MCCUNE SYDNEY ALEXANDRA. Ph.D. Donor Human Milk Uses Outside of the Hospitalized Preterm Infant. (2023)
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Human milk (HM) is the gold standard for infant feeding. This is supported by extensive research which has shown benefits of HM in infants including reduced gastrointestinal infections, respiratory disease, and mortality. When the mother's own milk (MOM) is unavailable, donor HM (DHM) is the recommended feeding option, especially for the preterm infant due to reduced risk of necrotizing enterocolitis. Despite a consensus of HM being the optimal nutrition for the newborn infant, there is a lack of consensus regarding analytical methods to study HM and clinical recommendations for using HM in high-risk populations. For example, it is unknown whether the terminal lactose in human milk oligosaccharides (HMOs) are impacting lactose measurement methods. Additionally, there is extensive research regarding HM feedings for hospitalized preterm infants, but little information on post-discharge feedings, including the frequency of DHM use and whether DHM needs the same safety standards as those used in the neonatal intensive care unit (NICU). Our research sought to explore emerging issues related to using DHM beyond the NICU.

In aim 1, we determined if human milk oligosaccharides (HMOs) influence common assays for measuring lactose in HM. BioVision Enzymatic Assay and Miris Human Milk IR Analyzer were influenced by HMOs ($p<0.05$), while Megazyme Enzymatic Assay and Ultra-High Pressure Liquid Chromatography Mass Spectrometry were not. In aim 2, we measured the nutrient and bacterial composition of DHM that has been rejected by milk banks after pasteurization due to the presence of bacteria over 4 days of refrigerated storage. DHM with bacterial presence after pasteurization did not significantly change in bacteria, protein, lactose, or immunoglobulin A content over storage duration ($p>0.05$). In aim 3, we investigated post-NICU

discharge preterm infant feeding regimens, infant feeding skills, and caregiver feeding perceptions and experiences in the first 4 weeks post-discharge. The number of infants receiving any human milk decreased from 70% at hospital discharge to 54% at 4 weeks post-discharge. Infant eating skills improved over time ($p=0.096$). Poor feeding behaviors were weakly correlated with poor caregiver experiences ($r=0.319$, $p=0.105$). Falling asleep during or soon after feeding was the most reported feeding behavior. Twenty-one percent of infants required nutritional intervention at their NICU follow-up visit. Any fortification at follow-up visit was moderately correlated with average weight gain since hospital discharge ($r=0.491$, $p=0.033$).

In conclusion, this research showed that some methods for measuring lactose in HM are influenced by HMOs while others are not. Given that HMOs are non-digestible, this has implications for the energy values estimated for human milk. We also showed that DHM with limited bacterial presence post-pasteurization maintains its bacterial and nutrient content over 4 days of refrigerated storage, with 81% of the DHM samples containing less aerobic bacteria as compared to the infant formula. Based on these findings, this DHM with limited bacterial presence may be an option as a supplement for healthy term infants to remain on an exclusively human milk diet. There was no DHM use reported in our study of post-discharge feeding. Preterm infant feeding behaviors improve over the first four weeks at home and poor caregiver experiences were weakly associated with higher infant feeding behavior difficulty. Additionally, changes in feeding regimens are common in the first 4 weeks after hospital discharge. Finally, any fortification at follow-up visit was positively associated with average daily weight gain since hospital discharge.

DONOR HUMAN MILK USES OUTSIDE OF THE HOSPITALIZED PRETERM INFANT

by

Sydney Alexandra McCune

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Approved by

Dr. Maryanne T. Perrin
Committee Chair

DEDICATION

To my 14-year-old self. We did it.

APPROVAL PAGE

This dissertation written by Sydney Alexandra McCune has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair

Dr. Maryanne T. Perrin

Committee Members

Dr. Jigna M. Dharod

Dr. Ron F. Morrison

Dr. Lenka H. Shriver

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February 16, 2023

Date of Final Oral Examination

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CHAPTER I: INTRODUCTION

Premature infants are defined as being born before 37 weeks gestation, and preterm births have been on the rise in recent years with 10.23% of US births in 2019 being preterm.[1] Preterm infants are at higher risk of morbidity and mortality and are over 9 times more likely to be admitted to the neonatal intensive care unit (NICU) as compared to term infants.[2–4] Preterm infants have immature gastrointestinal (GI) systems, therefore, their ability to digest and absorb nutrients is impaired. Because of this, nutrient deficiencies and poor growth are common, with close to 50% of NICU preterm infants being below the 10th percentile on growth charts at hospital discharge.[5–7] Mother's own milk (MOM) is considered the best feeding option for preterm infants. If MOM is unavailable, donor human milk (DHM) is the recommended feeding strategy. This is because human milk (HM) use has been shown to reduce the risk of necrotizing enterocolitis (NEC) as compared to formula use.[8] NEC is a severe and potentially fatal disease of the GI tract that is common among preterm infants and often requires surgery. NEC impacts 15% of NICU infants and 11% of infants born weighing less than 750 g.[9] Etiology of NEC is multifactorial; genetic predisposition, immature gastrointestinal system, abnormal microbiota, and highly immune-reactive intestinal mucosa all play a role in development of this disease.[9] While survival rates of NEC have improved over the decades, mortality rates still remain high.[10]

DHM in the US is primarily regulated and dispensed by 30 non-profit milk banks that are part of the Human Milk Banking Association of North America (HMBANA).[11] HMBANA was founded in 1985 and serves to accredit non-profit milk banks in the US and Canada as well as set international evidence-based guidelines for donor milk banking.[11] In the US, DHM undergoes Holder pasteurization where the milk is warmed to 62.5 degrees Celsius for 30 minutes. This process is effective in inactivating microbial agents that may be present while maintaining many

of the beneficial components of HM.[11,12] Safety standards of pasteurized DHM include testing each batch for presence of bacteria after pasteurization. If one bacterial colony forming unit (CFU) is present, the milk is discarded.[13] These stringent standards are in place to protect the vulnerable hospitalized preterm infant because milk banks have historically primarily served hospital NICUs.[14] However, other, less vulnerable populations have been increasingly using DHM which may not require such stringent standards.[15] No studies have investigated this DHM with bacterial presence after pasteurization, highlighting an important gap in the literature.

As previously stated, DHM is the preferred nutrition for NICU preterm infants when MOM is unavailable. However, studies have shown poor growth of hospitalized preterm infants fed with DHM alone compared to formula.[16] This may be due to over-reporting of DHM nutrients in the literature when using analytical methods developed for the bovine dairy industry. As compared to HM, dairy milk contains 3.5 times higher protein, a third less lactose, and very few oligosaccharides while HM is abundant with oligosaccharides.[17] It is unknown if the oligosaccharides present in HM influence the accuracy of common analytical methods for measuring lactose.

Even though poor growth has been seen with DHM use, DHM is still recommended over formula, due to the decreased risk of NEC.[16,18–21] DHM is now used in 87% of NICUs in the US, with over 90% of NICUs using HM fortifiers with DHM.[22,23] Despite clear guidelines regarding nutrition for preterm infants while in the NICU, there are no clear recommendations for feeding preterm infants after they are discharged, and the extent of DHM use post-discharge remains unknown.[24] A recent study auditing a post-discharge NICU clinic found that 61% of preterm infants were not growing appropriately at their first follow up visit and required feeding interventions from a Registered Dietitian, though use of DHM was not assessed.[25] Moreover, this

study did not investigate what happened during the first several weeks at home highlighting the need for further research into preterm infant feeding during the early post-discharge period.

The purpose of this research was to ensure analytical methods for measuring lactose in HM are reliable as well was to evaluate preterm infant feeding products and post-discharge feeding practices. We hypothesized that the oligosaccharide profile of HM may influence certain methods for measuring lactose, DHM with bacterial presence will maintain its bacterial and nutrient profile during refrigerated storage, and there would be a variety of post-discharge feeding regimens that change as the infant and caregiver adjust to in-home feedings. The specific aims for this research are as follows: Aim 1: Determine if human milk oligosaccharides (HMOs) influence common assays for measuring lactose in HM. Aim 2: Measure the macronutrient and bacterial composition of previously rejected DHM (1+ colony forming unit(s) post-pasteurization) over 4 days of refrigerated storage. Aim 3: Investigate post-NICU discharge preterm infant feeding regimens, infant feeding behaviors, and caregiver feeding perceptions and experiences in the first 4 weeks post-discharge. Overall, the goal of this research is to inform evidence-based guidelines for measuring lactose in HM as well as infant feeding after hospital discharge.

CHAPTER II: DONOR HUMAN MILK USE IN POPULATIONS OTHER THAN THE PRETERM INFANT: A SYSTEMATIC SCOPING REVIEW

Sydney McCune and Maryanne T. Perrin, Breastfeeding Medicine
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Abstract

Introduction: Exclusive breastfeeding is recommended for an infant's first six months of life. If unable to breastfeed, expressed breastmilk including donor human milk (DHM) is recommended for optimal nutrition. Benefits of DHM in preterm infants have been established by extensive research. However, less is known about DHM use in other populations.

Objective: To conduct a scoping review of the literature regarding DHM use in populations other than preterm infants.

Methods: PubMed and Clinicaltrials.gov were used to search for articles and clinical trials published between January 1, 2000 and February 29, 2020. In total, 182 articles and reports were identified and screened by 2 independent reviewers.

Results: Twenty-six articles met inclusion criteria and were reviewed. Studies were mostly observational in design and included infants born > 35 weeks gestational age with health risks (9/26) and healthy infants (13/26). Most studies in infants with health risks (7/9) investigated clinical outcomes, with small, observational studies suggesting potential improvements in feeding tolerance and GI health. Regarding healthy infants, no studies addressed growth, only one study measured clinical outcomes, and findings related to

breastfeeding outcomes were conflicting. Over half of the studies reviewed (15/26) were not designed to establish a potential relationship between DHM use and relevant health-related outcomes.

Conclusion: The current evidence of DHM use in populations other than preterm infants is limited by lack of direct health measures and infrequent use of randomized trials. More research is warranted to investigate clinical, growth, and breastfeeding outcomes.

Introduction

The American Academy of Pediatrics (AAP) and World Health Organization (WHO) recommend exclusive breastfeeding for an infant's first 6 months of life and continued breastfeeding for at least 1 year.[26,27] These recommendations are backed by studies that have reported several benefits of breastfeeding for the infant including reduced risk of respiratory tract infections, gastrointestinal tract infections, allergic disease, and infant mortality.[26]

However, not all infants are able to be breastfed or receive their mother's expressed milk. Regardless of the mother's decision to breastfeed, preterm infants weighing <1500 grams are recommended to receive an exclusive human milk diet, either mother's own milk (MOM) and/or donor human milk (DHM).[28] These recommendations are supported by evidence of reduced necrotizing enterocolitis (NEC) in preterm infants receiving DHM compared to bovine formula.[8,16] DHM use with preterm infants has rapidly increased in United States (US) over the past 10 years with the majority of neonatal intensive care units (NICU) now utilizing DHM.[22,23]

For healthy, term breastfed infants the AAP and WHO also recommend feeding DHM if MOM is unavailable or inadequate, though the extent that this is practiced is not well understood.[26,27] The WHO recommendations are part of the Baby-Friendly Hospital Initiative (BFHI) that was launched in 1991 to ensure mothers receive proper breastfeeding education and

support in the first days postpartum. Step six of BFHI states that infants are not fed any fluids or foods other than breastmilk, which promotes the use of DHM when infants are unable to receive MOM.[29] The number of BFHI hospitals in the US increased from 60 in 2007 to more than 600 in 2019, with births in BFHI hospitals now accounting for almost 30% of US births.[29]

There is little known about the extent or impact of DHM use in populations other than the preterm infant. Therefore, the primary purpose of this scoping review was to describe the nature and findings of research using DHM in populations other than the preterm NICU infant and identify gaps in the existing literature.

Methods

We conducted a systematic scoping review of peer-reviewed research, conference abstracts, and registered clinical trials related to DHM use in populations other than premature infants in the NICU.[30] The Preferred Reporting Items for Systematic Reviews and Meta-Analyses checklist was utilized to guide the reporting process. Electronic sources used to identify articles were PubMed and Clinicaltrials.gov. Keywords and MeSH terms used in the electronic searches included: (("donor milk" or "donor human milk") NOT (preterm or VLBW or "very low" or review)) for PubMed, and (“donor human milk” or “donor milk”) for ClinicalTrials.gov. Additional studies were located through hand-review of references from the articles identified in the primary search and author familiarity.

Clinical reports, original research articles, conference abstracts, and registered clinical trials that were published or registered between January 1, 2000 and February 29, 2020 were included in this review. Two reviewers (SAM and MTP) independently assessed all registered trials and study abstracts for inclusion/exclusion criteria. Abstracts were excluded for the following reasons: intended DHM recipient was only premature infants < 35 weeks gestational

age; did not use DHM; was milk sharing only; review article or commentary; not written in English; or did not discuss intended recipients (milk only studies). Trials identified through ClinicalTrials.gov were included if the protocol described providing DHM to non-preterm infant populations. Publications from these trials were identified at ClinicalTrials.gov and by individual searches using the investigator name. Trials without published results were included as pending studies. Trials that had been canceled were excluded from the review. Publications that passed abstract review were subject to a full review by two independent reviewers using the same exclusion criteria applied to abstract reviews.

Included studies were abstracted by two reviewers for the following information: intended DHM recipient, intended DHM use, study design, study population, study location, funding source, outcomes measured, and results. Studies were categorized into five categories based on the DHM recipient: adult; child; infants born at least 35 weeks gestation with health risks; healthy infants born at least 35 weeks gestation; or post-discharge preterm infants. Six categories were created to organize outcomes measured: DHM use patterns (e.g. percent of level 1 hospitals offering DHM, reasons for DHM use, volume of DHM used); knowledge and beliefs about DHM (e.g. perceived benefits and risks); clinical-related outcomes (e.g. length of hospital stay, days parenteral feeding); biomarkers (e.g. bacterial abundance, intestinal inflammation, thymic size); growth (e.g. length, weight, head circumference); and breastfeeding status at or after hospital discharge. Outcomes for each study were coded as positive outcomes (+), negative/detrimental outcomes (-), neutral (=), or present, with no longitudinal or control-group comparison (P). All reviewer discrepancies were resolved through discussion. Considerations of bias were informed by the Agency for Healthcare Research Quality (AHRQ) methods guide on healthcare research reviews and included: poor and/or inadequate reporting bias (related to

unclear or missing reporting of results and/or methods); study design bias (limitations and weaknesses of methods used); and directness of outcomes bias (research links DHM to an important infant health outcome).[31] Studies that only measured patterns of DHM use or knowledge/attitudes about DHM were considered to have not measured direct infant health outcomes. Potential sources of bias for each study were identified and discussed by two reviewers.

