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Effect of two pasteurization methods on the protein content of human milk

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Material and methods
3.1. Samples
3.2. Monodimensional electrophoresis (SDS-PAGE)
3.3. Standard oxidation curves
3.3.1. Reduced BSA
3.3.2. Oxidized BSA
3.4. Western Blot on polyvinylidene fluoride (PVDF) membrane
3.5. DNP derivatization on membranes
3.6. Hydrophobic immunostaining
3.7. Western Blot on nitrocellulose (NC) membrane
3.8. Preparation of the positive control for 4-hydroxy-2-nonenal (HNE) immunostaining
3.9. HNE immunostaining
3.10. IgA Immunodetection
3.11. Mass spectrometry
3.12. N-Terminal Sequencing
3.13. Measurement of the lipase activity
3.14. Available lysine determination
4. Results
5. Discussion
5.1. Effect of pasteurization on lactoferrin
5.2. Effect of pasteurization on bile salt-stimulated lipase
5.3. Effect of pasteurization on components of the immune system
5.4. Effect of pasteurization on total available lysine content
6. Acknowledgements
7. References

1. ABSTRACT

The Holder method is the recommended pasteurization method for human milk banks, as it ensures the microbiological safety of human milk (HM). The loss of some biologically active milk components, due to the heat treatment, is a main limit to the diffusion of donor HM. High-temperature short-time (HTST) pasteurization may be an alternative to maintain the nutritional and immunological quality of HM. The aim of the present study was to compare the impact of Holder and HTST pasteurization on the HM protein profile. The protein patterns of HTST-treated milk and raw milk were similar. The Holder method modified bile salt-stimulated lipase, lactoferrin and components of the immune system. The HTST method preserved the integrity of bile salt-stimulated lipase, lactoferrin and, to some extent, of IgAs. Holder pasteurization decreased the amount of bile salt-stimulated lipase and inactivated the remaining molecules, while the HTST method did not alter its activity. Pasteurization increased the bioavailable lysine quantity. HTST pasteurization seems to better retain the protein profile and some of the key active components of donor HM.

2. INTRODUCTION

Increasing evidence shows that the confirmed short- and long-term benefits of human milk (HM) for term infants extend also to preterm and very low birth weight infants (VLBWIs) (1-3). Fresh mother's own milk is the best choice for feeding all newborns, including preterm and VLBWI. Pasteurized donor HM offers a safe alternative and it is recommended as the feeding of choice when mother's own milk is not available or when the quantity is not sufficient (4-5). In many countries, HM banking is well established and standardized, and donor HM has become a health care component for preterm infants (6-9).

Pasteurization ensures the microbiological safety of donor HM, and the Holder method (62.5° C, 30 min) is the recommended method in for use in HM banks (6-9). However, pasteurization inactivates some of the nutritive and biologically active compounds of HM (10-14), which are considered to be responsible for its clinical benefits. This leads to concern that pasteurization may reduce the nutritional quality of HM and its protective effects against

infections and necrotizing enterocolitis (NEC) (15), although this has not been observed clinically. Pasteurized donor HM feeding has been shown to reduce feeding intolerance, and to confer protection against NEC and long-term cardiovascular disease risk (16-18). Recently, a pasteurization method, based on heating at a higher temperature but for a shorter time (HTST or Flash pasteurization), and which is in use in the food industry, has been suggested as an alternative to the Holder method for HM pasteurization (19-21).

Among the biologically active components, HM proteins play a key role, as they have nutritive, enzymatic, trophic and immunomodulatory functions (22). During heat treatments, milk proteins can be oxidatively modified, both *via* the Maillard reaction, which mainly gives lactosylation on lysine residues, and *via* lipid oxidation, where HNE adducts (4-hydroxy-2-nonenal) can react with histidine, lysine, and cysteine residues. Reactive dicarbonyl compounds or α-β unsaturated aldehydes, originating from these two modification pathways, can lead to protein carbonylation. The occurrence of such modified residues may lead to decreased protein digestibility and reduced nutritional supply (23,24). In previous studies, the Holder method was found to induce carbonylation on amino acid residues (23,24), whereas only one investigation has been conducted to study the effects of the HTST method on the HM protein characteristics (20).

The aim of the present study was to compare the impact of two different pasteurization methods, Holder and HTST, on the protein profile of donor HM.

3. MATERIALS AND METHODS

3.1. Samples

HM samples were collected simultaneously at the Bufalini Hospital (Cesena) from four healthy donor mothers, 1-3 wk after term delivery. The milk specimens were obtained by completely emptying one breast using an electric pump. Immediately after collection, the samples were pooled and divided into 3 aliquots. One aliquot was stored at 4°C until other the two aliquots underwent two different heat treatments. One aliquot was pasteurized by the Holder method (62.5°C for 30 min) (Metallarredinox, Italy) and the other one by the HTST method (72°C for 15 s) (Lauda Ecoline R.E., Brinkmann Instruments, Canada). After these treatments, the three aliquots (from here onwards called Raw, Holder and Flash) were immediately frozen at -80°C for subsequent analyses. Part of each sample was skimmed by centrifugation at 2,000 *g* for 30 min at 4°C, in order to be used for SDS-PAGE and subsequent immunostaining analyses, while the remaining part of each sample was used for available lysine determination and lipase assay. Quantification of the total protein content on each sample was performed using 2D Quant kit (GE Healthcare).

3.2. Monodimensional electrophoresis (SDS-PAGE)

In order to perform homogeneous SDS-PAGE on the skimmed breast milk, 5 µg of the total proteins for each well were mixed with Laemmli sample buffer (28), without any reducing agent. SDS-PAGE was carried out on 12% polyacrilamide gel (8 x 8.5 cm), with Laemmli running buffer on a BioRad MiniProtean III Cell, at 125 V.

