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Background: The use of pasteurized donor human milk (PDHM) is growing among neonatal intensive care units. Addition of commercial fortifiers to better meet the nutritional needs of preterm infants is common, however limited information is available regarding nutrient stability in fortified PDHM (FPDHM). Objective: The purpose of this study is to measure the anti-microbial activity and protein content of FPDHM during refrigerated storage over 96 hours. Methods: Unfortified PDHM served as the control (CONTROL). PDHM was subjected to treatment with 3 different fortifiers: an acidic (F-ACID), a neutral (F-NEUT), and a human-milk derived (F-HUM) fortifier. Samples were stored at 4°C, and every 24h, a 1-mL aliquot was removed for analysis. Samples were analyzed for total protein, immunoglobulin A (IgA), and lysozyme. Results: At baseline, there was a significant difference in protein (mean, standard deviation) concentration (g/dL) between control (1.3, 0.14) and all other treatments (F-ACID = 2.0, 0.19; F-NEUT = 2.2, 0.14 ; F-HUM = 2.5, 0.12; $p<0.001$). 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 time ($p>0.9$ all variables), nor was there a significant interaction effect between time and treatment ($p>0.9$ all variables). Conclusion: The type of fortifier has a more significant impact on bioactive components in fortified PDHM than storage time.

THE EFFECTS OF REFRIGERATED STORAGE TIME AND FORTIFICATION ON PASTEURIZED DONOR HUMAN MILK

by

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CHAPTER I

INTRODUCTION

Human milk (HM) is the ideal source of food for the majority of infants. This idea has been well-studied and global initiatives are in place supporting the expanded adoption of breastfeeding practices.[1–4] A diverse array of benefits are conferred to infants through breastmilk, ranging from the development of the immune system and protection against infections to emotional and cognitive advantages throughout the life cycle.[5,6]

Population Overview: Preterm Infants

Infants born before 37 weeks of gestation are considered preterm. More than 10% of infants born worldwide are premature and preterm birth rates are rising. Along with social, emotional, and financial burdens, preterm birth is associated with a host of health complications, along with higher rates of hospital readmission and longer hospital stays.[7,8] Immediate, short-term health concerns include compromised immunity,[9] feeding challenges,[10] and poor growth.[11] Long-term health consequences include neurological impairment and chronic lung disease, as well as an increased risk of developing non-communicable disease

later in life, such as hypertension and diabetes.[7,8,12] Many of the early challenges faced by premature infants can be addressed with proper nutrition intervention which are imperative to combat the consequences of poor growth that persist into childhood and beyond.[13]

Feeding Recommendations for the Preterm Infant

Providing preterm infants with sufficient nutrients to support growth and development is of tantamount importance. Kumar et al summarized the European Society of Pediatric Gastroenterology, Hepatology, and Nutrition's (ESPGHAN) recommendations for enteral nutrient intakes for preterm infants in their 2017 review.[10] (Table 1)

Table 1. ESPGHAN's Recommendations for Enteral Nutrient Intake for Preterm Infants[10]

Nutrient	Per kg of body weight per day
Fluid, mL	135-200
Energy, kcal	110-135
Protein, g	3.5-4.5
Fat, g	4.8-6.6
Carbohydrate, g	11.6-13.2

Preterm infants gain many benefits from a diet comprised primarily of HM. The use of expressed breastmilk from mom is the top choice among experts, with PDHM ranking second when mother's own milk is unavailable.[2,10,14] While infant formulas are associated with more impressive growth parameters, they confer none of the immunity-building components or innate defense mechanisms found

in HM. When compared to infant formula, the use of HM is associated with a lower risk of developing sepsis, late-onset sepsis, and necrotizing enterocolitis (NEC),[2,15,16] improved feeding tolerance,[2,15] and fewer hospital readmissions.[17,18]

Pasteurized donor human milk (PDHM) distributed by milk banks is recommended for feeding infants weighing less than 1500g when a mother's own milk is unavailable or insufficient.[14] Its use is associated with reduced healthcare costs,[17] better health outcomes,[2,19,20] and a reduced risk of the development of NEC.[21] PDHM is prescribed for a number of medical conditions, including prematurity, malabsorption syndrome, feeding intolerance, immunologic deficiencies, trophic feeds/gut priming, among others.[22] In neonatal hospitals in the United States, the use of PDHM has been steadily on the rise since the Human Milk Banking Association of North America's (HMBANA) inception in 1985,[23] with 68% and 73% of level 3 and 4 facilities reporting usage, respectively, in 2015.[24]

The pasteurization of donor HM is common practice among milk banking organizations, primarily to remove infectious contaminants and reduce the growth of harmful bacteria.[25,26] Optimizing the safety of banked HM is an appropriate concern given that it is often fed to preterm infants who have compromised immune systems.[27] The pasteurization of HM has been well-studied. A systematic review of 44 studies reported that, while pasteurization does indeed partially diminish several important biological components, such as

immunoglobulins and the activity of lipase enzymes, macronutrients are almost fully retained.[26]

While HM remains the ideal food for the majority of infants, its protein and energy contents are too low at all stages of lactation to support the increased needs of low and very low birth weight infants.[28] This led to the development of HM fortifiers (HMF), which are recommended when feeding preterm infants in order to meet their increased protein, energy, and micronutrient needs.[2,29] Fortification with HMF is now standard practice for the preterm neonate, with over 90% of NICU hospitals reporting use of bovine-milk-based or human-milk-based fortifiers.[24] However, the routine use of HMF has not eliminated postnatal growth failure.[30]

Storage of Pasteurized Donor Human Milk

Less is known about what happens to PDHM over the course of long-term storage. While the Academy of Breastfeeding Medicine has issued storage recommendations for raw HM fed to healthy infants in home settings,[31] recommendations regarding the storage of PDHM for use in a clinical setting with medically fragile infants are scarce. Existing guidelines recommend that: 1) thawed PDHM be stored at 4°C and used within 24 hours,[32] and 2) the maximum frozen storage (-20°C) time for PDHM is 3-6 months,[32–36] or one year after the earliest pumping date of milk within the pool.[37] Understanding appropriate storage conditions for PDHM in a clinical setting is an important area of research.

PDHM is a valuable commodity with a short shelf-life. Affordability was the most frequently cited barriers of non-use in a 2013 survey of 183 Level 3 NICUs.[38] Research to evaluate the feasibility of extending PDHM expiration dates has the potential to reduce cost barriers associated with a short shelf-life and product waste.

Study Aims

While the use of HMF has increased, there is scant research examining the effects of refrigerated storage time on the retention of macronutrients and other bioactive components in fortified HM. This is an important area of research and a major gap in the literature that this study aims to address.

The first aim of this study is to compare total protein content, lysozyme activity, and immunoglobulin A (IgA) activity in unfortified PDHM and PDHM fortified with three commercially available fortifiers at baseline. The second aim of this study is to evaluate changes in total protein, lysozyme and IgA activity over 96 hours of refrigerated storage. Both bovine- and human-milk-based fortifiers contribute additional protein and it can therefore be hypothesized that total protein content will increase in all fortified PDHM samples compared to unfortified. As bovine-milk-based fortifiers contain limited lysozyme and IgA, it can be hypothesized that only the human-milk-based fortifier will cause an increase in these antimicrobial proteins. Acidic fortifiers have been shown to reduce the pH of PDHM[39] and changes in pH can impact protein stability;

therefore, it was hypothesized that PDHM fortified with acidic Enfamil HMF would show greater losses of lysozyme and IgA compared to neutral Similac HMF, human-milk based Prolact+8, and unfortified.

Overall, we hypothesized that all fortified samples will have higher total protein concentrations than unfortified samples. Fortified PDHM will be as stable as unfortified samples over time, and all PDHM samples will be unaffected by storage time.