Results

An overview of the review process is detailed in Figure 1. The initial PubMed search yielded 151 articles, the ClinicalTrials.gov search yielded 16 studies, 7 articles were found through hand bibliography review of PubMed included articles, and 8 articles were identified from researchers' previous knowledge. After review of the abstracts and study descriptions, 144 articles were excluded leaving 33 articles and 5 clinical trials for full review. Twelve articles/trials were excluded after a full text review leaving 26 included in this systematic scoping review of DHM use in populations other than the premature, NICU infant.[32–57] Included studies were predominantly conducted in the US in healthy, term or late preterm infants (Table 1). Study methods were predominantly observational and descriptive, with only three studies using an experimental design. Study population, intended DHM use, outcomes measured, and key findings are summarized in Table 2 by recipient type and intended use. Reasons for DHM use included cancer therapy in adults; pre- and post-operative feedings in infants and children; primary feeding of foster children; and supplementation for healthy breastfeeding infants in the

Figure 1. PRISMA flow diagram of the literature search process used to identify studies addressing the use of donor human milk in non-premature infant populations. PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

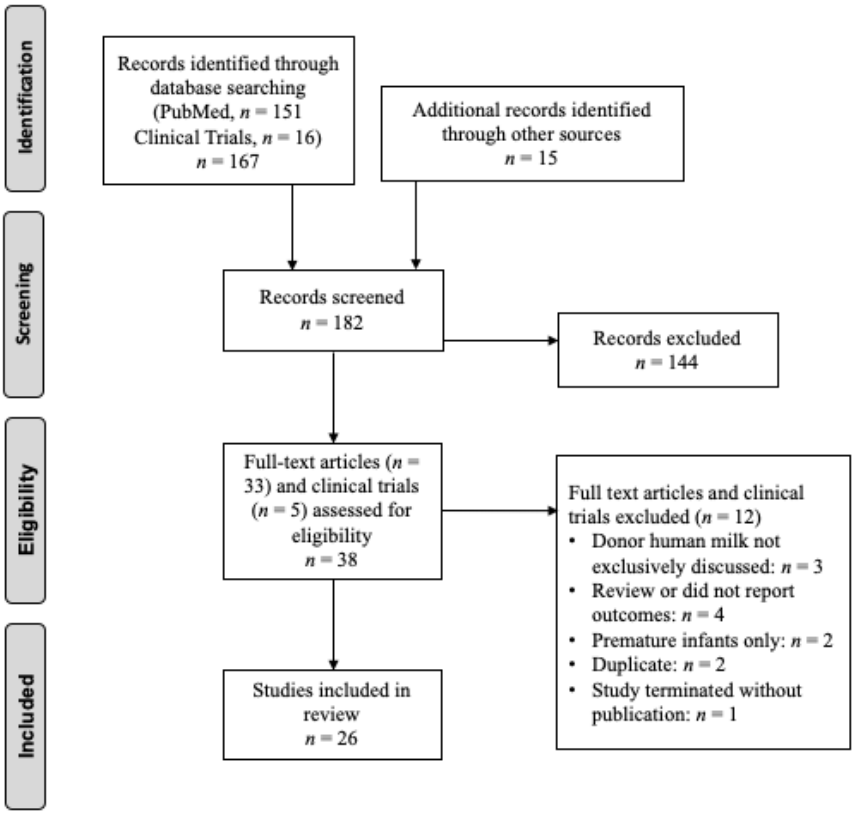


Table 1. Summary of studies included in the systematic scoping review of donor human milk use in non-premature infant populations.

Year	Author	Intended DHM recipient	Study Design	Study Location	Funding Source
2003	Jeppesen[32]	Uninfected infants born to HIV+ mothers	Observational cohort	Denmark	Not addressed
2009	Rough[33]	Adult cancer patients	Observational qualitative	United States	San Jose State University
2009	Szucs[34]	Post-discharge preterm quintuplets	Case study	United States	Not addressed
2013	Jeppesen[35]	Uninfected infants born to HIV+ mothers	Observational cohort	Denmark	Foundations of Sygekassernes Helsefond, Det Sundhedsvidenskabelige Forskningsra˚d, and Fonden
2014	Kair[36,38]	Healthy term and late-preterm infants experiencing hypoglycemia	Case reports	United States	Not addressed
2017	Alexander[37]	Term infants with Neonatal Abstinence Syndrome	Case control	United States	Medolac Laboratories provided milk for the study
2017	Kair[38]	Healthy term and late preterm infants	Observational qualitative	United States	University of Iowa Stead Family Department of Pediatrics
2018	Belfort[39]	Healthy infants born at least 35 weeks gestation	Cross-sectional observational	United States	National Institutes of Health; Brigham and Women's Hospital; and W.K. Kellogg Foundation
2018	Lewis[40]	Infants in the level 1 mother baby unit	Retrospective observational	United States	W. K. Kellogg Foundation
2018	Mannel[41]	Healthy late pre-term infants	Retrospective observational	United States	No funding relationships
2018	Rabinowitz[42]	Healthy term infants	Observational qualitative	United States	No funding relationships
2018	Reimers[43]	Medically vulnerable infants	Case reports	South Africa	Not addressed
2018	Sen[44]	Healthy term and late preterm infants	Retrospective observational	United States	National Institutes of Health
2019	Cognata[45]	Infants of all gestational ages with an isolated cardiac lesion	Retrospective cohort	United States	Texas Children's Hospital (Evie Whitlock Grant) and National Institutes of Health
2019	Drouin[46]	Healthy infants born at least 35 weeks gestation	Cross-sectional observational	United States	Brigham and Women's Hospital
2019	Ferrarello[47]	Healthy term infants experiencing hypoglycemia	Observational qualitative	United States	No funding relationships

2019	Ferrarello[48]	Healthy term infants experiencing hypoglycemia	Observational (quality initiative)	United States	Not addressed
2019	Kair[49]	Clinically stable, breastfeeding infants born at least 35 weeks gestation and weighing at least 1,750 g at birth	Retrospective cohort	United States	The University of Iowa and National Institutes of Health
2019	Kair[50]	Healthy, term infants who lost at least 4.5% of their birthweight in the first 36 hours of life	Randomized controlled trial	United States	The Gerber Foundation, the Children's Miracle Network, and National Institutes of Health
2019	Khandelwal[51]	Children 5 years and younger who received bone marrow transplant	Unblinded randomized trial	United States	Prolacta Bioscience
2019	Lehman[52]	Term NICU infants when mother's milk not available	Retrospective observational	Poland	Not addressed
2019	Mannel[53]	Term AGA foster infant with formula intolerance experiencing failure to thrive	Case report	United States	No funding relationships
2019	Meeks[54]	Late preterm and term infants admitted to the NICU	Observational	New Zealand	Not addressed
2019	Merjaneh[55]	Healthy infants in Level 1 nursery	Retrospective observational	United States	Not addressed
2020	Hoban[56]	Infants born at least 33 weeks gestation and/or weighing at least 1500 g admitted to the NICU for gastroschisis and/or intestinal atresia (75% born > 36 weeks gestational)	Retrospective cohort	Canada	No funding relationships
N/A[a]	Thoene[57]	Healthy term or later preterm infants experiencing hypoglycemia	Randomized controlled trial	United States	University of Nebraska

[a]Registered clinical trial with no results published yet.

AGA, appropriate for gestational age; DHM, donor human milk; HIV, human immunodeficiency virus; NICU, neonatal intensive care unit.

Table 2. Results of studies investigating donor human milk for non-preterm population by intended recipient and use.

Intended Recipient Type	Author	Year	Intended DHM use	Study population (n)	Outcomes Measured	Results		Types of Results				
							Use Patterns	Knowledge	Clinical	Biomarkers	Growth	Breastfeeding
Adults	Rough[33]	2009	Cancer therapy	Adult cancer patients (n=5) and family proxies (n=5)	Motivations and experiences of DHM use, duration and dose of DHM	Daily doses of DHM ranged from 3.5-16oz; duration of DHM use ranged from 1-62m; majority reported perceived benefits of DHM use	P	P				
Children (0-5 years old)	**Khandelwal[51]**	**2019**	Pre- and post-bone marrow transplant feeding	Children fed DHM (n=18), mother's milk (n=6) or standard formula feeding (control; n=14)	Markers of inflammation, stool microbiome and metabolomics	Higher markers of intestinal inflammation in controls; higher abundance of some bacteria in controls				+		
Infants born at least 35 weeks gestation (with health risks)	Hoban[56]	2020	Post-gastrointestinal surgery supplementation	Infants supplemented post-operatively with formula (n=70) or DHM (n=70)	Hospital length of stay, growth, NEC and sepsis risk, days on parenteral nutrition, days with central line	No difference in any outcomes between groups; when excluding large bowel atresia, infants with small bowel atresia and gastroschisis had significantly shorter hospital stays and central line days when receiving DHM			= +		=	
	Cognata[45]	2019	Pre-cardiac surgery feedings	Infants with cardiac abnormalities (n=546), including those who received exclusive human milk diet (n=198)	Incidence of NEC	An exclusive unfortified human milk diet was associated with a reduced risk of NEC			+			
	Reimers[43]	2018	Primary feeding (at home)	Foster infants in transition home in South Africa (n=7)	Duration of DHM use, health outcomes, growth	DHM use duration ranged 2 weeks – 2 years; variety of improvements reported including growth, feeding tolerance, and decreased infections and eczema	P		+		+	
	Mannel[53]	2019	Primary feeding (at home)	Foster infant (n=1) diagnosed with Failure to Thrive (weight dropped from 56 to 1 percentile) fed DHM for > 200 days	Feeding tolerance and weight gain	Vomiting, fussiness, emesis, or abdominal discomfort decreased; growth remained below 5th percentile			+		-	

	Jeppesen[32]	2003	Primary feeding (at home and in hospital)	HIV exposed-uninfected infants fed DHM (n=12), HIV unexposed infants by feeding type (n=47)	Thymic size at birth and 4 months	No significant difference between thymic size at birth when adjusting for weight; greater thymic size at 4 months was seen in HIV exposed-uninfected infants fed DHM compared to HIV-unexposed infants fed formula (23.8 vs 18.3)				+		
	Jeppesen[35]	2013	Primary feeding (at home and in hospital)	HIV -exposed-uninfected infants fed DHM (n=18), HIV unexposed infants by feeding type (controls; n=47)	Thymic size and clinical outcomes	Thymic size was not different at 8 and 12m age; between 8-12m, DHM fed infants had significantly fewer infections than controls			+	=		
	Alexander[37]	2017	Primary feeding (in hospital)	Term infants with Neonatal Abstinence Syndrome fed DHM (n=9) and matched control infants fed formula (n=9)	Infant growth, Finnegan's scores, and gastrointestinal sub-scores	Head circumference gains was lower among DHM group; Finnegan scores were not significantly different; DHM group had fewer infants with gastrointestinal sub-scores greater than 2 ($p<0.001$)			= +		-	
	Lehman[52]	2019	Supplementation (in hospital)	Term NICU infants (n=17)	Days and volume of DHM used, feeding mode at hospital discharge	Infants received on average almost 900 mL DHM for 4.4 days; 82.4% of infants were receiving mom's milk at discharge	P					P
	Meeks[54]	2019	Supplementation (in hospital)	Term infants in NICU (n=unknown)	Days of DHM	NICU term infants received DHM for an average of 5-6 days	P					
Healthy infants born at least 35 weeks gestation	Kair[36]	2014	Supplementation (hypoglycemia)	Term small for gestational age (n=1) and late-preterm (n=1) infants	Amount of DHM received and breastfeeding at 1-2 weeks post-discharge	45-147 mL of DHM used in the hospital; mothers were breastmilk feeding at 1-2 weeks post-discharge	P					P
	Ferrarello[47]	2019	Supplementation (hypoglycemia)	Term mother-infant dyads (n=83)	Prevalence of mothers choosing DHM for supplementation and breastfeeding rates at discharge	76% of parents chose DHM for supplementation; 98% of infants were still breastfeeding at discharge; 52% of infants who received DHM were exclusively breastfeeding at discharge compared to 0% of infants who declined DHM	P					+
	Ferrarello[48]	2019	Supplementation (hypoglycemia)	Nurses working in labor & delivery or on the mother & baby unit at Baby-Friendly Hospital (n=20)	Nurses' opinions and knowledge of DHM	Nurses lacked knowledge of DHM, but presumed safe; logistical concerns for implementing a DHM policy in a well-baby nursery		P				

	Thoene[57]	**Not yet published**	Supplementation (hypoglycemia)	Infants with hypoglycemia (n=62)	Blood glucose levels, exclusive breastfeeding duration	No results yet (registered clinical trial)						
	Kair[38]	2017	Supplementation (in hospital)	Post-partum mothers of healthy term and late preterm infants (n=30)	Themes related to maternal perspectives of supplementation with DHM versus formula	4 themes identified: formula is familiar and DHM is not, DHM is costly and logistically challenging, DHM is temporary and formula is ongoing, and DHM is "healthier"		**P**				
	Belfort[39]	2018	Supplementation (in hospital)	Massachusetts Hospitals and/or hospitals serviced by Mother's Milk Bank Northeast (n=71)	DHM utilization, clinician knowledge and opinions of DHM, exclusive breastfeeding rates at discharge	32% hospitals were using DHM for healthy infants; 78% of clinicians believed that studies show benefits of DHM use in healthy infants; hospitals using DHM for healthy infants reported higher rates exclusive breastfeeding at discharge compared to those who do not (77% vs. 56%, p=0.02)	P	P				+
	Lewis[40]	2018	Supplementation (in hospital)	Healthy infants in an inner-city Baby Friendly Hospital mother-baby unit (n=unknown)	Uses of DHM	Three most common uses of DHM: excessive weight loss, mother - child separation, and small for gestational age	P					
	Mannel[41]	2018	Supplementation (in hospital)	Late preterm infants at a single center breastfeeding only (n=27), supplemented with DHM (n=20), supplemented with any formula (n=93), or formula only (n=43)	Hospital length of stay (LOS) and breastfeeding status at discharge	LOS did not differ in breastfed infants by type of supplement; infants receiving formula supplementation were less likely to be breastfeeding at discharge than those supplemented with expressed human milk or DHM (RR = 0.84; 95% CI 0.77-0.92)			=			+
	Rabinowitz[42]	2018	Supplementation (in hospital)	Post-partum mothers of term newborns (n=24)	Maternal perspectives of DHM	58% mothers preferred DHM over formula; themes emerged regarding concerns of safety and lack of information about DHM	P	P				
	Sen[44]	2018	Supplementation (in hospital)	Healthy infants who received DHM (n=363) between 2013-2016	Trends in DHM use	Percent of healthy newborns using DHM increased 0.04% to 4.7% and average bottles per infant increased from 0.6 to 4.6 (100 mL bottles) throughout the duration of the study; indications for DHM: infant excessive	P					

						weight loss/dehydration (17%), late preterm birth (15%), poor latch (13%), and delayed lactogenesis (11%)						
	Drouin[46]	2019	Supplementation (in hospital)	Northeast US Level I Maternity hospitals using DHM (n=15)	Hospital policies on DHM use	87% of policies stated criteria for DHM use ; all required consent; 27% stated that DHM was the preferred supplementation; 53% of policies indicated DHM use for hypoglycemia and/or hyperbilirubinemia	P					
	Kair[49]	2019	Supplementation (in hospital)	Mother-infant dyads who supplemented with formula (n=376) or DHM (n=306)	Maternal characteristics by supplemental feeding type	DHM use less likely in mothers who were non-white, publicly insured, and non-English speaking	P					
	Merjaneh[55]	2019	Supplementation (in hospital)	Infants in level 1 nursery who were supplemented with formula (n=39) or DHM (n=33)	Exclusive breastfeeding at 6 months of life	Infants supplemented with DHM had 5 times greater odds of exclusive breastfeeding at 6 months of life after adjusting for delivery type and WIC (CI 1.37, 19.23, p=0.015)						+
	Kair[50]	**2019**	Supplementation (in hospital and at home)	Healthy term infants with ≥ 4.5% weight loss randomized to limited DHM (n=30) or exclusive breastfeeding (control; n=30)	Formula use a 1 week, prevalence of any breast milk feeding and breastfeeding without formula at 1, 2, 3 months	No significant differences in formula use at 1 week; control group had greater Any Breast Milk feeding at 1 month, and greater Feeding at the Breast at 1 month and 3 months compared to DHM group						= -
Post-discharge preterm infants	Szucs[34]	2009	Supplementation (at home)	Preterm quintuplets discharged at 3 months and fed DHM as a supplement to mother's milk until at least 6 months (n=5)	Mother's experience, volume of DHM supplementation, infants' health status at 6 m of life	Mother strongly advocated for DHM; used 420 oz DHM per week; clinicians reported that infants were healthy developing normally at 6 months of life	P	P	P			

Notes: Studies listed in **bold** are randomized controlled trials. Types of Results: **P** present (no comparisons); **+** present with favorable outcomes for DHM; **-** present with unfavorable outcomes for DHM; **=** present with no difference in outcomes. DHM – donor human milk; HIV – human immunodeficiency virus; NEC – necrotizing enterocolitis; NICU – neonatal intensive care unit; RR – relative risk; CI – confidence interval

hospital and at home. The frequency of studies by type of outcomes reported was as follows: patterns of use (n=13), knowledge and beliefs (n=6), clinical (n=8), biomarkers (n=3), growth (n=4), and breastfeeding (n=7). Over half of the studies (15/26) were not designed to establish a potential relationship with DHM use and health-related outcomes because they did not compare DHM use to an alternative feeding strategy, they combined DHM with MOM for analysis, or they did not measure infant health-related outcomes.