In order to conduct the gradient SDS-PAGE, one well, 4-12% gradient Nupage Novex Bis-Tris [Bis (2-hydroxyethyl) imino-tris (hydroxymethyl) methane-HCl] precast gels (Invitrogen) were used to separate skimmed breast milk samples (360 µg total proteins for each sample), with MES [2-(N-morpholino) ethane sulfonic acid] (Invitrogen) as running buffer, on a Novex Mini-cell (Invitrogen) at 200 V. The gels were stained with a freshly home-made prepared Blue Coomassie Colloidal stain (29) and digitized with a GS-800 Densitometer (Bio-Rad).

3.3. Standard oxidation curves

In order to evaluate the degree of carbonylation of the HM proteins before and after pasteurization, a specific immunostaining was performed. A compound, 2,4-Dinitrophenylhydrazine (DNPH), that specifically binds to carbonyls was used to derivatize the blotted membranes obtained from the homogeneous SDS-PAGE of raw and heat treated milk. A 0-40% gradient of oxidized BSA over fully reduced BSA was used as the reference compound (standard curve). The standard curves were built by mixing variable amounts of HOCl-oxidized BSA (Fluka) with fully reduced BSA, while maintaining a constant total protein concentration, in order to create a 0-40% gradient of oxidized BSA over reduced BSA.

3.3.1. Reduced BSA

Commercially available BSA contains carbonyls that can be reduced by sodium borohydride (NaBH4). Fully reduced BSA has been prepared by reacting a 10 g/L PBS solution (pH 7.4) containing 140 mmol/L of NaCl with 20 g/L NaBH4 for 40 min, followed by neutralization at pH 7.2 with 1 mol/L HCl. After overnight dialysis against 10 mmol/L PBS (pH 7.2), the protein concentration was brought to 4 g/L (30)

3.3.2. Oxidized BSA

105 mg of BSA were dissolved in 15 mL of 10 mmol/L PBS (pH 7.4) containing 0.1 mol/L NaCl, and incubated for 1 h with 7 g/L of sodium hypochlorite (NaOCl) at 37°C, under argon. The fully oxidized BSA solution was passed over a PD10 column (GE Healthcare) to remove unreacted NaOCl, and eluted with PBS to reach a final protein concentration of 4 g/L (31).

3.4. Western Blot on polyvinylidene fluoride (PVDF) membrane

PVDF membranes (Invitrogen) were soaked in methanol for 30 s, then washed with distilled water, and left for few minutes before Western blotting in 0.5X Towbin transfer buffer (32) added with 5% methanol. After homogeneous SDS-PAGE, the proteins were transferred at 30 V from the gel to the PVDF membranes using a tank transfer Xcell II Blot Module (Invitrogen), with 0.5X Towbin transfer buffer added with 5% methanol. The membranes used for the immunochemical analysis were soaked in methanol for 15 s before derivatization, while the negative control membranes were air-dried at room temperature.

3.5. DNP derivatization on membranes

The methanol activated membranes were equilibrated for 5 min in 20% methanol-80% PBS (pH 7.2), and then incubated in 2 mol/L HCl for 5 min, prior to protein derivatization. The membranes were then incubated for 5 min in a 0.5 mmol/L solution of DNPH in 2 mol/L HCl, and washed three times in 2 mol/L HCl for 5 min and seven times in methanol for 5 min each (33,34). Continuous shaking was used during all the incubation and washing steps. The membranes were finally dried overnight at room temperature.

3.6. Hydrophobic immunostaining

The sample and negative control membranes were immunostained using the protocol described by Mansfield (1995) (35), with minor modifications. The derivatized PVDF membranes were incubated for 1 h at 25°C with the primary antibody solution, which consisted of a 1:10,000 dilution of an anti-DNP-KLH rabbit IgG fraction antibody (Molecular Probes) in an incubation solution containing PBS, 0.05% Tween-20 and 1% gelatine (Bio-Rad). the membranes were the washed three times for 2 min with a washing solution containing PBS and 0.05% Tween-20. They were then incubated with a 1:2,500 dilution of horseradish peroxidise (HRP)-Goat Anti-Rabbit IgG (H+L) Conjugate (Invitrogen) in the incubation solution, for 30 min at 25°C. The membranes were washed 3 times for 2 min in the washing solution prior to visualization. HRP-3,3',5,5'Tetramethylbenzidine (TMB) (Molecular Probes) stain was used to visualize the reactive components. Membranes were digitized using a GS-800 Densitometer (Bio-Rad).

3.7. Western Blot on nitrocellulose (NC) membrane

After homogeneous SDS-PAGE, the proteins were electroblotted on NC membranes (Invitrogen) at 25 V using the tank transfer Xcell II Blot Module (Invitrogen), with Towbin 0.5X transfer buffer added with 5% methanol. The proteins separated by the 4-12% gradient SDS-PAGE gels were electroblotted on NC membranes (Invitrogen) at 30 V, using the tank transfer Xcell II Blot Module (Invitrogen), with the NuPAGE Transfer buffer (Invitrogen) added with 5% methanol.

3.8. Preparation of the positive control for 4-hydroxy-2-nonenal (HNE) immunostaining

In order to evaluate the degree of single protein carbonylation due to binding to the oxidized lipids (indirect pathway of carbonylation), a specific immunostaining of the HNE adducts was performed on the NC membrane. A 4-hydroxy-2-nonenal dimethylacetal (HNE-DMA) hexane solution (Sigma) was used to obtain a positive HNE control. The HNE-DMA was evaporatedunder a gentle stream of nitrogen at room temperature to eliminate the hexane, and 400 µL of 1 mmol/L cold HCl at 4°C were added and stirred for 45 min. This solution was added to a mix of the three milk samples and used as the positive control.

