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Effect of Alternative Pasteurization Techniques on Human Milk's Bioactive Proteins

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Aceti, A., Cavallarin, L., Martini, S., Giribaldi, M., Vitali, F., Ambretti, S., et al. (2020). Effect of Alternative Pasteurization Techniques on Human Milk's Bioactive Proteins. *JOURNAL OF PEDIATRIC GASTROENTEROLOGY AND NUTRITION*, 70(4), 508-512 [10.1097/MPG.0000000000002598].

Availability:

This version is available at: <https://hdl.handle.net/11585/789483> since: 2021-01-18

Published:

DOI: <http://doi.org/10.1097/MPG.0000000000002598>

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(Article begins on next page)

Effect of alternative pasteurization techniques on human milk's bioactive proteins

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Word count: 2511

Number of figures: 2

Number of tables: 1

Conflicts of Interests and Source of Funding: L.Ca. and M.G. are the inventors of a pending patent on the HTST pasteurizer for human milk used in the paper (Patent no. EP 15176792.8-1358). For the remaining authors, no conflict of interest is declared.

The work presented in the present paper was supported by the Italian Ministry of Education, University and Research Agrifood National Cluster in the frame of the project “PROS.IT- Promotion of consumer’s health: nutritional implementation of Italian agroindustrial products”.

Authors’ contributions

A.A., L.Ca., M.G., V.Z., and L.Co. conceptualized the study.

A.A., L.Ca., M.G., and L.Co. discussed and agreed on study methodology.

L.Ca., M.G. and S.A. performed data analysis.

S.M. and F.V. were in charge of data curation.

A.A., L.Ca. and M.G. wrote the original draft of the paper.

A.A., L.Ca., S.M., M.G., F.V., S.A., V.Z., and L.Co all contributed to paper’s review and editing.

Each author has approved the submitted version and agrees to be personally accountable for the author’s own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature.

1 **Abstract**

2 *Objectives*

3 Human milk (HM) feeding leads to improved outcome for preterm infants. When mother's milk is
4 unavailable, pasteurized donor HM (DHM) is the recommended alternative over formula. The
5 Holder pasteurization (HoP) method is universally performed in HM banks; however, it is known to
6 impair several functional HM components.

7 The aim of this study was to compare the efficacy of HoP vs. two innovative processing methods
8 (High-Temperature-Short-Time [HTST] pasteurization and High-Pressure-Processing [HPP]) in
9 preserving some bioactive HM protein components.

10 *Methods*

11 HM samples from donors of the Bologna HM bank were collected and divided into 4 subsamples:
12 one was kept raw, and each of the others was processed using a different technique (HoP, HTST,
13 and HPP). Total protein (TPC), secretory immunoglobulin A (sIgA), and lactoferrin content were
14 compared.

15 *Results*

16 Both HM lactoferrin and sIgA content were negatively affected, but to a different extent, by each
17 method: sIgA was better preserved by HTST (-23.3%) with only HPP leading to a significant
18 reduction (-35.8%); lactoferrin content was strongly reduced by HoP (-87.5%) and HTST (-83.5%),
19 and preserved by HPP (-24.7%). Variations in protein profile were seen for all processing methods,
20 being more relevant for HoP, followed by HTST and, finally, by HPP. All the three methods
21 reduced starting HM microbial counts to undetectable level.

22 *Conclusions*

23 Both HTST and HPP better preserved the original HM protein profile, compared to HoP. However,
24 they affected differently some bioactive HM components involved in immune response and
25 antibacterial activity.

26

27 **Keywords**

28 Donor human milk, human milk pasteurization, protein profile, lactoferrin, secretory IgAs

29

30 **What is known**

31 • Donor human milk (DHM) represents the best alternative to own mother's milk for preterm
32 infants

33 • To comply with microbiological safety standards, DHM is usually pasteurized in HM banks
34 using the Holder (HoP) method, which is known to impair several DHM bioactive properties

35 • Pasteurization methods alternative to HoP such as high-temperature-short-time (HTST) and
36 high-pressure-processing (HPP) are currently under investigation

37

38 **What is new**

39 • Both HTST and HPP comply with microbiological safety standards

40 • Secretory immunoglobulin A are better preserved by HTST; lactoferrin content is strongly
41 reduced by both HoP and HTST, and preserved by HPP.

