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

This work was supported by the Fondazione Cassa di Risparmio di Torino [grant number 2014.0850]; and the Italian Association of Donor Milk Banks.

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, García-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

105 stainless steel tubing systems submerged in thermostated water baths, through which HM was
106 pumped (Dhar, Fichtali, Skura, Nakai, & Davidson, 1996; Goldsmith, Eitenmiller, Toledo, &
107 Barnhart, 1983; Terpstra et al., 2007); others injected milk through a sterile water stream in a plate-
108 type industrial heat exchanger (Goldblum et al., 1984); some research studies were conducted by
109 directly heating and rotating small aliquots of milk, to simulate the typical thin-layering of dairy
110 industry HTST devices (Goeltz et al., 2009; Hamprecht et al., 2004). Several studies involved
111 simply heating small aliquots (from 40 μL to 4 mL) of HM in a bulk process (Mayayo et al., 2014;
112 Mayayo et al., 2016; Silvestre et al., 2006; Silvestre et al., 2008). Moreover, variable heating times
113 (5-15 s) and temperatures (71-75 °C) were adopted. All the reported processes, with the exception
114 of that of Goldblum et al. (1984), are substantially different from industrial HTST processes.
115 However, the introduction of HTST into the HMB routine has not been possible to date, due to the
116 lack of specific low-volume designed instrumentation. In most of studies concerning the HTST
117 processing of HM, quantitative comparisons, with respect to standard pasteurization, have been
118 described, but HoP was often simulated on small aliquots, rather than being performed according to
119 real HMB-implemented protocols, thus representing a possible bias for the generalization of the
120 comparison with novel technologies (Peila et al., 2015).
121 When projecting, creating, testing and patenting a new type of low-volume HTST pasteurizer
122 (Cavallarin et al., 2015), it was considered that: i) it was intended for HMBs, and thus consistency
123 with guideline requirements was mandatory; ii) the dairy industry HTST standards (72 °C holding
124 temperature for 15 s holding time) had to be fulfilled; iii) temperature control had to be ensured by
125 means of adequate probes; iv) a comparison with the HoP process had to be made using the HMB
126 implemented device.
127 The present research is aimed at reporting the efficacy of a new, low-volume, continuous flow
128 commercial HTST pasteurizer on HM quality in terms of: (i) bacterial inactivation, and (ii)
129 preservation of the main immunological and nutritional components.
130

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.

156 The HM samples were pooled separately for the two experiments, which were referred to as
157 Experiment 1 and Experiment 2. Panel A and panel B in Figure 1 show the experimental workflows
158 for Experiment 1 and Experiment 2, respectively.
159 In Experiment 1, frozen samples from individual HM donors were thawed overnight in a
160 refrigerator, and then in tap water, and were pooled to achieve a final volume of about 2 L in a
161 sterile Pyrex bottle. They were then mixed carefully, divided into 100 mL aliquots and placed in
162 sterile bisphenol-free polypropylene bottles. One aliquot of original HM (OHM) was used directly
163 to determine the HM background microflora. The remaining aliquots were pasteurized intensively
164 in a water bath for 1 hour at 63 °C (prolonged Holder Pasteurization) to kill all of the existing
165 vegetative forms of microorganisms (SHM). One aliquot was kept for about 20 h in the refrigerator,
166 and then analysed to verify the absence of microbial contaminants. The other aliquots were
167 immediately frozen and used later for inoculation (IHM) (section 2.4.3).
168 In Experiment 2, samples from individual HM donors were obtained frozen from the HMB, thawed
169 overnight in a refrigerator, and then in tap water, and were pooled to achieve a final volume of
170 about 400 mL in a sterile Pyrex bottle. They were mixed carefully and divided into three sterile
171 bisphenol-free polypropylene bottles. Two aliquots were subjected to standard HoP, in two separate
172 trays, in the HMB facilitiesThe two samples were processed in the same batch and differed only for
173 their position in the pasteurizer. One aliquot was subjected to HTST pasteurization using the new
174 instrument. The Raw (RHM), Holder (HoP-HM) and HTST (HTST-HM) pasteurized samples were
175 kept frozen at -20°C until analysis, with the exception of 10 mL *per* sample, which was kept
176 refrigerated for about 20 h before being used for microbial screening.
177