3.9. HNE immunostaining

The NC membranes were incubated for 1 hour at room temperature in a blocking solution (PBS buffer containing 1% gelatin) (BioRad), and washed for 5 min in a washing solution containing PBS and 0.05% Tween-20. The membranes were then incubated overnight with the primary antibody solution, that consisted of a 1:5,000 dilution of the rabbit anti-HNE antiserum in an incubation solution containing PBS, 0.05% Tween-20 and 1% gelatine (Bio-Rad) at 4°C, while this passage was omitted in the negative control. The membranes were then washed twice for 5 min in the washing solution. They were incubated with a 1:2,500 dilution of the HRP-Goat Anti-Rabbit IgG Conjugate (Invitrogen) in the incubation solution for 2 hours at room temperature. The membranes were then washed twice for 5 min in the washing solution, and once for 5 min with PBS alone. The 4-chloro-1-Naphtol (Sigma) stain was used to visualize the reactive components. The membranes were digitized using a GS-800 Densitometer (Bio-Rad).

3.10. IgA Immunodetection

Four-mm slices were cut from the NC membranes of the gradient gels and incubated twice for 15 min at room temperature with a TBS blocking solution (50 mmol/L Tris, 150 mmol/L NaCl, pH 7.4) containing 0.3% Tween 20. The membranes were then incubated overnight at room temperature with the primary antibody IgA heavy chain rabbit anti-human Polyclonal Antibody Lifespan Biosciences diluted 1:3,000 with the incubation solution (TBS, containing 0.05% Tween-20 and 0.05% gelatin, Bio-Rad). The membranes were then washed 3 times for 5 min in a washing solution containing TBS and 0.05% Tween-20, incubated with a 1:5,000 dilution of alkaline phosphatase-labelled goat anti-rabbit IgG (PerkinElmer) in the incubation solution for 1 h at room temperature, and then washed 3 times for 5 min in the washing solution. The immunoblots were developed with the phosphatase substrate BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) (AP Conjugate substrate kit, Bio-Rad). The membranes were digitized using a GS-800 Densitometer (Bio-Rad).

3.11. Mass spectrometry

The bands of interest from SDS PAGE have been identified by means of mass spectrometry analysis. For MALDI TOF and LC-nano-ESI-ion trap analyses, the bands were cut and destained overnight with a solution of 50 mmol/L ammonium bicarbonate and 40% ethanol, then washed three times for 10 min with acetonitrile and dried in a Speedvac. After, the proteins bands were reduced with 50 µl of a solution of of DTT 200mM/100 mM NH_4HCO_3 for 1h at 56°C and then alkylated with 500 µl of a solution of 200mM iodoacetamide/100 mM NH_4HCO_3 for 45 min, RT. The proteins were then digested *in gel* with trypsin (Promega), as described by Hellmann and co-workers (38). For the MALDI-TOF mass spectrometry, 0.5 µL of each peptide mixture was applied to a target disk, and allowed to dry. Then 0.5 µL of HCCA matrix solution (α-cyano-4-hydroxycinnamic acid) was applied to the dried sample and allowed to dry again. Spectra of protein digests were obtained using a Bruker Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). Mascot (39) and MS-Fit (40) software packages were used to interpret the MS spectra through the peptide mass fingerprinting method

(PMF) (41). Both software packages were searched against the NCBInr_20090123 database (42). The parameters used for the search were: S-carbamidomethyl derivate on cysteine as fixed modification, oxidation on methionine as variable modification and two missed cleavage sites for trypsin digestion. The peptide mass tolerance was 30 ppm.

Alternatively, the tryptic peptide mixtures were analyzed by means of LC-MS/MS using an Agilent-Technologies 1100 series nano HPLC, coupled to an Agilent XCT Plus ion trap fitted with a nano electrospray nebulizer. The chromatographic separations were run on a 150 x 0.075 mm C18 nanocolumn Zorbax 300SB (Agilent) using a linear 5-70% gradient of acetonitrile-0.1 % formic acid for 55 min, with a flow of 0.3 µL/min. The injection volume was 1 µL. The mass spectrometry parameters were: capillary voltage 1,600 V, fragmentation voltage 1.3 V. The Data Analysis software (Agilent) was used to elaborate the LC and MS/MS data. The Mascot search engine (39) was used to identifiy the proteins against the NCBInr_20090123 database (42). The parameters used for the search were: S-carbamidomethyl derivate on cysteine as fixed modification, oxidation on methionine as variable modification and two missed cleavage sites for trypsin digestion. The peptide mass tolerance was set to 0.5 Da and the fragment mass tolerance to 0.8 Da. The criteria used for protein identification were: 20 ion score with a protein Mascot score of more than 100 and at least two unique peptide sequences.

3.12. N-Terminal Sequencing

The bands of interest were excised and passively eluted as already described (43). The membranes were then microsequenced on a Procise 492 protein sequencer (Applied Biosystems). All the chemicals used in the procedure were from Applied Biosystems. The obtained amino acid sequences were compared with the protein sequences in the Uni-PROT database, using BLAST software (42).