42 • The highest variations in protein profile are seen after HoP.

43

44

45 *Introduction*

46 Human milk (HM) represents the optimal feeding for preterm infants, especially for those born with
47 a very low (<1500 g) birth weight (1). Mother's own milk is uniquely tailored for each newborn,
48 both in its nutritional composition and in the non-nutritive bioactive factors that promote survival
49 and healthy development (2). When mother's own milk is unavailable or insufficient, the use of
50 donor HM (DHM) is recommended (3): recent studies have shown that DHM-feeding is associated
51 with a reduction of neonatal morbidities including necrotizing enterocolitis (4,5). Furthermore, it
52 has been documented that the availability of DHM has a paradoxical beneficial effect in increasing
53 the rates of breastfeeding among mothers who deliver prematurely (4,6). To ensure its quality and
54 safety, DHM must be provided by a HM bank (HMB) (3): specific recommendations for the
55 preparation, pasteurization, and distribution of DHM exist in many countries worldwide (7–12) . As
56 for the process of pasteurization, the Holder pasteurization (HoP) method is universally
57 recommended by all HMBs, because, at present, it is the only method for which validated devices
58 are commercially available, and for which an extensive amount of evidence on safety and efficacy
59 exists (8,13–15). However, it is well known that HoP, which is a thermal process performed on
60 bottled DHM at 62.5°C for 30 minutes, followed by fast cooling to a temperature below 4°C,
61 impairs several functional HM components, including immunoglobulins (Ig), lactoferrin, lipases, as
62 well as other enzymatic activities, some cytokines and some vitamins (8,13–15). For this reason,
63 further research is currently being aimed at testing pasteurization methods alternative to HoP, which
64 would be capable of retaining the largest variety of HM bioactive properties to the highest extent,
65 without affecting DHM microbiological safety (8,13–16).

66 Several alternative pasteurization methods are being investigated in recent years: these include
67 thermal processes, such as High-Temperature-Short-Time pasteurization (HTST), and non-thermal
68 methods, such as High-Pressure Processing (HPP) and ultraviolet irradiation, as well mixed
69 techniques, such as (thermo-)ultrasonic processing (8,13,15–17). HTST and HPP are considered as
70 the most promising alternatives to HoP for DHM (16,17), being the most widely studied at present.

71 The main limitation to their applicability as routine pasteurization techniques in HMBs is the lack
72 of specific instrumentation, validated in relevant HMB environment. This technical gap is being
73 progressively filled, since specific devices to be operated with small milk volumes have been
74 recently developed for the use in HMBs (18,19).

75 The aim of the present study is to contribute to the innovation in the field by directly comparing the
76 efficacy of the two most promising techniques, HTST and HPP, to standard HoP. To this aim,
77 prototyped pasteurization equipment and commercial devices are used. Changes in the protein
78 profile of DHM, as well as specific HM bioactive components (lactoferrin and secretory IgA
79 content [sIgA]), are addressed.

80

81 *Methods*

82 • *Ethics*

83 The study protocol was approved by the Ethical Committee of Sant'Orsola-Malpighi Hospital,
84 Bologna, Italy (study # 165/2015/U/Tess). HM samples were collected from donors of the Bologna
85 HMB, after signing an informed written consent.

86

87 • *Collection of HM samples*

88 HM samples were collected, approximately 3 months after delivery, from five HM donors
89 following the requirements of the Bologna HMB. Mothers were asked to express milk by a breast
90 pump after carefully washing their hands and breast. HM was collected using sterile, single-use
91 breast pump kits into sterile polypropylene bottles. HM samples were stored at -20°C at the HMB
92 until processed, and then handled following the routine protocol used for DHM (11): they were
93 removed from the freezer several hours before the analyses and processed only when completely
94 thawed.

