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(Article begins on next page)
Pasteurization of human milk by a benchtop High-Temperature Short-Time device.

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ABSTRACT

A new small-scale continuous-flow High-Temperature Short-Time (HTST) pasteurizer has been designed for treating human milk. The efficacy of the new HTST device was assessed on inoculated *Listeria monocytogenes*, *Staphylococcus aureus* and *Chronobacter sakazakii*, as well as on raw human milk bacteria. The milk biochemical quality after HTST pasteurization was assessed in comparison to a standard Holder pasteurization, by determining the secretory IgAs (sIgAs) content, the protein profile, lysozyme and the bile salt stimulated lipase (BSSL) activities. No pathogen or bacterial growth was detected after HTST pasteurization with the new instrument. Changes in the protein profile were observed in the milk pasteurized according to both processes. The sIgAs content and BSSL activity were significantly higher in the milk pasteurized with the new device than in the same milk treated by the standard Holder pasteurization. In conclusion, the new HTST apparatus: (i) can effectively pasteurize human milk with a better retention of sIgAs content and BSSL activity; (ii) comply to human milk banking safety requirements.

Keywords: Donor milk; HTST; Bile Salt Stimulated Lipase; Challenge tests; Secretory IgAs.

INDUSTRIAL RELEVANCE

Currently, 206 active human milk banks are located in Europe (and 13 more are planned). The majority of the European banks still use Holder-based pasteurizers, which, despite efficacy in ensuring microbiological safety, are known to reduce/disrupt important nutritional and non-nutritional biological factors. Although already widely established in food industry, the advantages of HTST technology were tested only at small laboratory scale for human milk. The device tested in the present research was specifically designed to provide human milk banks with the technology they need to ensure a safe and lower-impact pasteurization process, that is suitable for processing different volumes of donations. The device can pasteurize up to 10 l of milk per hour, with a
minimum volume of 100 ml. The system is designed to be cleaned-in-place (CIP) after each pasteurization run and sanitized immediately prior to the next use, being thus more suitable for treating pools of milk from different donors than milk from single donations. Italian and EU patents have been filed for the device, within a partnership between public research institutions, stakeholders (Italian association of donor milk banks), and a private company in the sector of dairy processing equipment. The device has achieved a Technology Readiness Level (TRL) 6 (Prototype demonstration in a relevant environment). The cost of the new device will be comparable to that of a typical human milk Holder pasteurizer.

Abbreviations:

- Bile Salt Stimulated Lipase (BSSL)
- Colon Forming Units (CFU)
- High-Temperature Short-Time (HTST)
- Holder pasteurization (HoP)
- Human milk (HM)
- Human Milk Bank (HMB)
- Inoculated human milk (IHM)
- Original human milk (OHM)
- Pasteurized human milk (PHM)
- Raw human milk (RHM)
- Secretory IgAs (sIgAs)
- Sterile human milk (SHM)
- Total Viable Bacteria Count (TVC)
Funding

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Conflicts of Interests

LC MG SA EB AC have competing interests since they are the inventors of a pending patent application on the HTST pasteurizer for human milk described in the paper (Patent application no. EP 15176792.8-1358/2014). No conflict of interest exists for the remaining authors.
1. INTRODUCTION

Thermal treatments are commonly applied for food processing, because of their ability to kill pathogens and inactivate potentially detrimental enzymes, such as lipases and proteases. The Holder pasteurization (HoP) method (62.5 °C for 30 min) is currently the recommended pasteurization method for human milk banks (HMBs), as it ensures that the human milk (HM) is microbiologically safe (Arslanoglu et al., 2010). However, heat processing, particularly under severe conditions, may give rise to chemical and physical changes that can impair the organoleptic properties and reduce the content or bioavailability of some nutrients (Arslanoglu et al., 2013; Tully, Jones, & Tully, 2001). HM has an elevated biological value, thanks to the casein-to-whey ratio, high essential amino acid concentration and immunological components, such as immunoglobulins, lysozyme, and lactoferrin, which convey relevant antimicrobial properties (Andreas, Kampmann, & Mehring Le-Doare, 2015; Giribaldi, et al. 2012). Thermal treatments may also cause the unfolding of milk fat globule membrane proteins and whey proteins, whose end products are typically associated with off-flavours (Contador, Delgado, Garcia-Parra, Garrido, & Ramírez, 2015). The loss of some biologically active components, including immunological components, due to HoP, is a main limit to the spread of donor human milk (Moro & Arslanoglu, 2012; Tully et al., 2001). The maximization of the biological and nutritional quality of donor HM is considered a scientific and social priority: the ESPGHAN Committee on Nutrition has pointed out that “future research should focus on the improvement of milk processing in human milk banks, particularly of heat treatment” (Arslanoglu et al., 2013).