CHAPTER II

THE EFFECTS OF REFRIGERATED AND FROZEN STORAGE ON HOLDER-PASTEURIZED DONOR HUMAN MILK: A SYSTEMATIC REVIEW

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Storage of Holder Pasteurized Donor Human Milk

Introduction

According to a 2017 policy statement published by the American Academy of Pediatrics, the use of pasteurized donor human milk (PDHM) distributed by milk banks is recommended for infants weighing less than 1500g when a mother's own milk is unavailable or insufficient.[14] The use of PDHM is associated with reduced healthcare costs,[17] better health outcomes,[2,19,20] and a reduced risk of the development of necrotizing enterocolitis (NEC).[21] Results from the Center for Disease Control's 2015 Maternity Practices in Infant Nutrition and Care (mPINC) survey, indicated that two-thirds of neonatal intensive care hospitals were using donor human milk, and over 90% were using fortifiers to increase the nutrient content of human milk.[24]

The pasteurization of donor HM is common practice among milk banking organizations, primarily to remove infectious contaminants and reduce the growth of harmful bacteria.[25,26] This is an appropriate concern given that PDHM is often fed to preterm infants who have compromised immune systems.[27] The Holder method of pasteurization is the predominating procedure used by milk banks internationally and involves heating the HM to 62.5 – 63°C for 30 minutes.[32–34,41] Holder pasteurization of HM has been well-studied. While the process does indeed partially diminish several important biological components, such as immunoglobulins and the activity of lipase enzymes, macronutrients are almost fully retained.[26]

Less is known about what happens to Holder pasteurized donor human milk (HPDHM) over the course of long-term storage. While the Academy of Breastfeeding Medicine has issued storage recommendations for raw HM fed to healthy infants in home settings,[31] recommendations regarding the storage of HPDHM for use in a clinical setting with medically fragile infants are scarce. Understanding appropriate storage conditions for HPDHM in a clinical setting is an important area of research.

In 2011, the Human Milk Banking Association of North America (HMBANA) issued guidelines recommending that thawed HPDHM should be stored at 4°C and used within 24 hours.[32] Based on international guidelines outlining milk banking protocols, the maximum recommended frozen storage

(-20°C) time for HPDHM is 3-6 months.[33–36] According to the 2018 HMBANA guidelines, frozen HPDHM expires 1 year after the earliest pumping date of milk within the pool.[37] HPDHM is a valuable commodity with a short shelf-life. Affordability was the most frequently cited barrier of non-use in a 2013 survey of 183 Level 3 NICUs.[38] Research to evaluate the feasibility of extending HPDHM expiration dates has the potential to reduce cost barriers associated with a short shelf-life and product waste.

The purpose of this paper is to review the current evidence for the storage of Holder pasteurized donor human milk under refrigerated and frozen storage conditions.

Methods

Search process

This review of published literature was conducted through electronic searches of PubMed, Scopus, Science Direct, Google Scholar, ProQuest Central, and WorldCat Discovery. The electronic search included the following keywords and MeSH terms: (i) human milk; (ii) breast milk; (iii) donor milk; (iv) pasteurized donor human milk; (v) milk banks AND storage; (vi) (donor milk OR human milk) AND pasteurization; (vii) (donor milk OR human milk) AND storage; (viii) (donor milk OR human milk) AND refrigerated storage; (ix) (donor milk OR human milk) AND frozen storage; (x) (donor milk OR human milk) AND (storage OR pasteurization); (xi) (donor milk OR human milk) AND (storage OR bank)

AND pasteurization; (xii) (human milk OR human milk) AND (storage OR pasteurization) AND (bioactive OR immune OR antimicrobial); and (xiii) (donor milk OR human milk) AND (storage OR freez* OR refrig* OR processing) AND (pasteuriz*).

Inclusion and exclusion criteria

In order to be included in this review, a study must have been published between 1985 and May 2018, when the search was conducted. This cut-off date was chosen based on the establishment of HMBANA and its milk processing protocols. Studies were required to be peer-reviewed and include the primary outcome of the effects of extended storage, either under frozen (typically -20°C) or refrigerated (typically 4°C) conditions. Only studies examining donor HM that had undergone the Holder method of pasteurization were included, whether explicitly stated or as evidenced by the processing protocol used at the milk bank from which it was acquired. HPDHM fortified with HM fortifiers were also included. Studies assessing colostrum were not included. Studies were also excluded if they did not describe the length of storage time at a given temperature or the method of pasteurization employed, if they did not report outcomes specifically for HPDHM, or if the heat-sterilization process differed from the Holder method, such as high-temperature short-time (HTST), ultra-high temperature (UHT), or extended shelf life (ESL).

Data extraction

The following information was extracted for each study: author; year; title; type of milk; sample size; fortification status; storage temperature; storage duration; outcomes measured; findings. Two researchers independently reviewed all studies for inclusion/exclusion criteria and results, and differences were resolved via discussion.

Results

Initially, 19 studies that included HPDHM were identified. Three (16%) did not describe the length of storage time,[42–44] one (5%) did not report outcomes specifically for HPDHM,[45] and one (5%) was not peer-reviewed,[46] leaving a final total of 14 studies included in this review (Table 2).

Table 2. Summary of Refrigerated and Frozen Storage Studies Using Holder Pasteurized Donor Human Milk

Author(s)	Year	Storage conditions	Storage duration	Sample size	Outcome(s) measured
Lepri et al.[47]	1997	-20°C	90 days	n = 16 single-donor samples	Lipids: modified Folch method, and thin-layer and gas chromatography L-lactate: biosensor Degree of lipolysis: gas chromatography
Silvestre et al.[48]	2008	4-6°C	72 hours	n=10 single-donor samples	Bactericidal capacity: E. coli viability assay
Vieira et al.[49]	2011	-20°C	24 hours	n=57 single-donor samples	Macronutrients: Infrared human milk analyzer (MilkOScan by Foss)

Cohen et al.[50]	2012	4°C	122 hours	n=22 previously pooled samples	Bacterial growth: standard plate count method
García-Lara et al.[51]	2013	-20°C	180 days	n=34 individual samples from 28 donors	Macronutrients: Infrared Human Milk Analyzer (MIRIS, Sweden)
Vázquez-Román et al.[52]	2014	-20°C	90 days	n=36	Fat and energy content: creamatocrit (Lucas method)
Borgo et al.[53]	2015	-18°C	240 days	n=1 sample from single donor	Saturated and unsaturated FA: gas chromatography, nuclear magnetic resonance, infrared spectroscopy
Vickers et al.[54]	2015	4°C	0-96 hours, 9 days	n=42 previously pooled samples (2-5 donors per pool)	Bacterial growth: HMBANA Standard Operating Procedure for Culturing PDHM
Marinković et al.[55]	2016	-20°C	30 days	n=10 single-donor samples	Antioxidative properties: static oxidation-reduction potential (ORP) measurement; oxygen radical absorbance capacity (ORAC) assay; Reflectoquant ascorbic acid test; electron paramagnetic resonance spin-trapping spectroscopy
Vázquez-Román et al.[56]	2016	-20°C	3 months	n=40 previously pooled samples	Dornic acidity: titration
Meng et al.[57]	2016	4°C	7 days	n=13 single-donor samples	Aerobic bacteria and coliform count: Petrifilm Total protein: Bicinchoninic acid (BCA) assay Lysozyme activity: *Micrococcus lysodeikticus* based turbidimetric assay IgA activity: kinetic indirect ELISA

Kanaprach et al.[58]	2018	-20°C	6 months	n=40 single-donor samples	Intestinal cell growth-promoting activity: fetal intestinal growth assay Antimicrobial effect against *E. coli*: antimicrobial assay
Salcedo et al.[59]	2018	4°C	90 days	n=5 single-donor samples	Gangliosides concentrations: ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS)
de Waard et al.[60]	2018	-20°C	12 months	n=34 single-donor pools	Bacterial growth: blood and CLED agar Macronutrients: Human Milk Analyzer (MIRIS, Sweden)

Refrigerated storage of Holder pasteurized donor human milk

Five studies examined the storage of unfortified HPDHM under refrigerated conditions (Table 3). Storage times ranged from 24 hours to 90 days, and analytes assessed included microbial growth, total protein, bactericidal capacity, lysozyme activity, secretory immunoglobulin A (sIgA) activity, and total gangliosides concentrations. One of these studies included pilot information on fortified HPDHM, though significance was not independently assessed.[50]

Table 3. Effects of Extended Refrigerated Storage on Components of Unfortified Holder Pasteurized Donor Human Milk

Component	Duration	Findings	Author
Microbial growth			
	24-122 hours	No significant change	*Cohen[50]
	7 days	No significant change	Meng[57]
	9 days	No significant change	Vickers[54]
Total protein			
	7 days	No significant change	Meng[57]
Bactericidal capacit			
	72 hours	No significant change	Silvestre[48]
Lysozyme activity			
	7 days	No significant change	Meng[57]
IgA activity			
	7 days	No significant change	Meng[57]
Gangliosides			
GM3	90 days	No significant change	Salcedo[59]
GD3	90 days	No significant change	Salcedo[59]
Total gangliosides	90 days	No significant change	Salcedo[59]