3.13. Measurement of the lipase activity

The measurement of the lipase activity was performed by quantifying the reaction product p-nitrophenol (36). In a test tube, 50 µL of Raw, Holder, Flash and boiled (5 min at 95°C) unskimmed milk samples were mixed with 1 mL of 0.05 mol/L barbital buffer pH 7.6 (0.2 mol/L sodium barbital pH 7.6 with 0.2 mol/L HCl) and incubated at 37°C for 15 min. Then, 5 µL of substrate (50 mmol/L p-nitrophenyl butyrate in acetonitrile) were added, and the reaction mixtures were incubated at 37 °C for 10 min. Finally, 1 mL of Clarifying Reagent (Prolabo) was added to each tube, which was then stirred and incubated at 37 °C for 3 min. Absorbance at 420 nm was read within 15 min. The absorbance values were converted to µmol of p-nitrophenol using a standard curve in the 20 -200 µmole range. The boiled milk was used as a negative control to check the lipase inactivation, as reported by Williamson *et al.* (1978) (37) and Tully *et al.* (2001) (11). The determinations were replicated nine times. Statistical analysis was performed by a pairwise Student T-Test ($p \leq 0.05$).

3.14. Available lysine determination

The o-phthaldialdehyde (OPA) and N-α-acetyl-lysine were purchased from Sigma. All the reagents were of analytical grade, and Millipore Milli-Q water was used. The total available lysine content was measured using the OPA method (25,26). The OPA reagent (80 mg OPA dissolved in 1 mL ethanol 95%) was prepared immediately before use, and mixed with 99 mL of 0.05 mol/L sodium tetraborate buffer (pH 9.5) containing 5 mL of 20% SDS and 0.2 mL of 2-mercaptoethanol. A standard curve with a concentration ranging from 0.0125 to 0.375 mmol/L was obtained using N-α-acetyl-lysine as a reference compound, dissolved in 0.1 mol/L phosphate buffer pH 7.0 (27). The reference N-α-acetyl-lysine samples and 25 µL of unskimmed milk samples were added to 2 mL of the OPA reaction solution, briefly stirred, and left at room temperature for 2 min to incubate. The OD at 340 nm was measured against OPA reaction solution using an UV-VIS DU730 spectrophotometer (Beckman Coulter).

In order to eliminate the interference due to milk opalescence, blanks containing 25 µL of unskimmed milk in OPA reagent and not containing OPA (0.05 mol/L sodium tetraborate buffer pH 9.5, 1% SDS, 0.95% ethanol and 0.2% 2-mercaptoethanol) were used. The OD from each blank was subtracted from the OD obtained for the respective milk sample mixed with OPA-containing reaction buffer. This procedure allows the milk opacity due to both the casein micelles and the milk fat globules dispersion to be removed. The determinations were replicated seven times. The statistical analysis was performed by a pairwise Student T-Test and the results were considered statistically significant when associated to a probability of less than 5%.

4. RESULTS

A monodimensional SDS-PAGE of Raw, Holder and HTST milk samples was run, in order to discover the changes induced by pasteurization on the protein pattern. The resulting SDS-PAGEs and the corresponding grey-scale intensity chromatograms are shown in Figure 1. The HTST milk protein pattern appeared to be very similar to that of Raw milk, while the Holder pasteurized milk showed major differences. Heating milk at 62.5°C for 30 min reduced the intensities of the A, B and C bands. On the other hand, the E and F bands only appeared in the Holder milk and not in the other samples.

All the bands of interest were subjected to PMF by mass spectrometry analysis in order to identify the corresponding proteins. As shown in Table 1, the B and C bands were found to correspond to the oncofetal isoform of bile salt-stimulated lipase and to human lactoferrin, respectively. The A, E and F bands could not be identified.

The degree of protein carbonylation was assessed on the same homogeneous SDS-PAGE gels by DNP immunostaining. The results are shown in Figure 2. No aspecific reaction was detected in the control membrane (Figure 2, III). All the samples showed some degree of immunoreaction (Figure 2, II). Compared to the Raw and

Table 1. Protein identification by mass spectrometry of bands of interest from homogenous SDS PAGE (Figure 1)

Band	Protein name	UniProt KB/ TrEmbl Entry	N. MW[1]/ Experimental MW[2] (Da)	PMF Number of peptides/ Sequence coverage (%)
A	Not identified	-	-	-
B	Human bile salt-stimulated lipase	O75612	66,363/100,000	12/77 25%
C	Human lactoferrin	P02788	80,046/85,000	16/62 26%
E	Not identified	-	-	-
F	Not identified	-	-	-

Abbreviations: Nominal molecular mass of the entry[1], Molecular mass deduced from SDS-PAGE[2]