Adults

A study of 10 adult cancer patients or their proxies reported on DHM use patterns and beliefs.[33] A range of 3.5 to 16 oz. of DHM was used per day and the majority of participants reported perceived benefits from DHM use; however, no actual clinical outcomes were measured.[33]

Children

A single randomized controlled trial (RCT) was conducted in children age 0-5 undergoing bone marrow transplants.[51] Compared to the controls who received standard formula feeding (n=14), those who received DHM (n=18) or MOM (n=6) had lower markers of intestinal inflammation and lower abundance of certain bacteria post-transplant.

Infants born > 35 weeks gestational age with health risks

Patterns of Use

Three studies reported DHM use patterns among infants with health risks. In a case study of South African foster children with HIV or malnutrition, DHM was used as a primary source of feeding for 2 weeks – 2 years.[43,52,54] In a study of term infants admitted to the NICU (n=17), Lehman et al. reported infants were supplemented an average of 900 mL DHM for 4.4 days.[52]

Meeks et al. assessed supplementation among term infants admitted to the NICU and found that they received DHM for an average of 5-6 days.[54]

Clinical Outcomes

Six observational studies measured clinical outcomes in infants with a variety of illnesses and reported a combination of neutral and positive effects with DHM use. Hoban et al. found that infants supplemented with DHM undergoing gastrointestinal surgery (n = 70) did not differ in hospital length of stay, NEC and/or sepsis risk, parenteral nutrition days, or central lines days compared to infants supplemented with formula (n=70); however, in a sub-analysis including only small bowel atresia and gastroschisis surgeries, infants receiving DHM (n=58) had significantly shorter hospital stays and central line days than those who received formula (n=47).[56] Cognata et al. studied infants undergoing cardiac surgery (n=546) and found that an exclusive unfortified human milk diet (n=198), including MOM and DHM, was associated with a significantly lower incidence of NEC.[45] Alexander et al. reported fewer infants with neonatal abstinence syndrome (NAS) fed DHM (n=9) had Finnegan gastrointestinal sub-scores above 2 compared to those fed formula (n=9), but overall Finnegan scores were not different.[37] Mannel et al. and Reimers et al. investigated foster infants (n = 1 to 7) receiving DHM and reported improved feeding tolerance, reduced eczema, and lower infection incidence.[43,53] Jeppesen et al. reported that HIV-exposed uninfected infants receiving DHM (n=18) had fewer infections at 8-12 months compared to HIV unexposed infants receiving formula (n=47).[35]

Biomarkers Outcomes

Two studies reported thymic size in HIV-exposed uninfected infants.[32,35] Infant's thymic size was greater in HIV-exposed uninfected infants who received DHM (n=12) at 4 months of

age compared to HIV-unexposed infants fed formula (n=47).[32] However, a later study found no difference in thymic size at 8 and 12 months of age.[35]

Growth Outcomes

Four observational studies reported mixed findings related to growth. Among infants undergoing gastrointestinal surgery, there was no difference in weight or head circumference between infants supplemented post-operatively with DHM (n=70) and formula (n=70).[37,43,53,56] In a case study of 7 foster infants, Reimers et al. reported improved weight gain after introduction of DHM as their primary feeding.[43] In contrast, a case study of a foster infant whose birth weight dropped from the 56th percentile to the 1st percentile due to formula intolerance, reported that the infant's weight remained below the 5th percentile after being fed DHM for over 200 days.[53] In a case-control study of hospitalized infants with NAS, head circumference gains were lower in infants fed DHM (n=9) compared to infants fed formula (n=9).[37]

Healthy infants born > 35 weeks gestational age

Patterns of Use

Two studies reported patterns of DHM supplementation for hypoglycemia among healthy infants.[36,48] A case study of two infants reported a range of 45-147 mL of DHM was utilized for supplementation.[36] Ferrarello et al. found that among 83 mother-infant dyads, 76% of parents chose DHM as a supplement for hypoglycemia over formula.[48]

Six studies investigated DHM patterns of use for other supplementation reasons among healthy infants.[39,40,42,44,46,49] In a 2018 regional survey of 71 maternity hospitals, 32% of hospitals reported that they were using DHM for healthy infants.[39] Sen et al. investigated supplementation patterns at a single US hospital from 2013-2016 and found that the percentage of healthy infants

receiving DHM increased from 0.04% to 4.7%, with common indications for use including excessive weight loss, late preterm birth, poor latch, and delayed lactogenesis.[44] Lewis et al. investigated indications for DHM in a level 1 mother baby unit and reported the three most common reasons for supplementation among healthy infants were excessive weight loss, mother-infant separation, and small for gestational age.[40] Rabinowitz et al. surveyed breastfeeding mothers of healthy infants (n=24) and reported that 58% preferred DHM supplementation over formula.[42] Regarding hospital policies, Drouin et al. surveyed level 1 maternity hospitals in the Northeast using DHM (n=15) and found that 87% had policies that stated criteria for DHM, 27% stated DHM was preferred, and all required consent.[46] Kair et al. investigated maternal characteristics by supplemental feeding type and found that dyads that received formula supplementation (n=376) were more likely to be nonwhite, publicly insured, and/or non-English speaking than dyads who received DHM supplementation (n=306), raising concerns about healthcare inequities in DHM access.[49]

Knowledge of DHM

Four studies investigated clinician and family knowledge and opinions about DHM supplementation for healthy infants.[38,39,42,47] Rabinowitz et al. interviewed postpartum mothers (n=24) and reported maternal concerns related to safety of DHM and a lack of information.[42] I In another study interviewing postpartum mothers (n=30), Kair et al. identified 4 themes including that formula is familiar and DHM is not, DHM is costly and logistically challenging, DHM is temporary and formula is ongoing, and DHM is "healthier."[38] Ferrarello et al. reported that nurses (n=20) lacked knowledge of DHM and presented logistical concerns for implementing a DHM policy in a well-baby nursery.[47] In a survey of clinicians at maternity hospitals in the

Northeastern US (n=71), 78% believed that studies showed benefits of using DHM in healthy term infants, and 94% believed that DHM improved exclusive breastfeeding rates.[39]

Clinical Outcomes

One study reported clinical outcomes for infants receiving level 1 care. Mannel et al. found that hospital length of stay did not differ in late preterm infants (n=183) by feeding type (MOM, DHM and formula).[41] Thoene is currently investigating DHM supplementation among term infants experiencing hypoglycemia in an RCT that is registered to report blood glucose outcomes.[57]

Breastfeeding Outcomes

Six studies reported on how DHM use influenced breastfeeding outcomes in healthy infants with conflicting findings between observational studies and a single RCT.[36,39,41,48,50,55] Ferrarello et al. reported that among infants supplemented with DHM for hypoglycemia (n=63), 52% were exclusively breastfeeding at discharge compared to 0% of infants supplemented with formula (n=20).[47] Belfort et al. found that hospitals who reported using DHM to supplement healthy infants had higher rates of exclusive breastfeeding at discharge compared to those who did not (77% vs. 56%, p=0.02).[39] Mannel et al. found that the risk ratio of breastfeeding at discharge was 0.84 for infants who received any formula supplementation (n=93) compared to infants who received expressed human milk (n=20) (95% CI 0.77-0.92).[41] Merjaneh et al. found that breastfed infants supplemented with DHM (n=33) had 5 times greater odds of exclusive breastfeeding at 6 months compared to infants supplemented with formula (n=39) (95% CI 1.37, 19.23; p=0.015).[55] Conversely, Kair et al. conducted an RCT of healthy infants with > 4.5% weight loss in first 36 hours of life and found that infants randomized to receive early, limited-

volume DHM supplementation (n=30) were not more likely to be breast milk feeding at 3 months than the control group who were assigned to exclusive breastfeeding (n=30).[50]

Post-Discharge Preterm Infants

Scuzs et al. studied supplementation of DHM among post-discharge preterm quintuplets and reported they received approximately 420 oz. DHM per week in addition to mother's milk, and were developing normally at 6 months.[34]

Discussion

In this systematic scoping review of research using DHM in populations other than hospitalized preterm infants, we found the majority of studies were conducted in healthy infants (14/26), or infants > 35 weeks gestation with health complications (9/26). More than half of the studies (15/26) were not intended to establish a potential relationship between DHM use and health-related outcomes, which suggests a high level of directness of outcomes bias in the existing literature.

DHM Use in Non-Infant Populations

Research into DHM use in non-infant populations is scarce, with only a single study identified in adult cancer patients that was influenced by selection bias (all participants had independently sought DHM). Additionally, no clinical outcomes were directly measured so potential efficacy of DHM as an adult cancer therapy could not be evaluated. Studies are also limited regarding DHM use in children, with only one RCT investigating DHM use during bone marrow transplants. While the study reported positive outcomes related to intestinal inflammation and bacterial abundance, interpretation is limited by reporting bias due to combining infants who received DHM with those who received MOM when reporting findings.

DHM Use in Infants Born > 35 weeks Gestational Age with Health Risks

The majority of studies in infants with health risks (7/9) measured health-related outcomes including clinical findings, biomarkers, and/or growth; however, due to high heterogeneity between populations studied (including infants with NAS, feeding intolerances, HIV-exposure, and cardiac and gastrointestinal anomalies), there was limited ability to synthesize findings across studies. In small case studies of infants experiencing failure-to-thrive, there was consensus of improved feeding tolerance.[43,53] In studies measuring growth outcomes, there were inconsistent results, with 2/4 studies reporting inferior growth with DHM feedings.[37,43,53,56] Findings of slower growth is in agreement with studies among preterm infants that have reported slower growth with DHM feedings compared to preterm formula,[16,58–60] suggesting that future studies should investigate growth outcomes and also measure the macronutrient content of the DHM as this was not addressed in any of the growth studies included in this review. Future research is also needed in how DHM use among ill infants influences breastfeeding outcomes as limited studies have considered this.

Limitations of research in infants with health risks include the use of study designs that were predominantly observational, cross-sectional and contained less than 20 participants who received DHM. Moreover, three studies compared infant groups from two eras separated by multiple years.[32,35,56] Due to the time difference, changes in standard of care may have confounded study findings. Of final note is the reporting bias present in two studies that grouped MOM and DHM feedings together when reporting results, which inhibits any conclusions specific to DHM.[35,45]

DHM Use in Healthy Infants Born > 35 Weeks Gestational Age

Fourteen studies were conducted in the last 6 years using DHM in healthy infant populations, suggesting this is a growing topic of interest. A 2018 regional study of 71 maternity hospitals reported that 32% of hospitals were using DHM for healthy infants, though whether these patterns hold nationally remains to be established.[39] Reported uses of DHM included hypoglycemia and/or hyperbilirubinemia, excessive weight loss, delayed lactogenesis, small for gestational age, and maternal-infant separation.[40,44,46] Differential patient access to DHM was reported in one study that found non-white, publicly insured, and/or non-English speaking mothers received DHM as a supplement less often, raising concerns about healthcare inequities.[49]

The most common health-related outcome studied among infants in level 1 care receiving DHM was the impact on breastfeeding, with conflicting findings reported.[36,39,41,47,50,55] Four observational studies reported a positive impact on breastfeeding outcomes at hospital discharge or up to 6 months postpartum. In observational studies, reverse-causation may have been present when DHM use is counted towards definitions of exclusive breastfeeding at discharge, thus confounding findings. Conversely, one RCT showed no improvement in breastfeeding rates at 3 months postpartum in infants receiving early, limited-volume DHM supplementation for excessive weight loss.[50] Recent RCTs have found that early, limited-volume formula supplementation in the hospital did not impact breastfeeding status at 1 month and 6 months among healthy, term infants which aligns with the findings of the RCT in this review.[61,62] Collectively, these RCTs suggest that limited-volume supplementation in hospital, regardless of whether it is formula or DHM, may have minimal impact on breastfeeding outcomes.

Only a single study considered clinical outcomes (length of hospital stay) among healthy infants receiving DHM,[19] and no studies investigated growth outcomes. Directness of outcomes bias is prevalent in the current body of research, with the majority of studies in healthy infants not designed to establish a relationship between DHM use and health-related outcomes. Additionally, this is a rapidly evolving field, and given the time it takes to conduct the search only articles published through February 2020 were included, therefore, there is a lag with the current literature. Future research should include randomized trials to investigate the impact of DHM versus formula supplementation on health-related outcomes including breastfeeding status, clinical outcomes, and growth.

Conclusion

The studies presented in this scoping review identify that DHM is increasingly being used in infant populations other than the preterm, NICU infant. Overall, studies are limited due to directness of outcomes bias, with 15/26 studies not designed to establish a relationship with DHM use and health-related outcomes. More research is warranted regarding DHM use in non-preterm infants and should include randomized trials investigating clinical, growth, and breastfeeding outcomes.

Author Contributions

SAM and MTP contributed to study conception, study design, data collection, and data interpretation. SAM had primary responsibility for writing the manuscript. Both authors reviewed, edited, read, and approved the manuscript.

