as the HTST treatment ($p < 0.05$).

Concerning the total aerobic mesophilic bacteria (TAMB, Fig. 1A) evaluated in fresh semi-skimmed sheep, the milk samples US2 and US3 presented low contamination (1.81 and 2.7 log cfu/mL, respectively) right after processing (day 0); moreover, TAMB was constant (1.7 and 2.7 log cfu/mL, respectively; $p > 0.05$) during shelf life (4 °C ± 0.5). Meanwhile, in US4 was not detected any bacterial growth after US processing or after cold storage of the fresh milk.

The total coliform count (TCC, Fig. 1B) of the sonicated samples, except the US1, resulted in absence of coliforms, which complies with the microbial quality standards required for this product [26], meaning that US parameters used on those milk samples were enough for this type of microorganisms.

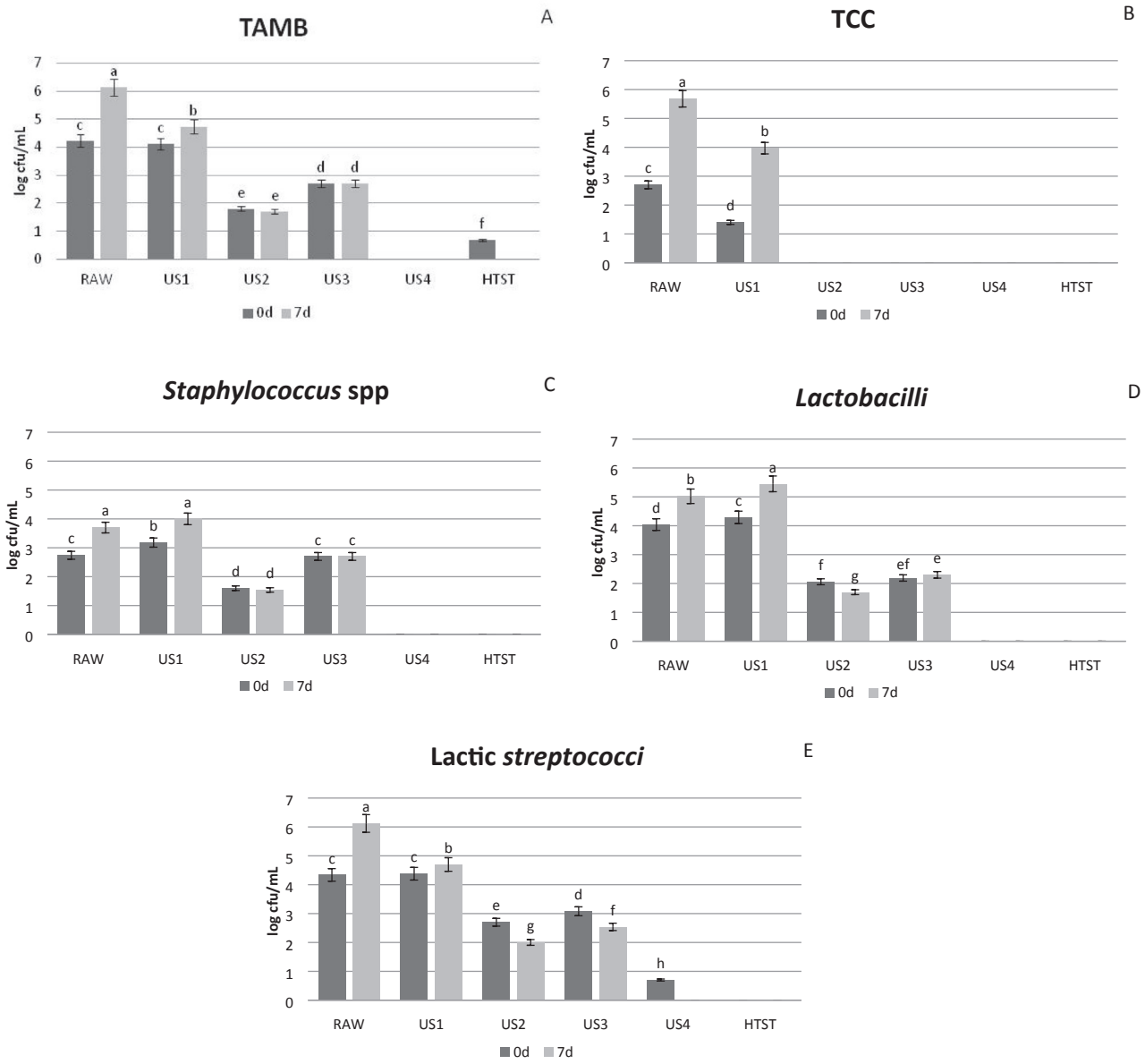

Fig. 1. Bacterial counts (log cfu/mL) of semi-skimmed sheep samples immediately after processing (0 d) and 7 days (7 d) of cold storage at 4 °C (± 0.5). RAW = raw; US1 = ultrasound treatment 1; US2 = ultrasound 2; US3 = ultrasound 3; US4 = ultrasound 4; and HTST = high temperature short time. TAMB: Total Aerobic Mesophilic Bacteria (A); TCC: Total Coliform Count (B); *Staphylococcus* spp. (C); Lactobacilli (D); and lactic *streptococci* (E). Different letters means significance difference $p < 0.05$.

For the *Staphylococcus* spp. inactivation (Fig. 1C), the US was in general effective, but with different intensities between the treatments. US2 (1.6 log cfu/mL) presented reduced contamination in comparison to RAW sample (2.74 log cfu/mL, $p < 0.05$), while US3 was not effective (2.7 log cfu/mL, $p > 0.05$) to this one. However, US prevented these bacteria growth during shelf life (4 °C ± 0.5), differently that observed in milk samples RAW and US1. US4 presented no bacterial growth, as for HTST processed milk ($p > 0.05$).