178 **2.3 Pasteurization equipment**

179 HoP was performed in an HM pasteurizer (Metallarredinox, Zingonia-Verdellino (Bg), Italy)
180 located in the HMB of the Città della Scienza e della Salute in Torino, Italy. A patent pending
181 HTST- based proprietary system (Giada s.r.l, Villafranca Piemonte (To), Italy) (Cavallarin et al.,

182 2015), specifically created for use in HMBs, was used for HM pasteurization at 72 °C for 15 s. The
183 new instrument is a bench-top device that consists of a system of tubular heat exchangers, which are
184 used for both heating and cooling. The thermostatic stay section has been designed to be isolated,
185 and temperature monitoring is achieved by means of specific digital probes placed at critical control
186 points. The new HTST pasteurizer was designed to meet the specific requirements of safety and
187 efficacy of HMBs: an increase in milk temperature in a few seconds, a thermostatic stay of 15 s
188 (max temperature drop of 1.5 °C during pasteurization) and fast cooling of the pasteurized milk (15
189 s to achieve a lower temperature than 20 °C).
190
191 **2.4 Experiment 1- Challenge tests**
192 *2.4.1 Microbiological profiling of the RHM and OHM background microflora.*
193 In order to assess the natural contamination of the milk, the OHM (Pool 1) and RHM (Pool 2)
194 background microflora were determined in duplicate. This process included enumeration of the total
195 aerobic mesophilic viable count (by Plate Count Agar (Merck, Darmstadt, Germany) – method
196 EN/ISO 4833, 2006), Enterobacteriaceae (by Crystal-Violet Neutral-Red Bile Glucose Agar
197 (Merck) - method AFNOR V08-054) and coagulase-positive *Staphylococci* (by Baird Parker RPF
198 Agar (Biolife Italiana, Milan, Italy) – method EN/ISO 6888, 1999). The same microbiological tests
199 were performed on SHM, to verify the efficacy of the prolonged Holder pasteurization before
200 inoculation.
201
202 *2.4.2 Bacterial test strains and preparation of the cell suspensions.*
203 *L. monocytogenes* (ATCC 7644), *S. aureus* (ATCC 33862) and *C. sakazakii* (ATCC 51329) were
204 used for the inoculation studies. Working stocks of *L. monocytogenes, S. aureus,* and *C. sakazakii*
205 were kept at −20 °C in Brain Heart Infusion nutrient broth (Oxoid Limited, Basingstoke, UK).
206 Nutrient broth was added with 20% glycerol as cryogenic protective agent. Fresh cultures were
207 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 x 10^9 CFU/mL for *L. monocytogenes*, 3x10^9 CFU/mL for *S. aureus* and 1.6 x 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.