Disclosures

The authors report no funding was received for this project. MTP serves on the Board of Directors for the Human Milk Banking Association of North America in an unpaid capacity. SAM has no conflicts of interest to report.

CHAPTER III: THE INFLUENCE OF HUMAN MILK OLIGOSACCHARIDES ON COMMON METHODS FOR MEASURING LACTOSE AND TOTAL CARBOHYDRATES IN HUMAN MILK

Abstract

Background: Lactose is the most abundant constituent in human milk and a predominant source of energy. Methods for assessing it are often borrowed from the bovine dairy industry. However, the carbohydrate matrices of bovine and human milk are quite different with bovine milk having very few oligosaccharides while human milk is abundant with oligosaccharides (HMOs), each with a terminal lactose unit that may be influencing analytical methods.

Methods: To determine if HMOs influence common analytical methods used for measuring carbohydrates in human milk, we assessed native (n=16) and HMO-spiked (n=16) human milk samples using four methods: BioVision enzymatic assay (measures lactose), Megazyme enzymatic assay (measures lactose), Ultra-High Pressure Liquid Chromatography Mass Spectrometry (measures lactose), and Miris Infrared Analysis (measures total carbohydrates).

Results: Native and spiked samples were not significantly different for lactose using Megazyme (mean difference (MD) = 0.0 g/dL; 95%CI 0.0 to 0.1) or UPLC-MS (MD = - 0.1 g/dL; 95%CI -0.4 to 0.1), suggesting no impact of HMOs. HMOs influenced lactose measurement with BioVision (MD = 0.2 g/dL, 95%CI 0.1 to 0.4, p = 0.005) and total carbohydrate measurements with Miris (MD = 0.4 g/dL, 95%CI 0.3 to 0.6, p = 0.000).

Conclusion: HMOs interfered with BioVision and Miris, but not UPLC-MS or Megazyme. Given that HMOs are non-digestible, this has implications for the energy values estimated for human milk.

Introduction

Human milk has been well characterized and is considered the optimal nutrition for infants by the American Academy of Pediatrics, the World Health Organization, and the United States Surgeon General.[27,28,63] This is supported by extensive research showing multiple benefits of human milk use in infants including reduced gastrointestinal infections, respiratory disease, allergic disease, and mortality.[1] However, despite being well characterized and extensive research, there are still large gaps in knowledge regarding human milk analysis including appropriate analytical methods for measuring carbohydrate content.

Lactose, a disaccharide, is the most abundant carbohydrate and organic constituent in human milk with a typical range of 6.7-7.8 g/dL. It has been shown to remain relatively stable after the early postpartum period.[64–67] Human milk oligosaccharides (HMOs) are the second most abundant type of carbohydrate in human milk and the third most abundant organic constituent in human milk, after lipids.[68] In contrast to lactose, HMOs tend to decrease over the duration of lactation.[66] Monosaccharides make up the smallest portion of carbohydrates in human milk, accounting for approximately 1% of the total carbohydrate content.[66] Lactose has several vital functions in the newborn infant which include supporting growth, innate immunity, providing roughly 40% of caloric intake, and development of the gut microbiota.[69–71] HMOs in human milk are non-nutritive to the newborn infant, but have several important functions including acting as a prebiotic for the infant's immature microbiota, acting as an anti-adhesive antimicrobial, and modulating intestinal epithelial cells and immune responses.[7] Because HMOs

are non-digestible to the infant, and are instead fermented in the gut by bacteria, they can lead to an over-reporting of energy in human milk when the same energy conversion factors (typical 4 kcal/g) are applied to HMOs as to digestible carbohydrates like lactose.[64,72] Instead, it is recommended by the Food and Agriculture Organization to use 2 kcal/g for fermentable fibers when determining the energy in foods.[73,74] AOAC, Inc. (formerly the Association of Official Agricultural Chemists) has an approved method for measuring lactose in milk that uses High-Performance Liquid Chromatography with Refractive Index Detection (HPLC-RID, AOAC 984.22).[75] AOAC also has an approved method for measuring lactose in milk that uses enzymatic reactions and spectrophotometry based on enzymes from Megazyme International (Megazyme, AOAC 2006.06).[75] These methods were originally developed for use with bovine milk but have also been utilized in human milk research. However, the carbohydrate matrices of bovine and human milk are quite different with bovine milk having trace amounts of oligosaccharides compared to human milk which is abundant with oligosaccharides (HMOs). Each HMO has a lactose unit at the reducing end and some HMOs have a β-1-3-linked galactose at the non-reducing end. A visual summary of common HMO structures can be found in the literature.[68] These terminal sugars could theoretically influence methods for measuring lactose in human milk, especially if enzymatic methods use enzymes that are capable of cleaving the terminal lactose or galactose units from HMOs. Recently, an infrared analyzer (IR) was approved by the FDA that measures the total carbohydrates in human milk (including lactose and HMOs).[76] However, no studies have assessed the impact of HMOs on any method to measure lactose in human milk.

The primary purpose of this study is to determine if common analytical methods for measuring lactose and total carbohydrates in human milk are influenced by the presence of

HMOs. We hypothesized that HMOs would not influence lactose measurements using the LC-MS method but would influence IR and enzymatic methods. The secondary purpose of this study was to compare lactose and total carbohydrate values across four commonly used analytical methods for human milk.

Methods

To determine if HMOs influence common analytical methods used in human milk research, we assessed native (n=16) and HMO-spiked (n=16) human milk samples using four common methods: BioVision enzymatic assay (measures lactose),[77] Megazyme enzymatic assay (measures lactose)[78], Miris IR analysis (measures total carbohydrates including lactose and HMOs)[79], and ultra-high pressure liquid chromatography mass spectrometry (UPLC-MS; measures lactose).[80]

Sample Preparation

Frozen, raw, de-identified human milk samples from unique donors (n=16) that were collected as part of another study were used to prepare native (n=16) and HMO-spiked (n=16) samples.[81] A Precision Shaking Water Bath 15 (SWB; TSSWB15; Thermo Fisher Scientific, Newington, NH USA) was used to thaw the frozen samples, at a setting of 55rpm and 35ºC until samples were completely thawed and reached a temperature of 30ºC to ensure we created representative samples in all study kits because lactose can precipitate out during long-term frozen storage.[82] Thawed, native samples were continuously mixed using a magnetic stirrer for five minutes, and while aliquoting into sterile microtubes for future analysis. For the HMO-spiked samples, native milk was spiked using a solution prepared with purified, powdered HMOs obtained from the University of California, San Diego. HMOs were isolated from pooled donor human milk. Proteins, lipids, salts, and monosaccharides were removed. Lactose concentration in

the generated HMOs was less than 1% with 99% HMOs. The spiking protocol was intended to increase the HMOs by 0.5 g/dL, which is approximately a 50% increase, while only diluting overall volume by 2%. Briefly, 1.105 g of purified HMO was mixed with 4.25 mL deionized water and gently agitated to dissolve. 260 uL of HMO solution was added to 13 mL of native milk samples. The HMO-spiked samples were mixed continuously using a magnetic stirrer for five minutes and while aliquoting into sterile microtubes. The test kits were stored in at -20 Celsius until analysis and/or shipment to collaborating labs.

Human Milk Oligosaccharide Analysis

To confirm samples were successfully spiked with HMOs, samples were sent to Bode Lab (University of California, San Diego) and measured using an established method for measuring HMOs in human milk (HPLC-FL).[83]

Enzymatic Analysis

The BioVision assay (K624, BioVision Incorporated, Milpitas, CA, USA) is a colorimetric assay kit that uses a proprietary enzyme mix (email communication with BioVision, 05/24/19) to hydrolyze lactose into the monosaccharides glucose and galactose.[10] Galactose is then oxidized, generating color, which is measured at OD 570 nm. A standard curve of known concentrations of lactose (provided as part of the kit) is used to determine lactose concentration of the samples. All samples were measured in triplicate using an Epoch Bio-tek Microplate Spectrophotometer Plate Reader (7091000; BIO-TEK, Winooski, VT, USA).

The Megazyme enzymatic assay (K-LAGCAR, Megazyme International, Wicklow, Ireland) Corporation) is based on the AOAC method 2006.06 which utilizes three reactions to quantify lactose in human milk using the galactose component of the lactose disaccharide.[9] The first reaction is the enzymatic breakdown of lactose into glucose and galactose using *Aspergillus*

Niger beta-galactosidase. The second reaction uses galactose mutarotase to convert alpha-D-galactose to beta-D-galactose. Once this reaction is complete, the plate is read at 340 nm. The final reaction is the reduction of NAD+ to NADH + H+ by beta-D-galactose using beta-galactose dehydrogenase. The plate undergoes a second reading at 340 nm. This reading is subtracted from the first to get final values. Lactose concentrations are calculated from equations generated from a standard curve of known lactose monohydrate (64044-51-5, Fisher Scientific, Fair Lawn, NJ, USA). All samples were measured in triplicate using an Epoch Bio-tek Microplate Spectrophotometer Plate Reader.

UPLC-MS Analysis

Lactose was quantified in human milk samples using the UPLC-MS methods of Fusch et al.[84] The diluent used for standards and samples was deionized water. Lactose monohydrate (64044-51-5, Fisher Scientific, Fair Lawn, NJ, USA) was used to create the standard curve at concentrations of 0, 2, 4, 6, 8, and 10 g/dL with a 50,000- dilution factor (DF). Samples were prepared using a 50,000 DF. The standard curve was calculated and used to determine lactose concentrations of the samples. Samples were analyzed using the Water's Acuity Ultra-High Performance Liquid Chromatography system (Waters, Milford, MA, USA) and the Thermo Fisher Scientific Q Exactive Plus (Thermo Fisher Scientific, Waltham, MA, USA) that are located at the Triad Mass Spectrometry Facility at the University of North Carolina Greensboro. The HMO solution was also measured with UPLC-MS to test for the potential presence of lactose in the spiking solution.

IR Analysis

Samples were sent to the Belfort Lab (Brigham Women's Hospital, Boston, MA) for IR analysis using the Human Milk Analyzer (Miris, Sweden) which is a mid-infrared milk analyzer

that measures the total carbohydrate concentration in human milk based on mid-IR absorbance of the hydroxyl group and validated against the difference method for measuring total carbohydrates (total solids minus fat, protein, and ash).[76,85] We sonicated 3 mL of milk for 5 seconds and warmed it in a Penguin hospital-grade milk warmer (Ameda, Buffalo Grove, IL, USA) with a custom temperature setting of 40 degrees Celsius. We analyzed each sample once. Miris check and control solutions were used to calibrate the analyzer according to manufacturer instructions.[76]

Statistical Analysis

Based on previous data on variation in lactose methods from our lab, the sample size necessary to detect an 8% change in lactose concentration with an 80% power and 95% confidence interval (CI) was 16. The sample size calculation was determined with a two one-sided test (TOST) using the statistical software R (R Core Team, 2017). Statistical analysis was conducted using IBM SPSS software (Version 26; IBM Corp., Armonk, NY) and Microsoft Excel (Version 16.42). Differences in lactose and HMO concentrations between pre- and post-spiked samples within an analytical method were evaluated using a paired t-test. Pair-wise comparisons of pre-spiked milk samples across the 4 analytical methods were evaluated using Pearson correlation coefficients. This research was classified as non-human subjects research by the Institutional Review Board at the University of North Carolina Greensboro (protocol 17-0523).

Results

HMO Spiking Confirmation

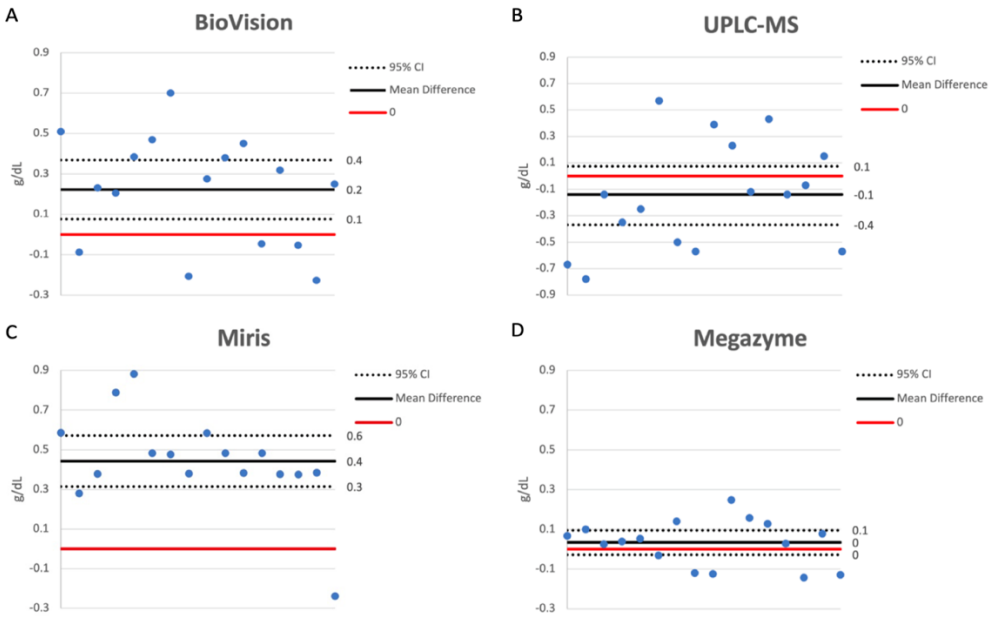
Average HMO concentration increased by 0.5 g/dL in spiked samples compared to native samples, indicating a successful spiking protocol. The range of actual HMO concentration

increase was 0.49 to 0.52 g/dL which indicates that the spiking protocol was consistent across all samples. The pure HMO spiking solution contained 0.8 g/dL of lactose per UPLC-MS. Based on our spiking protocol, this would translate to an 0.2% increase in lactose for a sample that contained 6.5 g/dL lactose in native milk, confirming that the HMO spiking solution did not contribute to measurable changes in lactose.

Impact of HMOs on Analytical Methods

As seen in Figure 1, native and spiked samples were not significantly different for lactose using Megazyme (mean difference (MD) = 0.0 g/dL; 95%CI 0.0-0.1) or UPLC-MS (MD = - 0.1 g/dL; 95%CI -0.4-0.1). There was a significant difference in native and spiked samples for total lactose measurements with BioVision (MD = 0.2 g/dL, 95%CI 0.1 to 0.4, p = 0.005), and total carbohydrate measurements with Miris (MD = 0.4 g/dL, 95%CI 0.3 to 0.6, p = 0.000).

Figure 1. Bland-Altman plots showing the mean difference and 95% Confidence Interval by analytical method for carbohydrate content after spiking human milk samples with human milk oligosaccharides. (A) BioVision Enzymatic Assay, (B) Ultra-High Pressure Liquid Chromatography Mass Spectrometry (UPLC-MS), (C) Miris Infrared Analyzer, (D) Megazyme Enzymatic Assay.



