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CE Human Milk Retains Important Immunologic Properties After Defatting

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Abstract

Background: In neonatal chylothorax, thoracic lymphatic drainage is ineffective. The resultant effusions often require drainage, leading to a loss of immune components. Affected infants can be managed with formula or defatted human milk feedings low in long-chain triglycerides to decrease lymph production. We hypothesized that there is no significant difference in the immunological profile or antibacterial effect of full-fat and defatted human milk. *Methods:* Milk from lactating mothers was divided into 1 aliquot that was defatted via centrifugation with the full-fat aliquot as control. Macronutrient content was analyzed with mid-infrared spectroscopy. Flow cytometry was used to measure immune cell populations. Lactoferrin, lysozyme, immunoglobulin (Ig)A, and IgG values were determined using enzyme-linked immunosorbent assay. The antibacterial properties were determined by inoculating paired full-fat and defatted milk samples with *Escherichia coli* or *Streptococcus pneumoniae* bacteria and performing colony counts. *Results:* Compared with full-fat milk, defatted milk demonstrated decreased total energy and fat and increased carbohydrate concentrations. Defatted milk demonstrated a significant decrease in all immune cell populations. There was no difference in IgA, IgG, lysozyme, or lactoferrin concentrations. Both aliquots demonstrated equivalent growth inhibition of *E. coli* and *S. pneumoniae*. *Conclusions:* Unexpectedly, defatted human milk contained significantly less leukocytes than full-fat milk. IgA, IgG, lysozyme, and lactoferrin concentrations were preserved. The ability of defatted milk to inhibit bacterial growth was unaffected, suggesting that the antibacterial benefits of human milk remain after the defatting process. Further investigation regarding the clinical effect of leukocyte loss in defatted milk is warranted. (*JPEN J Parenter Enteral Nutr.* 2020;44:904–911)

Keywords

cardiac disease; critical care; enteral nutrition; neonates; pediatrics

Clinical Relevancy Statement

Full-fat human milk (FFHM) provides many benefits for infants, including improved neurodevelopment and decreased infection risk. The current standard of care for infants with chylothorax is feeding with a formula that is low in long-chain triglycerides. Defatted human milk (DFHM) is an alternative for these infants but is labor intensive to prepare with unclear immunologic benefits. This study demonstrates that FFHM and DFHM contain equivalent immunologic properties, which supports the use of DFHM in infants with chylothorax.

Introduction

Neonatal chylothorax is a condition in which thoracic lymphatic drainage is ineffective, resulting in chylous pleural effusions. This can be congenital, as in lymphangiectasia, trisomy 21, or Turner syndrome, or acquired through surgical injury or trauma.[1] These effusions can compromise cardiorespiratory function, but drainage of effusions results in a loss of immune components and can lead to a secondary immunodeficiency.[2,3] Symptomatic chylothorax is initially managed by fasting, parenteral nutrition, and drainage of the effusions with a thoracostomy tube.[1,4] This is followed by formula feedings that are low in long-chain triglycerides (LCTs), as this decreases lymph production. An alternative

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feeding option is defatted human milk (DFHM), which is also low in LCTs. Studies have shown that feedings with DFHM are just as effective for chylothorax resolution as feedings with low LCT formula.[5-7] However, infants treated with DFHM have demonstrated reduced growth compared with those on low LCT formula in some studies.[5-7] This discordance can be addressed by close monitoring of infant growth and customization of DFHM fortification with low LCT formula supplementation.[5-10]