The lactic bacteria of the milk were also evaluated in this study, represented by *lactobacilli* and lactic *streptococci* counts. These counts were always significantly higher ($p < 0.05$) in RAW and US1 than the other samples (Fig. 1D, E). During shelf life (4 °C ± 0.5), the contamination in these samples (RAW and US1) increased considerably ($p < 0.05$). On contrary, during store (4 °C ± 0.5), lactic bacteria decreased ($p < 0.05$) in the other samples (US2, US3, US4 and HTST).

The effect of ultrasonic power and sonication duration were meaningful for the inactivation of bacteria. For example, the treatments US2 and US4, despite the application of similar energy densities (936 J/mL), they had different microbial counts after processing for TAMB, *Staphylococcus* spp., lactic bacteria ($p < 0.05$). This probably happens because in an uncontrolled environment (non-adiabatic conditions), the energy dissipation occurs differently when the power and duration of sonication are different. It can be seen by the measurements of the temperatures during processing, where the US4 reached higher ($p < 0.05$) temperatures (59–69 °C ± 1) in both cycles than US2 (53–63 °C ± 1).

In this study, the fresh semi-skimmed sheep milk US4 had similar microbial contamination after processing than HTST. Other studies evaluating the microbial inactivation of total aerobic mesophilic bacteria by high intensity ultrasound in dairy products encountered similar results, thus suggesting that US technology was as effective as HTST pasteurization for inactivation of microorganisms [23,37].

Table 2
Bacterial counts (log cfu/mL) of frozen stored (−20 ± 1 °C for 30, 90 and 180 days) semi-skimmed sheep milk samples immediately after processing (0 d) and 7-days cold storage (4 ± 0.5 °C).