In a previous study by our group (Baro et al., 2011), the HTST method (72 °C for 15 s) showed to better preserve, in comparison to HoP, the milk protein profile and some of the key active components of HM, with potential consequences on the availability of important nutritive compounds, such as fatty acids and available lysine.

In previous studies concerning the application of HTST to HM, other non-commercial devices were used, usually at a laboratory scale. Some authors used laboratory equipment, mainly consisting of
stainless steel tubing systems submerged in thermostated water baths, through which HM was pumped (Dhar, Fichtali, Skura, Nakai, & Davidson, 1996; Goldsmith, Eitenmiller, Toledo, & Barnhart, 1983; Terpstra et al., 2007); others injected milk through a sterile water stream in a plate-type industrial heat exchanger (Goldblum et al., 1984); some research studies were conducted by directly heating and rotating small aliquots of milk, to simulate the typical thin-layering of dairy industry HTST devices (Goeltz et al., 2009; Hamprecht et al., 2004). Several studies involved simply heating small aliquots (from 40 μL to 4 mL) of HM in a bulk process (Mayayo et al., 2014; Mayayo et al., 2016; Silvestre et al., 2006; Silvestre et al., 2008). Moreover, variable heating times (5-15 s) and temperatures (71-75 °C) were adopted. All the reported processes, with the exception of that of Goldblum et al. (1984), are substantially different from industrial HTST processes. However, the introduction of HTST into the HMB routine has not been possible to date, due to the lack of specific low-volume designed instrumentation. In most of studies concerning the HTST processing of HM, quantitative comparisons, with respect to standard pasteurization, have been described, but HoP was often simulated on small aliquots, rather than being performed according to real HMB-implemented protocols, thus representing a possible bias for the generalization of the comparison with novel technologies (Peila et al., 2015).

When projecting, creating, testing and patenting a new type of low-volume HTST pasteurizer (Cavallarin et al., 2015), it was considered that: i) it was intended for HMBs, and thus consistency with guideline requirements was mandatory; ii) the dairy industry HTST standards (72 °C holding temperature for 15 s holding time) had to be fulfilled; iii) temperature control had to be ensured by means of adequate probes; iv) a comparison with the HoP process had to be made using the HMB implemented device.

The present research is aimed at reporting the efficacy of a new, low-volume, continuous flow commercial HTST pasteurizer on HM quality in terms of: (i) bacterial inactivation, and (ii) preservation of the main immunological and nutritional components.
2. MATERIALS AND METHODS

2.1 Preliminary tests

In order to verify the pasteurization process, a preliminary experiment was run. A bovine milk sample was pasteurized and the alkaline phosphatase and peroxidase activities were determined in the treated sample. Alkaline phosphatase is a heat sensitive enzyme in milk that is used as indicator of pasteurization. If milk is properly pasteurized, alkaline phosphatase is inactivated. Lactoperoxidase is one of the most heat-stable enzymes found in milk and it is preserved after a correct HTST pasteurization. The raw bovine milk was purchased from a local automatic distributor, collected in a sterile Pyrex bottle, delivered refrigerated to the lab and stored refrigerated until it was processed. The milk (1 L) was divided into two aliquots: one was kept refrigerated until it was analysed; the other was subjected to HTST pasteurization by means of the new instrument (described in 2.3), collected in a sterile Pyrex bottle and stored refrigerated until the analysis. Alkaline phosphatase activity was tested by means of the enzymatic hydrolysis of p-nitrophenol phosphate, which yields p-nitrophenol and inorganic phosphate (ISO/TS 6090, 2004). Peroxidase activity was determined by means of Storch’s peroxidase test, which measures the oxygen transfer from hydrogen peroxide to other readily oxidisable substances (Council Directive 92/46/EEC).