Full-fat human milk (FFHM) has numerous benefits for the term and preterm infant and is the standard of care for infant nutrition.[11,12] These benefits include improved neurodevelopmental outcomes, decreased infection risk during the neonatal and infant periods, and decreased childhood obesity compared with formula-fed infants.[11,12] FFHM contains multiple immunologically important compounds, including maternal immunoglobulins (sIgA, sIgG), maternal immune cells (macrophages, neutrophils, lymphocytes), and antimicrobial compounds including free fatty acids, lactoferrin, and lysozyme.[13-15] These factors actively protect the breastfed infant from infection while simultaneously guiding neonatal and infant immune development.[13] Exclusively formula-fed infants do not receive these unique, individualized immunologic benefits. FFHM, which is rich in LCTs, is traditionally withheld in infants with chylothorax because of concerns that it may worsen the effusions. Because of the known benefits and immune protective properties associated with FFHM, some centers are using DFHM as the base nutrition to support infants with chylothorax rather than low LCT formula. Various methods of human-milk fat removal have been devised in order to remove LCTs.[8,9] The amount of fat in DFHM typically ranges from <0.5 g fat/dL to 0.9 g fat/dL depending on the study and method of measurement.[8,9] Whereas triglyceride content has been reported as the main outcome measure when comparing defatting methods, changes in immunological properties and other macronutrients have not been widely described.[5] Some immunologically important proteins, including lactadherin and immunoglobulins, are located within milk fat globules, so it is plausible that immune-related factors could be affected by the defatting process.[16,17] However, we hypothesized that there would be no significant difference in the immunological profile or antibacterial effect of FFHM and DFHM and sought to fill this gap in the literature.

In this study, we measured macronutrient content, immune cell populations, antimicrobial proteins, and bacterial growth inhibition in human milk before and after the defatting process. The DFHM samples contained less fat and total energy compared with the FFHM samples, whereas the remaining macronutrients were not significantly changed. The DFHM samples contained equivalent lactoferrin, lysozyme, sIgA, and sIgG compared with the FFHM samples, but had decreased immune cell populations, including T cells, B cells, monocytes, and neutrophils. Despite this, DFHM demonstrated equivalent growth inhibition of the bacteria *Escherichia coli* and *Streptococcus pneumoniae*.

Methodology

The University of Michigan Institutional Review Board approved this research protocol. Term (37–42 weeks' gestation) and preterm (<37 weeks' gestation) infants admitted to the C.S. Mott Children's Hospital were screened to determine eligibility. Exclusion criteria included mothers <18 years old and mothers who were not primarily English speaking. Lactating mothers were offered enrollment in the study, and written informed consent was obtained. Mothers provided a 30-mL morning expression of FFHM from 1 breast at least 2 hours after the most recent feeding. To ensure that near mature milk was obtained rather than colostrum, samples were collected between 10 and 60 days of lactation for term infants and between 10 and 28 days of lactation for preterm infants.[18,19] Samples were collected, de-identified, and assigned a study number. Sample sizes for individual experiments are detailed in the accompanying figures.

Samples were stored at 4°C and were processed within 4 days of expression, which is the recommended limit of storage time for fresh breast milk by the Academy of Breastfeeding Medicine.[20] Each maternal sample was divided into 2 aliquots for evaluation and comparison. One 10-mL aliquot remained whole for analysis (FFHM). The remaining 20 mL was processed in a centrifuge (2°C, 3000 rpm for 15 minutes) to separate the DFHM from the cream layer using the Hermle Z446 K centrifuge (Sayreville, NJ, USA). The DFHM was poured off to bypass the cream layer disk into a new bottle for testing. Creamatocrit testing on a Medela Crematocrit Plus (McHenry, IL, USA) was performed per manufacturer instructions to ensure the sample was adequately defatted with a value of <3% cream. FFHM and DFHM samples were either used fresh or were frozen at −20°C.

Macronutrients

Stored frozen FFHM and DFHM samples (5 mL) were thawed on ice, then warmed to 40°C using a bead bath (Miris heater; Miris, Uppsala, Sweden). Samples were sonicated with ultrasonic homogenization (Miris Ultrasonic Processor, 1.5 s/mL) immediately prior to total macronutrient analysis. FFHM and DFHM samples were analyzed for total macronutrient content using mid-infrared transmission spectroscopy Miris HMA (Human Milk Analyzer) according to manufacturer recommendations. Samples were run in duplicate for fat, crude protein, true protein, carbohydrate, total solid, and energy. Average macronutrient content was used for analysis.

