The effects of fortification and refrigerated storage on bioactive proteins in Holder-pasteurized donor human milk

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Abstract:

Objective: The aim of the study was to assess the total protein, lysozyme, and immunoglobulin A (IgA) content of unfortified and fortified Holder-pasteurized donor human milk (HPDHM) during 96 hours of refrigerated storage. **Study Design:** HPDHM was prepared in a hospital feeding room and subjected to treatment with 3 different fortifiers: an acidic, bovine-based (F-ACID), a neutral, bovine-based, and a human milk–derived (F-HUM) fortifier. Unfortified HPDHM served as the control (CONTROL). Samples were stored at 4°C, and every 24 hours, a 1-mL aliquot was removed for analysis. **Results:** At baseline, there was a significant difference in protein (mean, standard deviation) concentration (g/dL) between control (1.3, 0.1) and all other treatments (F-ACID = 2.0, 0.2; neutral, bovine-derived fortifier = 2.2, 0.1; F-HUM = 2.5, 0.1; $P < 0.001$). Lysozyme and IgA were significantly lower in the F-ACID group ($P < 0.001$). Lysozyme and IgA were significantly higher in the F-HUM group ($P < 0.001$). There was no significant effect of storage time ($P > 0.9$) for all dependent variables. **Conclusion:** The type of fortifier has a more significant impact on bioactive components in fortified HPDHM than does storage time. Our findings of lack of negative impact of refrigeration storage time on the protein and bioactive components of donor milk strengthen the recent recommendations to extend storage time to 48 hours.

Keywords: bioactives | donor human milk | donor milk | fortified | storage

Article:

What Is Known
- Unfortified Holder-pasteurized donor human milk retains lysozyme and immunoglobulin A activity for up to 7 days of refrigerated storage.
- Neonatal intensive care units in the United States routinely fortify human milk for very-low-birth-weight infants.

What Is New
- Acidic fortifiers significantly lower the lysozyme and immunoglobulin A activity in Holder-pasteurized donor human milk.
- Human milk–derived fortifiers significantly increase the lysozyme and immunoglobulin A activity of Holder-pasteurized donor human milk.
- Refrigerated storage for up to 96 hours had no impact on the total protein, lysozyme activity, and immunoglobulin A activity in fortified Holder-pasteurized donor human milk.

The use of pasteurized donor human milk is supported by the World Health Organization, the American Academy of Pediatrics, and the United States Surgeon General as an important strategy for improving health outcomes in premature infants when their mother's milk is not available [1–3]. In the United States, donor milk use is on the rise, with more than 65% of neonatal intensive care units (NICUs) reporting use in 2015 [4–6]. To ensure the safety of donor milk, the Human Milk Banking Association of North America (HMBANA) and other international milk bank networks have issued guidelines, including appropriate storage temperatures and durations. Current best practices for the refrigerated storage of Holder-pasteurized donor human milk (HPDHM) state that thawed milk should be stored in the refrigerator and used within 48 hours[7].

Evidence is emerging that unfortified HPDHM remains free of microbial growth during refrigerated storage over 4 days, suggesting an opportunity to extend the expiration date and reduce unnecessary waste of a costly and valuable resource [8–12]. Affordability was the most frequently cited barrier of nonuse in a 2013 survey of 183 level 3 NICUs [5]. Research to evaluate the feasibility of extending defrosted HPDHM expiration dates has the potential to reduce cost barriers associated with a short shelf life and product waste.

Although bacteria levels of HPDHM are one marker of product quality and safety, additional research is needed regarding the retention of nutrients and immune factors in HPDHM during extended refrigerated storage to inform evidence-based guidelines on appropriate clinical use. In addition, it is now common practice in the NICU to fortify mother's milk and HPDHM with human milk fortifiers (HMF) to increase protein and energy content [4]. The effects of refrigerated storage time on the retention of macronutrients and other bioactive components in fortified HPDHM remain largely unstudied [13]. The objective of this study is to assess total protein, lysozyme, and immunoglobulin A (IgA) in unfortified and fortified HPDHM during 96 hours of refrigerated storage. Lysozyme and IgA were selected for this study because of their antimicrobial properties [14] and their partial retention after pasteurization [15]. A 96-hour window was selected as the maximum amount of time a hospital might take to use a standard 4 oz bottle of HPDHM when starting preterm infants at a low feeding volume ($15–20 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) and advancing at 10 to $20 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$[16].

