Bacteria and bioactivity in Holder pasteurized and shelf-stable human milk products

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Running Title: Bioactivity in shelf stable human milk

Abbreviations: ANOVA, analysis of variance; CV, coefficient of variation; DHM, donor human milk; ELISA, enzyme linked immunosorbent assay; HMBANA, Human Milk Banking Association of North America; HP, Holder pasteurized samples; HRP, horseradish peroxidase; MOM, mother’s own milk; NEC, necrotizing enterocolitis; NICU, neonatal intensive care unit; PBST, phosphate buffered saline + 0.05% tween 20; RAW, raw human milk samples; sIgA, secretory immunoglobulin A; SS, shelf stable samples.

Word Count: 3,644
Abstract

**Background:** Historically, donor human milk available in a hospital setting has been pasteurized using Holder pasteurization. There is extensive research overviewsing the impact of Holder pasteurization on bioactive components of human milk. Recently, a shelf-stable human milk product, created using retort processing, has become available; however, little is published about the effect of retort processing on human milk.

**Objective:** We aim to assess the ability of retort processing to eliminate bacteria, and quantify the difference in lysozyme and secretory immunoglobulin A (sIgA) activity between Holder pasteurized and shelf-stable human milk.

**Methods:** Milk samples from 60 mothers were pooled. From this pool, 36 samples were taken; 12 samples were kept raw, 12 samples were Holder pasteurized (HP), and 12 samples were retort processed to create a shelf stable product (SS). All samples were analyzed for total aerobic bacteria, coliform, *Bacillus cereus*, sIgA activity, and lysozyme activity. Raw samples served as the control.

**Results:** One raw sample and 3 HP samples contained *B. cereus* at time of culture. There were no detectable bacteria in SS samples at time of culture. Lysozyme and sIgA activity were significantly greater in raw samples when compared to HP and SS samples (p < 0.0001). HP samples retained significantly more lysozyme and sIgA activity (54% and 87%, respectively) than SS samples (0% and 11% respectively).

**Conclusions:** Human milk processed using Holder pasteurization should continue to be screened for presence of *B. cereus*. Clinicians should be aware of the differences in the retention of lysozyme and sIgA activity in HP and SS products when making feeding decisions for medically fragile or immunocompromised infants to ensure patients are receiving the maximum immune protection.

**Keywords:** heat processing, infant nutrition, donor human milk, commercial sterilization, shelf-stable human milk
Introduction

The nutritional requirements of premature infants can be difficult to meet, and due to the increased nutritional value of human milk after delivering prematurely, their mother’s own milk (MOM) is the preferred food source [1,2]. Human milk contains bioactive components that help to protect the medically fragile infant from development of complications, such as sepsis, retinopathy of prematurity, and necrotizing enterocolitis (NEC) [3-5]. The protective benefits of human milk are maximized when an exclusively human milk diet is maintained, decreasing retinopathy rates by over 20%, and decreasing NEC rates by 12-14% [4-6]. When babies are born prematurely, the mother is at an increased risk of delayed onset of lactogenesis II and/or low milk volume [7-9]. In order to maintain an exclusively human milk diet, donor human milk (DHM) can be used until the mother’s milk supply is established [10,11].

Historically, the Human Milk Banking Association of North America (HMBANA) has been the provider of DHM in medical settings. To protect infants from potentially pathogenic bacteria, all donated milk is pasteurized and screened for bacteria. Holder pasteurization (62.5°C for 30 minutes) is the standard pasteurization method for HMBANA milk banks, and it has been shown to eliminate all pathogenic bacteria except Bacillus cereus [12]. Additionally, Holder pasteurization retains many bioactive compounds in human milk including 40-75% of lysozyme function and 50-100% of secretory immunoglobulin A (sIgA) function [12,13].

Recently, a shelf-stable DHM product developed with retort processing (121 °C, 20 PSI for 5 minutes) became available for use in a neonatal intensive care unit (NICU) setting in the United States. Retort processing differs from Holder and high-temperature short-time (HTST) pasteurization, which are the most widely used and researched thermal pasteurization methods. Retort processing differs from Holder and HTST in the temperatures utilized, time
of heat exposure, pressure utilized, and in the shelf life of the product [14].

Shelf-stable DHM may be an option for smaller facilities lacking storage and refrigeration space. However, only one study exists that investigates the effect of retort processing on bioactive components of human milk [15]. Results from this study indicate destruction of bioactive components in retort processed milk when compared to Holder pasteurized milk [15]. Unfortunately, only 3 samples were analyzed per treatment group and all samples originated from different donor pools. As levels of bioactive components in human milk can widely vary between mothers [16], the results from this study are difficult to draw definitive conclusions from.