95

96 • *HM processing*

97 HM pools (400 ml each) were obtained from individual donors by collecting milk during few
98 consecutive days. Each milk pool was divided into four sub-samples (100 ml each): one sample was
99 not pasteurized, one sample was pasteurized by HoP, one by HTST, and the last by HPP. Each sub-
100 sample was analysed for selected indicators of protein and microbial quality. HoP was performed at
101 the Bologna HMB, following the standard pasteurization procedure for DHM which is used in our
102 neonatal intensive care unit. Specifically, HoP was performed by a standard HM Holder pasteurizer
103 (S90 TES, Medicare Colgate LTD, Cullompton, UK). DHM was pasteurized using a temperature of
104 62.5°C for 30 minutes (tolerance $\pm 0.5^\circ\text{C}$), and then cooled to 4°C in 60 minutes (tolerance $\pm 0.5^\circ\text{C}$).
105 HPP was performed at HPP Italia, Traversetolo, Italy, using an industrial AV-30 device produced
106 by Avure Technologies, Inc. (Middletown, OH, United States); HPP was performed by applying a
107 6000 bar pressure for 3 minutes to bottled HM. HTST pasteurization was performed as previously
108 described (19), using a patented proprietary device (Patent number: EP 15176792.8-1358), which is
109 a bench-top device consisting in a system of tubular heat exchangers for heating as well as for
110 cooling. The temperature was monitored along the pasteurization steps by specific digital probes
111 (tolerance $\pm 0.5^\circ\text{C}$).

112

113 • *HM analyses*

114 Pasteurized (HoP, HPP and HTST), and unpasteurized HM were assayed for total protein, sIgAs
115 and lactoferrin content, and for protein profile. sIgAs were measured on 1:10,000 diluted samples,
116 using an ELISA kit (Biovendor, Brno, Czech Republic) and following the manufacturer's
117 instructions, in triplicate. Lactoferrin quantity was determined in triplicate on 1:20,000 diluted
118 samples (1:40,000 for unpasteurized HM), using an ELISA kit (Biovendor) and following the
119 manufacturer's instructions. Total protein content (TPC) was determined on samples skimmed by
120 centrifugation at 2,000g at 4 °C for 30 min, using 2DQuant kit (GE Healthcare Italia, Milan, Italy),
121 in duplicate, following the manufacturer's instructions. The protein profile (in non-reducing

122 conditions, 5 µg of proteins) was visualized by monodimensional electrophoresis on a 10-well 12%
123 Nu-PAGE® precast gel (Thermo Fisher Scientific, Waltham, MA, United States) with MES
124 (Thermo Fisher Scientific) as running buffer, on a Novex Mini-cell (Thermo Fisher Scientific) at
125 200 V. The gels were stained with Blue Coomassie Colloidal stain, following the protocol already
126 described in a previous study (20).

127 Microbiological safety analyses were performed following the Italian guidelines for HMBs (11), on
128 both unpasteurized and pasteurized HM samples, to verify the compliance of each pasteurization
129 technique to the HMB requirements. Each HM sample was assessed for total bacterial load (Plate
130 Count Agar [PCA] - Kima Meus, Piove di Sacco, Italy), presence of Enterobacteriaceae (Herellea
131 Agar - Kima Meus) and *Staphylococcus aureus* (Mannitol salt agar - Kima Meus). Bacterial counts
132 were reported as Colony Forming Units (CFU)/mL. In order to comply with the standards required
133 by HMB guidelines (11), in raw HM total viable bacteria count must be $<10^5$ CFU/ml, and both
134 Enterobacteriaceae and *S. aureus* $< 10^4$ CFU/ml; after pasteurization, no bacteria should be
135 detected.

136

137 • *Statistical analysis*

138 TPC, lactoferrin, and secretory IgA contents were reported as median values and interquartile
139 ranges. In order to evaluate difference among unpasteurized HM and the three different
140 pasteurization methods, one-way ANOVA with Tukey post-hoc comparison was used for TPC and
141 sIgA, and Kruskal-Wallis with Steel-Dwass post-hoc comparison for lactoferrin. A p-value $<.05$
142 was considered as statistically significant.

143

144 *Results*

145 • *Protein fraction analysis*

146 Results of protein fraction analyses are summarized in Table 1. TPC of defatted HM was quantified
147 before and after each processing method. TPC of the five analysed HM samples before

148 pasteurization varied between 5 and 12.1 g/L (median: 7.2 g/L); the total amount of HM proteins
149 was not affected significantly by any processing method.