TAMB	30 days of frozen storage		90 days of frozen storage		180 days of frozen storage	
	0 d	7 d	0 d	7 d	0 d	7 d
RAW	4.85^{b} ± 0.06	6.27^{a} ± 0.00	2.06^{b} ± 0.04	2.85^{a} ± 0.06	2.00^{b} ± 0.01	3.00^{a} ± 0.02
US2	nd	n.d.	n.d.	n.d.	n.d.	1.00^{c} ± 0.01
US3	0.70^{d} ± 0.09	1.00^{c} ± 0.04	1.00^{c} ± 0.04	1.00^{c} ± 0.04	n.d.	0.70^{d} ± 0.18
US4	1.00^{c} ± 0.04	1.00^{c} ± 0.04	n.d.	n.d.	n.d.	n.d.
HTST	0.70^{d} ± 0.09	n.d.	n.d.	n.d.	n.d.	n.d.
TCC	0 d	7 d	0 d	7 d	0 d	7 d
RAW	4.60^{b} ± 0.05	6.31^{a} ± 0.01	1.78^{b} ± 0.07	2.48^{a} ± 0.15	1.81^{a} ± 0.26	1.70^{a} ± 0.19
US2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
US3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
US4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HTST	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Staphylococcus spp.	0 d	7 d	0 d	7 d	0 d	7 d
RAW	3.25^{b} ± 0.02	4.45^{a} ± 0.02	1.90^{b} ± 0.05	3.02^{a} ± 0.04	1.74^{b} ± 0.12	3.00^{a} ± 0.04
US2	1.48^{d} ± 0.15	2.00^{c} ± 0.04	n.d.	n.d.	n.d.	n.d.
US3	1.00^{e} ± 0.04	1.40^{d} ± 0.19	n.d.	n.d.	$0,70^{d}$ ± 0.30	1.00^{c} ± 0.01
US4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HTST	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lactobacilli	0 d	7 d	0 d	7 d	0 d	7 d
RAW	3.74^{b} ± 0.01	6.16^{a} ± 0.01	2.33^{c} ± 0.02	4.00^{a} ± 0.11	2.54^{b} ± 0.20	3.60^{a} ± 0.44
US2	2.00^{c} ± 0.04	nd	0.70^{d} ± 0.09	n.d.	0.70^{c} ± 0.31	2.40^{b} ± 0.09
US3	1.49^{e} ± 0.15	1.70^{d} ± 0.09	2.70^{b} ± 0.04	2.81^{b} ± 0.03	0.70^{c} ± 0.18	2.22^{b} ± 0.01
US4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HTST	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lactic *streptococci*	0 d	7 d	0 d	7 d	0 d	7 d
RAW	5.46^{b} ± 0.01	6.10^{a} ± 0.03	3.30^{a} ± 0.02	3.28^{a} ± 0.02	2.98^{b} ± 0.12	3.60^{a} ± 0.01
US2	2.10^{e} ± 0.03	1.70^{f} ± 0.09	1.78^{c} ± 0.07	1.48^{d} ± 0.15	2.40^{c} ± 0.08	2.40^{c} ± 0.19
US3	2.74^{d} ± 0.01	3.13^{c} ± 0.03	2.31^{b} ± 0.02	2.30^{b} ± 0.02	2.38^{c} ± 0.37	2.54^{c} ± 0.20
US4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HTST	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

TAMB: Total Aerobic Mesophilic Bacteria. TCC: Total coliform count. n.d.: not detected. Different lowercase letters in the same column or row for each bacterial group and storage time means significance difference ($p < 0.05$).

3.1.2. Frozen stored semi-skimmed sheep milk processing

The second step of microbiological evaluation was carried out after processing by HTST and US of semi-skimmed sheep milk stored frozen (−20 ± 1 °C for 30, 90 and 180 days). The bacterial counts were realized immediately after processing (day 0) of each storage period and after 7 days of cold storage at 4 °C (± 0.5, shelf life; Table 2).

Firstly, it was observed that the freezing condition itself influenced the bacterial contamination. It could be observed that in RAW milk, without any other treatment, the microbial counts varied through time. From the fresh condition to the first 30 days of frozen storage, the counts increased (0.5–2 log cfu/mL, $p < 0.05$, data not shown) in every bacterial group tested, except *lactobacilli*, indicating that the freezing until 30 days of storage was not beneficial to the microbiological quality. However, after 90 days, the viable counts decreased (1.3–2.8 log cfu/mL, $p < 0.05$), and from 90 to 180 days of frozen storage the samples kept constant ($p > 0.05$).

Regarding the effects of the ultrasound treatment on the frozen semi-skimmed sheep milk stored for different periods (30, 90 and 180 days), generally US processing decreased significantly the viable bacteria in all tested media ($p < 0.05$, Table2), as well as HTST ($p < 0.05$).

During shelf life of defrosted samples, TAMB counts increased in RAW milk ($p < 0.05$), but this trend was not found in US milk samples and HTST. Coliforms (TCC) were below the detection limit in US and HTST samples immediately after processing and after 7 days.

Staphylococcus spp. was significantly reduced by the treatment, although they were below the detection limit in US4 and HTST in all samples.