* signifies that this study included fortified and unfortified HPDHM

Effects on microbial growth

Currently, there is no consensus definition of “acceptable levels” of bacteria in HM and special considerations must be made for infants in the NICU with compromised immune systems. Regarding healthy, term infants, Meng et al suggest two options: 1) use the levels set for Pasteurized Milk Ordinance (PMO) for Grade A pasteurized bovine milk (4.30 log CFU/mL), or 2) set the maximum level as that which is present in the milk in a feeding container immediately after exposure to the microflora in an infant’s mouth through bottle or cup feeding (average 2.8 log CFU/mL). In their 2016 study, Meng et al found that, after 7

days of storage at 4°C, HPDHM consistently had bacteria levels below both the PMO standard and the more stringent constraints set in option 2. The aerobic bacteria count for HPDHM stored at 4°C was 0.0 log CFU/mL at all time points up to 7 days.[57]

These data support similar findings by Cohen et al who, in a 2012 study, found no bacterial growth in 22 samples of HPDHM that were thawed and refrigerated for 24 to 122 hours of routine NICU handling.[50] However, 33% (2/6) bottles of fortified HPDHM exhibited bacterial growth. A 2015 study by Vickers et al found that there was no evidence of microbial growth in HPDHM when thawed and stored at 4°C for up to 9 days. This study utilized 42 randomly selected samples of HPDHM from a HMBANA milk bank. Study milk handling protocol aimed to mimic that which may be found in a NICU feeding room and, on average, the refrigerator was opened 27 times per day.[54] These data suggest that unfortified HPDHM maintains its antimicrobial defenses and remains free of microbial growth when stored at 4°C for up to 9 days.

Effects on macronutrient concentration

One published study has addressed the retention of macronutrients during refrigerated storage of HPDHM, and only protein concentration was assessed. In the 2016 study by Meng et al, HPDHM stored at 4°C exhibited no significant change in total protein concentration ($p=0.27$) between 0 – 7 days.[57]

Effects on bactericidal capacity and bioactive factors

There are two studies that examine the impact of refrigerated storage on bactericidal capacity and the activity of immunological factors in HPDHM. In their 2008 study, Silvestre et al determined that the bactericidal capacity of HPDHM against *E. coli* exhibited no significant changes during 72 hours of refrigerated storage (at 4-6°C).[48] In 2016, Meng et al reported no significant changes in the activity of lysozyme ($p=0.77$) and sIgA ($p=0.49$) after 7 days of refrigerated storage.[57]

In 2018, Salcedo et al published a study looking at the effects of heat treatment and storage time on the concentration of gangliosides in HM. Gangliosides are glycolipids primarily associated with the milk fat globule membrane, and their content and profile constituents vary throughout lactation. GD3 (Neu5Ac α2-8 Neu5Ac α2-3 Gal β1-4Glc β1-1 ceramide) is most abundant during the first few days of lactation, while GM3 (Neu5Ac α2-3 Gal β1-4Glc β1-1 ceramide) is found in the highest proportion in mature HM.[61] Salcedo et al found that storage for up to 90 days at 4°C had no significant impact on either total or specific ganglioside content in HPDHM.[59]

Frozen storage of Holder pasteurized donor human milk

While storing raw and pasteurized HM at -80°C minimizes changes to many properties, it is impractical for milk banks and neonatal units primarily due to its expense.[62,63] Freezer storage at -20°C is much more commonplace. This

section summarizes the findings from the 9 studies published examining the impact of frozen storage conditions on pH and microbial growth, as well as the retention of macronutrients, immunological activity, and enzymatic activity (Table 4).

Table 4. Effects of Extended Frozen Storage at -20°C on Components of Holder Pasteurized Donor Human Milk

Component	Duration	Findings	Author
Bacterial growth			
	8 months	No significant growth	de Waard[60]
Total protein			
	24 hours	Significant decrease	Vieira[49]
	8 months	Significant increase	de Waard[60]
Nitrogen			
	6 months	No significant change	García-Lara[51]
Total carbohydrate			
	6 months	Significant decrease	García-Lara[51]
	8 months	No significant change	de Waard[60]
Lactose			
	24 hours	No significant change	Vieira[49]
L-lactate			
	90 days	Decrease (significance not assessed)	Lepri[47]
Total fat			
	24 hours	Significant decrease	Vieira[49]
	3 months	Significant decrease	Lepri,[47] Vázquez-Román[52]
	6 months	Significant decrease	García-Lara[51]
	8 months	No significant change	de Waard[60]
Fatty acids			
	240 days	Varied by fatty acid	Borgo[53]
Degree of lipolysis			
	3 months	Increase (significance not assessed)	Lepri[47]
Energy			
	3 months	Significant decrease	Vázquez-Román[52]
	6 months	Significant decrease	García-Lara[51]
	8 months	No significant change	de Waard[60]
Bactericidal capacity			
	3 months	No significant change	Kanaprach[58]

	6 months	Significant decrease	Kanaprach[58]
Antioxidative properties			
Superoxide dismutase	30 days	No significant change	Marinković[55]
Glutathione peroxidase	30 days	No significant change	Marinković[55]
Glutathione reductase	30 days	No significant change	Marinković[55]
Ascorbate concentration	30 days	No significant change	Marinković[55]
Dornic acidity			
	3 months	Non-clinically significant decrease	Vázquez-Román[56]
Intestinal cell growth-promoting activity			
	6 months	No significant change	Kanaprach[58]

Effects on microbial growth

Only one study assessed microbial growth in HPDHM under frozen storage conditions. In a 2018 study, de Waard et al found that HPDHM stored at -20°C for 12 months remained free of microbial growth for the first 8 months.[60] Microbial analysis at 10 and 12 months revealed positive cultures in HPDHM samples from 17-28% of donors; however, study samples were drawn post-pasteurization and it was unclear whether this occurred under sterile conditions, which may have influenced results.

Effects on macronutrient concentrations

Six published studies have assessed the impact of frozen storage on macronutrient retention in HPDHM. Five studies examined total fats, 3 examined total protein or nitrogen, 3 assessed carbohydrates, and one assessed individual fatty acid profiles.

A 1997 study by Lepri et al found that, between 0 – 35 days of frozen storage, total fat content of HPDHM decreased only slightly (25.08 mg/mL ± 0.54 to 24.67 mg/mL ± 0.52), then notably after 70 (23.60 mg/mL ± 0.58) and 90 days (23.32 mg/mL ± 0.55) of frozen storage. This represented a -7.55% change between baseline and day 90.[47]

Others have also reported a decline in the fat content of HPDHM during extended frozen storage. In a 2011 study by Vieira et al, after 24 hours of storage at -20°C, HPDHM showed significant decreases in mean fat (5.5%, $p<0.001$) compared to never-frozen HPDHM.[49] In their 2013 study, García-Lara et al found that there were small but significant decreases in the fat (-0.13 g/dL, 2.8% relative decrease, $p = 0.001$) and energy (-1.55 kcal/dL, or -0.46 kcal/oz, 2.2% relative decrease, $p = 0.001$) content of HPDHM after 180 days of frozen storage. Importantly, authors noted that, while these declines were of low magnitude, when the impact of pasteurization on fat content was taken into account (3.5% relative decrease), the total reduction was 6.2%. This reveals a more clinically relevant issue with regard to the retention of energy for preterm infants who are the primary recipients of HPDHM, given a potentially cumulative detrimental impact associated with multiple processes including pasteurization and storage.

In their 2014 study, Vázquez-Román et al found that the fat content of HPDHM decreased by 0.39 g/dL (-15.08% relative change, $p=0.01$) after 30 days of frozen storage, but there was no significant change at 60 days (0% relative

decrease, p = 0.996) or 90 days (+6.5% relative change; p = 0.580) of frozen storage compared to baseline using creamatocrit as the method for fat assessment.[52] The aliquots prepared for the various storage conditions were homogenized by rocking them in an arc-like fashion ten times. This might not have been enough to thoroughly mix the study samples, which potentially explains why there were differences in the samples at 30 days, but not at 60 and 90 days compared to baseline. Additionally, while there is a strong correlation between the creamatocrit value and the lipid content of HM,[64] the authors point out the limitations of measuring fat content with this method. There is evidence that the fat globule ruptures during frozen storage and subsequent thawing.[65] This breakdown, along with the continued activity of lipoprotein lipase, causes an increase in free fatty acids. This results in a more tightly packed cream layer and, as this is what is measured in a creamatocrit test, can produce a false decrease in the creamatocrit reading, which would misrepresent the actual fat (and energy) content of the HM.[66]

Borgo et al, in a 2015 study, assessed the impact of extended freezer storage (-18°C) on the concentrations of specific saturated and unsaturated fatty acids in HPDHM over 8 months, with measurements taken every 30 days.[53] They reported upward, downward, and quadratic trends in several saturated and unsaturated fatty acids. However, there were major limitations to this work, including the fact that it involved a single sample from 1 donor, that two time

points were dropped because of inconsistent findings, and that the study did not describe how the sample was mixed during aliquoting, which may impact whether all sub-samples had similar fat content.