METHODS

HPDHM was acquired through an HMBANA milk bank (The New York Milk Bank, Valhalla, NY). Twelve unique batches were subject to 4 treatments: unfortified HPDHM (CONTROL) served as the control; fortification to 24 kcal/oz with a liquid, acidic, bovine milk–based whey hydrolysate fortifier (F-ACID) (Enfamil Human Milk Fortifier Acidified Liquid, Mead Johnson, Chicago, IL); fortification to 24 kcal/oz with a liquid, neutral, bovine milk–based fortifier (F-NEUT) (Similac Human Milk Fortifier Concentrated Liquid, Abbot, Chicago, IL); and fortification to 28 kcal/oz with a liquid, neutral, human milk–based fortifier (F-HUM) (Prolact+8, Prolacta Bioscience, City of Industry, CA).

Sample Preparation

Fortification and handling of the milk samples occurred in the hospital milk preparation room at Westchester Medical Center (Valhalla, NY) between September 2016 and December 2016 by a trained Milk Technician. Each unique batch of HPDHM was mixed according to the protocol outlined here: F-ACID was fortified with 1 vial (5 mL) of Enfamil HMF added to 25 mL HPDHM to reach 24 kcal/oz; F-NEUT with 1 envelope (5 mL) of Similac HMF fortifier plus 25 mL HPDHM to reach 24 kcal/oz; and F-HUM with Prolact+8 HMF (40 mL) plus 60 mL HPDHM, to reach 28 kcal/oz. Once mixed, all samples were stored in clean containers labeled with the HPDHM batch number and a unique letter code corresponding to fortification type. With the exception of the milk technician, all researchers were blinded to treatment type until after all samples were analyzed and all data were collected.

A 5 mL aliquot from each control and fortification group was stored in the refrigerator in a clean, glass container. Time 0 for the fortified samples began once the samples had been fortified and placed in the refrigerator. Time 0 for the CONTROL began once the unfortified HPHDM was aliquoted in the glass container and refrigerated. Samples were stored in the refrigerator throughout the study and a daily log of refrigerator temperatures was kept. Every 24 hours, each 5 mL aliquot was opened, and 1 mL of milk was removed using a sterilized pipette and transferred to a clean, glass bottle. The samples were resealed and returned to the refrigerator. The 1 mL aliquot was labeled with the storage time (0, 24, 48, 72, 96 hours) and immediately stored at −20°C in a specimen bag that contained the batch number and a unique letter code that identified the treatment group. When the 96-hour sample was added to the specimen bag, the bag was labeled with the date on which the last sample was collected. When all 12 batches had been processed, they were packaged on dry ice and shipped from Westchester Medical Center to the Nutrition Department at the University of North Carolina Greensboro (Greensboro, NC) for analysis. One batch was missing the F-NEUT treatment; therefore, the study generated 235 total samples for analysis, with a total of 60 samples across 5 time points for the unfortified, F-ACID, and F-HUM treatments, and 55 samples across 5 time points for the F-NEUT treatment.

In the laboratory, samples were thawed at room temperature and with the help of body heat, being held in the hands and pockets of the lab technician, mixed using a vortexer for approximately 3 seconds, divided into 125 μL aliquots, and then immediately refrozen at −20°C until analysis. Samples were stored frozen for between 4 and 15 months, and similar thawing techniques were employed before sample analysis.