Flow Cytometry

Fresh FFHM and DFHM samples were spun down at 600*g* for 15 minutes at 4°C to obtain a cell pellet. The cell pellet was washed twice with flow buffer (phosphate buffered saline with 1% fetal calf serum and 0.002 M EDTA). Fc receptors were blocked with purified human IgG (ThermoFisher Scientific, Waltham, MA, USA). Cells were labeled according to the manufacturer's instructions using the following antibodies (clones), each at a 1:100 dilution: from BioLegend (San Diego, CA, USA), CD3 (HIT3a, Pacific Blue), CD14 (M5E2, Brilliant Violet 650), and CD16 (B73.1, FITC); from BD Bioscience (San Jose, CA, USA), CD11b (ICRF44, APC); and from eBioscience (San Diego, CA, USA), CD19 (HIB19, PE). Flow cytometry was performed using a NovoCyte (Acea Biosciences, San Diego, CA, USA) with 405, 488, and 640 nm lasers. Data were analyzed using FlowJo version 10 (Becton Dickson, Franklin Lakes, NJ, USA).

Enzyme-Linked Immunosorbent Assay

Stored frozen FFHM and DFHM samples were thawed at room temperature. Samples were diluted within manufacturer recommended ranges as follows: IgA 1:20,000 dilution, IgG 1:1000 dilution, lysozyme 1:2000 dilution, and lactoferrin 1:200,000 dilution. Enzyme-linked immunosorbent assay (ELISA) analysis was performed for each sample per (Abcam, Cambridge, MA, USA) protocol for human milk in the following manuals: IgA Human SimpleStep ELISA Kit ab196263 (updated Oct 20, 2015), IgG Human SimpleStep ELISA Kit ab195215 (updated August 14, 2015), Lysozyme Human ELISA Kit ab108877 (updated May 18, 2016), and Human Lactoferrin SimpleStep ELISA Kit ab200015 (updated August 17, 2017).

Bacterial Growth Inhibition

Fresh FFHM and DFHM samples were inoculated with *E. coli* (American Type Culture Collection, Manassas, VA, USA [ATCC] 700973) or *S. pneumoniae* (ATCC 6303). The OD600 of the samples was approximately 0.257, which is equivalent to a McFarland standard 1, as previously published.[21] Fifty microliters of the bacteria were added per mL to full-fat milk, defatted milk, tryptic soy broth (*E. coli*), or Todd Hewitt broth with 0.1% yeast extract (*S. pneumoniae*). *E. coli*–containing samples were plated on tryptic soy agar, and *S. pneumoniae*–containing samples on sheep's blood agar in serial dilutions. Negative controls of both FFHM and DFHM not inoculated with bacteria were plated on tryptic soy agar. Prior to plating, *E. coli*–inoculated and negative control samples were incubated at 37°C with shaking at 200 rpm. *S. pneumoniae* samples were incubated at 37°C with 5% CO_2 without shaking. Negative controls and bacterial samples were plated at 2-hour intervals from 0 to 8 hours and incubated at 37°C with 5% CO_2. Bacterial colony-forming units (CFU) were assessed 24 hours after plating to determine the effect of the different milks on bacterial growth. Percent growth inhibition was calculated as [(bacterial CFU in control broth − bacterial CFU in breast milk)/bacterial CFU in control broth] × 100.

Statistical Analysis

Parametric paired *t*-tests were performed on macronutrients, lactoferrin, lysozyme, sIgG, and sIgA. Nonparametric Wilcoxon matched-pairs signed rank test was performed for all cell populations. Multiple paired *t*-tests were used for the bacterial inhibition arm of the study with statistical significance for each time point determined using the Holm-Sidak method. *P*-values of $<.05$ were considered significant. For each DFHM sample, the mother's own FFHM sample was used as its control.

