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Comparison of the Effect of Holder Pasteurization and High-Pressure Processing on Human Milk Bacterial Load and Bioactive Factors Preservation

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ABSTRACT

Objectives: This project aims at comparing the impact of Holder pasteurization (HoP) and high-pressure processing (HPP) on bacterial load and retention of immunological components in human milk.

Methods: Human milk samples discarded by the Public Mothers' milk bank (Montreal, Canada) for bacterial purpose were pooled (n = 6) and pasteurized either by heating in a water bath (62.5°C, 30 minutes) or by HPP treatment (425 MPa, four cycles of 6 minutes, initial milk temperature of 4°C or 37°C). Bacterial load, lysozyme activity, and levels of immunoglobulins, lactoferrin, lipase, and 26 cytokines were analyzed. Untreated milk samples from same pools served as control.

Results: HPP treatment of milk allows a similar elimination of bacteria than HoP; bacterial counts were under the detection limit [<3 colony-forming units (CFU)/mL] in 50% of milk pools after HPP treatment, compared to 17% for HoP. With initial heating of samples to 37°C before HPP treatment, inactivation to an extent under the detection limit was reached in 67% of pools. There is no significant difference in IgA, lysozyme, and cytokines concentrations between untreated milk and all treatment methods. While no significant difference was observed in the amount of lipase ($P > 0.07$) and IgG ($P > 0.11$) between untreated milk and HPP-treated milk samples, HoP seems to be damaging for these factors ($P < 0.04$). IgM is well preserved in HPP-4°C samples compared to untreated milk ($P = 0.07$) whereas a decrease is observed for this immunoglobulin levels in HPP-37°C and HoP samples ($P < 0.01$). Lactoferrin activity, is well maintained in HPP-37°C milk samples in comparison to untreated milk samples ($P = 0.52$). A decrease in activity of this molecule is noted for samples treated with HPP at 4°C ($P = 0.02$) and this decrease is even more pronounced for HoP samples ($P = 0.004$).

Conclusions: HPP is a promising alternative to HoP for treatment of human milk intended to preterm babies. Our results demonstrate that HPP treatment of human milk provides safe milk with less detrimental effects on the biochemically and immunologically active milk components than HoP.

Key Words: breast milk, high-pressure processing, human milk, thermal pasteurization

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What Is Known

- Holder pasteurization (HoP) is considered the standard method to ensure microbiological safety of donated human milk.
- Some human milk bioactive components are loosed by thermal treatment.
- Alternative methods for microbiological treatment of human milk, such as high-pressure processing (HPP), have been tested and showed promising results.

What Is New

- Four consecutive cycles of HPP at 4°C or 37°C (425 MPa, 6 minutes) provide safe human milk, similar to what is achieved by HoP.
- HPP treatment allows a better retention of human milk bioactive components than HoP.

Scientific data have confirmed that human milk provides nutrients and multiple immunological factors supporting the enhancement of host defenses, neurological development, and gastrointestinal function (1,2). Along the same lines, the World Health Organization (WHO) recommends breastfeeding for the first six months of life and identifies human milk as the optimal nutritional option for the term and preterm babies (3). Unfortunately, breastfeeding is not always possible and, in these cases, human milk banks (HMB) can provide human milk to preterm babies. Because the health of these babies is fragile, donated human milk must be pathogen-free, while providing an optimal nutritional value. The Human Milk Banking Association of North America (HMBANA) recommends to treat donor milk by Holder pasteurization (HoP) to inactivate bacteria and viruses in milk (4). An

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increasing number of studies demonstrate that HoP is (1) inefficient at destructing spore-forming bacteria such as *Bacillus cereus* (5,6), and (2) detrimental to many essential bioactive components of human milk (7–13). As a result, HMB discard large volumes of milk for safety concerns, resulting in important losses, while pathogen-free milk provided to preterm babies is bioactively suboptimal. Consequently, the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) Committee on Nutrition recently recommended that future research should be directed toward the ''development and evaluation of different pasteurization techniques to optimize microbiological safety and to maintain the biological and nutritional quality of human milk'' (14).