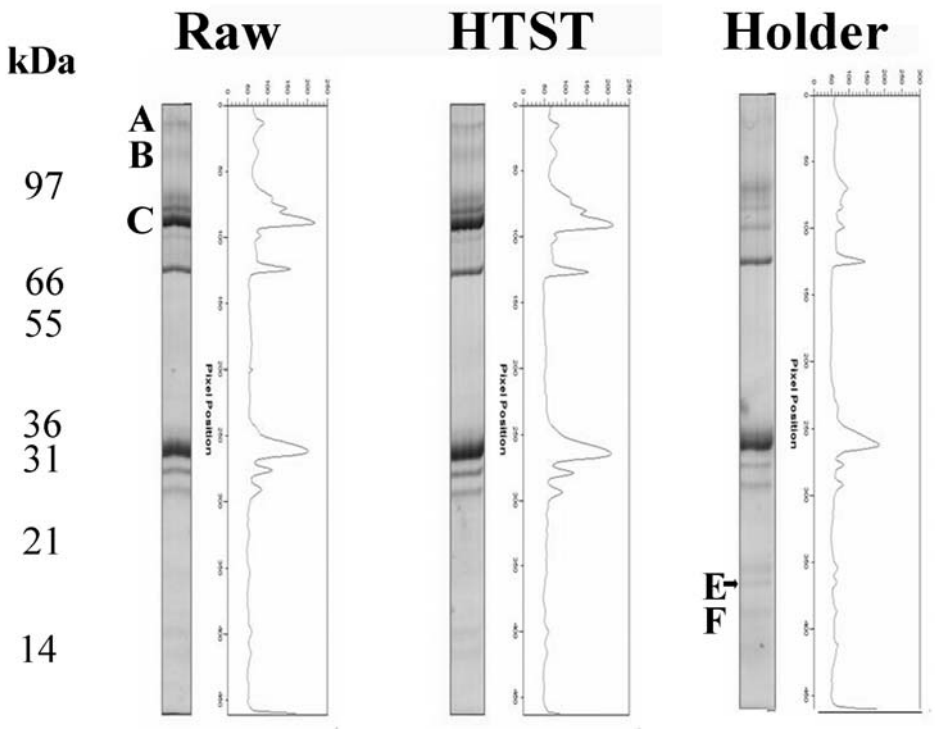

Figure 1. Homogeneous SDS PAGE and corresponding chromatograms obtained by densitometric scanning of the gels of raw and pasteurized HM samples (R: Raw; HT: HTST; Hd: Holder). The letters indicate the bands of interest.

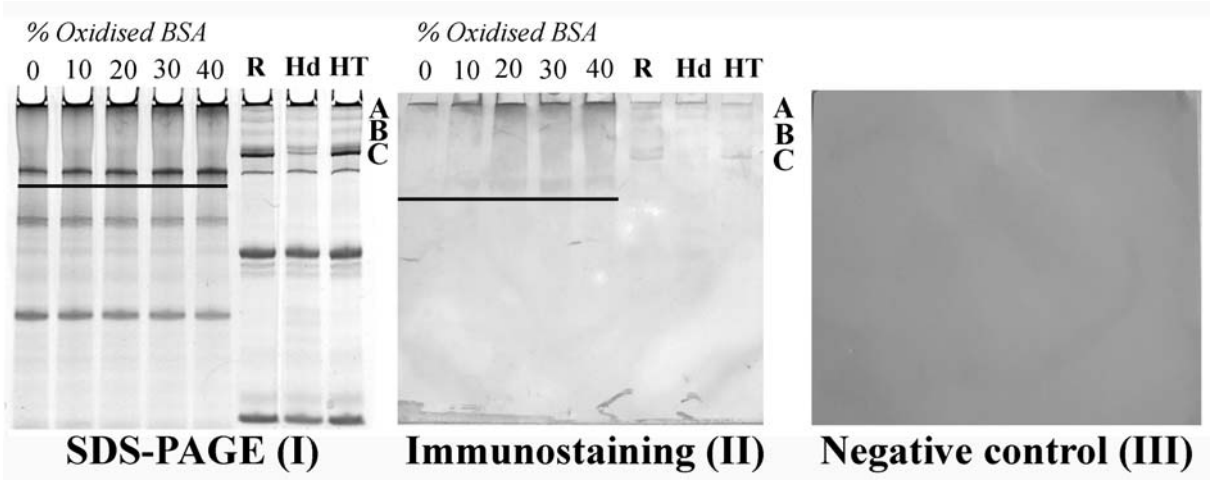

Figure 2. SDS PAGE (I) and DNP immunostaining (II) of control (R) and pasteurized (Hd: Holder; HT: HTST) HM. The letters indicate the bands of interest. Control membrane (III) was not derivatized with DNPH.

Table 2. Protein identification by mass spectrometry of band of interest from HNE immunostaining (Figure 3)

Band	Protein name	UniProt KB/ TrEmbl Entry	N. MW[1]/ Experimental MW[2] (Da)	PMF Number of peptides/ Sequence coverage (%)
P	Human β casein	P05814	25,382/30,000	3/20 19%

Abbreviations: Nominal molecular mass of the entry[1], Molecular mass deduced from SDS-PAGE[2]

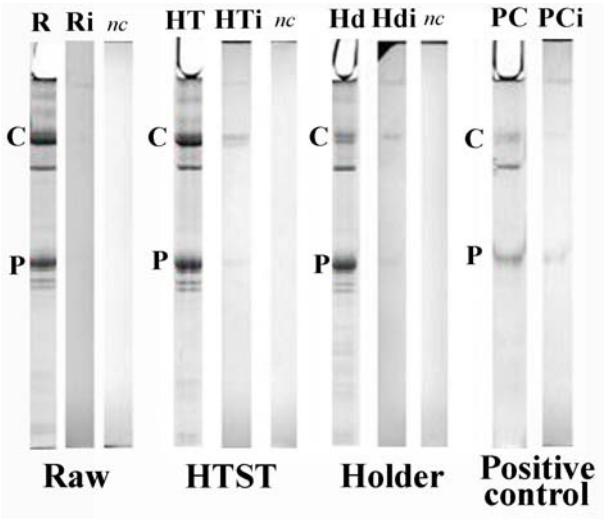

Figure 3. SDS PAGE (R: Raw; HT: HTST; Hd: Holder) and corresponding HNE immunostaining (Ri; HTi; Hdi) of the donor HM samples. C and P indicate the bands of interest. nc: negative control membrane, not incubated with anti-HNE antibody. PC: positive control, SDS PAGE and corresponding immunostaining (PCi).

HTST milk, the Holder milk sample showed a reduced immunoreaction on the B and C bands, which correspond to the bands that showed a decreased intensity in the SDS PAGEs (Figure 1 and 2,I). In addition, the lipid oxidation of proteins was assessed by revealing the HNE adducts, through specific immunostaining. The obtained results are shown in Figure 3. The pasteurized milk samples suffered from a certain degree of lipid oxidation. The modified bands, named C and P, were identified as lactoferrin and β-casein (Table 2), respectively.