In 2018, de Waard et al reported no significant changes in total fat or energy contents, during 8 months of frozen storage, but a significant increase in protein content (13.4% relative increase, $p=0.037$).[60] This is in contrast to findings by Vieira et al in 2011, who reported a significant decrease in protein concentrations (3.9%, $p<0.001$) after 24 hours of frozen storage.[49] In a 2013 study by García-Lara et al, there was no significant change in total nitrogen, which represents protein and non-protein nitrogen compounds, in HPDHM during 6 months of frozen storage.[51]

The 2011 study by Vieira et al reported no significant change in lactose concentrations ($p=0.427$) after 24 hours of frozen storage.[49] Similarly, de Waard reported stable carbohydrate composition over 8 months of frozen storage.[60] In the 2013 study by García-Lara et al, there was a small, but significant decrease in carbohydrate content (defined as lactose plus oligosaccharides, -0.08 g/dL, 1.7% relative decrease, $p = 0.006$).[51]

Overall, these studies suggest that total fat in HPDHM decreases by 3 – 8% between 24 hours and 180 days of frozen storage, including the nonsignificant reduction reported by de Waard et al. These decreases correspond to a significant reduction in energy content. Research reflects stable

carbohydrates during extended frozen storage and inconsistent effects on total protein in HPDHM.

Effects on bactericidal capacity and bioactive factors

As part of their 2018 study, Kanaprach et al assessed the effects of extended storage on the growth-promoting activity of fetal intestinal cells and the antimicrobial defenses against *E. coli* in raw and HPDHM.[58] The authors reported that the antimicrobial activity remained constant in HPDHM for up to 3 months of frozen storage (34.0% ± 13.5, compared to 35.9% ± 14.2 at baseline), but exhibited a significant decline at 6 months (-76.1% ± 23.5, $p<0.005$; -323.8% change), indicating an increase in bacterial growth. The HPDHM exhibited no significant changes in growth-promoting activity.

Effects on antioxidative capacity

There is only one published study assessing the antioxidative capacity of HPDHM during extended storage under any condition. In 2016, Marinković et al found that Holder pasteurization and storage at -20°C for 30 days did not affect static oxidation-reduction potential (ORP) or total nonenzymatic antioxidative capacity. While Holder pasteurization caused a significant reduction in ascorbate, superoxide dismutase, and glutathione peroxidase activity in HM, frozen storage did not lead to any further changes.[55]

Effects on pH, acidity, and osmolality

Two studies looked at changes in acidity in HPDHM under frozen storage conditions. In 1997, Lepri et al found that the concentration of L-lactate remained constant in HPDHM during the first 35 days of frozen storage, however after 70 and 90 days, there was an 18% decrease (significance not assessed).[47] Authors speculated that this decrease was due to degradation or a change in optical isomeric form. In a 2016 study, Vázquez-Román et al found that, from baseline to week 1 of storage at -20°C, there was a non-clinically significant decrease in the Dornic acidity (3°D to 2°D, $p<0.05$) of HPDHM, and this reading remained constant over the course of the 3-month study period.[56] Dornic acidity is an alternative measure of acidity specific to milk. This method determines the total titratable acidity and results are expressed in Dornic degrees, with milk measuring $\geqslant$8°D classified as too acidic for pasteurization.[67] The authors hypothesized that the limited change in Dornic acidity of HPDHM, compared to significant changes in raw HM, was due to inactivation of lipase enzymes during pasteurization.

Discussion

Summary of findings

It is impossibly difficult to condense the data presented in the existing literature into one tidy statement. There is very little overlap amongst these studies in regard to the components assessed, analytical methods used, and the

length of study period adopted. It would be safe to conclude that extended storage affects some aspects of HPDHM more than others.

Under refrigerated storage conditions, the most studied component of unfortified HPDHM was bacterial growth. All studies reported no bacterial growth in refrigerated unfortified HPDHM, with the longest study duration of 9 days.[50,54,57] Importantly, two of these studies were designed to reflect feeding room practices where the refrigerator and the HPDHM bottles were opened multiple times a day.[50,54] Not all studies reflected these relevant clinical conditions, which may have biased their results. There have been only single studies on other components in unfortified HPDHM, with no changes reported in total protein, lysozyme activity, sIgA activity, and gangliosides for 7 days or more in refrigerated conditions. While the growing body of evidence suggests that unfortified HPDHM may be safely stored in the refrigerator for longer than 24 hours, there is limited information on the refrigerated storage of fortified HPDHM, with one small study reporting bacterial growth in one-third of samples mixed with a powder fortifier.[50] Refrigerated storage of fortified HPDHM is an important area for future research given the ubiquitous use of HM fortifiers in the NICU setting.[24] Donovan et al studied fortified HM (raw and HoP) over 24 hours of refrigerated storage and reported that different fortifier types had the potential to change milk properties including pH and osmolality.[23]

Under frozen storage conditions, the most studied component of HPDHM was fat, with most studies reporting a small but significant decrease over periods of 24 hours to 8 months. The mixing and handling procedure for study samples was often not reported in the current body of research, which may bias findings about fat, given its propensity to separate from the aqueous layer. Additionally, many studies used milliliter volumes of samples, which may bias findings as it relates to fat, due to the high ratio of container surface area to sample volume compared to what occurs in multi-ounce bottles of HPDHM. Future studies, especially as they relate to fat, should describe mixing and handling protocols for HPDHM and test clinically relevant volumes. Carbohydrates appear to be stable during extended freezer storage, while findings for protein and other components have been inconsistent or only assessed in a single study, suggesting more research is needed.

Limitations and future implications

Several limitations were observed throughout the review process. Very few studies have been conducted that look exclusively at the effects of long-term storage (refrigerated or frozen) on HPDHM. Most address this issue in conjunction with other treatments and outcomes, and sometimes results specific to HPDHM were difficult to assess. Small samples size is another limitation, along with the use of samples from a single donor rather than samples from pooled HPDHM, which would more closely represent HPDHM found in the NICU.

Many studies were not designed to reflect clinical practices or typical storage volumes, which may bias results.

While Holder pasteurization is employed by the vast majority of milk banks,[32–34,41] future research should also focus on the storage of HM processed with techniques other than Holder, given the availability of other human milk products in the market. Differences in storage duration prior to processing should be accounted for in future studies, as the storage period prior to processing is also likely contributing to changes in milk characteristics. Study periods should extend to 12 months for frozen storage, and 4-7 days for refrigerated storage given the emerging evidence of microbial purity during these timeframes. Primary outcomes should include macro- and micronutrient retention, and the activity of bioactive proteins. Larger sample sizes and the use of pooled donor HM would give more power and relevance to study findings. It would fill a gap if future studies were to distinguish between preterm and term HPDHM. With the knowledge that the vast majority of NICUs utilize HM fortifiers,[24] there is a great need to assess the stability of fortified HPDHM beyond 24 hours of refrigerated storage.

Addendum: Fortification of Pasteurized Donor Human Milk

A variety of commercial HMF are available. While powdered forms of HMF provide additional essential nutrients, their use in the NICU setting is discouraged due to increased risk of contamination.[68] Liquid fortifiers are sterilized by heat-

treatment or acidification and are the preferred formula for use in a NICU. However, the use of acidified HMF is associated with increased clinical observations of feeding intolerance, metabolic acidosis, and poor growth.[69] In a 2014 retrospective analysis, Thoene et al examined the incidence of acidosis, growth, and the clinical outcomes of NICU infants fed either a powdered or liquid bovine-milk-based HMF. The use of an acidified liquid bovine-milk-based HMF resulted in a greater incidence of metabolic acidosis ($p=0.002$) and slower growth rates ($p \leq 0.0001$) compared to the powdered HMF.[69] While there is some evidence that fortification improves short-term growth outcomes in preterm infants,[21,30] this does not appear to hold true for long-term growth outcomes.