Results

DFHM Contained Significantly Less Fat and Total Energy Than FFHM

Human milk defatting significantly decreases total fat and triglyceride content while leaving electrolyte and trace mineral content largely unchanged.[5,22] The effect of human milk defatting on other macronutrients, including carbohydrate and protein content, remains unknown. Mid-infrared spectroscopy was used to compare the macronutrient content of FFHM and DFHM from the same donor, including total energy, fat, carbohydrate, and protein content. DFHM contained significantly less total energy and fat than FFHM in mothers of both term and preterm infants (Figure 1A and 1B, $P < .01$). The defatting process resulted in increased total carbohydrate content in both term and preterm human milk samples (Figure 1C, $P < .01$). The total protein content of DFHM remained unchanged in preterm samples, with a slight decrease noted in term samples (Figure 1D, $P < .01$). Overall, DFHM contained less total energy and fat with increased carbohydrate content compared with FFHM.

Immune Cell Populations Decreased During the Defatting Process, but Other Immune Factors Remained Unchanged

Human milk contains numerous maternally derived immune cell populations, including monocytes, T cells, B cells, natural killer cells, neutrophils, and eosinophils.[13,23] These cells are thought to protect the mammary gland from infection, provide active immunity for the infant, and guide the development of immunocompetence in the infant.[13,23] It is unclear what effect the defatting process has on human milk immune cell populations. Flow cytometry was used to

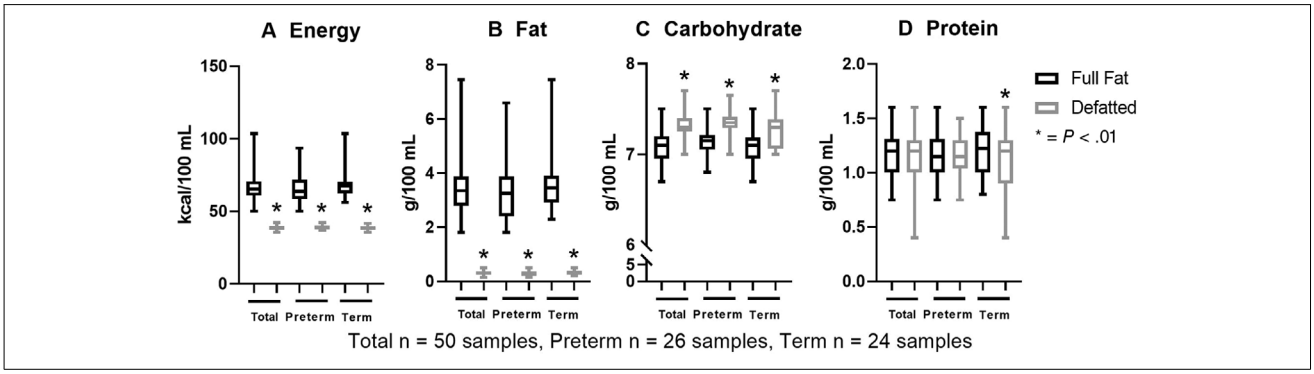

Figure 1. Difference in (A) kilocalories or (B–D) concentration of fat, carbohydrate, and protein per 100 mL in full-fat milk as compared with defatted milk. The boxes represent 25 percentile, median, and 50 percentile values obtained, whereas the whiskers represent the maximum and minimum values.