The lactic bacteria, in US4, as well as in HTST, were not detected in defrosted semi-skimmed sheep milk. Although, this group had a successful growth in US2 and US3 even in fresh and frozen period.

Engin and Yuceer [38] evaluated the US effects on microorganisms in milk and they also found significant reduction in numbers of total coliforms. However, US processing did not have a satisfactory reduction on mesophilic bacteria count, which was attributed to the power and frequency of the US instrument. Other studies [39–41] reported that freezing significantly decrease the viability of pathogenic microorganisms in milk. Moreover, the drop down of viable lactic bacteria counts was also reported in probiotic dairy products [42].

Our results obtained after freezing and frozen storage of the milk samples were expected. Low freezing rates associated with slow supercooling and low ice nucleation have been associated with plasmolysis, intracellular water loss driven by high osmotic pressure gradients. The bacteria cell membranes get damaged during freezing process due to mechanical stresses of the ice crystals formed in the external medium or inside the cells, thereby compromising the cell function and metabolic activity of some bacteria. In addition, mortality also takes place during thawing of the frozen products due to exposure of the microbial cells to osmotic effects [43,44]. However, the effects of milk composition on bacterial viability after a freeze thaw cycle are unknown. One possible mechanism may explain why some species grow better and others worse after freezing, is the response to the potential increase in mineral composition of milk, due to freezing extracellular water. This would expose bacterial cells to ice crystals and an osmotic gradient,

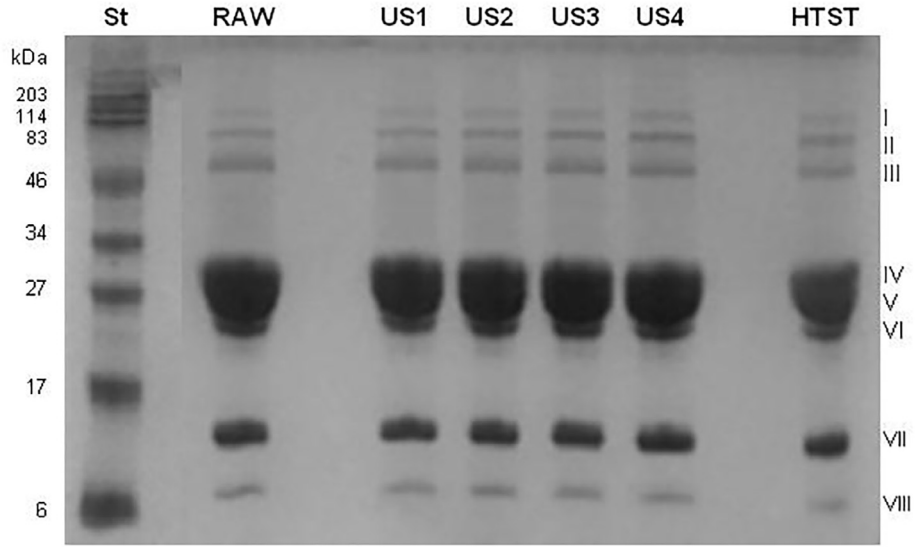

Fig. 2. Representative SDS-PAGE electrophoresis gel protein profile representation of semi-skimmed sheep milk samples. St = standard; RAW = raw; US1 = ultrasound 1; US2 = ultrasound 2; US3 = ultrasound 3; US4 = ultrasound 4; and HTST = high temperature short time. I = immunoglobulin; II = lactoferrin; III = serum albumin; IV = α-casein; V = β-casein; VI = κ-casein; VII = β-lactoglulin; VIII = α-lactalbumin.

which might lead to cell shrinkage and possibly cause membrane lesions [41].

3.2. Physico-chemical analyses

Concerning the proximate composition of raw, US and HTST skimmed sheep milk samples during storage, statistical difference was not observed between the different treatments and storage conditions ($p > 0.05$). The average values obtained in composition analysis were: 1.58% (v/v) fat, 6.18% (v/v) protein, and 4.75% (v/v) lactose.

The evaluation of pH on US samples fresh and stored frozen before processing (-20 ± 1 °C for 30, 90 and 180 days) showed no significant difference ($p > 0.05$) during shelf life.