There are a wide variety of commercial HMF on the market and over 90% of NICUs reported fortifying HM.[24] However, there is a general lack of comparative data available on their ability to supply the recommended quantities of macro- and micronutrients for VLBW infants. Relatively little research has been published evaluating the retention of bioactive components, and macro- and micronutrient contents of fortified HM, and on the effects of fortification on the osmolality and acidity of HM.

In their 2017 study, Koo and Tice compared the nutrient contents of multiple commercial human milk-based and bovine milk-based HMF (liquid and powder), as well as nutrient-enriched preterm infant formulas (PTF).[70] According to their findings, commercial HMF are able to provide adequate quantities of

protein, fat, and carbohydrates. However, in this analysis they assumed "average" values for HM macronutrients, which does not reflect the large macronutrient variability that is well supported in the literature.[28,71–73] At the same time, commercial HMF are drastically deficient in many essential micronutrients, yet contain others in excess. The use of PTF leads to exceptionally high intake of some micronutrients and does not compensate for those that are insufficient. The authors suggest that these inconsistencies are due to incomplete information on many of the currently recommended micronutrients at the time of these products' original formulation and call for reformulation of HMF to provide more appropriate nutrient profiles.

Effects of fortification on osmolality

The use of HMF raises some concern when assessing the osmolality and acidity of HM. Kreissl et al found that fortified HM had 147% higher osmolality compared to unfortified. Protein supplementation increased osmolality by 23.5 mOsm/L per 0.5-g step, with a maximum of 605 mOsm/L, and other therapeutic additives increased osmolality up to 868 mOsm/L, compared to unfortified HM at 297 mOsm/L.[74] Pasteurization appears to attenuate this increase in osmolality by 20-30 mOsm/L ($p<0.001$). High levels of osmolality are associated with increased risk of feeding intolerance and NEC,[75] and the AAP recommends a 450 mOsm/L maximum for infant formulas, although this suggestion is quite dated.[76] In a 2017 study, Donovan et al compared the effects of two different liquid bovine-milk-

based fortifiers - neutral Similac HMF (SHMF) and acidic Enfamil HMF (EHMF) - on pH, osmolality, and lipase activity.[45] Fortification with neutral SHMF resulted in significantly higher mean osmolality (525.48 mOsm per kg H_2O SHMF vs 337.16 mOsm per kg H_2O EHMF, $p<0.001$) and lipase activity (95596.1 U SHMF vs 88004.4 U EHMF, $p=0.002$). Fortification with acidic EHMF resulted in significantly higher concentrations of protein, fat, and calories compared to SHMF, and a significant decrease in pH (4.94 EHMF vs 6.32 SHMF, $p<0.001$). These findings suggest that the formulation of HMF has significant effects on the composition of HM as well as infant health outcomes.

Effects of fortification on acidity

Fresh HM typically has a pH range of 7.0-7.4.[39] The acidification of HM results in cellular changes that impact several important components. In a 2013 study, Erickson et al found that, when raw HM was acidified to pH 4.5 using citric acid, there was a 76% decrease in white cells ($p=0.001$), a 56% reduction in lipase activity ($p=0.01$), a 14% decrease in total protein ($p=0.001$), as well as a 36% increase in creamatocrit ($p=0.002$) compared to control.[39] Lipoprotein lipase activity decreases at <pH 5, which causes higher creamatocrit readings. This translates into more triglycerides and less free fatty acids, and results in partial fat malabsorption and lower caloric intake.[39] Preterm infants are already at risk for inadequate energy intake. Acidified HM would only exacerbate this problem and may cause others, such as decreased buffering capacity, decreased protein

digestion, weight loss, and dehydration.[39] EHMF, with a pH of 4.3, has been shown to decrease the pH of HM to 4.7 (significance not assessed).[39] HM with a pH <4.5 would constitute a non-physiological feeding due to changes to its biological components and properties.[39] These research findings suggest that some commercial HMF may reduce the bioactive properties of HM.

Storage of fortified, pasteurized donor human milk

As it stands, there is limited research addressing the storage of fortified PDHM. Donovan et al found that, over 24 hours of refrigerator storage, fortification status of both preterm and term HM and PDHM had a significant main effect on changes in mean protein ($p<0.001$), carbohydrate ($p=0.02$), pH ($p=0.03$), osmolality ($p<0.001$), and lipase activity ($p<0.001$). However, the authors concluded that it is unlikely that these changes are clinically relevant.[45]

No studies have examined changes in the antimicrobial proteins of fortified PDHM during extended refrigerated storage. Therefore, it is the aim of this study to address that gap in the literature by evaluating the total protein content and activity of lysozyme and IgA in unfortified and fortified PDHM over 96 hours of refrigerator storage using three commercially available fortifiers.

CHAPTER III

THE EFFECTS OF REFRIGERATED STORAGE TIME AND FORTIFICATION ON PASTEURIZED DONOR HUMAN MILK

Background

The use of pasteurized donor human milk (PDHM) is supported by the World Health Organization, the American Academy of Pediatrics (AAP), 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.[2,20,77] A recent Cochrane Review determined that preterm infants fed infant formula had a 2.77 relative risk (95% CI 1.40 – 5.46) of developing necrotizing enterocolitis (NEC), a life-threatening gastrointestinal disorder, compared to infants fed PDHM,[21] while a report from the Carolina Global Breastfeeding Institute estimated a healthcare savings of $2.90 for every $1.00 spent on PDHM.[17]

The use of PDHM in NICUs throughout the United States' is on the rise.[24,38,78] To ensure the safety of PDHM, HMBANA and other international milk bank networks have issued guidelines, including appropriate storage temperatures and durations. Current best practices for the safe storage and handling of PDHM state that thawed milk should be stored in the refrigerator and should be used within 24 hours,[32] and the maximum frozen storage (-20°C) time

for PDHM is 3-6 months,[32–36] or one year after the earliest pumping date of milk within the pool.[37] Evidence is emerging that PDHM remains free of microbial growth during refrigerated storage of 4 days during routine clinical use, suggesting an opportunity to extend the expiration date and reduce unnecessary waste of a costly and valuable resource.[50,54,57,79,80] Affordability was the most frequently cited barrier of non-use in a 2013 survey of 183 Level 3 NICUs.[38] Research to evaluate the feasibility of extending PDHM expiration dates has the potential to reduce cost barriers associated with a short shelf-life and product waste.

While bacteria levels of PDHM are one marker of product quality and safety, additional research is needed regarding the retention of nutrients and immune factors in PDHM during extended refrigerated storage in order 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 PDHM with human milk fortifiers (HMF) to increase protein and energy content to improve growth rates, therefore future storage studies should evaluate both fortified and unfortified PDHM. Currently, both bovine-milk-based and human-milk-based HMF are available in the United States.

While the use of HMF has become standard practice,[24] the effects of refrigerated storage time on the retention of macronutrients and other bioactive

components in fortified HM remain largely unstudied. This is an important area of research and major gap in the literature that this study aims to address.

The first aim of this study is to compare total protein content, lysozyme activity, and immunoglobulin A (IgA) activity in unfortified PDHM and PDHM fortified with three commercially available fortifiers at baseline. The bioactive proteins in HM help build the framework of an infant's innate immune system and are susceptible to partial degradation during heat-sterilization.[26] Lysozyme and IgA were selected for this study because of their antimicrobial properties[82] and their partial retention in PDHM. The second aim of this study is to evaluate changes in total protein, lysozyme and IgA activity over 96 hours of refrigerated storage. A 96-hour window was selected as the maximum amount of time a hospital might take to use a standard 4 oz bottle of PDHM when starting preterm infants at a low feeding volume (25-55 mL/kg/day) and advancing as tolerated.[81] Both bovine- and human-milk-based fortifiers contribute additional protein and it can therefore be hypothesized that total protein content will increase in all fortified PDHM samples compared to unfortified. As bovine-milk-based fortifiers contain limited lysozyme and IgA, it can be hypothesized that only the human-milk-based fortifier will cause an increase in these antimicrobial proteins. Acidic fortifiers have been shown to reduce the pH of PDHM[39] and changes in pH can impact protein stability; therefore, we hypothesized that PDHM fortified with

acidic Enfamil HMF will show greater losses of lysozyme and IgA compared to neutral Similac HMF, human-milk based Prolact+8, and unfortified.