High-pressure processing (HPP) is a nonthermal pasteurization procedure based on the application of high hydrostatic pressure (usually 400–800 MPa) for a short time. Since 1990, this emergent processing technology is increasingly adopted by the food industry and, more recently, by the pharmaceutical sphere (15,16). A number of studies suggest that HPP could be a viable alternative to HoP for the treatment of human milk. More importantly, adjustment of parameters such as temperature, number of cycles, and pression allows the destruction of bacterial spores (17) while preserving human milk nutritional and immunological factors (18–23).

The aim of the present study is to compare the effects of HoP and different temperature (4°C vs 37°C) for HPP treatments on bacterial load and retention of bioactive components in human milk.

METHODS

Preparation of Human Milk Pools

Unpasteurized, frozen human milk samples were obtained from Héma-Québec's Public Mother's Milk Bank (Montreal, Canada). Informed consent from the mothers was obtained in accordance with our approved institutional review board protocol. Milk samples were collected between 9 days and 11 months following birth delivery. Donated milk with total bacterial loads $> 10^5$ CFU/mL or showing the presence of enterobacteria ($>10^4$ CFU/mL), *Staphylococcus aureus* and/or *Bacillus* sp., was not processed and thus, used for this study. Milk samples were thawed by overnight storage at 4°C. As the composition of breast milk changes with baby's age, milk samples from four to six mothers were mixed with particular attention to their lactation stage to ensure optimal milk nutritional values in each pool. Samples were carefully transferred from their original storage containers to a sterilized glass flask and thoroughly mixed on a magnetic stirrer for at least 30 minutes to ensure a homogenous distribution of components. After pooling, samples were distributed into 100-mL aliquots in sterile plastic bottles (Sterifeed, United Kingdom) using a peristaltic pump. Each bottle was sealed with a Sealer500HA (Sterifeed, United Kingdom) and stored overnight at 4°C before HoP or HPP treatment. Control samples (untreated milk) were also stored overnight at 4°C before microbial analyses were performed. Samples of 1 mL were frozen at −20°C for biochemical testing. For the needs of this study, six different pools of milk were produced. Two independent samples per pool were treated by HoP or HPP (duplicate).

Holder Pasteurization

Milk samples (4°C) (n = 6) were immersed in an uncovered water bath heated to 63.5°C. One bottle was used to monitor milk temperature during thermal processing. Bottles were manually agitated every 5 minutes. When the inner temperature of the temperature-monitored bottle reached 62.5°C, the process was continued for 30 minutes. After treatment, milk bottles were submerged for 60 minutes in an ice-cold water bath to quickly reduce the temperature. Microbial analyses were immediately performed while samples of 1 mL were frozen at −20°C for biochemical testing.

High-Pressure Processing

For the HPP treatment, milk samples were pressurized in a hydrostatic pressure unit of 135 L (Hiperbaric 135; Hiperbaric, Burgos, Spain). Cooled water (8–10°C), without additives, was used as the pressure-transmitting fluid. Before HPP treatment, milk samples (n = 6) were either kept at 4°C (HPP–4°C) or warmed up at 37°C (HPP–37°C) in a water bath. We chose to compare these two temperatures based on very promising results published in 2012 by Demazeau et al (24). Since the water of the pressure unit system cannot be temperature-controlled and to ensure HPP treatments to 4°C or 37°C, milk bottles were immediately placed in separate closed containers (jars) filled with water at either 4°C or 37°C, depending on the tested condition. These closed containers, containing bottles of milk, were then treated at 425 MPa for four cycles of 6 minutes each. The delay between each cycle was from 11 to 15 minutes since jars containing water heated to 37°C had to be emptied and refilled each time to ensure the treatment to 37°C for each cycle. Following pressurization, samples from both experimental groups were removed from the containers and immediately placed at 4°C before microbial analysis. Samples of 1 mL were frozen at −20°C for biochemical testing.

Bacterial Counting

Milk bottles from each condition (untreated, HoP, HPP–4°C, and HPP–37°C) were gently mixed. A 100-μL aliquot from each bottle of pasteurized milk was plated, undiluted, on sheep blood agar (Oxoid Company, Nepean, ON, Canada). Untreated milk samples were diluted from 1/5 to 1/160 with nutritive broth before seeding 100 μL on sheep blood agar. Plates were prepared in triplicate and were incubated at 37°C for 24 hours before colony counting.