Sample Analysis

For each sample collected, IgA activity, lysozyme activity, and total protein content were assessed. IgA activity was measured by enzyme-linked immunosorbent assay, which has previously been described in detail [17,18]. *Escherichia coli* acquired from the STEC Center (Michigan State University, East Lansing, MI) were used to prepare an antigen for coating the wells of a microplate. IgA antibodies from human milk bind to the *E coli* antigens and also bind with anti-human-IgA antibody labeled with horseradish peroxidase (part number A0295, Sigma Aldrich, St Louis, MO). 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (part number A1888, Sigma Aldrich) was used as a colorimetric substrate. Absorbance was measured at 405 nm via spectrophotometry (Synergy HT, BioTek Instruments, Winooski, VT) and IgA activity was computed based on a known human colostrum IgA standard (part number I2636, Sigma Aldrich).

Lysozyme activity was analyzed by changes in turbidity to a microbial suspension of *Micrococcus lysodeikticus* (part number NC9310237, Fisher Scientific, Hampton, NH) at 450 nm over the course of 7 minutes, measured by spectrophotometry (Synergy HT, Bio-Tek Instruments) [19,20].

Total protein was measured by the bicinchoninic acid (BCA) method (part number PI23225, Fisher Scientific) [21]. This assay measures the reduction of Cu^{+2} by the acidic side chains of human milk proteins and the resulting color change, induced by BCA, which exhibits a strong absorbance at 562 nm. Samples were diluted 1:20 with deionized water and analyzed via spectrophotometry (Synergy HT, Bio-Tek Instruments) alongside known bovine serum albumin standards.

For each control sample, all corresponding fortified samples and all time points (4 treatments × 5 time points = 20 samples total) were measured on the same 96-well plate to eliminate interassay variability.

Statistical Analysis

Statistical analysis was performed using SAS software 9.4 Enterprise Edition (SAS Institute, Inc, Cary, NC). Descriptive statistics were computed for the main effects of treatment and time. Repeated measures were assessed using the mixed procedure to determine the effects of treatment, time, and an interaction between treatment and time. Main effects that were statistically different were evaluated using an analysis of variance with a Tukey adjustment for multiple comparisons. *P* values were set at 0.05. This study was classified as exempt by the institutional review board at the University of North Carolina Greensboro (protocol 16-0411).

RESULTS

Each analytical test was performed in triplicate and the resulting average coefficients of variation for assays were as follows: total protein, 2.9%; lysozyme activity, 9.4%; and IgA activity, 2.3%. For IgA activity, all R^2 values were >0.996, and for lysozyme, all R^2 values were >0.979. The F-NEUT treatment group was missing in sample 1; therefore, analysis was performed with the incomplete dataset of 12 samples, and with a complete dataset of 11 samples. None of the

significant conclusions changed; therefore, all data presented represent analysis using the 11 samples in which all treatments and time points were available.

Effect of Fortification at Baseline (Time 0)

Using the repeated measures analysis, there was a highly significant impact of treatment ($P < 0.001$) for all dependent variables. Descriptive statistics by treatment at baseline (time 0) are summarized in Table 1.

Table 1. Descriptive statistics by treatment at baseline (time = 0)

	Control	F-ACID	F-NEUT	F-HUM
Samples	11	11	11	11
Protein, g/dL	1.2 (0.1)*	2.0 (0.2)[†]	2.2 (0.1)[†]	2.5 (0.1)[‡]
Lysozyme, units/mL	5270 (890)*	3340 (1660)[†]	4530 (1150)[‡]	6230 (500)[§]
IgA, mg/dL	80.3 (22.4)*	70.0 (12.3)[†]	89.5 (18.1)*	144.4 (16.1)[‡]

Data represent means and standard deviations. Differences between groups were assessed by analysis of variance (ANOVA) analysis with a Tukey test for multiple comparisons. Entries in the same row with different superscripts are statistically different (P<0.05).

F-ACID = acidic bovine–derived fortifier; F-HUM = human milk–derived fortifier; F-NEUT = neutral bovine–derived fortifier; IgA = immunoglobulin A; UNFORT = unfortified.