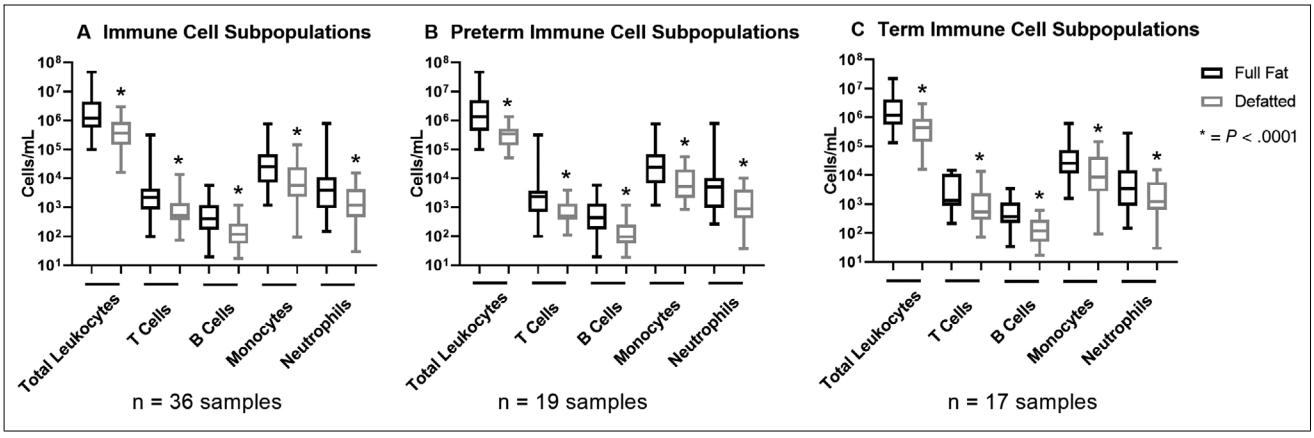

Figure 2. Differences in (A) total, (B) preterm, and (C) term immune cell subpopulations per 1 mL in full-fat milk as compared with defatted milk. The boxes represent 25 percentile, median, and 50 percentile values obtained, whereas the whiskers represent the maximum and minimum values.

compare populations of total leukocytes, T cells, B cells, monocytes, and neutrophils between FFHM and DFHM. DFHM contained significantly fewer total leukocytes, T cells, B cells, monocytes, and neutrophils than FFHM in both term and preterm samples (Figure 2A–C, $P < .0001$). Human milk contains many antimicrobial compounds, including lactoferrin, lysozyme, sIgA, and sIgG. Lactoferrin is one of the most abundant proteins in human milk. It binds free iron and makes it unavailable for bacterial growth and exerts iron-independent bactericidal activity.[24] Lysozyme is an enzyme that degrades both gram-positive and gram-negative bacterial cell walls, serving as a natural antibiotic.[25] sIgA and sIgG pass readily through the breast milk, providing the infant with protection against pathogens the mother has encountered.[25] We sought to determine if the defatting process had an impact on antimicrobial proteins found in human milk. ELISA was used to evaluate the levels of lactoferrin, lysozyme, sIgA, and sIgG in FFHM and DFHM. DFHM and FFHM from preterm mothers contained equivalent amounts of lactoferrin, whereas DFHM from term mothers contained slightly less lactoferrin than FFHM (Figure 3A, $P < .05$). There was no difference in the concentrations of lysozyme, sIgA, or sIgG between DFHM and FFHM (Figure 3B–D). Overall, DFHM contained significantly fewer immune cells but equivalent antimicrobial proteins compared with FFHM except for the decrease in lactoferrin in term DFHM samples.

DFHM and FFHM Demonstrated Equivalent Bacterial Growth Inhibition

Unpasteurized FFHM contains numerous bioactive compounds that inhibit bacterial growth.[21,26] To determine the effect of defatting human milk on bacterial growth, we inoculated FFHM and DFHM with *E. coli* or *S. pneumoniae* and measured bacterial growth inhibition. These bacteria were chosen because they are leading causes of neonatal sepsis and are some of the most likely pathogens neonates and infants might encounter while breastfeeding.[27] FFHM