2.2 Sample collection and pooling of specimens

The HM samples were obtained from the HMB of the Città della Scienza e della Salute in Torino, Italy, from eight healthy donor mothers. The donors cleaned their hands and breasts according to the HMB guidelines. The milk specimens were collected in sterile bisphenol-free polypropylene bottles (Flormed, Naples, Italy) using a breast pump and stored, by the HMB, at -20°C until processed.
The HM samples were pooled separately for the two experiments, which were referred to as Experiment 1 and Experiment 2. Panel A and panel B in Figure 1 show the experimental workflows for Experiment 1 and Experiment 2, respectively.

In Experiment 1, frozen samples from individual HM donors were thawed overnight in a refrigerator, and then in tap water, and were pooled to achieve a final volume of about 2 L in a sterile Pyrex bottle. They were then mixed carefully, divided into 100 mL aliquots and placed in sterile bisphenol-free polypropylene bottles. One aliquot of original HM (OHM) was used directly to determine the HM background microflora. The remaining aliquots were pasteurized intensively in a water bath for 1 hour at 63 °C (prolonged Holder Pasteurization) to kill all of the existing vegetative forms of microorganisms (SHM). One aliquot was kept for about 20 h in the refrigerator, and then analysed to verify the absence of microbial contaminants. The other aliquots were immediately frozen and used later for inoculation (IHM) (section 2.4.3).

In Experiment 2, samples from individual HM donors were obtained frozen from the HMB, thawed overnight in a refrigerator, and then in tap water, and were pooled to achieve a final volume of about 400 mL in a sterile Pyrex bottle. They were mixed carefully and divided into three sterile bisphenol-free polypropylene bottles. Two aliquots were subjected to standard HoP, in two separate trays, in the HMB facilities. The two samples were processed in the same batch and differed only for their position in the pasteurizer. One aliquot was subjected to HTST pasteurization using the new instrument. The Raw (RHM), Holder (HoP-HM) and HTST (HTST-HM) pasteurized samples were kept frozen at -20°C until analysis, with the exception of 10 mL per sample, which was kept refrigerated for about 20 h before being used for microbial screening.

### 2.3 Pasteurization equipment

HoP was performed in an HM pasteurizer (Metallarredinox, Zingonia-Verdellino (Bg), Italy) located in the HMB of the Città della Scienza e della Salute in Torino, Italy. A patent pending HTST-based proprietary system (Giada s.r.l, Villafranca Piemonte (To), Italy) (Cavallarin et al.,...
2015), specifically created for use in HMBs, was used for HM pasteurization at 72 °C for 15 s. The new instrument is a bench-top device that consists of a system of tubular heat exchangers, which are used for both heating and cooling. The thermostatic stay section has been designed to be isolated, and temperature monitoring is achieved by means of specific digital probes placed at critical control points. The new HTST pasteurizer was designed to meet the specific requirements of safety and efficacy of HMBs: an increase in milk temperature in a few seconds, a thermostatic stay of 15 s (max temperature drop of 1.5 °C during pasteurization) and fast cooling of the pasteurized milk (15 s to achieve a lower temperature than 20 °C).

2.4 Experiment 1 - Challenge tests

2.4.1 Microbiological profiling of the RHM and OHM background microflora.

In order to assess the natural contamination of the milk, the OHM (Pool 1) and RHM (Pool 2) background microflora were determined in duplicate. This process included enumeration of the total aerobic mesophilic viable count (by Plate Count Agar (Merck, Darmstadt, Germany) – method EN/ISO 4833, 2006), Enterobacteriaceae (by Crystal-Violet Neutral-Red Bile Glucose Agar (Merck) - method AFNOR V08-054) and coagulase-positive Staphylococci (by Baird Parker RPF Agar (Biolife Italiana, Milan, Italy) – method EN/ISO 6888, 1999). The same microbiological tests were performed on SHM, to verify the efficacy of the prolonged Holder pasteurization before inoculation.