233 The presence of *S. aureus* was determined using the method described in 2.4.1. *L. monocytogenes*
234 was evaluated by enriching with Demi-Fraser and Fraser (Sifin, Berlin, Germany) media incubated
235 at 37 ºC for 24 h (EN/ISO 11290, 1996); a subsequent growth on OCLA and PALCAM agar
236 mediums (Oxoid) was allowed for both enriching media at 37 ºC for 48 h. *C. sakazakii* was
237 determined by measuring the growth on the *Enterobacter sakazakii* Isolation Agar (ESIA) ready-
238 made plates (Liofilchem s.r.l. Roseto degli Abruzzi (Te), Italy) (AFNOR V08-054).
239
240 **2.5 Experiment 2: Evaluation of the biochemical profile of HTST and Holder pasteurized**
241 **milk.**
242 The RHM, HTST-HM and HoP - HM from trays 1 and 2 of the HMB Holder pasteurizer were
243 assayed to establish the sIgAs content, BSSL and lysozyme activity, as well as the protein profile.
244 The sIgAs were assayed on 1:10,000 diluted samples, using an ELISA kit (Biovendor, Kassel,
245 germany) following manufacturer's instructions, in triplicate. BSSL activity was measured in
246 triplicate according to a previously described protocol (Bertino et al., 2013), with minor
247 modifications. Briefly, an aliquot of 2 mL of substrate solution (25 M Tris-HCl pH 9.0, 0.25 mM 2-
248 methoxyethanol, 0.53 mM p-nitrophenyl myristate in DMSO, 5 mM sodium cholate) was added to
249 50 µL of undiluted (for all pasteurized samples) or diluted (1:1000 for RHM) milk samples, mixed
250 and left to incubate at room temperature for 60 min. Then, 1 mL of stop solution (acetone/n-heptane
251 5:2 vol/vol) was added, and immediately mixed by inversion. After centrifugation for 15 min at
252 6500*rpm* at 4 °C, absorbance of the lower aqueous phase was measured at 405 nm. Lysozyme
253 activity was tested in triplicate on 1:200 diluted samples using a fluorescence-based kit (EnzChek
254 Lysozyme Assay Kit, Thermo Fisher Scientific, Waltham, USA) following to the manufacturer's
255 instructions. The assay measures lysozyme activity as the increase in fluorescence resulting from
256 the enzyme activity on *Micrococcus lysodeikticus* cell walls labeled with fluorescein . Active
257 lysozyme enzyme hydrolyzes the b-(1-4)-glucosidic linkages between the N-acetylmuramic acid
258 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 2000*g* 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 mono dimensional 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.

285 **3.2 Microbiological and challenge tests on HTST pasteurized HM.**

286 In order to assess the natural contamination of the starting donated milk pool (OHM) from the
287 HMB, its background microflora was analysed. The total viable bacteria count (TVC), coagulase
288 positive *Stafilococci* and Enterobacteriaceae loads are reported in Table 2. No *L. monocytogenes* or
289 *C. sakazakii* were found.
290 After prolonged pasteurization by means of HoP, the absence of vegetative forms of
291 microorganisms was assessed, and the resulting milk was considered "sterile" (SHM). This milk
292 was used as the starting material for the challenge tests. The milk was inoculated (IHM) with *L.*
293 *monocytogenes, S. aureus* and *C. sakazakii* in order to perform the challenge tests; the bacterial
294 counts of the starting inocula are reported in Table 1. PHM did not show any pathogen growth after
295 the HTST pasteurization conducted with the new prototype, in any of the performed challenge tests,
296 as assessed by both qualitative and quantitative methods. At the same time, the rinsing water did not
297 present any growth of the inoculated microorganism. The efficiency of both the Holder and HTST
298 pasteurization was also tested in Experiment 2 (see Figure1B for the experimental design) by
299 measuring the bacterial loads before and after the pasteurization of naturally contaminated milk
300 (Table 1). No bacteria were detected in the milk following either pasteurization procedure.
301

302 **3.3 Evaluation of the biochemical profile of HTST and Holder pasteurized milk.**

303 The qualitative changes between RHM, HTST-HM and HoP-HM, as performed directly in an HMB
304 device, were determined in Experiment 2 by measuring the sIgAs content, and BSSL and lysozyme
305 activity, and by profiling the protein profile in both reducing and non-reducing conditions.
306 A monodimensional denaturing protein electrophoresis was run on all the samples, in order to
307 profile any major change induced by the different pasteurization methods on the protein pattern.
308 The resulting images are shown in Figure 2. The protein profiles seemed to be very similar, as far as
309 the reducing conditions are used (lanes 1-4), without any visible degradation of the specific bands
310 following any of the pasteurization processes. Accordingly, the lysozyme activity was measured