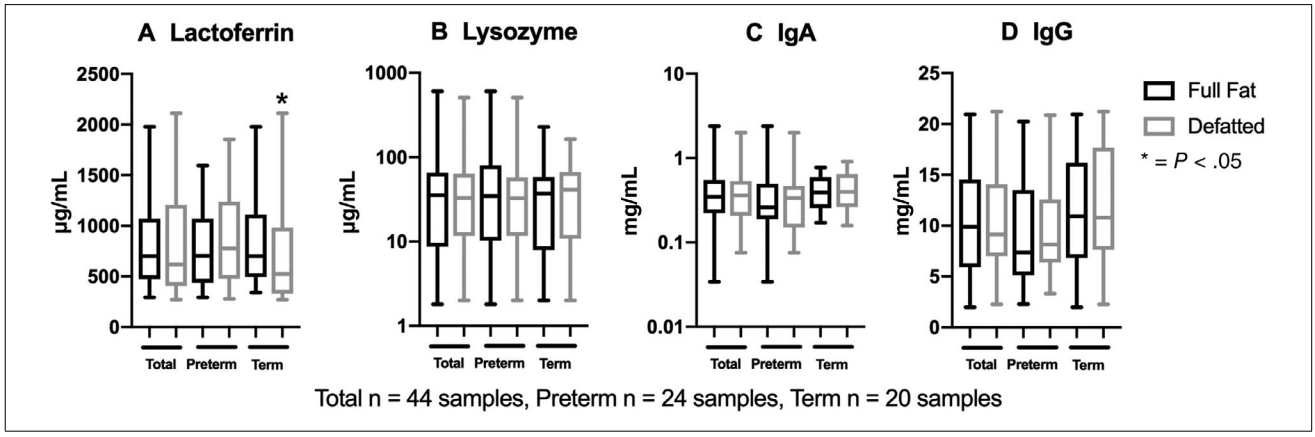

Figure 3. Difference in concentration of immune components A) lactoferrin, B) lysozyme, C) IgA and D) IgG per 1 mL in full-fat milk as compared with defatted milk. The boxes represent 25 percentile, median, and 50 percentile values obtained, whereas the whiskers represent the maximum and minimum values. Ig, immunoglobulin.

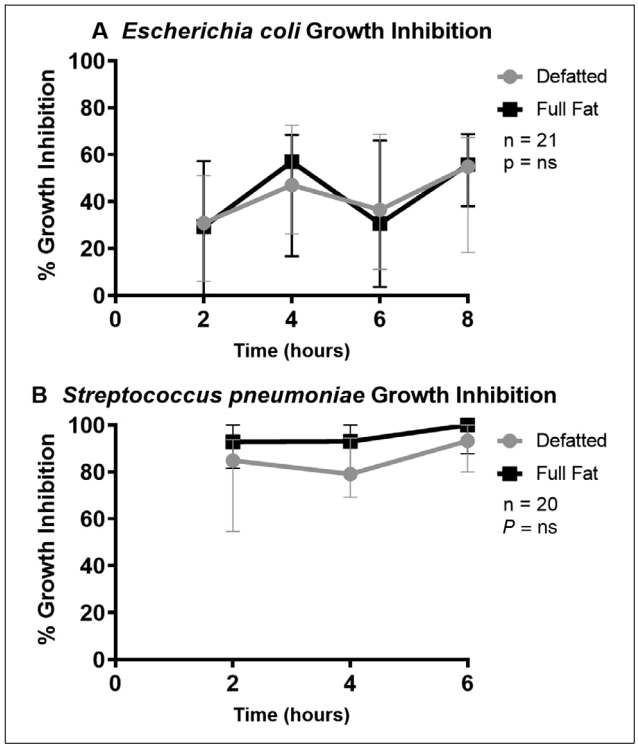

Figure 4. Percent of bacterial growth inhibition in full-fat and defatted milk as compared with control broth for (A) *Escherichia coli* and (B) *Streptococcus pneumoniae*. The points represent median growth inhibition with error bars representing 95% CIs. ns, not significant.

and DFHM demonstrated equivalent bacterial growth inhibition of both *E. coli* and *S. pneumoniae* up to 8 hours after inoculation (Figure 4A and 4B). Although DFHM contained significantly fewer immune cells, it demonstrated equivalent antimicrobial activity compared with FFHM.