Comparison Between Analytical Methods

Descriptive statistics for native and HMO-spiked samples are summarized in Table 3. Native samples contained 7.7 ± 0.3 g/dL of lactose per BioVision, 6.3 ± 0.3 g/dL of lactose per Megazyme, 8.7 ± 0.2 g/dL of total carbohydrates per Miris, and 7.7 ± 0.5 g/dL of lactose per UPLC-MS. The coefficient of variance for samples and standard curves measured in triplicate for BioVision was 0-4% for the samples and 0-5% for the standard curve, for Megazyme was 0-7% for the samples and 0-3% for the standard curve, and for UPLC-MS was 0-2% for the samples and for 0-9% for the standard curve.

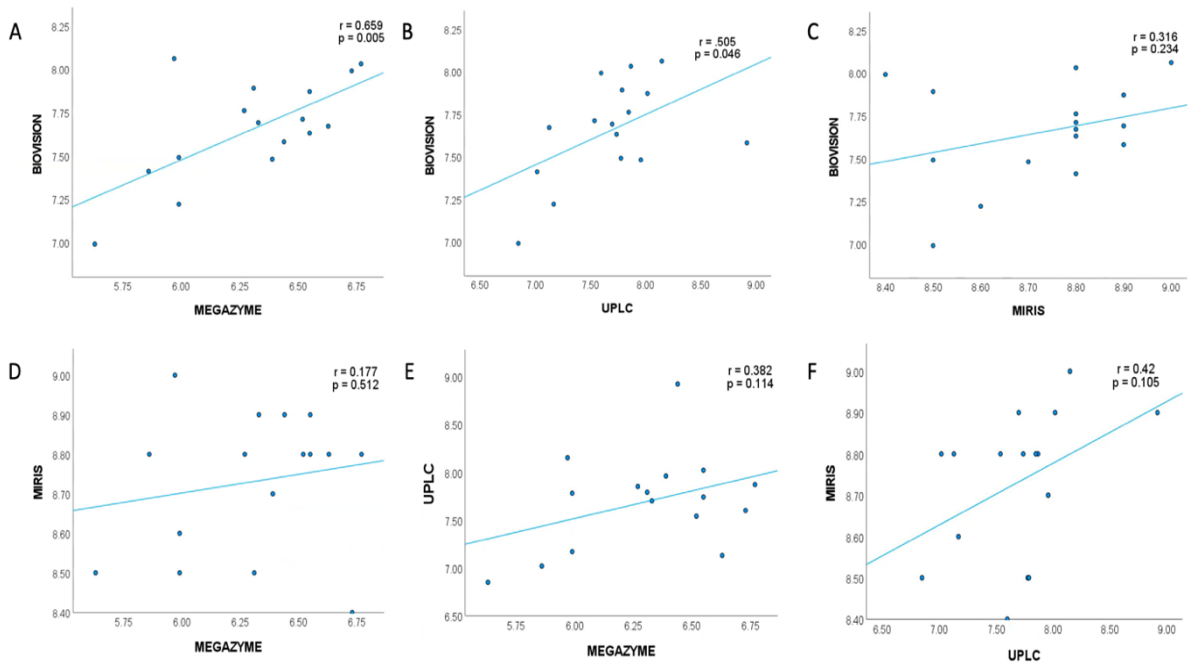
Table 3. Mean concentrations of carbohydrates in native and Human Milk Oligosaccharide (HMO)-spiked human milk samples by analytical method.

	Native Samples	**HMO-Spiked Samples**	
Method Type	**Mean ± SD (g/dL)**	**Mean ± SD (g/dL)**	**Mean Difference (95% CI)**
Lactose			
BioVision	7.7 ± 0.3	7.9 ± 0.4	0.2 (0.1, 0.4)
Megazyme	6.3 ± 0.3	6.3 ± 0.3	0.0 (0.0, 0.1)
UPLC-MS	7.7 ± 0.5	7.5 ± 0.5	-0.1 (-0.4, 0.1)
Total Carbohydrates			
Miris	8.7 ± 0.2	9.2 ± 0.3	0.4 (0.3, 0.6)

SD, Standard deviation; CI, Confidence interval; UPLC-MS, Ultra-high pressure liquid chromatography mass spectrometry

There was moderate correlation of Megazyme with BioVision ($r = 0.659$, $p = 0.005$), and of UPLC-MS with BioVision ($r = 0.505$, $p = 0.046$), but correlations were weak between Miris and BioVision ($r = 0.316$, $p = 0.234$), Megazyme and Miris ($r = 0.177$, $p = 0.512$), UPLC-MS and Megazyme ($r = 0.382$, $p = 0.114$), and UPLC-MS and Miris ($r = 0.420$, $p = 0.105$) (Figure 2).

Figure 2. Correlations of carbohydrate content of native human milk samples between the 4 analytical methods. (A) Megazyme & BioVision, (B) UPLC-MS & BioVision, (C) MIRIS & BioVision, (D), Megazyme & MIRIS, (E) Megazyme & UPLC-MS, (F) UPLC-MS & MIRIS.



Discussion

In this study that was designed to assess the impact of HMOs on a variety of analytical techniques for measuring lactose and total carbohydrates in human milk, we found that HMOs interfered with some lactose methods, and others were not, highlighting the need for reliable and accurate methods of analysis for human milk.

For our primary aim, we hypothesized that lactose measurements would not increase with HMO spiking for UPLC-MS methods but would increase for Miris and enzymatic assays. UPLC-MS measures using the mass of a substance whereas Miris measures total carbohydrates, and the enzymatic methods measure galactose which may be acting on the terminal galactose units on HMOs. We found that Miris had a mean increase in spiked samples of 0.4 g/dL which reflects approximately 80% of the spiked HMO content and BioVision had a mean increase of

0.2 g/dL which reflects approximately 40% of the spiked HMO content, suggesting these two methods are influenced by presence of HMOs in human milk. Megazyme and UPLC-MS did not change significantly between native and spiked samples, suggesting they are not influenced by HMOs in human milk.

We found mixed results regarding whether HMOs influenced enzymatic methods, with Megazyme not influenced by HMOs, and BioVision influenced by approximately 40% of the added HMOs. The nonsignificant Megazyme results may be due to the specific β-galactosidase utilized by the Megazyme Assay kit, which is produced by the fungus *Aspergillus niger*. Previous research has shown that the β-galactosidase from *A. niger* is able to cleave b-1-3 linked terminal galactose molecules effectively at temperatures of 35-70ºC, and after a 24 hour incubation.[86] Moreover, the optimum activity of the enzyme shifted to 65 ºC when bovine serum albumin (BSA) was present.[86] Human milk is abundant in the alpha-lactalbumin protein, which may have a similar effect of shifting the efficacy of the reaction to higher temperatures as BSA. These results suggest that the *A. niger* β-galactosidase used in the Megazyme assay may not be effective in cleaving terminal galactose molecules from HMOs due to the lower temperature of our assay (20-25 ºC), the shorter length of incubation (less than 1 hour), and the presence of alpha-lactalbumin in human milk. These factors make Megazyme less able to act on HMOs, and therefore, less error prone as an enzyme-based method as compared to BioVision which is also enzyme-based.

Our secondary aim was to compare four analytical methods commonly used for measuring carbohydrates in human milk. We found that BioVision was moderately correlated with Megazyme and UPLC-MS ($r = 0.659$, $r = 0.505$, respectively). Miris was not correlated with any other methods. This is likely due to Miris measuring total carbohydrates compared to

the enzymatic and UPLC-MS methods, which are intended to measure lactose.[76,84] Fusch et al. reviewed 12 studies that evaluated IR milk analyzers, including MIRIS, and found inconsistent results of IR analyzers in measuring lactose/carbohydrates in human milk.[87] Recently, Kwan et al. found poor correlation between IR methods and UPLC-MS for measuring lactose.[88] In the bovine milk industry, HPLC-RI and Megazyme are considered appropriate and reliable analytical methods.[75] In this study, we found Megazyme was not influenced by HMOs, but we did not test the HPLC-RI method. However, we found that UPLC-MS was not influenced by HMOs, but why the reported values were different from Megazyme remain unclear. This highlights an important gap in the literature to determine if HPLC-RI and UPLC-MS produce similar results for lactose content in human milk.

While neither the UPLC-MS protocol or the Megazyme enzymatic assay were influenced by HMOs, these methods were not significantly correlated and produced a greater than 20% difference in mean lactose values (7.7 g/dL vs 6.3 g/dL, respectively), suggesting other factors may have contributed to these differences. Rochow et al. used similar UPLC methods to our study and reported mean lactose values of 7.4 and IQR of 6.8 to 7.9 in mature milk, which is in line with our UPLC findings.[89] In contrast, Coppa et al. used HPLC combined with refractive index detection (HPLC-RID) based on AOAC 984.22 and reported mean lactose values in term mature milk of 6.4 g/dL, which is similar to the values we found with the Megazyme enzymatic assay.[66] A potential reason for the apparent difference between UPLC-MS and the Megazyme assay in this study could be due to differences in the standard and sample preparation. While both methods utilize the same standard, lactose monohydrate, the standards were prepared separately. Additionally, different dilution factors (50,000 for UPLC-MS and 100 for Megazyme) may have influenced accuracy as high dilution factors require multiple transfers

which can amplify small pipetting inaccuracies. While AOAC method 984.22 is an approved method for measuring lactose in bovine milk, there have been limited studies comparing this method with other analytical methods frequently used for measuring lactose in human milk, highlighting an important gap in the literature.

A strength of this study is the confirmation of HMO spiking of our samples and the limited contribution of additional lactose from the spiking protocol (approximately 0.2%). Additionally, we included two enzymatic methods. No previous studies have compared two enzymatic assays, and many do not report the specific enzymes used. This study found that Megazyme enzymatic assay was not influenced by HMOs, but BioVision enzymatic assay was. Moreover, BioVision reported higher lactose values in the native human milk samples than Megazyme which is likely due to the natural HMOs present in human milk that are being partially quantified by BioVision, but not by Megazyme. A limitation of this study was the use of individual standards for each method, instead of using one standard for all methods, which may have contributed to some of the differences between methods that we observed. We also did not measure lactose using the AOAC 984.22 method, which is important to include in future research that validates methods for human milk.

Conclusion

This study found that certain methods for measuring carbohydrates in human milk are influenced by the presence of HMOs, and others are not. HMOs are non-digestible for the infant, therefore, the choice of analytical methods for measuring carbohydrates in human milk will significantly influence energy estimates, which in turn may inform clinical interventions. Megazyme and UPLC-MS methods were not influenced by HMOs, therefore, these methods are

more appropriate for measuring lactose content in HM. However, further investigation is needed due to the differences seen in the reported lactose values of these methods.

CHAPTER IV: THE EFFECTS OF REFRIGERATED STORAGE ON NUTRIENTS IN DONOR HUMAN MILK WITH LIMITED BACTERIAL PRESENCE AFTER HOLDER PASTEURIZATION

Abstract

Background: The Human Milk Banking Association of North America (HMBANA) sets the purity and quality standards for donor human milk (DHM) in the United States. These standards include zero bacterial presence after Holder pasteurization. The milk is discarded if any bacteria remain, despite no evidence that bacterial presence is related to infection risk in infants. This study aimed to determine if nutrient and bacterial composition of DHM with limited bacterial presence after pasteurization change over 4 days of refrigerated storage.

Methods: Twenty-five unique samples of DHM that had been rejected due to post-pasteurization bacterial growth were collected from 2 HMBANA milk banks. Gerber Good Start GentlePro Powder Infant Formula, Stage 1 was used as a comparison. A portion of milk was removed at 24-hour intervals beginning at hour 0 to hour 96. The process for removing milk mimicked home-use. Aerobic bacteria content was measured using 3M™ Petrifilm™ Aerobic Count Plates. Total protein content was measured using a bicinchoninic acid kit. Lactose content was measured using Megazyme Enzymatic Assay. Immunoglobulin A (IgA) was measured using an enzyme-linked immunoassay. Longitudinal changes in the composition of bacteria, protein, lactose, and IgA in each sample over storage duration were analyzed using repeated measures analysis of variance and mixed models tests. $P<0.05$ was deemed significant.

Results: There were no significant differences in lactose, protein, bacteria, or IgA content over storage duration (P=0.649, P=0.690, and P=0.385, P=0.805 respectively). Total aerobic bacteria were less than 10^2 CFUs in 81% of the timepoints tested for DHM samples. Total aerobic bacteria were too many to count (> 300 CFUs) in the infant formula sample at all time points.

Conclusion: There were no significant changes in the total bacterial count, protein, IgA, or lactose over 4 days of refrigerated storage in DHM that had been rejected post-pasteurization. The majority of DHM samples contained less than 10^2 CFUs of aerobic bacteria, while the powdered formula control contained too many to count at all time points. In periods of high demand for DHM, DHM with low bacteria growth post-pasteurization may be an option as a supplemental food for the growing number of healthy infants who receive DHM.

Introduction

Human milk has been well characterized and is considered the optimal nutrition for newborn infants by the American Academy of Pediatrics, the World Health Organization, and the United States (US) Surgeon General.[63] Current policies recommend infants be exclusively breastfed for the first six months of life, then introduction of complementary foods with continued breastfeeding for at least one year.[90] When mother's own milk is unavailable, donor human milk (DHM) is the recommended feeding strategy, especially for the preterm infant who is at risk for necrotizing enterocolitis.[16,28,90]

Guidelines for the production and distribution of DHM in the US are primarily determined by the Human Milk Banking Association of North America (HMBANA).[91] In the US, DHM undergoes Holder pasteurization where the milk is warmed to 62.5 degrees Celsius for 30 minutes which is effective in inactivating microbial agents that may be present while

maintaining many of the beneficial components of HM.[11,12] Safety standards of pasteurized DHM include testing each batch for presence of bacteria after pasteurization. If one bacterial colony forming unit (CFU) is present, the milk is discarded.[13] These stringent standards are in place to protect the vulnerable hospitalized preterm infant because milk banks have historically primarily served hospital Neonatal Intensive Care Units (NICU), with 88% of level 3 and 4 NICUs across the US utilizing DHM.[14,22] However, other, less vulnerable infant populations have been increasingly using DHM which may not require such stringent standards.[15] Two recent studies investigated the percentage of DHM rejected due to post-pasteurization bacterial growth and found that up to 12.6% of DHM is discarded because of bacterial presence.[92,93] Studies have reported no relationship between presence of bacteria in HM and infection in premature infants which is in contrast to HMBANA's current guidelines for zero bacterial presence.[94,95]

As a comparison, the dairy industry allows up to 20,000 CFUs per milliliter of dairy milk as part of the Grade A pasteurized milk ordinance.[96] CFUs are not addressed in the FDA regulations for powdered infant formula (PIF), but PIFs must be assessed for presence of *Cronobacter* and *Salmonella* species.[97] All storage studies to evaluate shelf-life of DHM have been conducted in DHM with no bacterial presence after pasteurization; no studies have looked at DHM that contains minimal bacterial growth post-pasteurization. This DHM has the potential to be repurposed to less vulnerable populations instead of being discarded.

The purpose of this study was to determine if nutrient content (protein, lactose, and IgA) and bacterial count of DHM with limited bacterial presence after pasteurization change over 4 days of refrigerated storage. We hypothesized that protein, lactose, IgA, and total bacterial count would not significantly change over 4 days of storage at 4°C.