The protein profile of the processed samples was carried out in order to evaluate possible changes in the milk proteins, caused by acoustic cavitation effects. Fig. 2 represents the protein profiles of RAW, US (1–4) and HTST processed semi-skimmed sheep milk of the fresh and frozen stored milks obtained by SDS-PAGE electrophoresis gel.

The molecular weight of sheep milk protein fractions presented on SDS-PAGE electrophoresis gel was identified according to the milk protein molecular weight [30]. Thereby, it could be visualized that immunoglobulin (96.7 kDa), lactoferrin (85.5 kDa) and serum albumin (70.8 kDa) appeared on the top of electrophoresis gel, respectively, because these proteins have higher molecular weight. Right below in gel, it appeared the casein fractions: α (36.9 kDa), β (30.8 kDa), and κ (28.2 kDa), respectively. The division between α and β-casein are hardly visible at electrophoresis gel (Fig. 2) due to higher amount of those proteins in sheep milk [3]. Also the darker intensity of caseins fractions (α = 23. 2; β = 32.8; and κ = 14.4% saturation) indicates the greater concentration of casein on sheep milk (4.45% ± 0.10 v/v; data not shown), when compared to the whey proteins. The β-lactoglobulin (19.7 kDa) and α-lactalbumin (16.4 kDa) appeared below in gel due to the lower molecular weight. Additionally, β-lactoglobulin is presented in greater amount (18.7% saturation) than others whey proteins (immunoglobulin = 1.02%; lactoferrin = 2.6%; serum albumin = 4.1%; α-lactalbumin = 3.3% saturation) in sheep milk, which is in accordance with Selvaggi et al. [45].

The determination of the FAA profile after processing was carried out in order to investigate the release of free amino acids due to ultrasound effect. Firstly, HPLC showed a FAA profile of 16 amino acids and their concentration (mg/100 g) in semi-skimmed sheep milk, namely: alanine (Ala: 0.69–1.13), arginine (Arg: 4.07 – 6.22), aspartic acid (Asp: 0.02 – 0.06), cystine (Cys: 0.63 – 2.78), glutamic acid (Glu: 0.08 – 0.33), glycine (Gly: 0.74 – 1.01), histidine (His: 0.31 – 1.15), isoleucine (Ile: 0.04 – 0.55), leucine (Leu: 0.02 – 0.60), lysine (Lys: 0.20 – 1.06), methionine (Met: 0.82 – 1.74), phenylalanine (Phe: 0.31 – 0.75), proline (Pro: 0.11 – 0.27), serine (Ser: 0.10 – 0.20), tyrosine (Tyr: 0.26 – 1.01) and valine (Val: 1.19 – 2.31). Sheep milk presented greater content of FFA than cow milk [26], mainly Arg (3.36 – 6.22 mg/100 g), Cys (0.63 – 2.78 mg/100 g), Val (1.19 – 2.31 mg/100 g) and Met (0.82 – 1.73 mg/100 g).

There was not a significant release of FAA in samples when comparing RAW, US (1–4) and HTST ($p > 0.05$) in the fresh period, meaning that neither US nor HTST processing were responsible for amino acids release. However, it was observed an increasing concentration ($p < 0.05$) of FAA in samples stored frozen for at least 90 and 180 days (-20 ± 1 °C). Fortunately, the increased amount of FAA, which occurred after 90 and 180 days of frozen storage, did not interfere on amino acids levels enough to change the nutritional quality of the milk in respect to amino acids daily intake [46].

A principal component analysis (PCA) at the samples fresh and frozen stored (days 90 and 180) was performed with the 10 higher FAA concentrations for a better presentation of the FAA data (Fig. 3). PCA bidimensional map explained 64.36% of variability in FAA using two components, first dimension (D1) referred to 33.28% and second dimension to 31.08%. The different times of frozen storage were allocated in distinct quadrants of PCA, being fresh in third, 90 days in forth and 180 days in the first quadrant. Each amino acid point appears near the treatment that better explained its variability. As well, samples near to specific amino acid suggest it contributed the most to the release of that amino acid. Therefore, as the fresh milk stayed away from the amino acid points, it indicates lower release of amino acids, differently from the frozen stored samples, which presented an important association with the free amino acids. Moreover, the different frozen storage times were associated with different amino acids.