Bacterial Identification

After isolation on sheep blood agar plates, bacterial identification of *B cereus* was performed by conventional phenotypic techniques and particularly by the characteristic beta-hemolysis pattern of the bacteria on these agar plates. When necessary, identification was performed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS; VitekMS, bioMérieux, France).

Biochemical Assays

Immunoglobulins

IgA, IgG, and IgM were quantified by the capture enzyme-linked immunosorbent assay (ELISA) developed by our laboratory. All controls and samples were analyzed in triplicate. 96-well plates were coated overnight at 2–6°C with appropriate antibody (5 μg/mL; goat anti-human IgA, goat anti-human IgG Fγ fragment-specific, goat anti-human IgM Fc fragment specific, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Plates were washed with a solution of Tween-20 (0.05%) and phosphate-buffered saline (PBS)–0.25% casein–0.05% Tween-20 blocking solution was added to each well (200 μL/well). Plates were incubated for 30 minutes at 37°C for IgA, and 1–2 hours for IgG and IgM, then washed. Milk samples were centrifuged at 800 × *g* for 10 minutes and the aqueous

phases were diluted with the relevant PBS–casein–Tween-20 solution and added to plates (100 μL/well). Human standard serum (Cedarlane, Burlington, ON, Canada, or Accurate Chemical & Scientific Corporation, Westbury, NY, USA) was diluted in the same diluent to prepare calibration curves. Diluent was used as blank. Plates were next incubated at 37°C for 60 minutes before being washed four to six times with Tween-20 (0.05%). Horseradish peroxidase (HRP)-coupled goat anti-human IgA+G+M (Jackson ImmunoResearch Laboratories) was diluted in the relevant PBS–casein–Tween-20 solution and added to each well (100 μL/well). Plates were incubated at 37°C for 60 minutes before being washed four times.

A volume of 100 μL of TMB peroxidase substrate solution (ScyTek Laboratories Inc., Logan, UT, USA) was added to each well (100 μL/well) and plates were incubated in the dark at ambient temperature for 30 minutes. Finally, 100 μL/well 1N H_2SO_4 was added, and absorbance was immediately read at 450 nm on a Spectramax 384+ spectrophotometer using the SoftMax Pro software (Molecular Devices, San Jose, CA, USA).

Lysozyme

Lysozyme activity in whole milk was determined using a *Micrococcus lysodeikticus*-based turbidimetric microplate assay (Sigma-Aldrich, St. Louis, MO, USA), as described by Helal et al (25). The activity was measured by reading the turbidity of the bacterial suspension at 450 nm every minute for 5 minutes.

Lactoferrin

Human lactoferrin was measured using an ELISA kit (Abcam, Cambridge, UK), following the manufacturer's instructions. Before performing the ELISA, milk samples were centrifuged at 800 × *g* for 10 minutes and the fat layer was removed. Samples were diluted 1/200,000 in the diluent supplied by the manufacturer.

Lipase

Human bile salt-stimulated lipase (BSSL) was measured using an ELISA kit (Cloud-Clone Corp., Katy, TX, USA), following the manufacturer's instructions.

Cytokine Determination

Milk samples were centrifuged at 800 × *g* for 10 minutes and the fat layer was removed. Concentrations of GM-CSF, TNF-α, IL-1β, IL-4, IL-6, MIP-1α, IL-8, IL-15, IFN-α, IL-2R, IP-10, MIP-1β, Eotaxin, RANTES, MIG , IL-12 (p40/p70), IL-1RA, IFN-γ, IL-13, MCP-1, IL-7, IL-17, IL-10, IL-5, and IL-2 were determined in milk aqueous phase using a Human Cytokine 25-plex Assay kit (ThermoFisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Concentrations of TGF-β2 were also measured using an ELISA kit (Bio-Rad, Hercules, CA, USA), following the manufacturer's protocol. Samples were analyzed using a Luminex system (Bio-Rad).