In order to characterize the protein profile more clearly at high and low molecular weights, all the milk samples were also subjected to a gradient SDS-PAGE. The resulting gels and chromatograms are shown in Figure 4. In the Raw milk, the C band was divided into two portions, one on the upper side (C_1) and one on the lower side (C_2). The C_1 and C_2 bands, and C band from the Holder milk, were all identified as human lactoferrin. Bands L, M, and N from Holder milk resulted to be fragments of β-casein (Table 3). The J and K bands, that completely disappeared in the Holder milk, were identified in the raw milk as components of the immune system (Ig λ-light chain variable region, MHC class I antigen) (Table 3). Finally, the O and Q bands, which were more intense in the Raw milk, detectable in the Flash milk and almost absent in the Holder milk, were identified through specific immunoreaction as human IgA (Table 3).

In order to assess whether the pasteurization method effected the level of lipase activity, a specific assay was conducted and the results are shown in Figure 5. The Holder milk showed a lack of lipase activity, while a comparable ($p \geq 0.05$) level of activity was detected for the Raw and HTST milk.

In addition, a specific assay was performed in order to investigate how the two pasteurization treatments effect the availability of lysine in milk. The mean contents of the available lysine of Raw, Holder and HTST milk samples are reported in Figure 6. The results indicate that the Holder milk had a significant ($p \leq 0.05$) higher content of available lysine.

5. DISCUSSION

Donor HM is the preferred feeding for preterm infants, when mother's own milk is not available in sufficient quantity. The supply of donor milk should come from established HM banks, which adhere to specific quality control guidelines (6-9). These guidelines (6-8) recommend that the Holder pasteurization method (62.5°, 30 min) is used to ensure the microbiological safety of donor HM. It has been shown that key nutritional and biological components, such as oligosaccharides, vitamins A, D and E, lactose, long chain PUFAs and the epidermal growth factor (which is important for intestinal maturation) are preserved by the Holder method (11, 13, 44-46). However, this process leads to the loss of some biologically active milk components: secretory IgAs, total IgAs, lactoferrin, lysozyme, lymphocytes, lipase, alkaline phosphatase, cytokines, and some growth factors. (10-14). The loss in these biological components in donor milk following pasteurization is a well-known problem, and

Table 3. Protein identification by mass spectrometry, N-terminal sequencing and immunoblotting of the bands of interest from gradient SDS PAGE (Figure 4)

Band	Protein name	UniProt KB/ TrEmbl Entry	N. MW[1]/ Experimental MW[2] (Da)	PMF Number of peptides/ Sequence coverage	MS/MS sequences/charge state	N-term sequencing	Immunoblot
C	Human lactoferrin	P02788	80,228/ 85,000	-	ESTVFEDLSDEAERDEYELLCPDNTR / 3+ IDSGLYLGSGYFTAIQNLR / 2+ SQQSSDPDPNCVDRPVEGYLAVAVVR / 3+	-	-
C_1	Human lactoferrin	P02788	80,228/ 85,000	-	ADAVTLDGGFIYEAGLAPYK/ 2+ CAFSSQEPYFSYSGAFK/ 2+ EDAIWNLLR/ 1+ FQLFGSPSGQK/ 1+ IDSGLYLGSGYFTAIQNLR/ 2+ CGLVPVLAENYK/ 1+ SQQSSDPDPNCVDRPVEGYLAVAVVR/ 2+ LADFALLCLDGK/ 1+ YLGPQYVAGITNLK/ 2+		
C_2	Human lactoferrin	P02788	80,228/ 85,000	-	DLLFK / 1+ DSAIGFSR / 1+ CFQWQR / 1+ GQFPNLCR / 1+ DGAGDVAFIR / 1+ EDAIWNLLR / 1+, 2+ THYYAVAVVK / 1+ FCLFQSETK / 2+ FQLFGSPSGQK / 1+, 2+ SDTSLTWNSVK / 1+ FFSASCVPGADK / 2+ LADFALLCLDGK / 1+, 2+ CGLVPVLAENYK / 1+, 2+ GGSFQLNELQGLK / 1+, 2+ RSDTSLTWNSVK / 1+, 2+ KGGSFQLNELQGLK / 2+ DEYELLCPDNTR / 1+ YLGPQYVAGITNLK / 1+, 2+ NLLFNDNTECLAR / 1+, 2+ SVQWCAVSQPEATK / 1+, 2+ DSPIQCIQAIAENR / 1+ YLGPQYVAGITNLKK / 2+ CSTSPLLEACEFLR / 1+, 2+ GEADAMSLDGGYVYTAGK / 1+ SNLCALCIGDEQGENK / 1+, 2+ FDEYFSQSCAPGSDPR / 2+ CAFSSQEPYFSYSGAFK / 2+, 3+ DVTVLQNTDGNNNEAWAK /2+ NGSDCPDKFCLFQSETK / 2+, 3+ ADAVTLDGGFIYEAGLAPYK / 2+, 3+ IDSGLYLGSGYFTAIQNLR / 2+, 3+ FFSASCVPGADKGQFPNLCR / 3+ SQQSSDPDPNCVDRPVEGYLAVAVVR / 2+, 3+ CNQWSGLSEGSVTCSSASTTEDCIALVLK / 3+ KCNQWSGLSEGSVTCSSASTTEDCIALVLK / 3+		
J	Ig λ-light chain variable region	Q5NV79	10,755/ 130,000	3/29 30%	SSVKLTCTLSSR1/+ GSGIPDR/1+ FSGSSSGADR/1+		
K	MHC class I antigen	Q9TQB8	20,895/ 130,000	4/40 29%	TNTQTYRVSLR/1+ NLRGYYNQSEAGSHTLQRMYGCDVGPDGR/1+ RHLENGKETLQR/1+ HLENGKETLQRA/1+		
L	Human β casein	P05814	25,295/ 30,000		SPTIPFFDPQIPK / 1+, 2+ VLPIPQQVVPYPQR / 2+ AVPVQALLLNQELLLNPTHQIYPVTQPLAPVHNPISV / 3+		
M	Human β casein	P05814	25,382/ 30,000	3/29 19%			
N	Human β casein	P05814	6,000/ 30,000			^{161}LALPPQPL	
O							Positive reaction against Human IgA
Q							Positive reaction against Human IgA