Characterizing the bioactivity in different forms of DHM will allow informed choices regarding nutritional interventions with premature infants. Lysozyme and sIgA were chosen for analysis due to their roles in immune protection in the gastrointestinal tract. Lysozyme degrades the outer cell wall of gram-positive bacteria [17] and contributes to destruction of gram-negative bacteria in vitro [18]. Secretory IgA is synthesized by the mother’s immune system in response to environmental cues, binds microbes in the infant’s gastrointestinal tract to prevent their passage into other tissues [19].

Fragile infants who receive MOM or DHM with active sIgA and lysozyme have increased protection against pathogens within their environment [1,20]. Holder pasteurization and retort processing may yield DHM with different bioactive profiles. Research has shown that as the heat of the treatment increases, the destruction of bioactive components in human milk also increases [21]. This study assesses the ability of retort processing to eliminate bacteria and quantifies the difference in lysozyme and sIgA activity between Holder pasteurized and shelf-stable human milk.

**Methods**

The study received ethical approval from the North Carolina State University Institutional
Review Board. Raw human milk was obtained from 60 approved donors through WakeMed Mothers’ Milk Bank in Cary, NC. When donating to WakeMed Mothers’ Milk Bank, donors consent that if their milk is unable to be used for medical purposes, their milk may be used for research studies. One sample from each mother was obtained and used to create a pool of raw human milk totaling 260 ounces. The samples were pooled in the WakeMed Mothers’ Milk Bank by a trained technician using a standard pooling protocol. Each individual sample was thawed in the refrigerator prior to pooling. Proper personal protective equipment was worn during handling of the milk per HMBANA guidelines. When samples were adequately thawed, they were moved under a sanitized laminar flow hood to prevent contamination during the pooling process. Samples were removed from original milk storage containers and transferred into 4000 mL beakers prior to complete thawing to minimize fat separation and ensure maximum transfer of contents. During thawing, temperatures were maintained at or below 4 °C to discourage additional bacterial growth. When all individual samples were completely thawed, they were combined into multiple wide mouth Erlenmeyer flasks and mixed using a pour down method six times (Figure 1a). Once mixed, samples were swirled gently to homogenize and they were strained prior to bottling (Figure 1b).

Twenty-four three-ounce samples were taken from the pooled milk and stored in Orthofix Axifeed 100 mL bottles (Product Number 022001010, Nolato Jaycare Limited, Portsmouth, United Kingdom). Twelve samples received no further treatment (RAW) and 12 samples were processed using Holder pasteurization (HP; 62.5 °C for 30 minutes) in an Ace Intermed Special Feed Pasteuriser (Model HMP2070-40HCUL, Handover, Hampshire, England) at the WakeMed Mothers’ Milk Bank, Cary, NC.

All milk was kept refrigerated whenever not being pooled or processed. The remaining pooled milk was transferred into 2000 ml Erlenmeyer flasks and put on ice for transport to North Carolina State University [less than 30 minutes] for bottling and retort processing to
create a shelf-stable product. Upon arrival, the remaining pooled milk was gently swirled to homogenize (Figure 1b), and then poured into 12 ten-ounce aluminum cans, leaving 3-6 mm of air space at the top. Cans were sealed using a can sealer (Dixie Canner Company, Athens, GA) and retort processed to create a shelf-stable product (SS; 121 °C, 20 PSI for 5 minutes) using a Stock America, Inc. full water immersion retort processor (PR-I900, Raleigh, NC). After all processing was completed, all samples were aliquoted and stored at -80 °C until analysis. Note that all milk only underwent one freeze/thaw cycle to mimic the freeze/thaw cycle that occurs in a HMBANA milk bank. For analysis, each sample was analyzed for bacterial content per standard protocols at WakeMed Pathology Lab, sIgA activity, and lysozyme activity, as described below.

**Bacterial Screening**

Bacterial analysis was completed at the WakeMed Pathology Lab in accordance with the HMBANA guidelines for quantitative bacterial analysis for mother’s milk (Raleigh, NC). HP and SS samples underwent a full post-processing culture to identify any present bacteria. RAW samples were screened for presence of Bacillus cereus, Escherichia coli, general appearance of Enterococcus, gram-negative rods, yeast, and Staphylococcus aureus. Due to the large variety of bacteria potentially present in RAW samples [22], we chose to only screen for bacteria that may be of concern in a DHM setting, specifically with use in the NICU.