Statistical Analysis

Differences between untreated, HoP- and HPP-treated samples were assessed using a nonparametric paired Wilcoxon signed-rank test. The level of significance was set at $P < 0.05$. All statistical analyses were done with SAS Enterprise Guide version 9.4 software (SAS Institute Inc., Cary, NC, USA). Results are presented as box and whiskers graphs.

TABLE 1. Bacterial load (CFU/mL of milk) in human milk samples as a function of treatment

	Treatment*			
Batch number	Untreated milk	HoP	HPP–4°C	HPP–37°C
1	6950	3	BDL†	BDL†
2	37,300	1448	493	213
3	5816	3	BDL†	BDL†
4	3683	BDL†	3	BDL†
5	573	37	3	BDL†
6	9025	163	10	3

CFU = colony-forming units; HoP = Holder pasteurization; HPP = high-pressure processing.
*All bacteria found in treated samples were *Bacillus* sp.
†BDL: below the detection limit of the method (3 CFU/mL).

RESULTS

Bacterial Loads

Table 1 reports bacterial loads recovered in untreated, HoP, HPP–4°C and HPP–37°C milk samples in the six milk pools. Even if all treatment conditions significantly reduced bacterial counts compared to untreated milk ($P < 0.008$), no differences were observed in bacterial loads among pasteurization methods ($P > 0.16$). These results suggest that HPP treatment is as effective as HoP in reducing bacterial load. Preincubation of milk at 37°C before HPP treatment tends to facilitate the destruction of bacteria in milk but no statistical difference is observed.

Immunoglobulins

Figure 1 shows IgA, IgG, and IgM levels in the six pools of milk according to the treatment applied. Results are presented in box and whiskers graphs. No significant difference is observed in IgA levels between untreated milk and treated milk, no matter of the pasteurization method used. A significant decline in IgG levels in HoP-treated samples is noticeable compared to untreated milk and HPP-treated samples. Unprocessed milk samples had a median IgG concentration of 21.2 μg/mL (MIN–MAX = 18.7–23.6) whereas median IgG level of 14.2 μg/mL (MIN–MAX = 12.3–15.8) was measured for HoP milk samples which represent a decreased of about 33%. HPP treatment does not cause an IgG level decrease when compared to untreated milk. No significant differences in IgM concentrations were observed either between untreated and HPP–4°C milk samples whereas a significant decrease in IgM level is observed when milk samples were treated by HPP at 37°C when compared to untreated milk. More importantly, this diminution is higher in HoP samples which are also statistically different from those treated by HPP. These results suggest that, except for IgM levels in the HPP-37°C-treated milk samples, HPP treatment cause no significant reduction in IgA, IgG, and IgM retention when compared to untreated milk. On the other hand, processing of human milk by HoP causes substantial losses of these crucial immunological factors.

Lysozyme

HPP treatment allows a good recovery of lysozyme activity in milk, as demonstrated by the results presented in Figure 2A. A comparable level of lysozyme activity was observed in HPP–4°C, HPP37°°C, and untreated milk samples, where retention of