2.4.2 Bacterial test strains and preparation of the cell suspensions.

*L. monocytogenes* (ATCC 7644), *S. aureus* (ATCC 33862) and *C. sakazakii* (ATCC 51329) were used for the inoculation studies. Working stocks of *L. monocytogenes*, *S. aureus*, and *C. sakazakii* were kept at −20 °C in Brain Heart Infusion nutrient broth (Oxoid Limited, Basingstoke, UK). Nutrient broth was added with 20% glycerol as cryogenic protective agent. Fresh cultures were prepared in their early stationary growth phase for each experiment by inoculating a loop of the
frozen culture in 9 mL of the respective sterile culture medium and then incubating the cultures at 37 °C for 24 h. The microbial counts were monitored by measuring the optical density at 600 nm and confirmed using a culture-based method. The resulting L. monocytogenes, S. aureus and C. sakazakii cell suspensions contained about $10^9$ colon forming units (CFU)/mL (OD 0.90 ± 0.03). The culture-based method involved spreading 0.1 mL of the appropriate serial dilutions in peptone water (Merck) onto specific appropriate media, incubated at 37 °C, and the colonies were counted after 24 h: final concentration of $1.5 \times 10^9$ CFU/mL for L. monocytogenes, $3 \times 10^9$ CFU/mL for S. aureus and $1.6 \times 10^9$ CFU/mL for C. sakazakii were confirmed by means of plate counts on appropriate selective media.

2.4.3 SHM inoculum.

L. monocytogenes, S. aureus and C. sakazakii were inoculated in 500 mL of SHM in sterile Pyrex bottles (see Table 1 for the inoculum loads). Each pathogen was inoculated in a single batch. Inoculated samples (IHM) were kept refrigerated until they were pasteurized, for a maximum of 30 min.

2.4.4 HTST pasteurization of IHM and cleaning of the system.

IHM samples of each pathogen were HTST pasteurized (PHM) and collected in sterile Pyrex bottles. The samples were kept refrigerated for about 20 h before the microbiological analyses. After each pasteurization cycle, the HTST equipment was cleaned by pumping 3% v/v Divoflow (JohnsonDiversey S.p.A., Milan, Italy) detergent solution at 50 °C (1 L) through the system in the CIP (cleaning-in-place) mode for 10 min, and this was followed by tap water for 5 min. The day after each experiment, rinsing water (500 mL) was allowed to recirculate in the system for 10 minutes, collected in a sterile Pyrex bottle and checked for pathogenic growth.

2.4.5 Microbiological analyses of the HTST pasteurized IHM.

Colony counts of PHM were performed in triplicate. As the absence of pathogen growth is required after pasteurization in HMB, undiluted samples were also subjected to microbiological screening.
The presence of *S. aureus* was determined using the method described in 2.4.1. *L. monocytogenes* was evaluated by enriching with Demi-Fraser and Fraser (Sifin, Berlin, Germany) media incubated at 37 °C for 24 h (EN/ISO 11290, 1996); a subsequent growth on OCLA and PALCAM agar mediums (Oxoid) was allowed for both enriching media at 37 °C for 48 h. *C. sakazakii* was determined by measuring the growth on the *Enterobacter sakazakii* Isolation Agar (ESIA) ready-made plates (Liofilchem s.r.l. Roseto degli Abruzzi (Te), Italy) (AFNOR V08-054).