Methods

Twenty-five unique samples of DHM that had been rejected due to post-pasteurization bacterial growth were collected from 2 HMBANA milk banks, Mother's Milk Bank Northeast (Newton, MA, USA) and King's Daughters Milk Bank at CHKD (Norfolk, VA, USA). Powdered infant formula (Gerber Good Start GentlePro Powder Infant Formula, Stage 1) was used as a comparison because formula is the recommended food when human milk is not available. Samples were shipped overnight on dry ice and stored at -20°C until sample preparation.

Sample Preparation

The 100-120mL bottles of DHM were defrosted in the refrigerator at 4°C. Once samples were defrosted, bottles were gently mixed by inverting 10-12 times by hand. Baseline samples, which represent time 0, were removed (~10mL), aliquoted into appropriate amounts, and stored in the freezer (at approximately -20°C) until analysis. The remaining DHM was returned to the refrigerator at 4°C and stored for 96 hours to simulate home storage. Four days of storage was chosen for this study because it is twice as long as the current HMBANA guidelines for defrosted DHM storage which is 48 hours.[91] In order to simulate a "home environment," the primary investigator washed hands, but did not wear gloves, a lab coat, or other PPE, or disinfect the countertop prior to each removal. Once in the refrigerator, at hours 24, 48, 72, and 96, the primary investigator gently mixed each bottle by inverting 10-12 times by hand and removed approximately 10 mL of sample that was aliquoted into necessary amounts, labeled with time of removal, and stored in a freezer (approximately -20°C) until analysis. Temperature of the refrigerator was recorded at each removal (0, 24, 48, 72, and 96 h).

Sample Analysis

Lactose was measured using the Megazyme enzymatic assay (K-LAGCAR, Megazyme International, Wicklow, Ireland), based on the AOAC method 2006.06 which utilizes three reactions to quantify lactose in human milk.[72] Lactose concentrations were calculated from equations generated from a standard curve of known lactose monohydrate (64044-51-5, Fisher Scientific, Fair Lawn, NJ, USA). Standards and samples were measured in triplicate using an Epoch Bio-tek Microplate Spectrophotometer Plate Reader (7091000; BIO-TEK, Winooski, VT, USA).

Total protein was measured using Pierce™ Bicinchoninic Acid (BCA)Protein Assay Kit (23225, Thermo-Scientific, Waltham, MA, USA).[98] This assay uses the peptide bonds in proteins to reduce Cu^{2+} to Cu^{1+}. Cu^{1+} is then chelated by two molecules of BCA which forms a purple complex that absorbs light at 562 nm. To determine the concentration of protein in the sample, it is compared to the absorbances of a standard bovine serum albumin with known protein concentrations. Standards and samples were measured in triplicate using an Epoch Bio-tek Microplate Spectrophotometer Plate Reader.

Total aerobic bacteria were measured by a plate enumeration method with a range of detection of 0-300 CFUs (3M Petrifilm® Aerobic Plate Counts, 70200572124; 3M, St. Paul, MN, USA). Sterile peptone was used as a control. Samples were undiluted and measured on a single plate. Plating was performed under a biosafety bench with sterile pipette tips. Plate counting was conducted under a biosafety hood with a hand counter and magnifier. If CFUs were too numerous to count (>300), they were designated a CFU count of 300 for analysis.

IgA activity was measured using an enzyme-linked immunoassay (ELISA) developed by Chen.[99,100] The IgA first binds to an *Escherichia coli* antigen, developed using 8 E. coli strains

acquired from the STEC Center (Michigan State University, East Lansing, MI, USA), which is then bound to an anti-human-IgA antibody. The antibody is labeled with Horseradish Peroxidase enzyme (HRP, A0295, Sigma, Aldrich, St Louis, MO, USA) which, in the presence of hydrogen peroxide, catalyzes the conversion of the *e. coli*-IgA complex in the presence of 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS; A-1888-5G, Sigma Aldrich) into a colored product. A spectrophotometer measures the color intensity at 405 nm which is then compared to known concentrations of human IgA standards (I2636, Sigma Aldrich). Standards and samples were measured in triplicate using an Epoch Bio-tek Microplate Spectrophotometer Plate Reader (Synergy HT, BioTek Instruments, Winooski, VT).

Statistical Analysis

Sample size was calculated based on the number of samples necessary to detect a 20% change in lactose concentration with an 80% power and an alpha value of 0.05 was found to be 14. We chose a sample size of 25 which aligned better with similar previous studies. Lactose was chosen for power calculations because if bacteria is growing in the DHM, they would likely be fermenting the lactose, thus decreasing its content in the samples.

Statistical analysis was conducted using SPSS software (version 28) and Microsoft Excel (Version 16.42). Longitudinal changes in nutrients, bioactive factors, and bacteria over 96 hours of storage were evaluated with repeated measures analysis of variance test and linear mixed model. $P < 0.05$ was deemed significant. Sample ID was treated as a random effect.

Results

Lactose, IgA, and protein analytical methods were conducted in triplicate and the average coefficients of variation were as follows: 2.7% for lactose, 5.7% for IgA, and 2.5% for protein. R^2 values were >0.997 for IgA, >0.999 for lactose, and >0.989 for protein. There was zero

aerobic bacterial growth for the sterile peptone control. Descriptive statistics for DHM samples are reported in Table 4.

Table 4. Mean (range) values for nutrient and total bacterial count of donor human milk over 96 hours of refrigerated storage.

	Hr 0	Hr 24	Hr 48	Hr 72	Hr 96
Lactose (g/dL)	6.7 (5.7-7.5)	6.7 (5.9-7.3)	6.7 (5.7-7.2)	6.7 (5.5-7.2)	6.7 (5.5-7.4)
Active IgA (g/L)	1.4 (1.0-1.7)	1.4 (1.0-1.8)	1.4 (1.0-1.7)	1.4 (1.0-1.8)	1.4 (1.0-1.8)
Protein (g/dL)	0.7 (0.5-0.9)	0.7 (0.5-0.8)	0.7 (0.5-0.9)	0.7 (0.5-0.8)	0.7 (0.5-0.9)
Bacteria (CFUs)	55 (0-300)	55 (0-300)	66 (0-300)	65 (0-300)	66 (0-300)

Notes: IgA – Immunoglobulin A; Longitudinal changes in nutrients, bioactive factors, and bacteria over 96 hours of storage were evaluated with repeated measures analysis of variance test and linear mixed model. There were no significant changes in nutrient or bacterial composition throughout storage duration.

Changes over refrigerated storage duration

Linear changes in content of lactose, IgA, protein, and bacteria are shown in Figure 3. There were no significant differences in lactose, IgA, protein, or bacteria content in DHM over 96 hours of refrigerated storage (P=0.649, P=0.805, P=0.690, and P=0.385, respectively). Total aerobic bacteria were less than 10^2 CFUs in 81% of the timepoints tested for DHM samples. Total aerobic bacteria were too many to count (> 300 CFUs) in the control sample (infant formula) at all time points. The infant formula contained an average 1.2 g/dL lactose and 0.9 g/dL protein higher content compared to the DHM samples and no IgA.

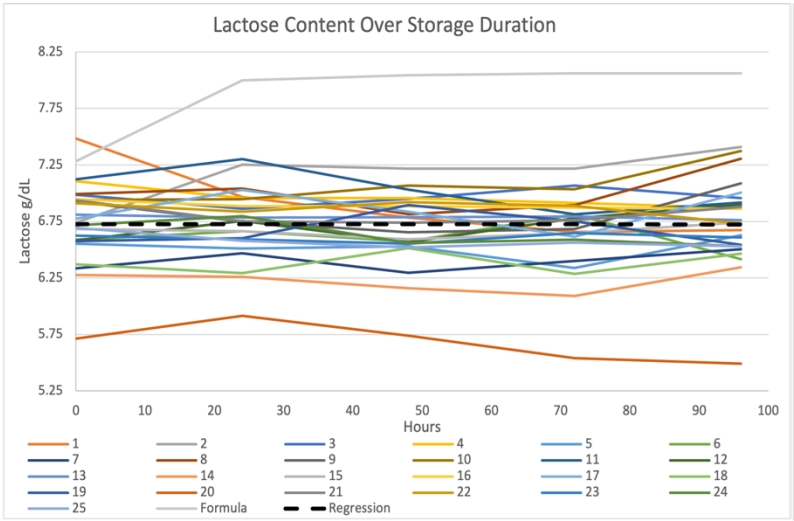
Discussion

There have been no studies to investigate the quality and storage of DHM that has bacterial presence after Holder pasteurization. Historically, this DHM has been discarded. This

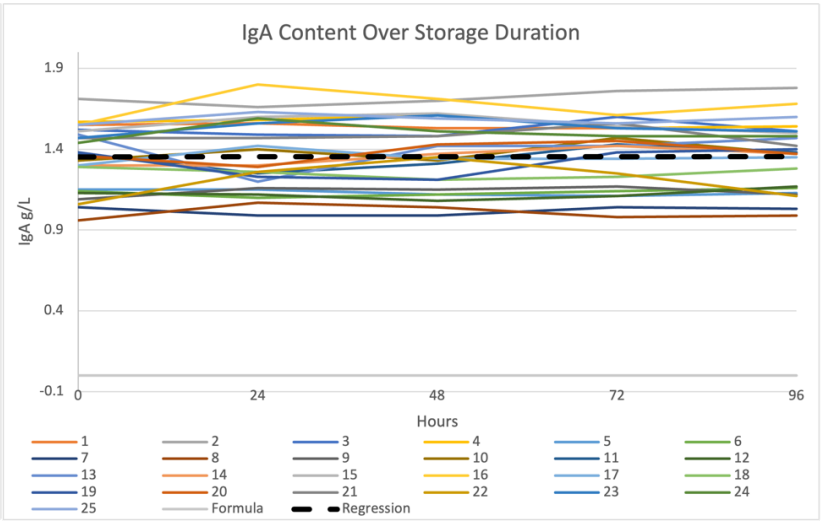
study aimed to investigate bacterial and nutrient changes in DHM that had been rejected by HMBANA milk banks due to presence of at least one bacterial colony forming unit. Over 4 days of storage at 4°C we found no significant change in aerobic bacteria, IgA, lactose, or protein content. These findings are in agreement with previous literature that investigated refrigerated storage of DHM that had been accepted post-pasteurization (without bacterial presence). Mandru et al. and Vickers et al. found that in pasteurized DHM there was no change in total bacterial count over 4-9 days of refrigerated storage under clinical handling and storage settings.[101,102] Moreover a review by Schlotterer et al. found that 3 studies reported that DHM maintains its quality and there was no change in total bacterial count over 4-9 days of refrigerated storage with one conducted using home settings.[103] The results from previous studies along with the results of this study suggest that both DHM with and without bacteria post-pasteurization maintains some of its antimicrobial properties and prevents bacterial growth over 4 days of refrigerated storage under clinical and home settings. Additionally, Schlotterer et al. investigated protein, lysozyme, and IgA in DHM that had no bacterial growth post-pasteurization and found no significant changes over 96 hours of refrigerated storage.[100] These align with the results from this study, suggesting that DHM with and without bacteria post-pasteurization maintains its nutrient composition over 4 days of refrigerated storage.

Figure 3. Nutrient or total bacterial count by sample ID over storage duration (96 hours). (A) Lactose, (B) IgA, (C) Protein, (D) Bacteria.

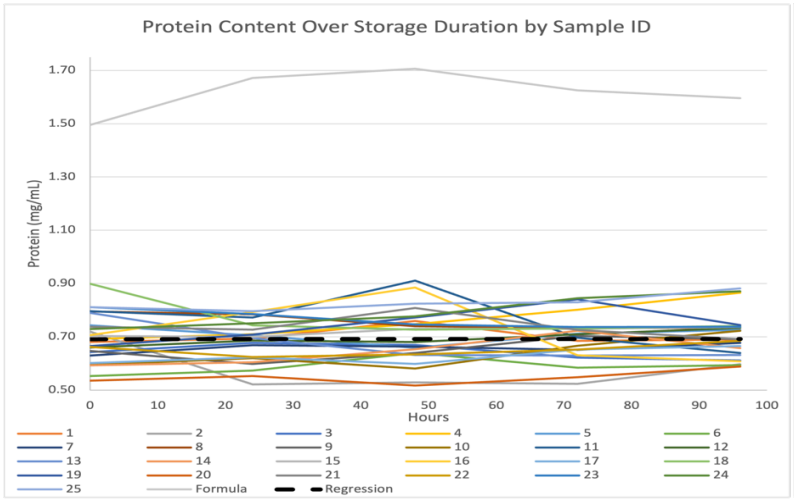
A.



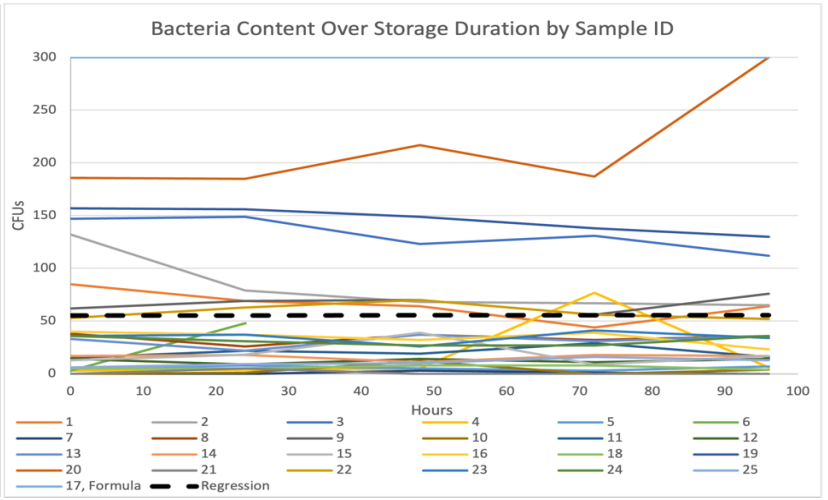
B.



C.



D.



The majority (81%) of the DHM samples in our study contained less aerobic bacteria as compared to the infant formula. Powdered infant formula is not analyzed for bacterial levels. Instead, it is analyzed for certain strains of bacteria that are known to be pathogenic, specifically Salmonella and Cronobacter species.[97] Bacteria is innate in human milk and previous research of Schanler et al. found no correlation between total bacterial count in mother's milk and infection risk in extremely premature infants.[94] This suggests that limited bacteria that remains in DHM post-pasteurization may be of no greater risk than bacteria found in powdered infant formula. However, a limitation of this study is that we did not analyze the DHM for the strains of bacteria in the milk, including spore forming bacteria such as *Bacillus cereus.* Future studies should analyze the bacteria strains present in DHM that contains bacteria post-pasteurization to determine if any are potentially pathogenic such as *Cronobacter* and *Salmonella* species.

Of important note is the recent formula shortage in the United States that began in February 2022. While the American Academy of Pediatrics released a statement urging the White House to take action to address this crisis and offered potential solutions for caregivers feeding their infants formula, many caregivers still struggled to find formula to feed their infants, with some attempting to make their own infant formula.[104] By not discarding the DHM with bacteria after pasteurization, this milk could be utilized by term infants during periods when mother's own milk and/or formula is unavailable.