**Secretory Immunoglobulin A (sIgA) Activity**

The activity of sIgA in our samples was measured using a modified indirect enzyme linked immunosorbent assay (ELISA). Briefly, flat-bottom, high-binding, 96-well plates were incubated for 12 to 18 hours with an Escherichia coli antigen. After completion of the incubation period, plates were washed 3 times with phosphate buffered saline + 0.05% tween
20 (PBST). Human milk samples and human IgA from colostrum standards (Sigma-Aldrich #I-2636) were then plated in triplicate and incubated for 3 hours at room temperature. Plates were washed with PBST after the incubation period and then loaded with horseradish peroxidase (HRP) anti-human IgA (Sigma-Aldrich #A-0295) and incubated for 1 hour at room temperature. After, plates were washed a final time with PBST. The substrate solution (20 mL 0.05M citrate buffer, 0.1 mL 3% hydrogen peroxide, 0.5 mL 40 mM ABTS) was then added and immediately read on a plate reader at 405 nm at time 0 and every 2 minutes for 20 minutes. To determine sIgA activity, the changes in absorption over time were graphed, and a regression line was computed for each of the samples and the standards. The samples were then compared to the IgA standards to determine activity. Coefficients of variation (CV) for triplicates were between 1 and 6%.

**Lysozyme Activity**

Lysozyme activity was measured using the change in turbidity of a microbial suspension of *Micrococcus lysodeikticus*, a method developed and adapted for use in a 96 well plate [23,24]. One unit of lysozyme is equal to a decrease in turbidity of 0.001 angstroms per minute at 450 nm at pH 7.0 and 25 °C [23]. Briefly, 25 µL of human milk samples were plated in triplicate in a 96-well plate (1:20 diluted RAW samples, 1:10 diluted HP samples, undiluted SS samples) and 200 µL of bacterial suspension, reading approximately 1.00 at 450 nm on a spectrophotometer, was added to each well using a multi-channel pipette. The plate was read on a plate reader at 450 nm every 30 seconds for 6 minutes. R-square values were calculated to ensure appropriate function of assay and coefficient of variation was used to determine reliability. CV’s for triplicates were between 5 and 7%. Lysozyme activity was then calculated using the following equation:
lysozyme (units/mL) = [Average Change of Absorption/(0.001 * volume of sample in mL)]*dilution factor of milk samples.

Statistical Analysis

Independent processing treatments were performed in triplicate for analysis of lysozyme and sIgA activity. A statistical comparison of lysozyme activity and sIgA activity between raw milk, mothers’ own milk, Holder pasteurized milk and shelf-stable milk was done by one-way ANOVA. Differences between means were tested for significance (α = 0.05) by the Tukey HSD test.

Results

Bacteria

Raw milk samples (RAW) were screened for the presence of Bacillus cereus, Escherichia coli, general appearance of Enterococcus, gram-negative rods, yeast, Staphylococcus aureus, and Pseudomonas sp. All RAW samples contained Enterococcus sp., gram-negative rods, yeast, and Pseudomonas sp. One RAW sample was found to contain B. cereus. These results were typical of raw human milk and served as a control for Holder pasteurized (HP) samples and shelf-stable (SS) samples (Table 2).

HP samples and SS samples went through a complete post-processing screen to characterize any bacteria present. Three samples of HP milk had growth of B. cereus. No other growth was observed in HP milk. Shelf-stable samples had no bacterial growth (Table 2).

Secretory Immunoglobulin A Activity

Secretory IgA was measured in all samples, using RAW samples as a control. The
analysis showed an average of $1.04 \pm 0.09$ mg active sIgA/mL in RAW samples, and was significantly more than HP and SS human milk from the same pool ($p < 0.0001$; $0.90 \pm 0.03$ and $0.11 \pm 0.07$ mg active sIgA/mL respectively; Figure 2).

Lysozyme Activity

Lysozyme activity was measured in all samples using RAW samples as the control. RAW samples had an average lysozyme activity of $7969 \pm 1394$ units/mL and was significantly greater ($p < 0.001$) than both HP lysozyme activity ($4269 \pm 963$ units/mL), and SS lysozyme activity (no activity detectible; Figure 3).

Discussion

In a neonatal intensive care unit (NICU) it is imperative to scrutinize nutritional interventions to avoid expensive, life threatening complications [6,25]. With emerging options for human milk based feeding, evidence is needed on the full spectrum of feeding choices in order to inform best practice. Mother’s own milk is unequivocally the best option for premature infants [1,2]. Whenever possible, breastfeeding should be supported and encouraged if the mother’s goal is to breastfeed. When the mother faces obstacles establishing an adequate milk supply for her infant(s), donor human milk (DHM) provides a way for the medically fragile infant to maintain exclusively human milk feedings. The effects of various human milk processing methods on nutrient and bioactive retention may impact health outcomes and is an important area of future research.

Compared to raw human milk, our results show that human milk processed via Holder pasteurization retains more sIgA activity and lysozyme activity (HP; 90%, 54%) than shelf-stable human milk (SS; 10%, 0%). When compared to raw human milk, the reduction of activity observed in HP human milk is consistent with ranges reported in the literature.
As our study looked specifically at biological activity rather than concentration of lysozyme and sIgA, there was no published literature to use as a reference for expected values or ranges. Meredith-Dennis et al. found lower concentrations of IgA and no difference in lysozyme concentrations when comparing Holder and retort processed milk. These results may or may not be in agreement with our findings, as measured protein concentration can remain the same even when there is a loss of biological activity due to partial denaturation. In addition, this was a cross-sectional study with different donor pools represented in each treatment group; therefore, differences in milk composition cannot be specifically attributed to processing effects.