Discussion

DFHM is becoming a widely accepted alternative to low LCT formula in the treatment of neonatal chylothorax, but no studies have evaluated the global macronutrient composition and immunologic properties of DFHM. In this study, we demonstrated that DFHM contained less total energy and fat than FFHM, but that carbohydrate and protein content were equal or slightly increased. DFHM contained significantly fewer total immune cells and immune cell subpopulations than FFHM, but the antimicrobial proteins lactoferrin, lysozyme, sIgA, and sIgG were conserved. Most significantly, bacterial growth inhibition was preserved in DFHM.

As expected, DFHM contained less total energy and fat than FFHM.[5-7] Consistent with a previous report, we found that DFHM contained an increased concentration of carbohydrates, with an average increase of 0.2 g/100 mL compared with FFHM.[28] This increase is unlikely to be clinically relevant and is most likely due to a reduction in the total volume of liquid available for suspension after the defatting process, though this is speculation. After defatting, DFHM is deficient in not only fat but total energy, essential fatty acids, and fat-soluble vitamins.[29] In practice, these deficiencies can be managed by supplementing the DFHM with low LCT formula and vitamins.[7,9] It is important to note that excessive carbohydrate intake increases the respiratory quotient and stimulates lipogenesis and fat deposition, which could have long-term consequences for infant growth and metabolism.[30,31] Infants who are fed DFHM may receive a slightly higher concentration of carbohydrates compared with infants fed FFHM, so they should be carefully monitored for growth. Fortification should be tailored to each individual infant's needs to optimize growth and development and to ensure they are receiving appropriate ratios of macronutrients.

Total leukocytes and all immune subpopulations were significantly decreased in DFHM. This is likely due to the method of defatting, as it involved centrifugation followed by decanting of the non–fat-containing milk. This decanting method left the immune cell pellet in the bottom of the centrifugation tube rather than in the DFHM. Although we did not evaluate for the presence of epithelial, progenitor, or stem cells in DFHM, it is likely that they were also decreased.[23,32] FFHM is rich in leukocytes, which are believed to protect both the mammary gland and infant against infection while guiding development of the neonatal and infant immune system.[13,14,33] Colostrum contains significantly more leukocytes than mature milk, but viable leukocytes have been recovered from the feces of infants fed mature human milk, suggesting that these cells remain intact and functional in the infant intestine.[13] Breast milk monocytes and macrophages perform phagocytosis, secrete immunoregulatory factors, and release engulfed sIgA upon contact with bacteria in the infant intestine.[13,34] Breast milk–derived lymphocytes traffic from the infant's intestine to the lymph nodes, spleen, and liver and are thought to promote maturation of the infant's lymphocytes.[14] All of these functions are likely important for neonatal and infant immune system maturation, and it is unclear if the immune cell populations remaining in DFHM are capable of performing these functions adequately. The presence of immune cell populations in DFHM, although reduced, is likely still superior to exclusive low LCT formula feeding for neonates and infants with chylothorax. Additionally, other methods of defatting should be investigated for effectivess of retaining the full FFHM immune cell pellet. This study reinforces the need for the development of evidence based procedures for processing DFHM.