DHM is increasingly being used for term infant.[15] However, it is still unknown if DHM is nutritionally adequate for the term infant. Castro et al. found that vitamin C levels are significantly reduced in DHM as compared to mother's milk, suggesting that infants receiving primarily DHM should receive a vitamin C supplement.[105] Future studies should investigate

other nutrients in DHM that are necessary for optimal growth and development of infants before DHM is recommended as an alternative primary source of food for the term infant.

Conclusion

DHM with limited bacterial presence ($<10^2$ CFUs) post-pasteurization maintains its bacterial and nutrient content over 4 days of refrigerated storage, with 81% of the DHM samples containing less aerobic bacteria as compared to the infant formula. Based on these findings, this DHM with limited bacterial presence may be an option as a supplement for healthy term infants to remain on an exclusively human milk diet. Further investigation is warranted to determine the species of bacteria present in this milk.

CHAPTER V: PREMIEFEED: INVESTIGATING PRETERM INFANT FEEDING BEHAVIORS AND CAREGIVER EXPERIENCES IN THE FIRST WEEKS AT HOME

Abstract

Background: Preterm infants face significant nutritional risk due to their immature gastrointestinal systems and underdeveloped feeding coordination. Despite these risk factors for poor feeding outcomes, there is limited research into the first weeks after hospital discharge. The aim of this study was to investigate longitudinally the feeding regimen, feeding behaviors, and the caregiver perceptions and experiences with feeding in the first four weeks following hospital discharge of the preterm infant.

Methods: Caregivers of preterm infants at Novant Health Forsyth Medical Center were recruited to participate in a weekly telephone survey for the first 4 weeks following the infant's hospital discharge. Answers for infant feeding behaviors and caregiver experiences were scored on a 3-point Likert scale. An infant eating skill index was used to analyze overall feeding behaviors and a caregiver feeding experience index was created to analyze overall caregiver experience with feeding their infant. Growth and feeding data were collected from the infant's first NICU follow up visit. $P < 0.05$ was deemed significant.

Results: Twenty-four caregivers completed the study. The number of infants receiving any human milk decreased from 70% at hospital discharge to 54% at 4 weeks post-discharge. Infant eating skills improved over time ($p=0.096$). Poor feeding behaviors were weakly correlated with poor caregiver experiences ($r=0.319$, $p=0.105$). Falling asleep during or soon after feeding was the most reported feeding behavior. Twenty-one percent of infants required

nutritional intervention at their NICU follow-up visit. Any fortification at follow-up visit was moderately correlated with average weight gain since hospital discharge (r=0.491, p=0.033).

Conclusion: This study found that feeding skills improve over the first four weeks at home and that poor caregiver experiences were weakly associated with higher infant feeding behavior difficulty. Future studies should further investigate the period after hospital discharge to better establish how preterm infant feeding behaviors and caregiver experiences change over time.

Introduction

Preterm birth rates in the United States (US) have been on the rise since 2015, accounting for 10.2% of all births in 2019.[106] Premature infants face significant health risks which are influenced by their impaired ability to digest and absorb nutrients due to their immature gastrointestinal systems. Human milk is regarded as the preferred food for the preterm infant as it has been shown to reduce risk of necrotizing enterocolitis.[8] However, studies have shown poor growth with human milk alone, so it is frequently fortified.[16] Despite extensive research into feeding the hospitalized preterm infant, there is minimal research regarding feeding the post-discharge preterm infant, specifically in the first weeks after discharge.

A recent study conducted by Zhang et al. audited a post-discharge neonatal intensive care unit (NICU) clinic and found that 61% of preterm infants were not growing appropriately at their first follow up visit 4-5 weeks after discharge and required feeding interventions from a Registered Dietitian.[25] This suggests that feeding problems are common in the first weeks after discharge; however, this study did not capture what happens during those first weeks at home highlighting an important gap in the literature. Additionally, they found that 52% of infants were discharged with human milk as part of their feeding regimen, but did not differentiate between

mother's own milk and donor human milk (DHM).[25] Our recent scoping review on DHM use found that DHM is increasingly being studied for use outside of the NICU, including in the post-discharge feeding of preterm infants.[15]

Few studies have investigated post-discharge eating behaviors of premature infants and caregiver's perceptions and experiences with feeding their infant at home. Howe et al. developed a survey to measure preterm infant feeding difficulties and its relationship with maternal distress compared to term infants in the first 2 years of life.[107] This study found that mothers of preterm infants perceived significantly more feeding-related issues compared to mothers of term infants, suggesting that feeding difficulties continue beyond hospital discharge.[107] DeMauro et al. investigated the incidence of post-discharge feeding dysfunction in the first year of life in late and early preterm infants.[108] This study used a questionnaire that was completed by parents assessing infant feeding behaviors as well as parent's experiences with feeding their preterm infant. Results from this study indicated that parents of early preterm infants reported higher oromotor dysfunction and more avoidant feeding behaviors at 3 months compared to late preterm infants and that parental discomfort was correlated with nearly all types of feeding dysfunction.[108] However, these studies had longer follow up periods and did not capture the time immediately after discharge when caregivers are transitioning into being the primary feeder.

The purpose of this study was to investigate longitudinally the feeding regimen, feeding behaviors, and the caregiver perceptions and experiences with feeding in the first four weeks following hospital discharge of the preterm infant. We hypothesized that there would be a variety of feeding regimens between infants, and longitudinal changes in regimens within an individual infant as caregivers adjust to in-home feeding. We also hypothesized that poor infant feeding behaviors would be associated with poor caregiver feeding experiences and poor growth and that

higher percent human milk feedings would be associated with lower caregiver reported feeding discomfort. Finally, we hypothesized that very few infants would be receiving DHM feedings post-discharge.

Methods

In this longitudinal study, a weekly telephone survey was administered to caregivers of preterm infants to collect data on in-home feeding practices in the first 4 weeks post-hospital discharge. This study was reviewed and approved by the Novant Health Forsyth Memorial Institutional Review Board (IRB). Reliance agreements were issued with UNC Greensboro IRB and Wake Forest IRB.

Participant Recruitment

Eligible participants included the caregivers of preterm infants born less than 30 weeks' gestation and/or born less than 1500 g who were feeding orally prior to hospital discharge. Exclusion criteria included caregivers who did not speak English, and infants with surgical necrotizing enterocolitis (NEC), periventricular leukomalacia, or other serious medical condition that may interfere with feeding. If twin or triplet infants were eligible, infant "A" or the first infant who went home was selected for participation. This pilot study allowed up to 30 caregiver-infant dyads to complete the study. Completion of the study was defined as completing at least 3 of the 4 weekly interviews. To account for potential dropout, up to 45 caregiver-infant dyads were allowed to be enrolled in this study. Participants were recruited at a level 3B NICU (Novant Health Forsyth Medical Center, Winston-Salem, NC). The feeding protocol at this institution included preterm infants born less than 34 weeks gestation were eligible to receive DHM until 34 weeks corrected gestational age if MOM was unavailable and parental consent was obtained. Infants were transitioned to a preterm infant formula beginning at 34 weeks corrected gestational

age. Fortification of feedings was based on birth weight. All infants born weighing less than 1500 g received fortified feedings using a human milk fortifier while in the NICU. If infants were recommended to continue fortification after hospital discharge, infants were transitioned to fortification using a preterm infant formula 2-3 days before discharge to ensure tolerance.

Eligible individuals were approached by the neonatal nutritionist or the primary investigator approximately one week prior to infant discharge and were provided with an informational flyer about the study. Individuals interested in participating were provided with a copy of the consent form and infant feeding diaries for tracking at-home feeding regimens. Written consent was obtained prior to discharge. Demographic and contact information were also collected, and the first phone interview was scheduled. To reduce attrition, a small incentive was offered to participants. The incentive consisted of $10 gift card for completion of each of the first 3 surveys for a total of $30. An additional $20 gift card was given for completion of the 4th survey. The total amount of gift incentive after completion of all 4 surveys was $50.

Data Collection

Beginning one week after infant discharge, study participants completed a weekly telephone survey assessing infant feeding regimen, infant feeding behaviors, and caregiver feeding perceptions and experiences. Prior to the scheduled telephone survey, study participants completed a one-day food diary using a paper log given at enrollment for their infant to ensure accuracy in diet data and reduce potential recall bias. Surveys were conducted by telephone and administered by the primary investigator. To reduce potential attrition, the primary investigator sent a text reminder to participants the day before their phone interview. Medical record ID was used to identify participants for data abstraction from medical records. Survey data and abstracted data from the medical record was entered into REDCap, a HIPAA-compliant, web-

based application that securely captures and stores research data. Study duration was 4 weeks, so caregivers completed 4 phone surveys. Each survey interview was 15-20 minutes in duration, for a total time commitment of approximately 1.5-2 hours. Data abstracted from the Novant Health medical records included gestational age (GA) at birth, birth anthropometrics (weight, length, head circumference), medical diagnoses, sex, race, insurance type, discharge anthropometrics (weight, length, head circumference), and feeding regimen at discharge. The following data was collected from the medical record after the first post-NICU follow-up at Amos Cottage (Wake Forest University): weight, length, head circumference, description of any nutritional interventions made at follow-up clinic, and recommendation for future healthcare (return visit to feeding clinic, discharge to pediatric care, other). Upon study completion, all identifying information including medical record ID was removed from the REDCap database prior to analysis.

Survey Development

A preliminary telephone survey for this study was developed using questions from previous infant feeding surveys including the Center for Disease Control's (CDC) Infant Feeding Practice Study II (IFP II) Month 2 questionnaire, and The Behavior Based Feeding Questionnaire, a feeding behavior questionnaire developed for preterm infants.[107–109] To ensure content and face validity, the survey was reviewed by infant feeding experts and caregivers involved in feeding infants, and revisions were made prior to administering. The survey is shown in Table 5.

Table 5. Survey Questions.

Domains	Questions
Feeding Regimen	Did your baby receive breast milk through direct at-the-breast feeding? Y/N. If Y- how many times per day?

	Did your baby receive expressed/pumped breast milk? Y/N. If Y- how many times per day? If Y, please indicate approximately what % of the milk was: - Your milk - Milk provided to you from another mom - Milk from a human milk bank Did your baby receive formula? Y/N. If Y- how many times per day? If your baby received formula, please specify what brand: In the last day, about how long did a feeding typically last? In the past day, did you regularly add anything to the human milk or formula? In the past day, did your baby receive any other foods, beverages, or supplements (e.g. vitamins, cereal)? In the past day, about how many ounces of milk/formula did your baby drink at each feeding? In the past week, did you change the type of milk (for example, switching from breastmilk to formula, or changing the brand of formula)? If yes, why did you make this change? In the last week how often was your baby encouraged to finish a bottle if he/she stops drinking before the milk/formula is all gone? In the last week what was your infant's bowel movement frequency per day? What was the quality of your infant's stool? Has your child experienced signs of colic (e.g. gassy)? Has your child experienced signs of reflux (e.g. spit ups)?
Oral Motor	Over the past week: Did your infant have any trouble with sucking during feeding? Did your infant have any trouble with coughing or choking during feeding? Did your infant have any trouble with swallowing during feeding?

	Did milk/formula leak out from your infant's mouth during feeding?
Regulation	How often did your infant have the following behaviors during feeding time over the past week: Pushes milk/ bottle away Turns head away from milk/bottle Closes mouth when you try to feed him/her Gags when he/she sees the milk or when the milk/ bottle is placed in the mouth Holds milk in mouth Spits out milk
Endurance	Did your infant feel tired easily or fall asleep during or after feeding?
Respiration	Did your infant seem to have difficulty breathing during feeding?
Muscle Tone	Did you have any difficulty holding your infant while feeding them? Did your infant's tongue push outside the mouth exaggeratedly during feeding?
Appetite	How was his/her appetite?
Healthcare	In the last week, has your baby been hospitalized due to feeding difficulties? In the last week, has your baby been seen by a specialty clinic due to feeding difficulties?
Caregiver Experiences	In the past week, do you think your baby has been feeding enough? In the past week were feeding times for you usually stressful, average, or relaxed? In the past week, have you contacted a health care provider with concerns about feeding your baby? In the past week, how comfortable did you feel feeding your baby?

Discharge Education	Did a doctor/health professional teach you how to prepare formula? Did a healthcare provider discuss the risks of improperly preparing formula? Did a healthcare provider discuss the safe handling of breastmilk? Did a doctor/health professional teach you how to prepare fortified milk (breastmilk and/or formula)?

Data Analysis

Descriptive statistics were utilized for the numeric and categorical data. Answers from the survey on infant eating skills and caregiver experiences were scored on a Likert scale. An infant eating skill (IES) index was computed that was a composite of all eating skills assessed in the survey including oral motor, regulation, endurance, muscle tone, appetite, and respiration. The range of scores was 15-53, with a higher score indicating fewer feeding skills or more feeding difficulty. Additionally, a caregiver feeding experience (CFE) index was computed that was a composite of all caregiver experiences and perceptions assessed in the survey with a range of scores 4-11. Higher scores indicated higher feeding discomfort. The IES and CFE Indices were used to explore relationships between infant eating skills, caregiver perceptions and experiences, feeding types, and infant growth.

The percent of diet from human milk was computed based on the ratio of number of human milk feeds (mother's milk and DHM) divided by number of total feeds and analyzed to determine if there was a relationship between percent human milk feeds and feeding tolerance/discomfort. Percent of feeds that received fortification was computed based on the number of fortified feeds divided by total number of feeds over the 4 weeks and analyzed to determine if there was a relationship between percent fortified feeds and growth outcomes at follow up visit. Additionally, any fortification (Yes/No) at follow-up visit was analyzed to

determine if there was a relationship of fortification at follow-up and growth outcomes. Growth outcomes at follow-up visit were defined as growth-per-day since hospital discharge. Daily weight gain from hospital discharge to follow-up was calculated by subtracting weight at discharge from weight at follow-up and dividing by the number of days from discharge to follow-up visit. The same calculation was done for length and head circumference. Changes in milk offered were assessed on a weekly basis and reasons for change were recorded. The number of changes in milk offered was calculated based on the number of participants who changed their infant's feeds.

Statistical Analysis

Repeated measures ANOVA was used to analyze changes over the 4 weeks, Pearson's and Spearman's correlations were used to analyze correlation between two variables. $P < 0.05$ was deemed significant. Analysis was conducted using SPSS Version 28 (IBM, Armonk, NY).

Results

This longitudinal exploratory preterm infant feeding study was conducted at Novant Health Forsyth Medical Center where participants were recruited on a rolling basis from February 2022-December 2022. Thirty-seven caregivers agreed to participate and were enrolled in the study. Twenty-four caregivers completed the study.

Patient Demographics and Baseline Feeding at Discharge

Patient demographic information is reported in Table 6. The mean birth GA was 29.4 weeks, mean birthweight of 1,120 g, mean birth length 36.9 cm, and mean birth head circumference 26.4 cm. Eleven (46%) patients were male and 50% had public health insurance. 33% (8/24) of infants were classified as extremely low birth weight < 1000 g, 58% (14/24) very

low birth weight <1500 g, and 8% (2/24) low birth weight <2500 g. One patient did not have a reported race in their medical record, 7 were Black, 12 white, 2 Hispanic, and 2 mixed race.