311 and no significant difference was found between HTST- and HoP-HM (Tab. 3). In addition, the
312 lysozyme activity value measured in both of the pasteurized samples did not differ significantly
313 from the value found in RHM (50.2±0.2 U/μL). However, the protein profiles in the non-reducing
314 conditions (lanes 6-9) displayed some differences in the high molecular weight bands following all
315 the pasteurization processes.
316 The sIgAs content of HTST- and HoP-HM was determined and retention rates with respect to RHM
317 were calculated. HoP-HM was found on average to retain 46.3% sIgAs, although a noteworthy
318 difference was observed between the two samples pasteurized in the two different trays
319 (36.8±21.6% vs. 55.8±3.1%). HTST-HM showed a significantly higher sIgAs retention. As for the
320 BSSL activity retention, in both HoP and HTST the enzymatic activity resulted to be almost
321 completely destroyed with respect to RHM (145±22 μmol/mL/min); nevertheless, HTST-HM
322 displayed a significantly higher activity than HoP-HM (Tab. 3). No significant difference in HoP-
323 HM was observed between the two trays (data not shown).
324
325 **4. DISCUSSION**
326 A new apparatus for the HTST pasteurization of HM has been tested in order to verify its ability to
327 eliminate selected pathogens as well as to assess the effect of pasteurization on the milk protein
328 profile and selected nutritional and immunological parameters. The instrument was preliminarily
329 tested to assess its ability to perform a proper HTST pasteurization cycle on bovine milk, and some
330 commonly used technological parameters confirmed that the test was successful (positive
331 peroxidase and negative alkaline phosphatase reactions in the pasteurized milk). The raw human
332 milk used for the experiment resulted to be highly contaminated by coagulase-positive
333 *Staphylococcus* spp. Human milk contains commensal bacteria, and in particular non-pathogenic
334 coagulase-negative *Staphylococci* from the bacterial flora of the maternal skin. The transmission of
335 pathogens, such as *Staphylococcus aureus,* has also been reported (Heikkila & Saris, 2003). The
336 starting raw milk pools delivered from the bank showed a similar background microflora to that

337 already observed in a previous experiment by our group on HM from a Neonatal Intensive Care
338 Unit (Giribaldi et al., 2013).
339 The milk was completely pasteurized by prolonged Holder treatment, thus constituting SHM for
340 inoculation. The inocula for the challenge tests (*L. monocytogenes, S. aureus* and *C. sakazakii)* were
341 chosen on the grounds of being high-risk pathogens that cause the contamination of milk and
342 neonatal infections. All the pathogens were eliminated by the HTST pasteurization performed with
343 the new instrument. When HoP and HTST were used to eradicate the natural background microflora
344 of the HM in the present study, no bacterial growth was observed, even in the presence of high
345 microbial loads, including Enterobactericeae. These results are in agreement with the first reports
346 that measured the efficacy of HTST on naturally contaminated HM: Goldblum et al. (1984) found
347 complete pasteurization of HM after HTST by using a plate-type industrial heat exchanger and
348 injecting HM in a sterile water stream. Challenge tests by Dhar et al. (1996) and by Terpstra et al.
349 (2007) confirmed the efficacy of small-scale laboratory devices, based on continuous flow HTST
350 pasteurization, on inoculated *Escherichia coli, S. aureus* and *Streptococcus agalactiae.* Terpstra et
351 al. (2007) also investigated the efficacy of HTST on HM samples inoculated with 3 lipid-enveloped
352 and 2 non-enveloped viruses. The system was shown to be highly effective against lipid-enveloped
353 viruses (HIV and marker viruses for hepatitis B and C). The efficacy of a low cost simulated HTST
354 to be used in resource-limited settings (FoneAstra system) has also been reported. The FoneAstra
355 system resulted to be a reliable, low-cost pasteurization monitoring and reporting systems to be
356 used worldwide for treating donor milk (Naicker et al., 2015)
357 The biochemical parameters used to assess the quality of pasteurized milk were selected from
358 among the most commonly measured in previous studies on the effect of HoP on HM. Attention
359 was focused in particular on parameters that are significant from an immunological and/or
360 nutritional point-of-view, and which had previously been reported to be decreased by HoP
361 pasteurization (Tully et al., 2001). Both sIgAs and BSSL resulted to be significantly higher in the
362 milk pasteurized with the new HTST pasteurizer than in the milk treated with the standard HoP,