Other than a slight decrease in lactoferrin in DFHM from term lactating mothers, concentrations of lactoferrin, lysozyme, sIgA, and sIgG were preserved after the defatting process. Previous investigators have reported both equivalent and different concentrations of lactoferrin in breast milk from mothers of preterm and term infants during different stages of lactation.[35-37] The variability of findings within the literature may be due to the small sample sizes and different methods used to measure lactoferrin levels.[37] Moreover, lactoferrin in breast milk has also been positively associated with symptoms of infant illness and variably affected by freezing.[38-40] Concentrations of lactoferrin range from 240 to 6501 μg/mL in mature preterm milk and from 240 to 4590 μg/mL in mature term milk.[24,35-37] The lactoferrin concentrations in our samples fell well within these published ranges (292–1595 μg/mL in preterm FFHM, 278–1854 μg/mL in preterm DFHM, 355–1977 μg/mL in term FFHM, 270–2111 μg/mL in term DFHM). Because of this, we believe the slight decrease in lactoferrin detected in the DFHM samples from mothers of term infants is unlikely to be clinically significant and could be the result of the confounding factors noted above. Importantly, DFHM and FFHM demonstrated equivalent bacterial growth inhibition of both of the common neonatal pathogens, *E. coli* and *S. pneumoniae*, suggesting that the defatting process does not disrupt human milk antimicrobial function.

Infections caused by the loss of immunoglobulins in lymphatic fluid are a potential risk of congenital and postsurgical chylothorax.[41] Human milk's many immunologic benefits may protect infants who have these conditions. The human-milk fat globule plays a role in the infant's antimicrobial defense by releasing free fatty acids and monoglycerides via the digestive process, which disrupt cell membranes of lipid-coated microorganisms including bacteria, viruses, and protozoa.[15] Our findings showed that the loss of milk fat in the centrifugation process did not affect the antimicrobial function of human milk against *E. coli* and *S. pneumonia*. However, Ogundele observed decreased bactericidal activity against a serum-sensitive strain of *E. coli* in DFHM compared with FFHM.[42] Further evaluation of DFHM's antimicrobial function against free fatty acid and monoglyceride targeted organisms is warranted to further understand the effects of defatting.

Our study has several limitations. First, not all assays were run on fresh milk. The macronutrient analysis and ELISA assays were run on previously frozen samples. Freezing milk has been shown to impact the concentrations of lactoferrin and IgA.[39,40,43,44] However, a strength of the study is that each mother's FFHM served as a control. As both FFHM and DFHM samples were frozen for the same length of time, we expect that any protein degradation would affect these samples equally and not significantly impact our main findings. This study was conducted at a single center, and our patient demographic is specific to our institution. Therefore, the results may not be widely generalizable. Although we assessed a range of antimicrobial proteins in this study, there are many additional immunologically important compounds present in human milk that were not included. These include cytokines, microRNAs, oligosaccharides, and prebiotics, among others.[33,45,46] Although the bacterial growth inhibition of *E. coli* and *S. pneumoniae* was preserved in DFHM, the role that the human-milk fat globule and other immune components play in supporting infant immune function needs to be investigated. Longer-term prospective studies need to be performed to assess the impact that defatting human milk has on the risk of other infections caused by bacteria and viruses in infants with chylothorax.

Conclusion

Although DFHM contained significantly fewer immune cells than FFHM, other antimicrobial properties were preserved, including lactoferrin, lysozyme, sIgA, and sIgG

levels and bacterial growth inhibition. On the basis of these results, we recommend DFHM be used as the standard of care for neonates and infants with chylothorax so they can receive the known developmental and immunologic benefits of human milk. Special care should be taken to ensure appropriate fortification of DFHM with low LCT formula to prevent growth failure in this patient population.

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Statement of Authorship

B. A. Jackson, B. E. Gregg, S. D. Tutor, J. R. Bermick, and K. P. Stanley contributed equally to the conception and design of the research; B. A. Jackson, B. E. Gregg, S. D. Tutor, J. R. Bermick, and K. P. Stanley contributed to the acquisition and analysis of the data; B. A. Jackson, B. E. Gregg, S. D. Tutor, J. R. Bermick, and K. P. Stanley contributed to the interpretation of the data; B. A. Jackson drafted the manuscript. All authors critically revised the manuscript, agree to be fully accountable for ensuring the integrity and accuracy of the work, and read and approved the final manuscript.

Supplementary Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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