All 3 fortifiers significantly increased the total protein concentration of HPDHM (F-ACID 2.0 g/dL, F-NEUT 2.2 g/dL, F-HUM 2.5 g/dL, $P < 0.05$) compared to control (1.2 g/dL). Total protein was not significantly different between F-ACID and F-NEUT treatments ($P = 0.15$). As expected F-HUM treatment group had a significantly higher protein concentration compared with all other treatments ($P < 0.0001$).

Lysozyme activity was significantly different in all treatment groups ($P < 0.001$). Fortification with F-ACID resulted in a significant decrease in lysozyme activity compared to all other treatments ($P < 0.001$), with a 37% reduction compared to control. Lysozyme was undetectable at all time points in the F-ACID treatment group of sample 5. Mean lysozyme activity was significantly higher in F-HUM group compared with all other treatments ($P < 0.001$), with an 18% increase compared to control.

IgA activity was not significantly different between F-NEUT and control samples ($P = 0.23$). There was significant decrease in mean IgA activity with F-ACID treatment ($P < 0.001$) compared to all other treatments, with a 13% reduction compared to control. There was a significantly increase in mean IgA activity in F-HUM treatment group ($P < 0.001$) compared to the other treatments, with an 80% increase compared to control. Box and whisker plots of the baseline data (time 0) are presented in Figure 1.
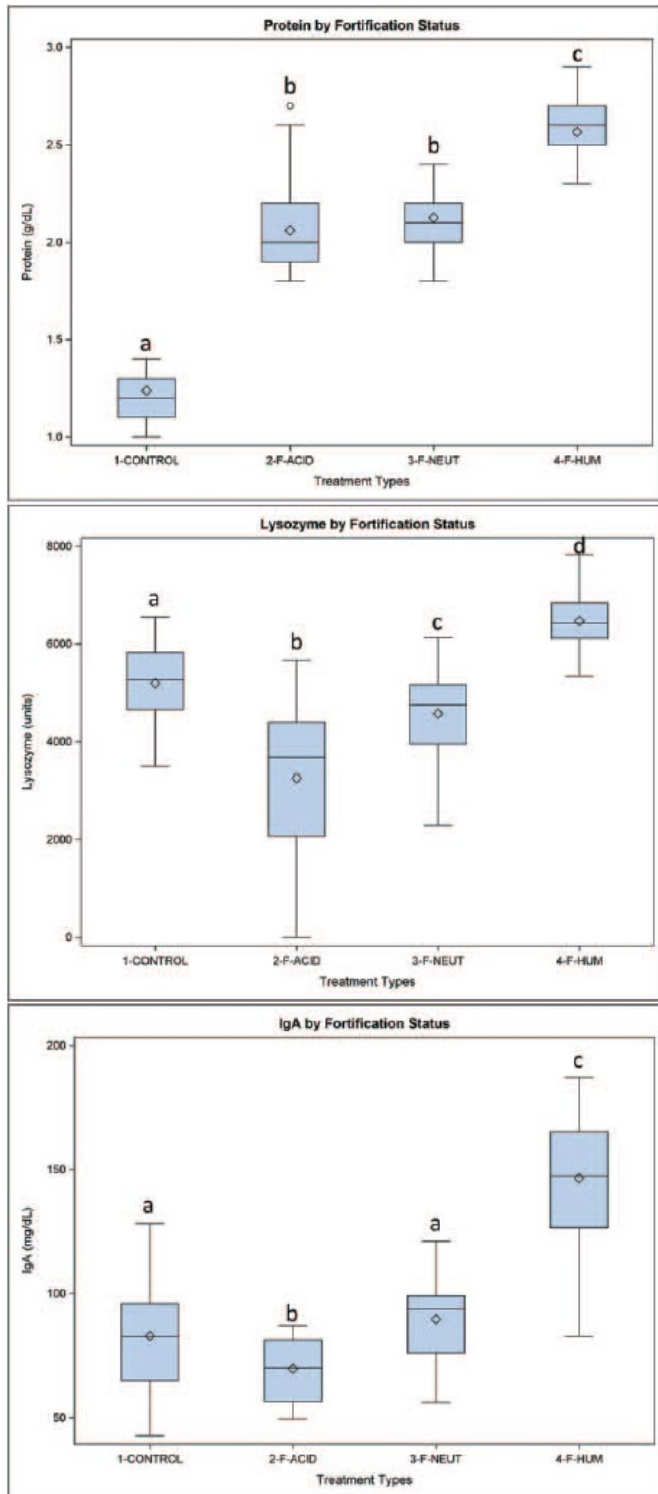
Figure 1. Holder-pasteurized donor human milk (n = 11 per treatment) at baseline (time 0) based on fortification status. Distributions with the same letter are not statistically different ($P > 0.05$) using an analysis of variance (ANOVA) analysis with a Tukey test for multiple comparisons. In the box and whisker plots, the box spans the interquartile range, the line represents the median, the diamond represents the mean, and the top and bottom lines extend to the highest and lowest observations.