Paniwnyk [14] states that US may destabilize casein, enhancing protein solubilization and delaying serum separation with no change of quality or sensory parameters of the final product, because US may increase rennet gelation rate, curding rate, curd firmness, gel firmness, coagulum strength, final storage modulus, cohesiveness and water holding capacity. However, studies indicates that protein solutions are not significantly modified upon sonication [47], which could be verified in the present study, where the protein profiles after processing maintained similar in all samples during the entire experimental period ($p > 0.05$). Our findings indicate that ultrasound processing did not produced any technological or nutritional harm to semi-skimmed sheep milk proteins, which is in accordance to Monteiro et al. [20]. Freezing milk might induced protein degradation and release free amino acids

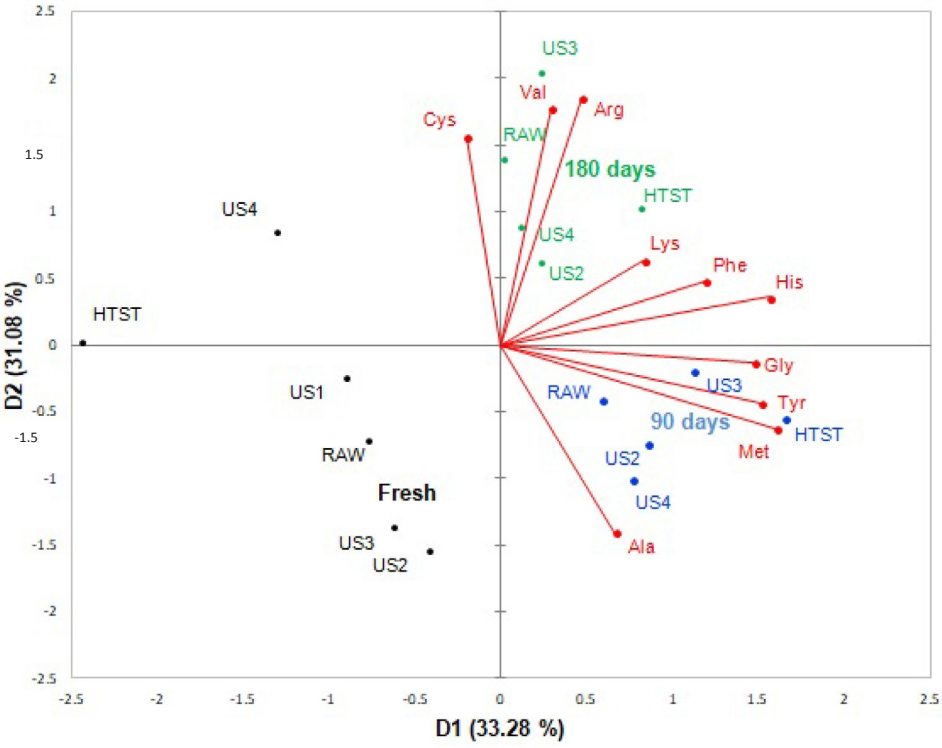

Fig. 3. Principal Component Analysis of free amino acids with higher concentration in semi-skimmed sheep milk samples (fresh, 90 and 180 days of frozen storage, −20 ± 1 °C). RAW = raw; US1 = ultrasound 1; US2 = ultrasound 2; US3 = ultrasound 3; US4 = ultrasound 4; and HTST = high temperature short time.

from 90 days of frozen storage, even if it has no importance from nutritional point of view, due to very low FAA content released.

4. Conclusions

The ultrasound parameters tested in this study seemed to be promising to achieve bacterial inactivation in semi-skimmed sheep milk, eliminating or maintaining the low contamination, acceptable for drinking milk. This finding presented interesting, as similar bacterial inactivation was obtained when it compared to the conventional treatment with the advantage of using small temperature processing.

US-treated sheep milk, namely US2 and US3, could be advantageous comparing to HTST pasteurization, since the ultrasound processing could preserve some lactic acid bacteria while inactivate satisfactory amount of contaminant bacteria in the frozen stored milk. In addition, no relevant change was noted on protein or free amino acid profile of pasteurized or US-treated semi-skimmed sheep milk, proving that this technology could be used in milk to produce sheep milk products such as cheeses, maintaining the product quality.

Overall, ultrasound processing should be considered by the sheep milk industry.

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