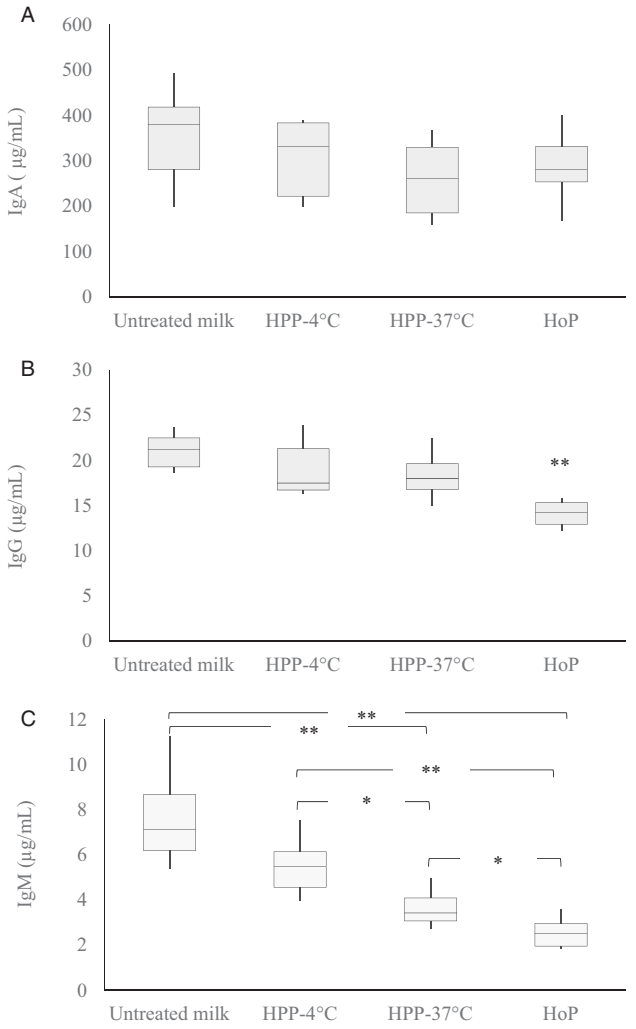

FIGURE 1. IgA (A), IgG (B), and IgM (C) contents of milk samples treated by Holder pasteurization or HPP. In each box and whisker graph, the box is defined by the 25% and 75% quartiles, and median of data is indicated inside; whiskers correspond to the minimum and maximum values. Statistical differences between groups are indicated with a symbol: $^{**}P < 0.01$. For IgG (B), HoP-treated samples are statistically different ($^{**}P < 0.01$) from each other groups. Median values of raw milk were: IgA: 379.92 μg/mL; IgG: 21.17 μg/mL; IgM: 7.12 μg/mL; n = 6. HoP = Holder pasteurization; HPP = high-pressure processing.

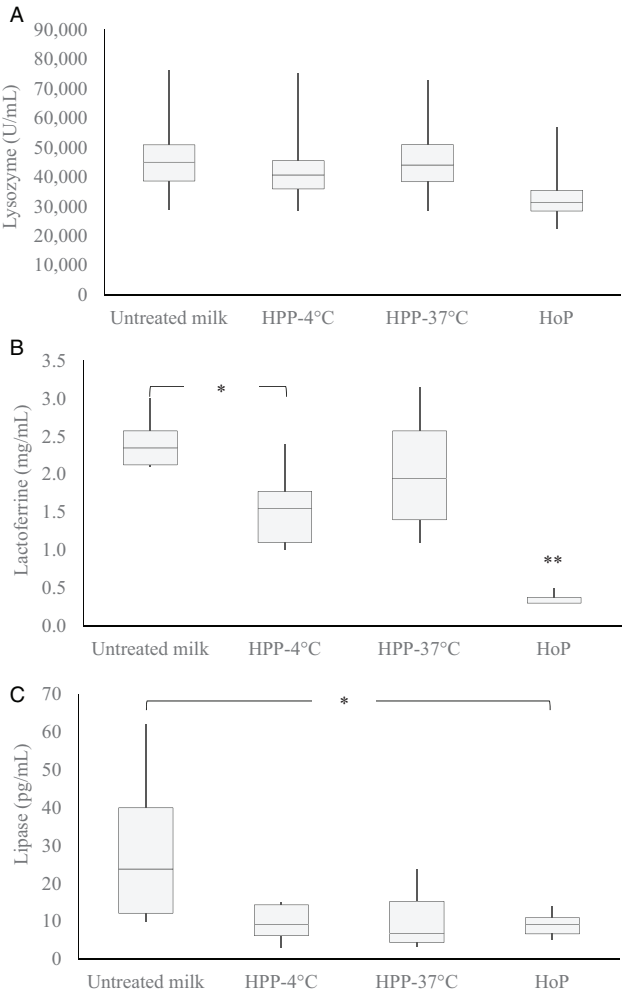

FIGURE 2. Lysozyme (A), lactoferrin (B), and lipase (C) content in milk samples treated by Holder pasteurization or HPP. In each box and whisker graph, the box is defined by the 25 and 75% quartiles, and median of data is indicated inside; whiskers correspond to the minimum and maximum values. Statistical differences between groups are indicated with a symbol: $^{*}P < 0.05$, or $^{**}P < 0.01$. For lactoferrin (B), HoP-treated samples are statistically different ($^{**}P < 0.01$) from each other groups. Median values of raw milk were: lysozyme: 45,067 U/mL; lactoferrin: 2.35 mg/mL; lipase: 23.83 pg/mL; n = 6. HoP = Holder pasteurization; HPP = high-pressure processing.

lysozyme activity in treated samples was 94% and 98%, respectively, relative to untreated samples.