363 conducted according to the HMBs guidelines and using an HMB pasteurizer. sIgAs represents the
364 majority of IgA in HM (Goldsmith et al., 1983). In early reports, the retention of sIgAs and /or the
365 total IgAs following HTST was found to be complete (Goldblum et al., 1984), decreased with
366 respect to raw milk (about 60% for Dhar et al., 1996) and equal to that of HoP with respect to raw
367 milk (63% according to Goldsmith et al., 1983; 80% for Hamprecht et al., 2004; 40% for Mayayo et
368 al., 2016). In the present experiment, the amount of HTST-retained IgAs following the treatment of
369 the milk with the new apparatus was almost twice as high as the IgAs content of the same milk
370 processed with HoP (as measured in a real HMB implemented pasteurizer), and higher than the
371 levels reported in previous investigations. However, the results concerning the IgAs content
372 following the two pasteurization systems need to be confirmed on a larger number of samples, since
373 it is well documented that the range of variation of IgAs values as affected by Holder pasteurization
374 is very high (Peila et al., submitted).As for lysozyme activity, no significant difference was found in
375 the present study in either of pasteurized samples, compared to raw milk. This result is in contrast
376 with previous findings, which reported a decrease in lysozyme as an effect of HoP (Sousa, Santos,
377 Fidalgo, Delgadillo, & Saraiva, 2014; Viazis, Farkas, & Allen, 2007), as well as of HTST
378 pasteurization (Mayayo et al., 2016). In all these reports, lysozyme activity was determined using a
379 *Micrococcus lysodeikticus*-based turbidimetric assay, which measures to what extent bacterial
380 growth is prevented by the addition of lysozyme-containing samples. However, HM is a complex
381 mixture of several anti-bacterial factors, whose degradation may also have an impact on the lower
382 rates of the measured "lysozyme activity", when it is measured as previously described. In the
383 present study, an attempt was made to overcome this problem using an assay in which the specific
384 lysozyme muramidase activity is measured by quantifying the fluorescence released from labeled
385 *M. lysodeikticus* cell walls. This difference in the analytical approach can explain the discordance
386 from literature and points out the need for appropriate analytical tools and a correct interpretation of
387 the results.

388 Following both types of pasteurization, BSSL displayed an activity almost 1,000-fold lower in
389 comparison to the activity recorded in the RHM sample. The almost complete disruption of lipase
390 activity following any pasteurization treatment, confirms previous reports (Goldblum et al., 1984;
391 Hamprecht et al. 2004), although the measured activity in those investigations was not exclusively
392 due to BSSL. The lipase activity in the present study has been much lower than that observed in a
393 previous study by our group (Baro et al., 2011). Again in that case, lipase activity was measured
394 without the induction of bile salts. Nevertheless, the BSSL bioactivity resulted to be significantly
395 higher in the HTST treated samples than in the HoP samples.
396 As far as the protein profile modification in pasteurized HM is concerned, no qualitative difference
397 was found between RHM, HoP and HTST in the reducing conditions. This result is in contrast with
398 previous findings of our group (Baro et al., 2011) and of Mayayo et al. (2014) for HoP, which were
399 obtained in non-reducing electrophoretic conditions. Conformational differences, if any, may be
400 detected more easily when non-reducing electrophoresis is used. This is confirmed by the fact that
401 Mayayo et al. (2014) were not able to profile any change in the protein pattern following HoP when
402 reducing conditions were used. Therefore, a non-reducing electrophoresis was run, and some slight
403 degree of polymerization for high molecular weight protein bands was observed (probably
404 ascribable to lactoferrin and immunoglobulins), thus confirming the results of Baro et al. (2011) and
405 Mayayo et al. (2014). In conclusion, the new bench-top HTST apparatus can effectively pasteurize
406 HM with a better retention of the sIgas content and BBSL activity, compared to standard Holder
407 pasteurization.