Effect of Refrigerated Storage Time

Neither unfortified nor fortified HPDHM samples showed any significant changes over 96 hours of refrigeration. Time had no significant impact on protein concentration or the activity of lysozyme or IgA in any of the samples ($P > 0.97$ for all variables). Furthermore, there was no significant interaction between fortification type and storage time ($P > 0.05$ for all variables). Descriptive statistics for the study time points are summarized in Table 2.

Table 2. Descriptive statistics for all treatments by time

	Protein, g/dL	Lysozyme, units/mL	IgA, mg/dL
Hour 0	2.0 (0.5)	4840 (1520)	96.0 (33.7)
Hour 24	1.9 (0.5)	4920 (1570)	97.3 (33.4)
Hour 48	2.0 (0.5)	4820 (1490)	97.6 (35.1)
Hour 72	1.9 (0.5)	4830 (1560)	97.8 (36.0)
Hour 96	1.9 (0.5)	4930 (1700)	97.8 (36.3)

    Data represent means and standard deviations (n¼44 samples per time point). There were no statistically significant differences between groups ($P>0.05$).
    IgA = immunoglobulin A.

DISCUSSION

There is limited information regarding the effects of refrigerated storage on fortified HPDHM[13]. In this study, 96 hours of refrigerated storage had no significant impact on the total protein concentration and the activity of lysozyme and IgA in unfortified and fortified HPDHM. Studies of refrigerated storage of unfortified HPDHM support our findings. Meng et al [10] reported no significant change in total protein, lysozyme activity, or IgA activity in unfortified HPDHM that was stored for up to 7 days in the refrigerator. Silvester et al [22] studied the bactericidal capacity of unfortified HPDHM against *E coli* for 72 hours of refrigerated storage and found no significant changes.

Regarding differences among treatments at baseline, fortification with commercial HMF resulted in a 62% to 103% increase in protein concentration compared to control. Although this result is unsurprising, it is important to acknowledge that these products indeed do what they claim to do, which is to boost the protein content of human milk. Other studies have described similar increases. Donovan et al [23] reported significant, although somewhat greater, increases in protein concentrations of both MOM and HPDHM fortified with F-NEUT and F-ACID HMF compared to unfortified.

Similarly, as it was the only human milk–based HMF included in the study and the only treatment innately containing lysozyme and IgA, it is no surprise that only F-HUM treatment affected significant increases in both lysozyme and IgA activity in HPDHM. IgA in human milk has been associated with reduced illness in breast-feeding infants [24]. Similarly, in models of neonatal malnutrition using a piglet, dietary human lysozyme was associated with improved gut barrier function [25]. Increasing the antimicrobial proteins in HPDHM for the preterm infant may have clinical benefits and warrants further investigation. Fortification with F-HUM also resulted in a significantly higher protein concentration compared to the other fortifiers. This is very likely

due to the fact that these samples were mixed to 28 kcal/oz due to clinical need at the time of sample collection, compared to the other treatments, which were mixed to 24 kcal/oz.