Lactoferrin

Results of lactoferrin measurements are presented in Figure 2B. HPP−37°C treatment of milk samples do not alter lactoferrin concentrations, as shown by a median value of 2.0 mg/mL compared to control samples (2.4 mg/mL). However, a reduction in the temperature of the HPP treatment seems to impact the levels of lactoferrin, as HPP−4°C milk samples show a median value of 1.6 mg/mL which is different from untreated milk. HoP seems to greatly alter lactoferrin levels in human milk since the average recovery of lactoferrin in HoP samples was only 15% of the levels measured in untreated milk samples with a slight median concentration of 0.30 mg/mL.

Lipase

Results of lipase measurements in HoP- and HPP-treated human milk samples are presented in Figure 2C. When compared to untreated milk samples, significant declines in lipase levels were found in HoP-treated samples. A range of values from 5.1 to 14.0 pg/mL was obtained for these samples while concentrations from 10.0 to 62.0 pg/mL were obtained in control samples. This represents an average recovery rate of 47% after HoP treatment. HPP−4°C and HPP-37°C milk samples also showed a reduction in lipase concentration when compared to untreated milk samples, but this difference was not statistically significant.

TABLE 2. Cytokine concentrations (pg/mL) in milk samples as a function of treatment

	Treatment			
Cytokine	Untreated milk	HoP	HPP−4°C	HPP−37°C
IL-8	119.0 (106.1–123.1)	197.6 (128.5–280.1)	83.3 (63.3–153.7)	105.1 (86.5–133.8)
IP-10	30.9 (30.3–35.4)	35.6 (30.6–39.4)	18.8 (13.1–29.9)	27.4 (18.7–31.7)
MCP-1	524.2 (410.9–688.0)	434.5 (335.1–698.4)	460.1 (292.8–621.2)	363.9 (306.0–623.8)
MIG	25.7 (19.4–29.3)	29.3 (29.3–29.4)	18.4 (15.6–24.1)	15.9 (12.2–23.8)
TGF-β2	844.7 (671.9–956.8)	703.3 (511.2–932.4)	605.1 (424.8–746.0)	767.1 (428.8–864.4)

Results are expressed as median ± interquartile range (IQR). No statistically significant differences were seen between untreated milk and HoP or HPP-treated milk for any of the tested cytokines.

HoP = Holder pasteurization; HPP = high-pressure processing.

Cytokines

Among the 26 cytokines analyzed by Luminex technology, only IL-8, IP-10, MCP-1, MIG, and TGF-β2 were detected at measurable levels. Results are presented in Table 2. No significant differences were observed between treated milk samples (HoP, HPP−4°C, and HPP−37°C) and untreated milk samples.

DISCUSSION

There is no doubt that human milk is a unique nutrient and is the most adapted to the needs of a newborn infant. Scientific evidence point to the benefits of human milk for suitable growth and development of term and preterm babies (2,3,26). While fresh mothers' milk does not need to be heat-treated when administered within 24 hours, processing human milk seems to considerably impact its most valuable constituents. Emerging data indicate that pasteurization's heat treatment affects human milk quality by reducing some bioactive and immunologically active components (7–13). More importantly, several studies have shown that sporulating bacteria such as *B cereus* resist the temperature applied during HoP (5,6,27), which could ultimately lead to serious complication for preterm babies (28). In this study, we have also identified sporulated *B cereus* by MALDI-TOF technology in five out of six pools following HoP treatment (83.3%). The fact that nonconforming milk, discarded by the milk bank because of high bacterial contamination concerns, was used for this study can explain the presence of *Bacillus* sp. in pools. Since bacterial spores are so difficult to eliminate could explain a large number of sporulated *Bacillus* sp. found in processed milk samples, even if the milk had been pasteurized.