Methods

PDHM was acquired through a HMBANA milk bank (The New York Milk Bank, Hastings-on-Hudson, NY) that employs Holder pasteurization. Twelve unique batches were subject to 4 treatments: 1) unfortified PDHM (CONTROL) served as the control; 2) fortification with a liquid, acidic, bovine-milk-based fortifier manufactured by Mead Johnson (F-ACID) and mixed to 24 kcal/ounce; 3) fortification with a liquid, neutral, bovine-milk-based fortifier manufactured by Abbot (F-NEUT) and mixed to 24 kcal/ounce; and 4) fortification with Prolact+8 (F-HUM) manufactured by Prolacta Bioscience, a liquid, neutral, human-milk-based fortifier which provides an estimated 29 kcal/ounce when mixed to manufacturer's instructions. Details of the nutrient content of each fortifier are found in Table 5 and the predicted macronutrient content of HM when mixed per instructions are found in Table 6. These estimates assume an average nutrient content of mature HM and do not take into account the variability that has been documented in the literature.

Table 5. Nutrient Content of Study Human Milk Fortifiers

	Enfamil (F-ACID)[83]	Similac (F-NEUT)[84]	Prolact+8 (F-HUM)[85]
Volume of fortifier (mL)	5	5	40
Volume of human milk (mL)	25	25	60
Fortifier protein (g/serving)	0.55	0.35	2.4
Fortifier fat (g/serving)	0.575	0.27	3.6
Fortifier carbohydrate (g/serving)	0.25	0.81	3.6

Table 6. Predicted Macronutrient Content of Unfortified and Fortified Human Milk When Mixed with Study Human Milk Fortifier

	Average (UNFORT)[86]	Enfamil (F-ACID)[83]	Similac (F-NEUT)[84]	Prolact+8 (F-HUM)[85]
Protein (g/100mL)	1	2.7	2.0	3.0
Fat (g/100mL)	3.5	4.8	3.8	5.7
Carbohydrate (g/100mL)	7	6.7	8.5	7.8

Note: F-ACID and F-NEUT were mixed to 24 kcal/oz; F-PRO was mixed to 29 kcal/oz. Values were calculated from the manufacturers' websites using mixing instructions and based on the nutrient profile of "average" HM in control.

Samples were housed under refrigerated storage conditions (approximately 4°C) for a total of 96 hours and were analyzed for the activity of lysozyme and IgA, and protein content. Fortifications and storage of samples were conducted at Westchester Medical Center (Vallhala, NY) and sample analyses were conducted at the University of North Carolina at Greensboro (UNCG) (Greensboro, NC).

Sample preparation

Fortification occurred in the hospital feeding room by a trained feeding technician. Each unique batch of PDHM was mixed according to the protocol outlined here. CONTROL, as the control, had no additional nutrients added to the sample. F-ACID was fortified with 1 vial of Enfamil HMF (5 mL fortifier plus 25 mL HM), F-NEUT was fortified with 1 envelope of Similac HMF (5 mL fortifier plus 25 mL HM), and F-HUM was fortified with Prolact+8 HMF (40 mL fortifier plus 60 mL HM plus). Once mixed, all samples were stored in a clean container labeled with the batch number and a unique letter code corresponding to treatment type. With the exception of the feeding technician, all researchers were blinded to treatment type until after all samples were analyzed and all data were collected.

An initial 5 mL aliquot from each treatment group was stored in the refrigerator in a clean, glass container. Time 0 began once the sample had been fortified and placed in the refrigerator. Samples were stored in the refrigerator throughout the study and a daily log of refrigerator temperatures were 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 sample was 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 UNCG (Greensboro, NC) for analysis. Eleven of the 12 unique batches generated 20 samples, with 4 treatments at 5 time points, and the twelfth generated 15 samples, with 3 treatments at 5 time points, for a total of 235 samples delivered for analysis. Due to the missing treatment from batch 1, data points from analyses of this batch were omitted. This left a total of 220 samples included in the results data.

In the lab at UNCG, samples were thawed at room temperature and with the help of body heat, being held in the hands and pockets of the lab technician, vortexed for approximately 3 seconds, divided into 125μL 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 prior to sample analysis.

Sample analysis

For each sample collected, IgA activity, lysozyme activity, and total protein content were analyzed. IgA activity was measured by enzyme-linked immunosorbent assay (ELISA), which has previously been described in detail.[87,88] *E. 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 HM bind to the *E coli* antigens, and also bind with anti-human-IgA antibody labeled with Horseradish peroxidase (HRP) (part number A0295, Sigma Aldrich, St. Louis, MO). 2,2'-azinodi-3-methylbenzothiazoline-6-sulfonic acid (ABTS) (part number A1888, Sigma Aldrich) is used as a colorimetric substrate. Absorbance is measured at 405nm via spectrophotometry and IgA activity measures the concentration of active IgA in the milk that is able to bind *E. coli* antigens. (Synergy HT, Bio-Tek Instruments, Winooski, VT)

Lysozyme activity was analyzed by changes in turbidity to a microbial suspension of *Micrococcus lysodeikticus* (part number NC9310237, Fisher Scientific, Hampton, NH) at 450nm over the course of 7 minutes, measured by spectrophotometry.[89,90] (Synergy HT, Bio-Tek Instruments, Winooski, VT)

Total protein was measured by the BCA method (part number PI23225, Fisher Scientific, Hampton, NH).[91] This assay measures the reduction of Cu^{+2} by the acidic side chains of HM proteins and the resulting color change, induced by bicinchoninic acid (BCA), which exhibits a strong absorbance at 562nm. HM was diluted 1:20 with deionized water and analyzed via spectrophotometry (Synergy HT, Bio-Tek Instruments, Winooski, VT) alongside known bovine serum albumin (BSA) standards.

For each control sample, all corresponding fortified samples and all time points (4 treatments x 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 ANOVA with a Tukey's adjustment for multiple comparisons. P-values were set at 0.05.

Results

Each analytical test was performed in triplicate and the resulting average CV for assays were as follows: 1) total protein, 2.9%; 2) lysozyme activity, 9.4%; and 3) IgA activity, 2.3%. For IgA activity, all R^2 values were >0.996, and for lysozyme, all R^2 values were >0.979. Using the repeated measures analysis, there was a highly significant impact of treatment ($p < 0.001$ for all dependent variables). There was no effect of time ($p > 0.7$ for all variables) and there was no interaction between treatment and time ($p > 0.9$ for all variables).

Effect of fortification at baseline on pasteurized donor human milk

Descriptive statistics by treatment at baseline are summarized in Table 7 and by time in Table 8. The F-NEUT treatment group was missing in Sample 1. Descriptive statistics were calculated for baselines means and standard deviations using both all 12 samples and 11 samples, dropping Sample 1. It was observed that none of the significant conclusions changed, therefore all data presented represent analysis using only the 11 samples where all treatments and time points were included.

Table 7. Descriptive Statistics of Treatment at Baseline (time=0)

	Control (UNFORT)	Enfamil (F-ACID)	Similac (F-NEUT)	Prolact+8 (F-HUM)
Protein concentration (g/dL)	1.3 (0.1)[a]	2.0 (0.2)[b]	2.2 (0.1)[b]	2.5 (0.1)[c]
Lysozyme activity (units/mL)	5270 (890)[a]	3340 (1660)[b]	4530 (1150)[c]	6230 (500)[d]
IgA activity (mg/dL)	80.3 (22.4)[a]	70.0 (12.3)[b]	89.5 (18.1)[a]	144.4 (16.1)[c]