### 2.5 Experiment 2: Evaluation of the biochemical profile of HTST and Holder pasteurized milk.

The RHM, HTST-HM and HoP - HM from trays 1 and 2 of the HMB Holder pasteurizer were assayed to establish the sIgAs content, BSSL and lysozyme activity, as well as the protein profile. The sIgAs were assayed on 1:10,000 diluted samples, using an ELISA kit (Biovendor, Kassel, Germany) following manufacturer's instructions, in triplicate. BSSL activity was measured in triplicate according to a previously described protocol (Bertino et al., 2013), with minor modifications. Briefly, an aliquot of 2 mL of substrate solution (25 M Tris-HCl pH 9.0, 0.25 mM 2-methoxyethanol, 0.53 mM p-nitrophenyl myristate in DMSO, 5 mM sodium cholate) was added to 50 µL of undiluted (for all pasteurized samples) or diluted (1:1000 for RHM) milk samples, mixed and left to incubate at room temperature for 60 min. Then, 1 mL of stop solution (acetone/n-heptane 5:2 vol/vol) was added, and immediately mixed by inversion. After centrifugation for 15 min at 6500rpm at 4 °C, absorbance of the lower aqueous phase was measured at 405 nm. Lysozyme activity was tested in triplicate on 1:200 diluted samples using a fluorescence-based kit (EnzChek Lysozyme Assay Kit, Thermo Fisher Scientific, Waltham, USA) following to the manufacturer's instructions. The assay measures lysozyme activity as the increase in fluorescence resulting from the enzyme activity on *Micrococcus lysodeikticus* cell walls labeled with fluorescein. Active lysozyme enzyme hydrolyzes the b-(1-4)-glucosidic linkages between the N-acetylmuramic acid and N-acetyl-D-glucosamine residues in the mucopolysaccharide cell wall, relieving the quenching
and yielding an increase in fluorescence that is proportional to lysozyme activity. The total protein content was determined in triplicate on HM samples, skimmed by means of centrifugation at 2000g at 4 °C for 30 min, using a 2DQuant kit (GE Healthcare Europe, Milan, Italy), following the manufacturer’s instructions. The protein profile (in reducing and non reducing conditions, 5 µg of proteins) was visualized by monodimensional electrophoresis on a 10-well 12% Nu-PAGE® precast gel, with MES as the running buffer, on a Novex Mini-cell (Thermo Fisher Scientific) at 200 V. The gels were stained with Blue Coomassie Colloidal stain, according to the protocol already described in a previous study (Giribaldi et al., 2013).

2.6 Statistical analyses

Data on the performed biochemical analyses were analysed using the KyPlot 2.0 statistical software (Kyens Lab Inc., Tokyo, Japan), with one-way ANOVA at a significance of below 0.05 to assess significant differences. When the calculated values of F were significant, Tukey’s post-hoc analysis was used to classify any significant difference between the mean values.

3. RESULTS

3.1 Setting up the pasteurization parameters.

The instrument was preliminarily tested on bovine milk in order to assess its suitability for performing a proper HTST pasteurization cycle, and the test was successful. The new HTST system correctly performed a pasteurization cycle (72 °C for 15 s), since the marker enzyme, that is, alkaline phosphatase, resulted to be inactivated in a pasteurized bovine milk sample. A shorter retention time (10 s) was also tested, and was found not to be sufficient to inactivate the alkaline phosphatase in the bovine milk. By setting the pasteurization conditions at 72 °C for 15 s, the lactoperoxidase activity was preserved in a pasteurized bovine milk sample, thus meeting the requirements for the HTST pasteurization of bovine milk.
3.2 Microbiological and challenge tests on HTST pasteurized HM.

In order to assess the natural contamination of the starting donated milk pool (OHM) from the HMB, its background microflora was analysed. The total viable bacteria count (TVC), coagulase positive *Stafilococci* and Enterobacteriaceae loads are reported in Table 2. No *L. monocytogenes* or *C. sakazakii* were found.

After prolonged pasteurization by means of HoP, the absence of vegetative forms of microorganisms was assessed, and the resulting milk was considered “sterile” (SHM). This milk was used as the starting material for the challenge tests. The milk was inoculated (IHM) with *L. monocytogenes*, *S. aureus* and *C. sakazakii* in order to perform the challenge tests; the bacterial counts of the starting inocula are reported in Table 1. PHM did not show any pathogen growth after the HTST pasteurization conducted with the new prototype, in any of the performed challenge tests, as assessed by both qualitative and quantitative methods. At the same time, the rinsing water did not present any growth of the inoculated microorganism. The efficiency of both the Holder and HTST pasteurization was also tested in Experiment 2 (see Figure1B for the experimental design) by measuring the bacterial loads before and after the pasteurization of naturally contaminated milk (Table 1). No bacteria were detected in the milk following either pasteurization procedure.