150 Lactoferrin content of unpasteurized HM varied between 0.54 and 1.59 g/L (median: 0.82 g/L) and
151 was affected negatively by all the three pasteurization methods. A high reduction in HM lactoferrin
152 content was observed following both thermal pasteurization methods, with HoP having the greatest
153 effect (-87.5% after HoP and -83.5% after HTST). The difference between the two thermal methods
154 was not significant. On the contrary, HPP was found to better preserve HM lactoferrin content (-
155 24.7%). Secretory IgA content of unpasteurized HM varied between 2.1 and 3 g/L (median: 2.3
156 g/L) and was negatively affected by all pasteurization methods, but to a different extent. In
157 particular, only HPP led to a significant reduction (-35.8%) of sIgA content, with an almost double
158 reduction if compared to HTST (-23.3%).

159 In Figure 1, the protein profile of the five pooled HM sub-samples in non-reducing conditions is
160 reported; for one sample, the reducing protein profile is also displayed. For all the HM samples, the
161 reducing conditions were not suitable to visualize any difference in protein band abundance. When
162 non-reducing conditions were used, variations on specific HM protein band abundance were seen.
163 Moreover, these changes were common to all individuals, despite minor differences in the baseline
164 HM profile. Variations seemed more relevant for HoP, followed by HTST and, finally, by HPP.

165 In Figure 2, one individual protein profile was magnified to highlight the bands accounting for
166 visible changes. The identity of the proteins contained in these bands was assessed by comparison
167 with previous publications by our group (19–21): protein aggregates (mainly lactoferrin, band a)
168 seemed to be increased by all pasteurization methods, although to a different extent. On the
169 contrary, components of the immune system (bands b and c, containing heavy constant chain
170 subunits and polymeric receptor of immunoglobulin) and lactoferrin (band d) were decreased in all
171 pasteurized HM samples compared to raw HM, in variable amounts according to the processing
172 method used. For thermal methods only (HoP and HTST), the protein profile showed an increase of
173 two low molecular mass bands (band e and f), corresponding to beta casein fragments. These

174 shorter beta casein fragments have been always found following thermal pasteurization (HoP and
175 HTST) in our previous (19–21) and ongoing experiments, although we cannot discriminate if they
176 are native beta casein variants or a degradation product of the main beta casein form.

177

178 • *Microbiological analyses*

179 Each pasteurization method proved to comply with safety standards required by HMB guidelines.
180 Specifically, total bacterial count in all the raw HM samples was lower than 10^5 CFU/mL and
181 Enterobacteriaceae and *S. aureus* count were lower than 10^4 CFU/mL. Bacterial count was
182 undetectable in all the pasteurized HM samples, treated by HoP, HTST, or HPP.

183

184 *Discussion*

185 The availability of DHM provided by HMBs has been recently included among the milestones for
186 enteral nutrition of preterm infants developed in the last century (22). However, despite a huge
187 number of research efforts, current treatment of DHM is still unsatisfactory in terms of retention of
188 those bioactive components which are believed to mediate the beneficial clinical effects of HM in
189 preterm infants, such as the reduction of necrotizing enterocolitis and the improvement of long-term
190 neurocognitive outcome (23,24). For this reason, current research is directed towards the
191 identification of novel pasteurization methods which would be capable to preserve HM bioactive
192 components without affecting microbiological safety. To date, a number of papers on the effects of
193 innovative processing techniques for DHM pasteurization have been published and the results of
194 these papers have been summarized in recent reviews (13–16). Evidences on the impact of
195 innovative technologies are currently being evaluated by several research groups (18,19,25,26). The
196 assessment of new pasteurization technologies is hampered by the increase in parameters to be
197 monitored, others than time and temperature, especially for HPP, which is being tested in a high
198 variety of pressure settings (26). In an effort to elucidate the relative advantages of the most
199 promising innovative technologies over the traditional HoP method, we designed a study to

200 compare the effects of HoP, as performed in HMBs, vs. HTST, performed with a patented
201 proprietary device validated for treating HM, and continuous HPP on individual HM samples. All
202 previous reports on the issue (26–28) were conducted by simulating the processing treatments on
203 small HM volumes or even on single protein fractions, but no report to date used real HMB
204 conditions for processing whole individual HM samples to compare the three methods. In the tested
205 conditions, none of the processing methods affected the total amount of proteins, thus confirming
206 previous observations (13–15).