In our study, Holder pasteurization eliminated all bacteria except *Bacillus cereus*. It is understood that Holder pasteurization does not kill *B. cereus* and causes *B. cereus* spores to sporulate during heating [12,26]. HMBANA milk banks have chosen to continue using this method, and screen for and discard any batches that are positive for *B. cereus* post-processing to preserve nutritional value of the milk. Retort processing used to create the shelf-stable product eliminated all bacteria. Our study provides evidence that retort processing is effective at eliminating all bacteria from human milk, while Holder pasteurized DHM must continue to be screened pre- or post-processing for *B. cereus* to ensure its safety for consumption by medically fragile infants. The results for SS samples confirm that retort processed DHM is a sterile product [27].

The small sample size was a limitation to our study. However, clear patterns emerged regarding bioactivity retention during Holder pasteurization and retort processing. Additionally, this study only looked at two of many possible heat-sensitive bioactive components. Additional research is needed on other components in human milk to provide a more complete understanding of the impact of retort processing.

Considering the observed differences in bioactivity of lysozyme and sIgA, a more
complete analysis should be performed to determine the impact of retort processing on all heat
sensitive components of human milk, including additional nutrients and bioactive
components. Furthermore, there is currently no peer-reviewed literature on health outcomes of
medically fragile infants fed retort processed human milk. Results from this study are
important for clinicians to consider when choosing a feeding method for any medically fragile
or immunocompromised infant.
Acknowledgments

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Author Contributions: HL, MP, and AF conceived and designed experiments; MWG collected samples; HL and MWG performed experiment; HL and MP analyzed samples; HL and AF analyzed data, HL and AF wrote the manuscript; HL, MWG, MP, and AF assisted in revision of the manuscript; all authors read and approved the final manuscript.
References

Table 1. Parameters for Holder pasteurization, high-temperature short-time (HTST) pasteurization, and retort processing.

<table>
<thead>
<tr>
<th></th>
<th>Holder Pasteurization</th>
<th>HTST Pasteurization</th>
<th>Retort Processing</th>
</tr>
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<tbody>
<tr>
<td>Temperature</td>
<td>62.5 °C</td>
<td>72-75 °C</td>
<td>121 °C</td>
</tr>
<tr>
<td>Pressure</td>
<td>0 PSI</td>
<td>0 PSI</td>
<td>20 PSI</td>
</tr>
<tr>
<td>Time</td>
<td>30 minutes</td>
<td>5-15 seconds</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Shelf-life of Product</td>
<td>6 months in freezer</td>
<td>6 months in freezer</td>
<td>3 years</td>
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</table>
Table 2. Results of bacterial analysis in raw human milk, Holder pasteurized human milk, and shelf-stable human milk

<table>
<thead>
<tr>
<th></th>
<th>Number of samples containing bacteria (%)</th>
<th>Pseudomonas species</th>
<th>Gram negative rods</th>
<th>Enterococcus species</th>
<th>Yeast</th>
<th>Bacillus cereus</th>
<th>Escherichia coli</th>
<th>Staphylococcus aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAW ¹</td>
<td></td>
<td>12 (100)</td>
<td>12 (100)</td>
<td>12 (100)</td>
<td>12 (100)</td>
<td>1 (8.3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>HP ²</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3 (25)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>SS ³</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

¹Raw samples (unpasteurized)
²Holder pasteurized samples (62.5 °C for 30 minutes)
³Shelf-stable, retort processed samples (121 °C, 20 PSI for 5 minutes)
Figure 1. (a) WakeMed Mothers’ Milk Bank, Cary, NC standard mixing protocol for combination of four flasks of human milk (b) WakeMed Mothers’ Milk Bank, Cary, NC standard homogenization, straining, and bottling protocol for human milk.

Figure 2. Secretory immunoglobulin A (sIgA) activity (± SD) in raw (RAW), Holder pasteurized (HP) and shelf-stable (SS) human milk. Bars with different letters were significantly different (p < 0.001)

Figure 3. Lysozyme activity (± SD) in raw (RAW), Holder pasteurized (HP), and shelf-stable (SS) human milk. Bars with different letters were significantly different (p < 0.001).
sIgA Activity in Raw, Holder Pasteurized, and Shelf-Stable Human Milk Samples

### Treatment

- **RAW**
- **HP**
- **SS**

### sIgA Activity (mg active sIgA/mL human milk)

- **A**
- **B**
- **C**