HPP is a method that shows promising potential as an alternative to HoP of human milk. In the present study, when a pressure of 425 MPa was applied to milk samples previously heated to 37°C for four cycles of 6 minutes, 66.7% of milk samples contained less than 3 CFU/mL of bacteria, which is our detection limit. Post HPP treatment, two pools of milk still contained remaining bacteria akin to *B cereus*. Black and colleagues have also reported bacterial spores resisting to HPP treatment using pressures higher than 400 MPa (29). This phenomenon was studied in more detail by Wuytack et al using *B subtilis* spores. The group observed incomplete spore germination after one 600-MPa treatment. At that pressure, the small acid-soluble spore proteins (SASPs) would not be degraded, which would interrupt the germination process (30). In contrast, when pressure between 50 and 300 MPa is applied at 40°C for about 30 minutes to sporulated bacteria, germination is activated via nutrient receptor stimulation, leading the bacterium to act as if the environment is no longer hostile and to germinate into vegetative cells, which would then be destroyed by subsequent HPP cycles (31). We confirmed this hypothesis by treating two pools of milk containing $>2.6 \times 10^5$ CFU/mL (background flora; the major part was sporulated *B cereus*) by HPP at 300 MPa. After four cycles of 10 minutes each, no bacteria were cultured from both milk samples (data not shown). The initial milk temperature also seems to influence HPP efficiency. Our results show that a pressure of 425 MPa tend to be more effective in destructing bacterial spores when milk samples are previously heated and treated at 37°C, than HPP-treated at 4°C. This synergy between pressure and temperature is also observed and well described by Van Opstal and colleagues (16). Authors demonstrated that germination of bacterial spores (6 log) is efficient when the temperature of the milk is above 30°C even if the pressure is low (200 MPa for 30 minutes). Bacteria are then eliminated by subsequent treatment.

Immunoglobulins probably the most studied human milk component, and many authors have reported variable losses of these immune factors after HoP. In this study, we also report significant reductions in IgG level (>32%) following HoP. Many studies have described losses in IgA (from 20% to 64%) and IgG (from 20% to 34%) after HoP, which is not surprising given that these proteins are heat-sensitive (7,13,22). Similar to Viazis et al and Sousa et al, this study shows that HPP treatment at 425 MPa allows the retention of IgA and IgG. At 4°C, losses from 7% to 10% are observed, which is consistent with others who have reported reductions from 5% to 20% in immunoglobulin concentrations (12,19,22). There is no many results concerning the effect of HPP on IgM level in literature. The present study shows no significant decrease when samples are treated by HPP at 4°C compared with untreated milk (Fig. 1C). Similar results were observed by Sousa et al with an HPP treatment to 8°C (12,22). A loss in this immunoglobulin level is however observed if samples are HPP-treated at 37°C (approx. 52% of loss) and to a greater extent if samples are treated by HoP (>70% of loss). IgM was also found by others to be affected in human milk following HoP with a loss from 50% to 80% (12,13,22). Given the crucial role of these bioactive factors in immune defense mechanisms, these observations highlight the importance of using a milk treatment technology which can reduce bacterial load while preserving the levels of immunoglobulins.

In maternal milk, lysozyme acts as a natural antibiotic by degrading the outer cell wall of Gram-positive bacteria such as *B cereus* (22). Our results suggest that HPP treatment has no significant impact on lysozyme activity, as a slight decline from 2% to 6% was observed in HPP-treated samples. On the other hand, the enzyme lost about 37% of its activity after HoP. While not significant, these outcomes are similar to results from other groups who have reported lysozyme activity retention rates from 90% to 100% after HPP treatment, versus a loss from 21% to 40% in pasteurized samples (7,11,12,19,32). These results suggest that HPP

treatment of human milk would allow superior conservation of this antimicrobial activity compared to HoP.

Milk lactoferrin plays many roles by supporting antimicrobial, immunomodulatory, and anti-inflammatory activities (33). This protein also promotes iron absorption in babies as well as the growth of *Bifidobacterium* species, which ensure healthy bowel colonization (34,35). An average of 2.4 mg/mL of lactoferrin was recovered from raw milk. This concentration sharply declined in HoP samples, in which only 0.4 mg/mL was recovered, suggesting that less than 15% of the initial lactoferrin is retained after this treatment. In a similar study, Czank and colleagues have reported lactoferrin retention rates ranging from 22% to 39% after HoP (32). HPP treatment of human milk samples allows the retention of 63% to 83% of lactoferrin, suggesting superior protein recovery. This result is similar to data from Pitino et al (36), who reported a 28% reduction in lactoferrin levels in HPP-treated maternal milk (500 MPa for 8 minutes at 4°C).