Abbreviations: Nominal molecular mass of the entry[1], Molecular mass deduced from SDS-PAGE[2]

often results in skepticism regarding its nutritional and immunological quality. Thus, the optimization of HM pasteurization has become one of the priorities of HM banks.

HTST pasteurization seems to be a valuable alternative to the Holder method as far as minimizing the loss of nutritional and immunological components while ensuring microbiological safety. Although there is no uniformity in the application of this method, with regards to the temperature (71°C to 75°C), duration (5-18.5 s), or the instrumentation, there is already a convincing body of evidence concerning its bacteriological and viral safety (19-21). Research is currently underway concerning its efficacy

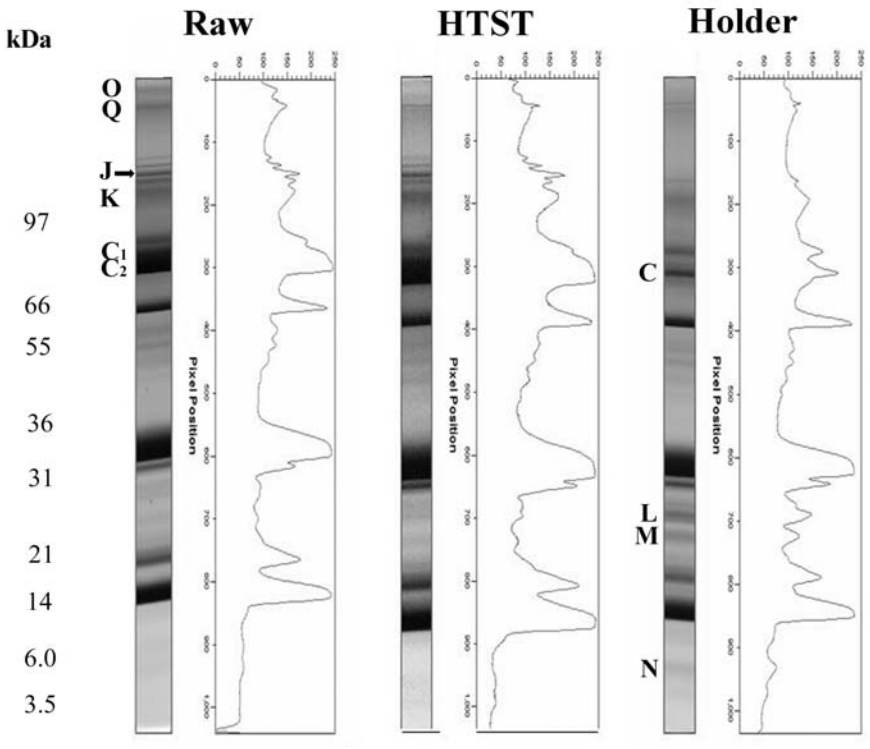

Figure 4. Gradient SDS PAGE (R: Raw; HT: HTST; Hd: Holder) of the donor HM samples and the corresponding chromatograms obtained by densitometric scanning of the gels. The letters indicate the bands of interest.

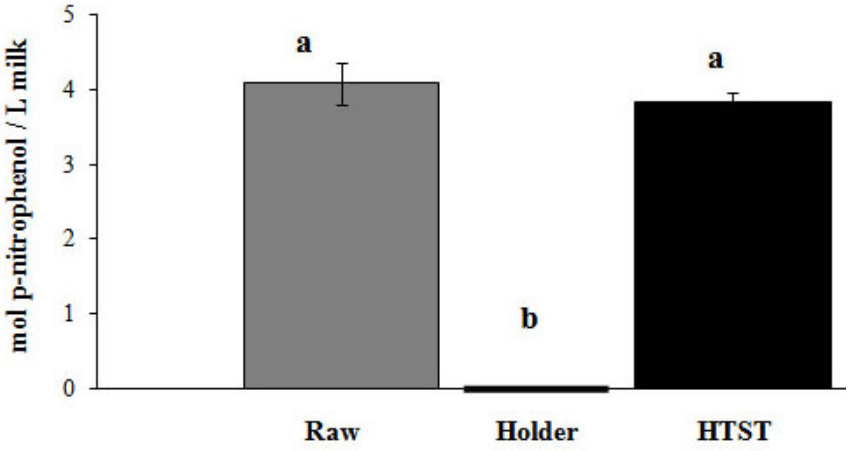

Figure 5. Determination of the lipase activity (mol p-nitrophenol/L milk) in Raw, Holder and HTST milk. Error bars represent s.e. of difference (n = 9). Bars not accompanied by a common letter differ significantly according to the Student T test ($p < 0.05$).

in retaining the nutritional and immunological quality of HM. The available data suggest that HTST pasteurization is better at preserving lactoferrin, IgA, IgG, and insulin like growth factors, and maintaining the antioxidant capacity of HM (13, 14, 19, 20).