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

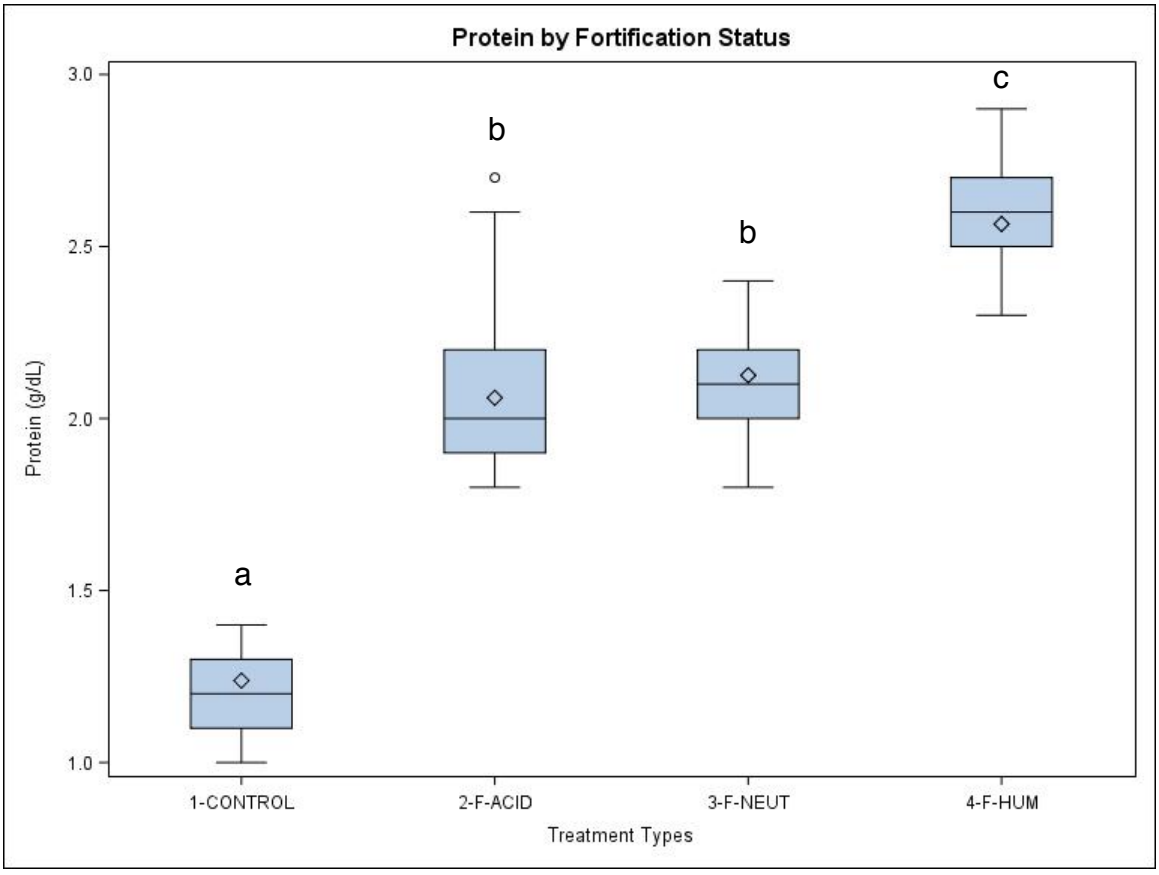
Table 8. Descriptive Statistics by Time

	Protein (g/dL)	Lysozyme activity (units/mL)	IgA activity (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)

Note: Data represent means and standard deviations. There were no statistically significant differences between groups ($p>0.05$).

All three fortifiers significantly increased the total protein concentration of PDHM (F-ACID 2.0 g/dL, F-NEUT 2.2 g/dL, F-HUM 2.5 g/dL, $p<0.05$) compared to control (1.3 g/dL). Total protein was not significantly different between F-ACID and F-NEUT treatments ($p=0.15$). F-HUM treatment group had a significantly higher protein concentration compared to all other treatments ($p<0.0001$). (Figure 1)

Figure 1. Effect of Fortification on Protein Concentration at Baseline

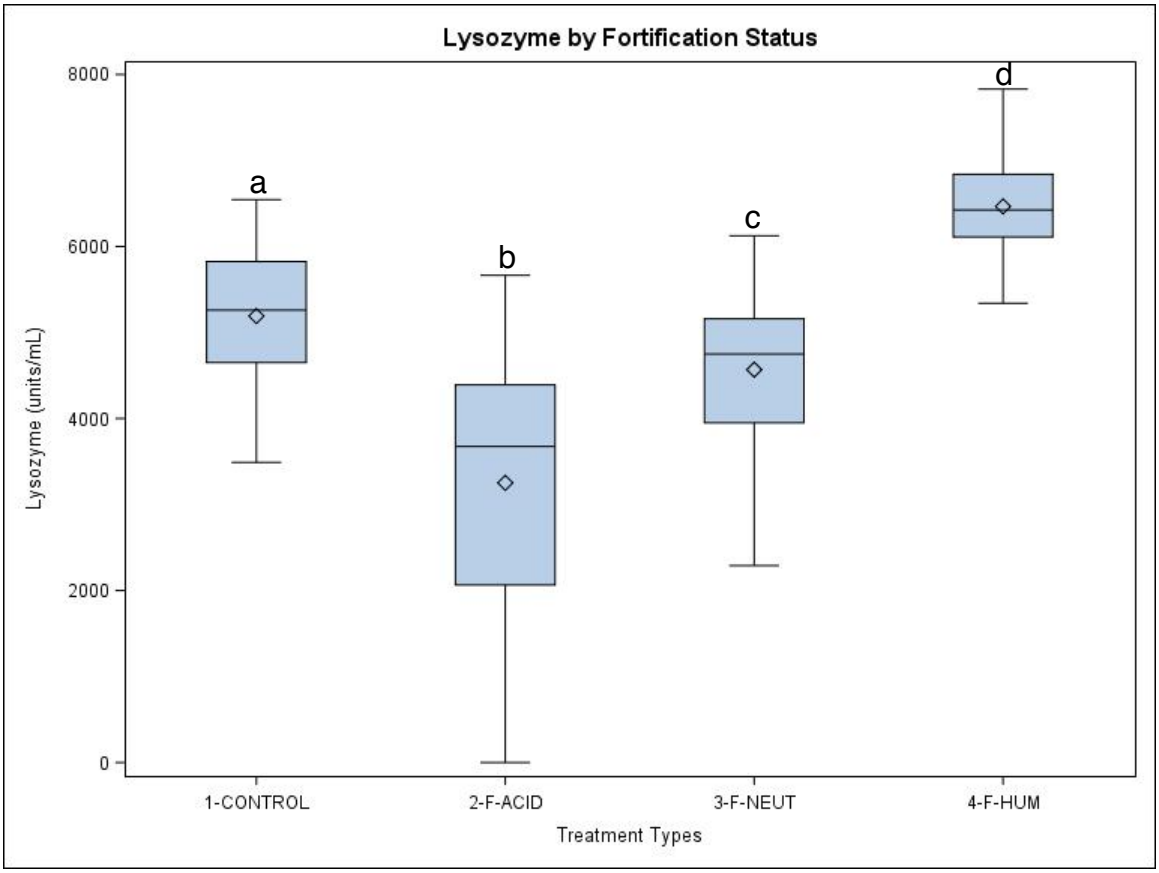


Note: Distributions with the same letter are not statistically different ($p>0.05$) using an ANOVA analysis with a Tukey test for multiple comparisons. In the box and whisker plots, the box spans the interquartile range, the line represents the median, the diamond represents the mean, and the top and bottom lines extend to the highest and lowest observations.

Lysozyme activity was significantly different in all treatment groups ($p<0.0001$). Fortification with F-ACID resulted in a significant decrease in lysozyme activity compared to all other treatments ($p<0.0001$), 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 to all other treatments ($p<0.0001$), with an 18% increase compared to control. (Figure 2)