At baseline, HPDHM fortified with F-ACID exhibited significant declines in both lysozyme and IgA activity compared to all other treatments. Anytime acidity is increased, the risk for deactivating and denaturing proteins increases. It is quite likely that this is the case here. Although the pH of the HPDHM in this study was not measured due to small sample volume, Donovan et al [23] found that fortification with an acidic HMF resulted in a pH of 4.96 in preterm HPDHM and a pH of 5.10 in term HPDHM. Quan et al [26] reported a 19% reduction in the lysozyme activity, and no change in IgA, in raw human milk fortified with a human milk fortifier that was available in the early 1990s, although no additional information on the fortifier characteristics were provided. Reduction of the bioactive factors in HPDHM through the use of acidic fortifiers may have immune consequences for the vulnerable preterm neonate. Emerging evidence also suggests acidic fortifiers lead to higher rates of feeding intolerance and metabolic acidosis in the preterm infant [27].

Study Limitations

In order to aliquot and complete analyses in the laboratory, the HPDHM samples underwent 4 freeze-thaw cycles, which is 2 more than traditionally encountered in normal use [12,28]. Each freeze/thaw cycle brings with it the risk of destabilized casein micelles and the altered quaternary structure of whey proteins, which can result in the formation of precipitates [29]. In our study, the F-ACID treatment resulted in precipitates in the samples which required additional handling steps so as not to interfere with spectrophotometry readings. These samples were thawed in a shaking water bath at 35°C and 80 rpm for 60 minutes to improve homogenization. It is possible that the multiple freeze-thaw cycles contributed to this phenomenon. That being said, all samples were subject to the same number of freeze-thaw cycles, which allows for comparison across the study. In the hospital feeding room, the F-HUM was mixed to 28 kcal/oz, although both the F-ACID and F-NEUT were mixed to 24 kcal/oz. The human milk–based fortifier used in this study was a higher calorie and protein fortification than originally planned due to clinical needs in the hospital during the study period, and it would be expected to provide more protein than the bovine milk–based fortifiers. This explains the average higher protein concentrations measured in this treatment group compared to the others. However, it is also likely that, had the human milk–based HMF been mixed to 24 kcal/oz like the F-ACID and F-NEUT HMF, the levels of lysozyme and IgA activity would still have been significantly higher than all other treatments, due to the fact that only F-HUM would likely contain meaningful quantities of these antimicrobial proteins. The BCA assay over-reports actual protein content; however, it has been validated as reliable for measuring human milk ($R^2 > 0.99$) and is therefore appropriate for assessing differences between groups [21]. We did not evaluate bacterial growth in our study, which is an important consideration in the NICU setting.

Gaps in Literature and Future Research Implications

Very few studies have specifically evaluated the impact of long-term refrigerated or frozen storage of HPDHM. Many address long-term storage in combination with other pasteurization methods, treatments, and outcomes, which can make the results specific to storage difficult to

assess. In addition, small sample size and the use of milk samples from a single donor rather than samples of pooled HPDHM are common limitations. In order to more accurately represent HPDHM found in the NICU, future studies should use pooled milk samples in clinically relevant volumes and be designed to emulate clinical practices, with the opening and closing of refrigerators and containers. More research is also needed into how fortifiers impact essential micronutrients in HPDHM.

CONCLUSIONS

As expected, fortification with liquid bovine and human milk–based fortifies significantly increases the protein concentration in donor human milk. Human milk–based fortifier provides additional IgA and lysozyme which may be beneficial. Our findings of lack of negative impact of refrigeration storage time on the protein and bioactive components of donor milk strengthen the recent HMBANA recommendation to extend storage time to 48 hours. The observed decline in IgA and lysozyme with acidic fortification is concerning and warrants further studies.

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