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

Inoculated pathogens	**Initial loads in IHM[1]** (CFU/mL)	**Final loads in PHM[1]** (CFU/mL)
Listeria monocytogenes	1.2 x 10^6	absent in 25 ml
Staphylococcus aureus	3.0 x 10^6	< 100
Chronobacter sakazakii	1.6 x 10^6	absent in 10 ml

504 [1] IHM = inoculated human milk; PHM = HTST pasteurized milk.

505

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

	Pool 1 (OHM)[1] (CFU/mL)	**Pool 2 (RHM)[1]** (CFU/mL)
Total viable count	1.0 x 10^6	7.7 x 10^4
Coagulase-positive *Staphylococci*	2.5 x 10^5	1.1 x 10^4
Enterobacteriaceae	< 10	2.1 x 10^2

507 [1] OHM = original human milk for Experiment 1; RHM = raw human milk for Experiment 2

508

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

Parameter (Unit)	**HoP-HM[1]**	**HTST-HM[1]**
sIGAs (% of RHM[1])	46.3±13.2[a]	78.9±2.4[b]
BSSL activity (μmol/min/mL)	0.09±0.03[a]	0.26±0.10[b]
Lysozyme activity (U/μL)	52.3±3.2	48.8±0.8

510 [1] HoP-HM = Holder pasteurized milk; HTST-HM = HTST pasteurized milk; RHM = raw milk

511 [a] The data represent the standard deviations of the means obtained from three independent replicate
512 experiments. Different superscript letters indicate significant Tukey's post-hoc mean differences
513 across each row.
514
515

Figure Captions

FIGURE 1. Experimental design and workflow for Experiment 1 (Panel A) and Experiment 2 (Panel B).