3.3 Evaluation of the biochemical profile of HTST and Holder pasteurized milk.

The qualitative changes between RHM, HTST-HM and HoP-HM, as performed directly in an HMB device, were determined in Experiment 2 by measuring the sIgAs content, and BSSL and lysozyme activity, and by profiling the protein profile in both reducing and non-reducing conditions. A monodimensional denaturing protein electrophoresis was run on all the samples, in order to profile any major change induced by the different pasteurization methods on the protein pattern. The resulting images are shown in Figure 2. The protein profiles seemed to be very similar, as far as the reducing conditions are used (lanes 1-4), without any visible degradation of the specific bands following any of the pasteurization processes. Accordingly, the lysozyme activity was measured
and no significant difference was found between HTST- and HoP-HM (Tab. 3). In addition, the lysozyme activity value measured in both of the pasteurized samples did not differ significantly from the value found in RHM (50.2±0.2 U/μL). However, the protein profiles in the non-reducing conditions (lanes 6-9) displayed some differences in the high molecular weight bands following all the pasteurization processes.

The sIgAs content of HTST- and HoP-HM was determined and retention rates with respect to RHM were calculated. HoP-HM was found on average to retain 46.3% sIgAs, although a noteworthy difference was observed between the two samples pasteurized in the two different trays (36.8±21.6% vs. 55.8±3.1%). HTST-HM showed a significantly higher sIgAs retention. As for the BSSL activity retention, in both HoP and HTST the enzymatic activity resulted to be almost completely destroyed with respect to RHM (145±22 μmol/mL/min); nevertheless, HTST-HM displayed a significantly higher activity than HoP-HM (Tab. 3). No significant difference in HoP-HM was observed between the two trays (data not shown).

4. DISCUSSION

A new apparatus for the HTST pasteurization of HM has been tested in order to verify its ability to eliminate selected pathogens as well as to assess the effect of pasteurization on the milk protein profile and selected nutritional and immunological parameters. The instrument was preliminarily tested to assess its ability to perform a proper HTST pasteurization cycle on bovine milk, and some commonly used technological parameters confirmed that the test was successful (positive peroxidase and negative alkaline phosphatase reactions in the pasteurized milk). The raw human milk used for the experiment resulted to be highly contaminated by coagulase-positive Staphylococcus spp. Human milk contains commensal bacteria, and in particular non-pathogenic coagulase-negative Staphylococci from the bacterial flora of the maternal skin. The transmission of pathogens, such as Staphylococcus aureus, has also been reported (Heikkila & Saris, 2003). The starting raw milk pools delivered from the bank showed a similar background microflora to that...
already observed in a previous experiment by our group on HM from a Neonatal Intensive Care Unit (Giribaldi et al., 2013).

The milk was completely pasteurized by prolonged Holder treatment, thus constituting SHM for inoculation. The inocula for the challenge tests (*L. monocytogenes, S. aureus* and *C. sakazakii*) were chosen on the grounds of being high-risk pathogens that cause the contamination of milk and neonatal infections. All the pathogens were eliminated by the HTST pasteurization performed with the new instrument. When HoP and HTST were used to eradicate the natural background microflora of the HM in the present study, no bacterial growth was observed, even in the presence of high microbial loads, including Enterobactericeae. These results are in agreement with the first reports that measured the efficacy of HTST on naturally contaminated HM: Goldblum et al. (1984) found complete pasteurization of HM after HTST by using a plate-type industrial heat exchanger and injecting HM in a sterile water stream. Challenge tests by Dhar et al. (1996) and by Terpstra et al. (2007) confirmed the efficacy of small-scale laboratory devices, based on continuous flow HTST pasteurization, on inoculated *Escherichia coli, S. aureus* and *Streptococcus agalactiae*. Terpstra et al. (2007) also investigated the efficacy of HTST on HM samples inoculated with 3 lipid-enveloped and 2 non-enveloped viruses. The system was shown to be highly effective against lipid-enveloped viruses (HIV and marker viruses for hepatitis B and C). The efficacy of a low cost simulated HTST to be used in resource-limited settings (FoneAstra system) has also been reported. The FoneAstra system resulted to be a reliable, low-cost pasteurization monitoring and reporting systems to be used worldwide for treating donor milk (Naicker et al., 2015)