207 On the other hand, some differences were found in the quantity of specific proteins, as assessed by
208 ELISA and by protein electrophoresis: HTST was found to better preserve the original content of
209 sIgAs, in comparison to HPP, to a higher extent in comparison to the standard processing method.
210 These results are in contrast with previous reports (28), which found a double rate of degradation of
211 IgAs after HoP and HTST, as simulated on very low amounts (40 μ l) of skimmed HM. It is to
212 mention that one of the reasons for this discrepancy is that, in our study, HTST is performed by a
213 prototyped continuous flow device, rather than in batch. Through this technique, the damage caused
214 by heat is minimized by forming a thin layer of milk flowing in a continuous tube system, requiring
215 very short time to reach the operating temperature, and, equally relevant, minimizing times for
216 cooling down the milk, which is not the case in batch processes.

217 The rate of retention of sIgAs in our study for HoP and HPP was similar to that reported by
218 Permanyer and colleagues (29). An opposite trend was found, in the tested conditions, for another
219 important antibacterial factor, lactoferrin. Lactoferrin content was strongly reduced by both thermal
220 pasteurization techniques, as measured by ELISA tests, while it was better preserved by HPP.
221 Recently, Wesolowska and colleagues (26) found that HP pasteurization at 600 MPa for 10 min
222 allowed retaining 55% of the original lactoferrin content. In the current study, HPP at same pressure
223 for 3 min was enough to bring microbial growth to undetectable level, thus retaining almost 65% of
224 the original lactoferrin content in raw HM. The tendency of native HM lactoferrin to be denaturated
225 following pasteurization was investigated (27), and the kinetics of HM lactoferrin denaturation as a

226 consequence of HPP reported in that study are in accordance to our present data. Nevertheless,
227 efficacy of HPP for 3 min at 600 MPa in guaranteeing microbial safety should be better confirmed
228 in future by inoculation and validation studies, such as those performed for the HTST prototype
229 used in the present study (19).

230 When evaluating protein band intensity by protein electrophoresis under reducing conditions, the
231 lactoferrin band intensity was comparable among all the samples, including raw milk. When
232 running the tests in non-reducing conditions, as in other reports (19,20,27), we observed that
233 conformational changes of lactoferrin occurred, driven by the thermal treatments, resulting in an
234 increase of high molecular weight aggregates, causing an apparent decrease of the original
235 lactoferrin band. This phenomenon occurred mostly in HoP-treated milk, while it seemed to be less
236 pronounced following HPP and HTST. This observation highlights the limits of techniques based
237 on antibody recognition to detect aggregated complexes of lactoferrin, thus limiting the exact
238 quantification of the absolute lactoferrin content in HM treated by thermal methods. The effect of
239 conformational changes of lactoferrin on its bioavailability and bioactivity remains to be assessed.

240 In conclusion, the results of the present study show that HTST and HPP affect differently some of
241 the bioactive HM components involved in immune response and antibacterial activity. In addition,
242 both methods demonstrate to better preserve the original protein profile of raw milk, compared to
243 standard HoP. Further studies should be aimed at characterizing residual protein bioactivity in HM
244 treated with the two different pasteurization methods.

245

246 **Acknowledgments:** The authors would like to thank Michele Morbarigazzi at HPP Italia for
247 performing High Pressure Processing on donor human milk samples and Sara Antoniazzi at the
248 Institute of Sciences of Food Production of the Italian National Research Council for helping in
249 performing protein analyses.

250

251

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- 324
- 325
- 326
- 327

328 **Figure legends**

329

330 **Figure 1:** NuPAGE total protein profile of human milk (HM) after different pasteurization
331 processes. Colloidal Coomassie brilliant blue stained. Each image (sample) is representative of one
332 pooled HM sub-sample. Std: mass markers Mark12 (Thermo Fisher Scientific). A: unpasteurized
333 HM; B: holder pasteurized HM; C: high pressure processed HM; D: high-temperature short-time
334 pasteurized HM. Sample 5 protein profile is represented in both absence (non-reducing) and
335 presence (reducing) of Dithiotreitol.

336

337 **Figure 2:** NuPAGE total protein profile of human milk (HM) after different pasteurization
338 methods. Colloidal Coomassie brilliant blue stained. Bands a-f were identified in previous studies.
339 (19–21). Std: mass markers (kDa) Mark12 (Thermo Fisher Scientific). A: unpasteurized HM; B:
340 holder pasteurized HM; C: high pressure processed HM; D: high-temperature short-time
341 pasteurized HM.