Figure 2. Effect of Fortification on Lysozyme Activity at Baseline

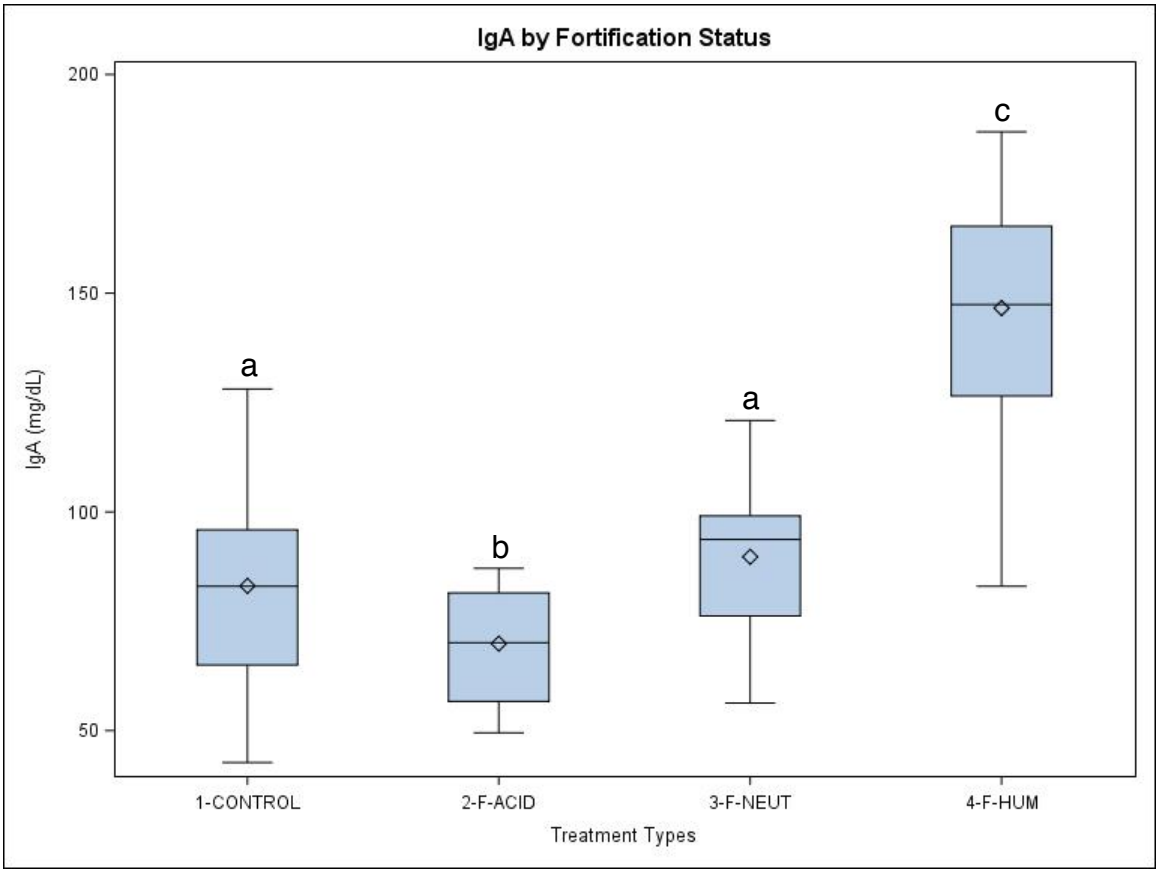


Note: Distributions with the same letter are not statistically different ($p>0.05$) using an ANOVA analysis with a Tukey test for multiple comparisons. In the box and whisker plots, the box spans the interquartile range, the line represents the median, the diamond represents the mean, and the top and bottom lines extend to the highest and lowest observations.

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 ($p<0.0001$) 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 ($p<0.0001$) compared to the other treatments, with an 80% increase compared to control. (Figure 3)

Figure 3. Effect of Fortification on IgA Activity at Baseline



Note: Distributions with the same letter are not statistically different ($p>0.05$) using an 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 storage time on pasteurized donor human milk

Neither unfortified nor fortified PDHM 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).

Interaction between time and treatment

There is no significant interaction between fortification type and storage time ($p>0.7$ for all variables).

Discussion

There is limited evidence regarding the effects of storage on fortified PDHM.[40] In this study, 96 hours of refrigerated storage had no significant impact on the total protein concentration and the activity of antimicrobial proteins in fortified PDHM. Regarding differences among treatments at baseline, fortification with commercial HMF resulted in a 62 – 103% increase in protein concentration compared to control. While 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 HM. Other studies have described similar increases. Donovan et al reported significant, although somewhat greater, increases in protein concentrations of both MOM and PDHM fortified with Enfamil and Similac HMF compared to unfortified.[45]

Similarly, as it was the only HM-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. Fortification with F-HUM also resulted in a significantly higher protein concentration compared to the other HMF. This is very likely due to the fact that these samples were mixed to 29 kcal/oz compared to the other treatments, which were mixed to 24 kcal/oz.

At baseline, PDHM 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. While the pH of the PDHM in this study was not measured, Donovan et al found that fortification with acidic Enfamil HMF resulted in a pH of 4.96 (0.18 s.d.) in preterm PDHM and a pH of 5.10 (0.25 s.d.) in term PDHM.[45] This would have been an interesting outcome to evaluate in relation to the decline in enzymatic activity seen exclusively in the F-ACID treatment group.

Study limitations

This study was not without its limitations. Regarding the milk samples, several issues were encountered. Throughout the aliquoting process, one treatment group within each sample was observed to contain a relatively large amount of white “grit” or precipitate. Researchers suspected this to be the F-

ACID treatment group due to previous research findings implicating the precipitation of casein micelles in an acidic medium.[39] A modified thawing technique was employed in order to improve homogenization and the accuracy of spectrophotometry readings of these samples, which was of particular concern regarding the changes in turbidity assessed in the analysis of lysozyme activity. This involved thawing samples in a shaking water bath at 35°C and 80 rpm for 60 minutes to help break up the precipitate matter. Several samples were reanalyzed once this thawing technique was established. Once unblinded to treatment group, researchers confirmed that the presence of precipitate did correspond to fortification with acidic Enfamil HMF.

In order to aliquot and complete analyses in the lab at UNCG, the PDHM samples underwent four freeze-thaw cycles, which is two more than traditionally encountered in a clinical setting.[22] 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.[62] It is possible that this phenomenon may have contributed to the “grit” encountered in the F-ACID group, which particularly impacted the analysis of lysozyme, and may have interfered with homogenization and spectrophotometry readings. 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 HMF Prolact+8 was mixed to 29 kcal/oz, while both the Enfamil and Similac HMF 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 Enfamil and Similac HMF, the levels of lysozyme and IgA activity would still have been significantly higher than all other treatments, due to the fact that only Prolact+8 contained meaningful quantities of these antimicrobial proteins.

Sample group 1 contained only three sets of treatments when received at UNCG for analysis. The F-NEUT treatment was not included in this sample group. Baseline means were calculated using all 12 samples and only 11 samples. There was no significant difference between the results, therefore data from sample group 1 was omitted.

CHAPTER IV

EPILOGUE

Challenges

As is the case with most research projects, this study was met with a number of challenges. During the aliquoting process, it was observed that one treatment group within each sample contained a relatively large quantity of white "grit" or precipitate. It was suspected that these samples comprised the F-ACID treatment group, as the precipitation of casein micelles has been seen in acidic mediums.[39] There was some concern that the presence of this precipitate would interfere with spectrophotometry readings, particularly regarding the changes in turbidity assessed in the analysis of lysozyme activity. Researchers attempted to "break up" the precipitate by modifying the thawing technique employed for this treatment group prior to analysis. Samples were thawed in a shaking water bath at 35°C and 80 rpm for 60 minutes. Once all analyses were complete and researchers were unblinded to treatments, researchers' suspicions were confirmed, and it was revealed that the "gritty" samples were indeed all fortified with acidic Enfamil HMF.

Sample 1 was missing a fourth treatment group, which turned out to be the F-NEUT treatment. Fortunately, after calculating means and standard deviations for data sets containing all 12 and only 11 samples, it was determined that the results were not significantly different; therefore, only data on the 11 complete samples were included in the analysis.

Gaps in the Literature and Future Research Implications

In terms of published studies evaluating the longevity of PDHM post-fortification, the body of research is lacking indeed. Research regarding the microbial purity of fortified PDHM during extended refrigerator storage would inform the feasibility of extending the current 24-hour storage time limit. If bacterial growth remained at a minimum, the results of the present study would be reinforced and provide justification for the extension of the current limited timeframe. There are no published studies assessing the effects of fortification on HM that has been pasteurized using methods other than Holder, nor is there research evaluating the impact of fortification on the micronutrient content of PDHM.

Very few studies have specifically evaluated the impact of long-term refrigerated or frozen storage of PDHM. 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. Additionally, small sample size and the use of milk samples from a single donor rather than samples

of pooled PDHM, are common limitations. In order to more accurately represent PDHM found in the NICU, future studies should utilize pooled milk samples in clinically-relevant volumes and be designed to emulate clinical practices, with the opening and closing of refrigerators and containers.

Conclusion

At baseline, all treatments effectively increased the protein concentration of PDHM when mixed per the manufacturer's instructions. Only treatment with F-ACID produced a significant decrease in the activity of lysozyme and IgA. HM-based HMF Prolact+8 affected a significant increase in lysozyme and IgA activity. Time had no significant impact on any of the samples, and there was no significant interaction effect between time and treatment. Overall, fortified and unfortified PDHM are equally stable over 96 hours of refrigerated storage. It appears the type of HMF has a greater impact on fortified PDHM than storage time.

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