The biochemical parameters used to assess the quality of pasteurized milk were selected from among the most commonly measured in previous studies on the effect of HoP on HM. Attention was focused in particular on parameters that are significant from an immunological and/or nutritional point-of-view, and which had previously been reported to be decreased by HoP pasteurization (Tully et al., 2001). Both sIgAs and BSSL resulted to be significantly higher in the milk pasteurized with the new HTST pasteurizer than in the milk treated with the standard HoP,
conducted according to the HMBs guidelines and using an HMB pasteurizer. sIgAs represent the
majority of IgA in HM (Goldsmith et al., 1983). In early reports, the retention of sIgAs and/or the
total IgAs following HTST was found to be complete (Goldblum et al., 1984), decreased with
respect to raw milk (about 60% for Dhar et al., 1996) and equal to that of HoP with respect to raw
milk (63% according to Goldsmith et al., 1983; 80% for Hamprecht et al., 2004; 40% for Mayayo et
al., 2016). In the present experiment, the amount of HTST-retained IgAs following the treatment of
the milk with the new apparatus was almost twice as high as the IgAs content of the same milk
processed with HoP (as measured in a real HMB implemented pasteurizer), and higher than the
levels reported in previous investigations. However, the results concerning the IgAs content
following the two pasteurization systems need to be confirmed on a larger number of samples, since
it is well documented that the range of variation of IgAs values as affected by Holder pasteurization
is very high (Peila et al., submitted). As for lysozyme activity, no significant difference was found in
the present study in either of pasteurized samples, compared to raw milk. This result is in contrast
with previous findings, which reported a decrease in lysozyme as an effect of HoP (Sousa, Santos,
Fidalgo, Delgadillo, & Saraiva, 2014; Viazis, Farkas, & Allen, 2007), as well as of HTST
pasteurization (Mayayo et al., 2016). In all these reports, lysozyme activity was determined using a
*Micrococcus lysodeikticus*-based turbidimetric assay, which measures to what extent bacterial
growth is prevented by the addition of lysozyme-containing samples. However, HM is a complex
mixture of several anti-bacterial factors, whose degradation may also have an impact on the lower
rates of the measured “lysozyme activity”, when it is measured as previously described. In the
present study, an attempt was made to overcome this problem using an assay in which the specific
lysozyme muramidase activity is measured by quantifying the fluorescence released from labeled
*M. lysodeikticus* cell walls. This difference in the analytical approach can explain the discordance
from literature and points out the need for appropriate analytical tools and a correct interpretation of
the results.
Following both types of pasteurization, BSSL displayed an activity almost 1,000-fold lower in comparison to the activity recorded in the RHM sample. The almost complete disruption of lipase activity following any pasteurization treatment, confirms previous reports (Goldblum et al., 1984; Hamprecht et al. 2004), although the measured activity in those investigations was not exclusively due to BSSL. The lipase activity in the present study has been much lower than that observed in a previous study by our group (Baro et al., 2011). Again in that case, lipase activity was measured without the induction of bile salts. Nevertheless, the BSSL bioactivity resulted to be significantly higher in the HTST treated samples than in the HoP samples.

As far as the protein profile modification in pasteurized HM is concerned, no qualitative difference was found between RHM, HoP and HTST in the reducing conditions. This result is in contrast with previous findings of our group (Baro et al., 2011) and of Mayayo et al. (2014) for HoP, which were obtained in non-reducing electrophoretic conditions. Conformational differences, if any, may be detected more easily when non-reducing electrophoresis is used. This is confirmed by the fact that Mayayo et al. (2014) were not able to profile any change in the protein pattern following HoP when reducing conditions were used. Therefore, a non-reducing electrophoresis was run, and some slight degree of polymerization for high molecular weight protein bands was observed (probably ascribable to lactoferrin and immunoglobulins), thus confirming the results of Baro et al. (2011) and Mayayo et al. (2014).