Fat absorption is essential to the development of babies, since approximately 50% of total energy intake is supplied by breastmilk or formula fat. Lipase is a heat-labile factor, which facilitates absorption and digestion of human milk and formula fat by breaking down fat molecules, thereby releasing their energy. This bioactive factor is crucial for preterm babies for whom fat absorption is lower than in older children and adults due to low levels of pancreatic lipase. Studies have shown that fat absorption from fresh human milk, either alone or combined with formula, is greater than that from heat-treated human milk (11,37,38). Heating human milk to 40–55°C for 39 minutes destroys the enzyme activity and decreases lipid absorption in premature babies (39). Consistent with reports from other research teams (36,39,40), our results show that HoP destroys more than 50% of lipase. Similar results were obtained with HPP treatment at 4°C. However, better retention of lipase was observed (65%) when milk samples were HPP-treated at 37°C. Demazeau et al have published similar findings, showing that 78% to 100% of initial lipase concentrations were retained after four cycles of HPP treatment (350 MPa; 5 minutes) at 38°C. Importantly, they have observed 0% recovery of lipase in HoP milk samples (41).

Many factors such as freezing, time of delivery, or lactation period influence retention of cytokines, chemokines, and growth factors in human milk. Among the 26 cytokines evaluated in this study, only five possibly involved in the development of the baby have been detected. Both HoP and HPP treatment do not seem to affect IP-10, MIG, MCP-1, TGF-β2, and IL-8. Similar results have been reported by other groups (9,42,43). These immune factors are involved in the regulation of immunity. IP-10 and MIG are involved in the migration and activation of intestinal T cells and increased mucosal immunity during the neonatal period (44). IL-8 and MCP-1, strong chemoattractants for neutrophils and monocytes, are responsible for cells trafficking and host defense but also protect intestinal cells against injury (45). TGF-β is involved in immunomodulation of neonatal gastrointestinal epithelium and also decreases tissue damage associated with NEC (46). This cytokine also modulates the secretion of IgA and control inflammatory response by inducing tolerance (45).

Limitations of the Study

We recognize some limitations in this study and these should be mentioned here. Firstly, to not use milk qualified for premature babies, we used milk discarded by the milk bank due to a high rate of bacterial contamination. We are aware that normally, the bank does not process milk containing so much bacteria with a huge presence of *Bacillus* sp. or other sporulating bacteria. This represents the worst-case scenario and the results of bacterial load could have been better if qualified pools of milk have been used. Moreover, it would have been interesting to inoculate pools of milk with a precise concentration of known bacteria to verify and compare the ability of the treatment to reduce bacterial load. However, this was not possible in this study since the establishment where HPP treatments were performed was a place where sterilization of food was done. In order to avoid contamination of the entire building with bacteria, it was prohibited to inoculate our samples. Finally, we used Sterifeed bottles (Sterifeed, United Kingdom) for both HoP and HPP treatments while these bottles are not suitable to resist to high-pressure treatments. As a result, several bottles exploded during treatments. Better bottles should also have allowed more efficient treatment and better results. Finally, the use of a temperature-controlled pressure unit system should allow better control of the temperature treatment.

This study highlights some of the drawbacks of the pasteurization method used in most HMB, and emphasizes the need for innovative pasteurization methods to ensure microbial safety while preserving the immunological and functional quality of donor milk. HPP appears to be a suitable alternative for the treatment of maternal milk. This relatively new technology allows to reduce the number of sporulating bacteria, which translates to lower milk losses by milk banks. HPP also confers a higher recovery of bioactive factors having potentially lifesaving properties for preterm babies. This process is also more rapid than HoP. Nevertheless, further studies are required to determine the optimal treatment parameters, such as pressure and temperature, that will optimize milk quality and safety.

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