5.1. Effect of pasteurization on lactoferrin

The present study is, to the authors' knowledge, the first to address the effects of different pasteurization methods, in terms of oxidation damage, on individual HM proteins. Human milk protein oxidation induced by thermal treatments, via the Maillard reaction, by direct oxidation on the amino acid residues, and by indirect reaction via lipid oxidation, has been investigated. The HM protein pattern of the HTST-treated milk and the raw milk resulted to be similar, whereas the Holder method substantially modified several proteins. The apparent decrease in lactoferrin quantity in Holder sample is likely to be due to the heat induced formation of carbonyls and the subsequent formation of disulfide-bonded high molecular aggregates

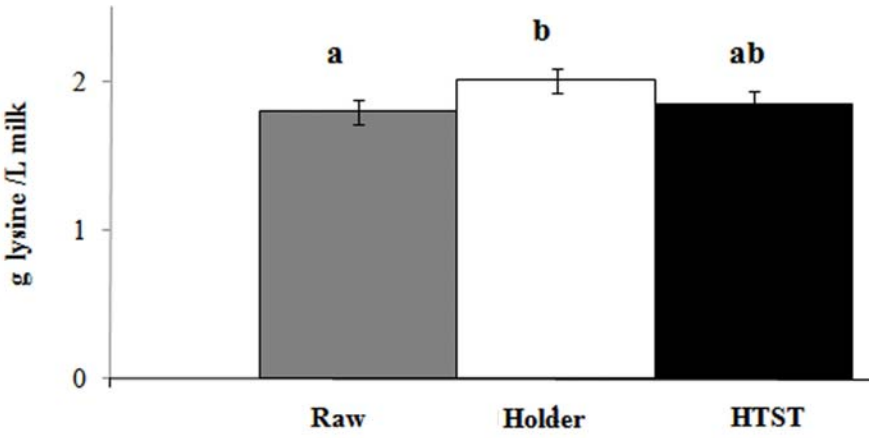

Figure 6. Content of available lysine (g/L of milk) in Raw, Holder and HTST milk. Error bars represent s.e. of difference (n = 9). Bars not accompanied by a common letter differ significantly according to the Student T test ($p < 0.05$).

which prevent lactoferrin from entering the SDS-PAGE gels and being visualized. This phenomena was previously reported for bovine milk (57,58), and could explain the apparent lower level of carbonylation on this protein in the Holder sample compared to both Raw and HTST milk. The decrease in lactoferrin in HM following pasteurization has been reported by several authors (11,49). Mata *et al.* (50) have reported a denaturation temperature of 67°C for lactoferrin as isolated in HM, with a peak at 71.85°C. Mata *et al.* have also confirmed the suitability of HTST pasteurization for partially retaining the iron-binding capacity of lactoferrin (although decreased by 25%), whilst a 20 min heat treatment at 80°C could reduce it by one half (50). Lactoferrin carbonylation has been observed in both raw and pasteurized HM. Lactosylation, by changing the protein conformation and making it less accessible to proteases, has been reported to partially prevent proteolysis on lactoferrin in bovine milk (51). This could also be expected to take place for HM lactoferrin, since about 10% of the ingested lactoferrin has been found intact in faeces of breast-fed infants (52).

5.2. Effect of pasteurization on bile salt-stimulated lipase

Bile salt-stimulated lipase is subjected to structural modifications which lead to a total loss of its enzymatic activity, as shown in Figure 1 and 5. The results demonstrate that the Holder method completely inhibits lipase, whereas the HTST method preserves both the integrity of the molecule (Figure 1) and its activity (Figure 5). In fact, as reported by Williamson and co-workers (37), even after only 1 min of pasteurization at 62.5°C, the lipase activity is completely lost. On the contrary, in the present experiment the HTST method based on a higher temperature (72°C) and shorter time (5-15 s) process, has been shown to preserve lipase activity and integrity.

5.3. Effect of pasteurization on components of the immune system

The HTST treated HM preserved the integrity of other native proteins, beside lactoferrin and bile salt-stimulated lipase, such as some components of the immune system (the J and K bands) (Figure 4, Table 3). The O and Q bands, which represent IgAs (Figure 4, Table 3), seem to be more sensitive to thermal treatment, being less intense in the heat-treated milk in comparison to the Raw HM. This is in agreement with what has been reported by Bjiorksten *et al.* (49) and Dhar *et al.* (20), who reported that a small increase in temperature causes a loss of IgA activity.

5.4. Effect of pasteurization on total available lysine content

As far as β-casein is concerned, carbonylation is not dramatically increased by either pasteurization method. The Holder method instead induces some protein degradation. Since caseins are considered to be the main source of available lysine in milk products (53), both these aspects – the formation of peptides and the low level of carbonylation - could explain the slightly higher availability of lysine residues in the Holder treated milk. The mean value of the total available lysine found in raw breast milk is consistent with a previous report (54). The slightly higher lysine content of the Holder treated milk found in our experiment is not consistent with a previous paper, which reported a strong reduction of lysine availability in thermal treated breast milk samples, and especially in those pasteurized by the Holder method (52).

In conclusion, different heat treatments have different impacts on HM proteins. The HTST treated HM resulted in a protein profile that was close to that of raw milk, with the preservation of the native forms of lactoferrin, and bile salt-dependent lipase, and to some extent of IgAs and some other components of the immunosystem. The HTST pasteurization method not only retained the integrity, but also the activity of the bile salt-stimulated lipase. This data could have important clinical implementations for donor milk use in terms of improved fat digestion and infection prevention for premature infants. The Holder treated HM seemed to have a higher content of bioavailable lysine. Briefly, HTST pasteurization seems to be better than the Holder method in retaining the protein profile, and some of the key biologically and immunologically active components of donor HM.

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