In conclusion, the new bench-top HTST apparatus can effectively pasteurize HM with a better retention of the slgas content and BSSL activity, compared to standard Holder pasteurization.
REFERENCES


### TABLE 1. Challenge tests: effect of HTST pasteurization on human milk.

<table>
<thead>
<tr>
<th>Inoculated pathogens</th>
<th>Initial loads in IHM&lt;sup&gt;1&lt;/sup&gt; (CFU/mL)</th>
<th>Final loads in PHM&lt;sup&gt;1&lt;/sup&gt; (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>$1.2 \times 10^6$</td>
<td>absent in 25 ml</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>$3.0 \times 10^6$</td>
<td>$&lt; 100$</td>
</tr>
<tr>
<td><em>Chronobacter sakazakii</em></td>
<td>$1.6 \times 10^6$</td>
<td>absent in 10 ml</td>
</tr>
</tbody>
</table>

<sup>1</sup> IHM = inoculated human milk; PHM = HTST pasteurized milk.

### TABLE 2. Background microflora of human milk (starting Pools 1 and 2)

<table>
<thead>
<tr>
<th></th>
<th>Pool 1 (OHM)&lt;sup&gt;1&lt;/sup&gt; (CFU/mL)</th>
<th>Pool 2 (RHM)&lt;sup&gt;1&lt;/sup&gt; (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total viable count</td>
<td>$1.0 \times 10^6$</td>
<td>$7.7 \times 10^4$</td>
</tr>
<tr>
<td>Coagulase-positive <em>Staphylococci</em></td>
<td>$2.5 \times 10^5$</td>
<td>$1.1 \times 10^4$</td>
</tr>
<tr>
<td><em>Enterobacteriaceae</em></td>
<td>$&lt; 10$</td>
<td>$2.1 \times 10^2$</td>
</tr>
</tbody>
</table>

<sup>1</sup> OHM = original human milk for Experiment 1; RHM = raw human milk for Experiment 2

### TABLE 3. Nutritional milk characteristics as affected by Holder and HTST pasteurization.

<table>
<thead>
<tr>
<th>Parameter (Unit)</th>
<th>HoP-HM&lt;sup&gt;1&lt;/sup&gt;</th>
<th>HTST-HM&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>sIGAs (% of RHM&lt;sup&gt;1&lt;/sup&gt;)</td>
<td>$46.3\pm13.2^a$</td>
<td>$78.9\pm2.4^b$</td>
</tr>
<tr>
<td>BSSL activity (μmol/min/mL)</td>
<td>$0.09\pm0.03^a$</td>
<td>$0.26\pm0.10^b$</td>
</tr>
<tr>
<td>Lysozyme activity (U/μL)</td>
<td>$52.3\pm3.2$</td>
<td>$48.8\pm0.8$</td>
</tr>
</tbody>
</table>

<sup>1</sup> HoP-HM = Holder pasteurized milk; HTST-HM = HTST pasteurized milk; RHM = raw milk
The data represent the standard deviations of the means obtained from three independent replicate experiments. Different superscript letters indicate significant Tukey's post-hoc mean differences across each row.
FIGURE 1. Experimental design and workflow for Experiment 1 (Panel A) and Experiment 2 (Panel B).

FIGURE 2. NuPAGE protein profile of the HM samples treated with the different pasteurization processes. Lanes 1 to 4, samples run in reducing conditions: 1) RHM = raw milk; 2) HoP-HM = Holder pasteurized milk Tray 1; 3) HoP-HM = Holder pasteurized milk Tray 2; 4) HTST-HM = HTST pasteurized milk; 5) Molecular weight standards. Lanes 6 to 9, samples run in non-reducing conditions: 6) RHM = raw milk; 7) HoP-HM = Holder pasteurized milk Tray 1; 8) HoP-HM = Holder pasteurized milk Tray 2; 9) HTST-HM = HTST pasteurized milk. Colloidal Coomassie Blue stain.