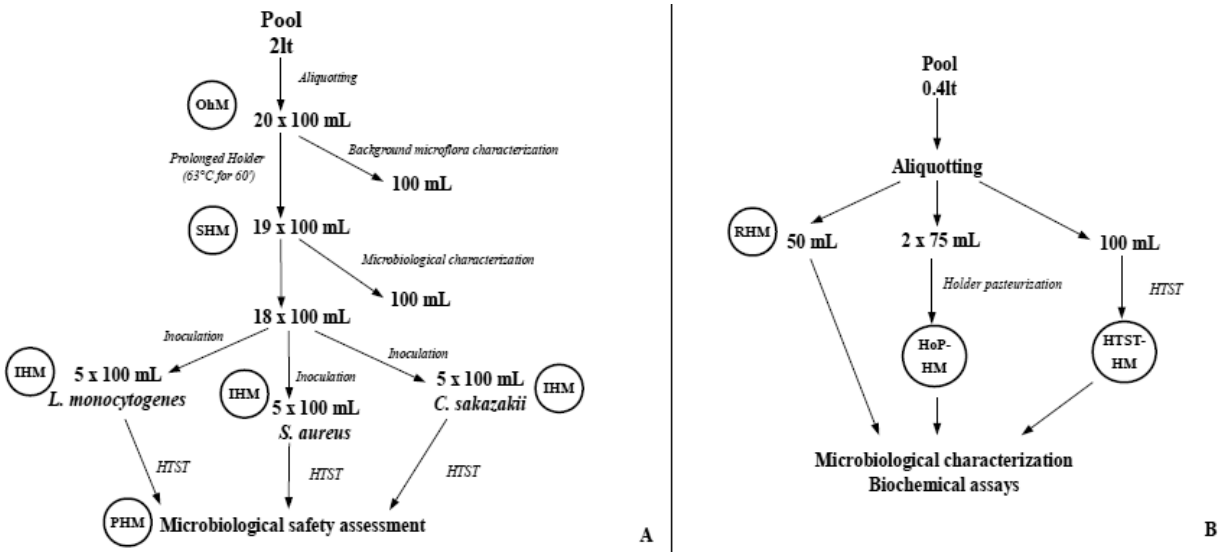
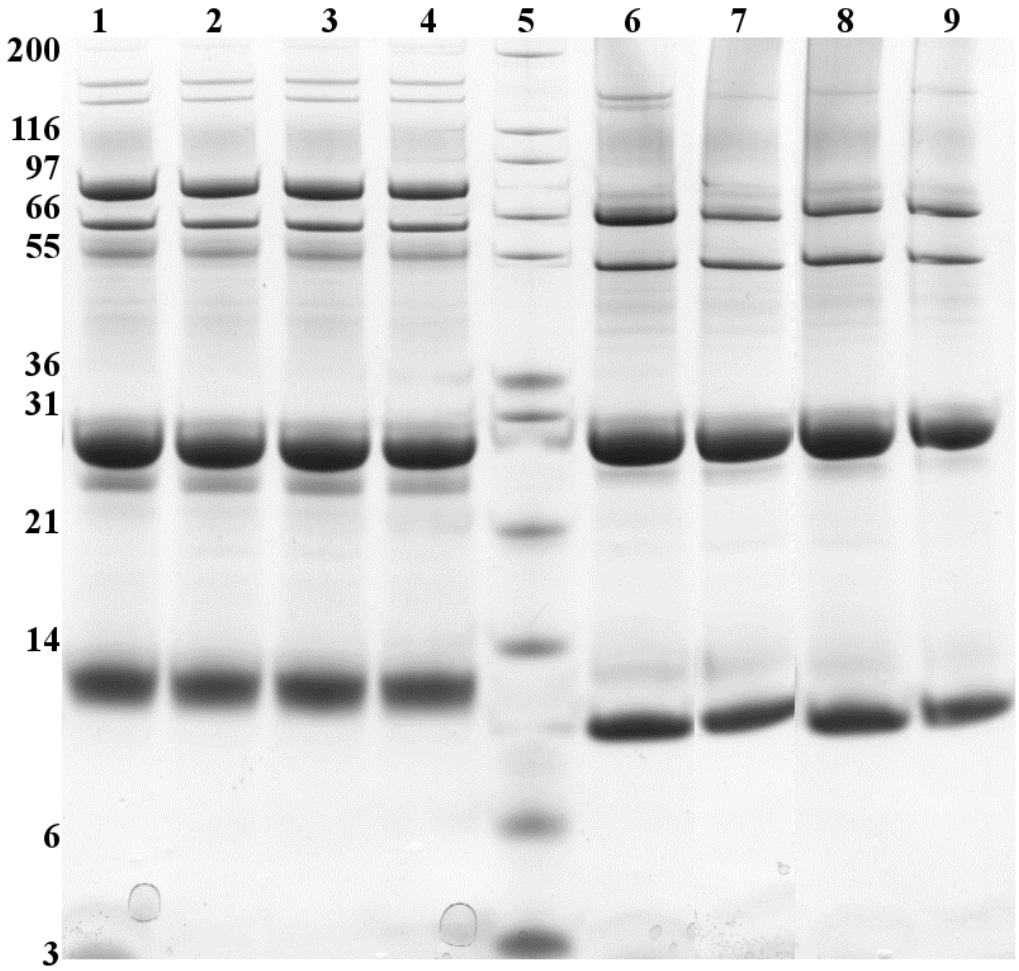


FIGURE 2. NuPAGE protein profile of the HM samples treated with the different pasteurization processes. Lanes 1 to 4, samples run in reducing conditions: 1) RHM = raw milk; 2) HoP-HM = Holder pasteurized milk Tray 1; 3) HoP-HM = Holder pasteurized milk Tray 2; 4) HTST-HM = HTST pasteurized milk; 5) Molecular 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.



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