The majority (17/24, 70%) of infants were receiving some type of HM per their discharge feeding regimen. Of the infants receiving HM, 100% of the milk was their mother's own milk (MOM). Six (25%) infants were receiving unfortified feeds, 3 were receiving unfortified MOM and 3 receiving unfortified formula. The majority (18/24, 75%) of infants in this study were discharged with fortified feeds. The most common caloric density was 24 kcals/oz (71%), but ranged from 20-30 kcals/oz. No infants were discharged on DHM feedings. Two infants were receiving feeds thickened with oatmeal.

Eighteen (75%) caregivers reported they received education regarding formula preparation, 12 (50%) reported they received education on the risks of improperly preparing formula, 18 (75%) reported they received education on safe handling of breastmilk, and 22 (92%) reported they received education on how to fortify their breastmilk and/or formula.

Table 6. Infant Demographics.

Characteristics (n=24)	**Mean (Range)**
Mean (range) gestational age at birth (weeks)	29.4 (25.9-34.0)
Mean birth weight (g)	1,120 (500-1,655)
Mean birth length (cm)	36.9 (28.0-43.0)
Mean birth head circumference (cm)	26.4 (22.0-29.5)
Male # (%)	11 (46%)
Race % (n) Black White Hispanic Mixed Not Reported	 7 (29%) 12 (50%) 2 (8%) 2 (8%) 1 (4%)

Public Insurance # (%)	12 (50%)
Mean hospital length of stay (days)	55.5 (21-119)
Mean change in weight from birth to hospital discharge (g/day)	24.3 (11.9-31.8)
Mean change in length from birth to hospital discharge (cm)	9.5 (3.0-19.0)
Mean change in head circumference from birth to hospital discharge (cm)	5.8 (0.5-12.5)
Feeding Regimens at Hospital Discharge # (%)	
MOM only	1 (4%)
Fortified MOM	11 (46%)
Unfortified MOM or Formula	3 (13%)
Fortified MOM or Formula	2 (8%)
Formula Only	2 (8%)
Fortified/Concentrated Formula	5 (21%)

MOM – mother's own milk

Feeding Regimens

The percent of infants receiving any human milk decreased over the 4 weeks from 67% at week 1 (16/24), 58% at week 2 and 3 (14/24), and 54% (13/24) at week 4. The percent of infants receiving exclusive HM feedings (100% of all feedings) was 46% (11/24) at weeks 1-3 and 42% (10/24) at week 4. The percent of total feeds that were HM (0-100%) for each infant is shown in Figure 4. Infants receiving any fortification of feeds over the 4 weeks were 67% (16/24) at weeks 1 and 4, 63% (15/24) at week 2, and 71% (17/24) at week 3. The percent of daily feeds receiving fortification (0-100%) for each infant over all timepoints is shown in Figure 5. During the study period, 8/24 caregivers (33%) reported changing the type of milk they fed their infant, with 2 changing more than once during the first four weeks at home for a total of 10 changes in milk type offered over the 4 weeks. Reasons for changing feedings included switching formula due to intolerance (3/10), formula availability (1/10), weight gain issues (1/10), and stopped

breastfeeding/pumping and started formula (3/10), stopped fortifying HM due to intolerance (2/10). Eleven caregivers (46%) contacted a healthcare provider regarding their infant's feeds during the first 4 weeks at home.

Figure 4. Percent of total feedings that used Human Milk over 4 weeks. Each row indicates a unique participant.

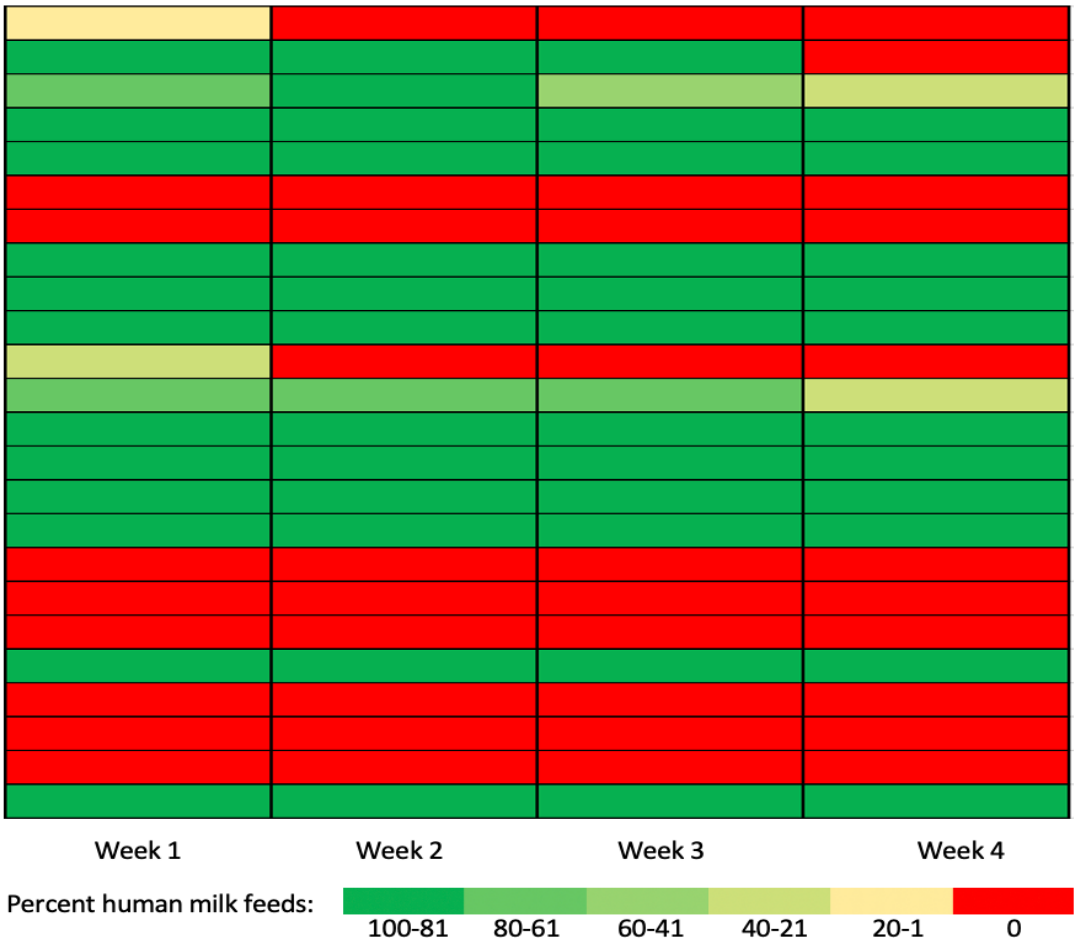
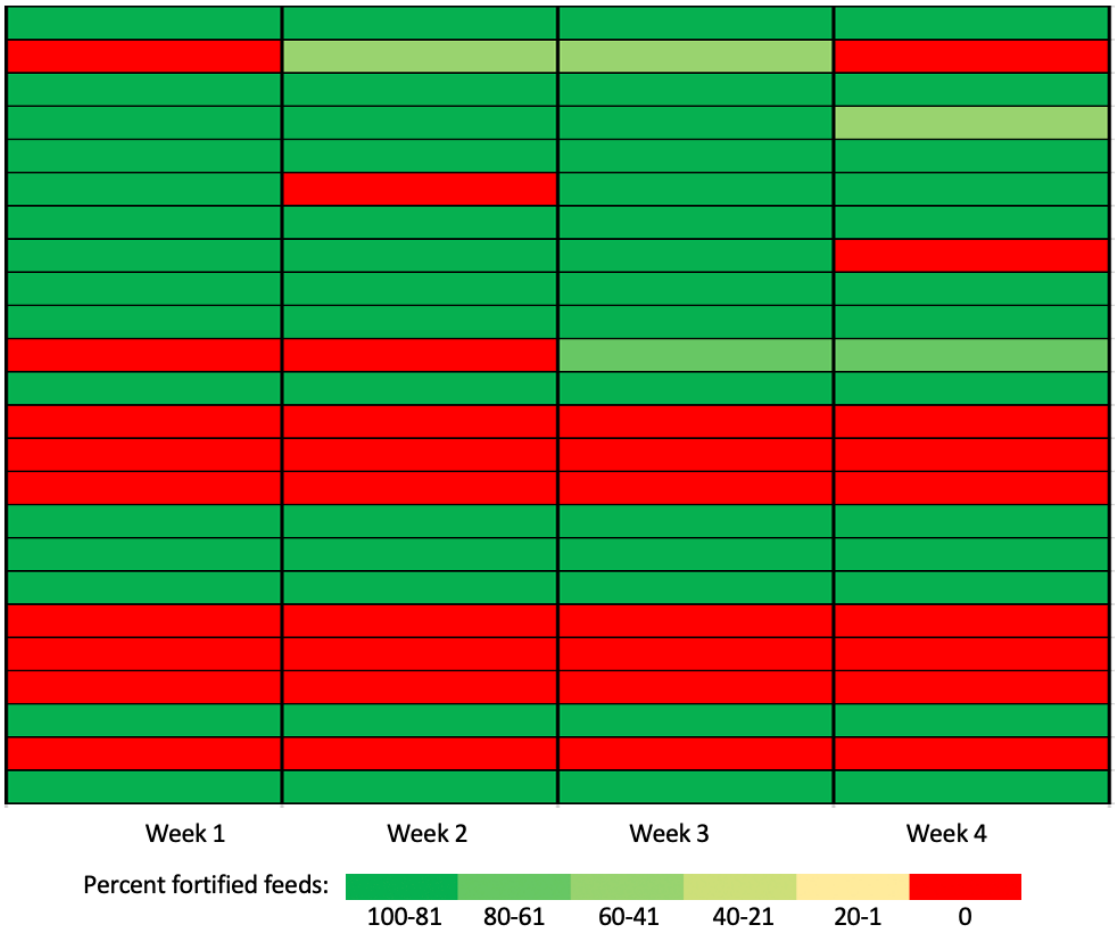


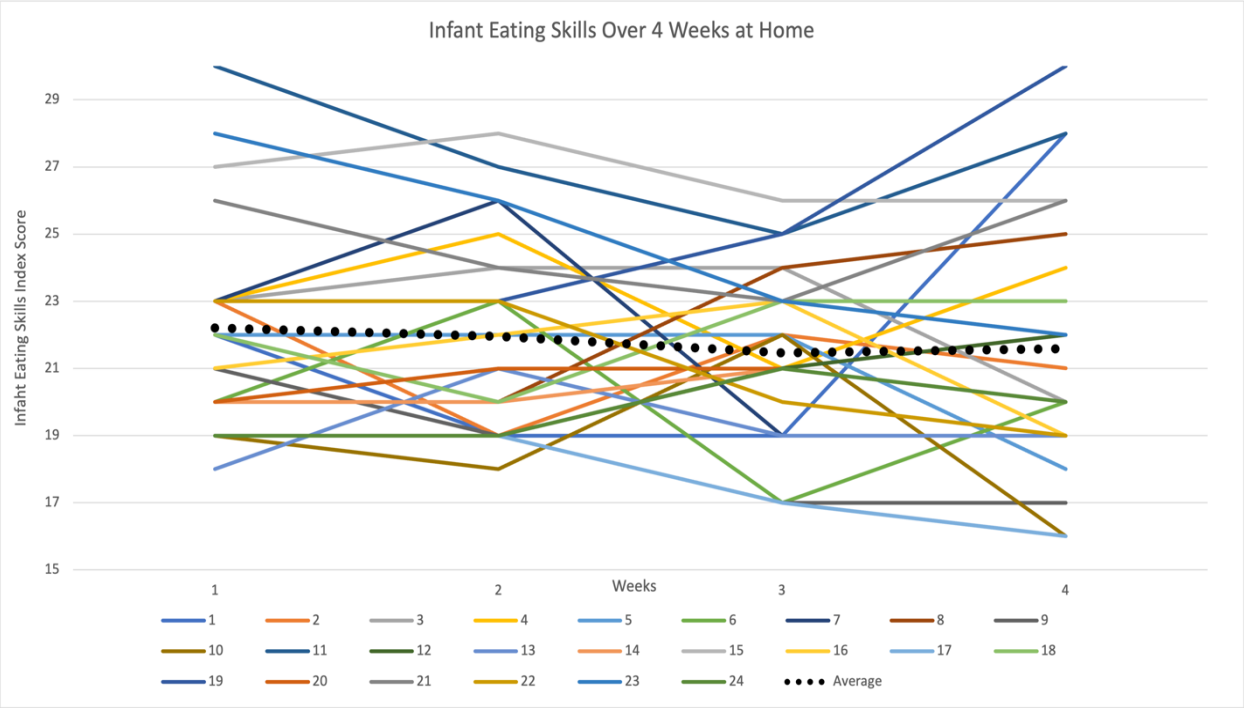
Figure 5. Percent of fortified feedings over 4 weeks. Each row indicates a unique participant.



Infant Eating Behaviors

The range of IES Index scores was 16-30, with higher scores indicating greater feeding difficulties. Average IES index scores decreased over the 4 weeks from 22.2 to 21.6 (Figure 6). However, the scores did not decrease significantly over time (p=0.096). Scores were highest for infant receiving mixed formula and MOM feedings, followed by formula, with MOM fed infants having the lowest IES Index scores. Lower scores were seen in infants who were born weighing more than 1000 g and higher scores in infants born weighing less than 1000 g.

Figure 6. Infant eating skills over the first 4 weeks at home. Higher infant eating skills index scores indicate higher infant feeding difficulties. Each line represents a unique participant.



Milk leaking from mouth was the most prevalent reported issue within the oral motor domain, with 19/24 (79%) infants in week 1 reporting issues sometimes or often, 16/24 (67%) infants in week 2, 17/24 (70.8%) infants in week 3, and 16/24 (67%) infants in week 4. Spitting up was the most prevalent reported issue within the regulation domain, and its prevalence changed weekly: 13/24 (54%) infants in week one, 19/24 (79%) infants in week 2, 15/24 (63%) in week 3, and 21/24 (88%) in week 4. Falling asleep during or soon after feeding was the most common behavior overall that occurred sometimes or often. Reports of falling asleep during feeding decreased over time, with 21/24 (88%) infants in week 1, 19/24 (79%) in week 2, and 16/24 (67%) in week 3 and 4. Average oral motor skills decreased over the four weeks with an average score at week 1 of 4.3 to a score of 3.8 at week 4 and approached significance ($p=0.053$). There were no significant changes in scores over time for any other domain.

Caregiver Experiences

Mean CFE Index scores did not change over the 4 weeks with a mean score of 5.5 (Figure 7). There was a weak positive correlation between infant eating skills and caregiver experiences (r=0.339, p=0.105, Figure 8).

Figure 7. Caregiver feeding experiences index scores over 4 weeks. Higher caregiver feeding experience index scores indicate higher levels of discomfort. Each line represents a unique participant.

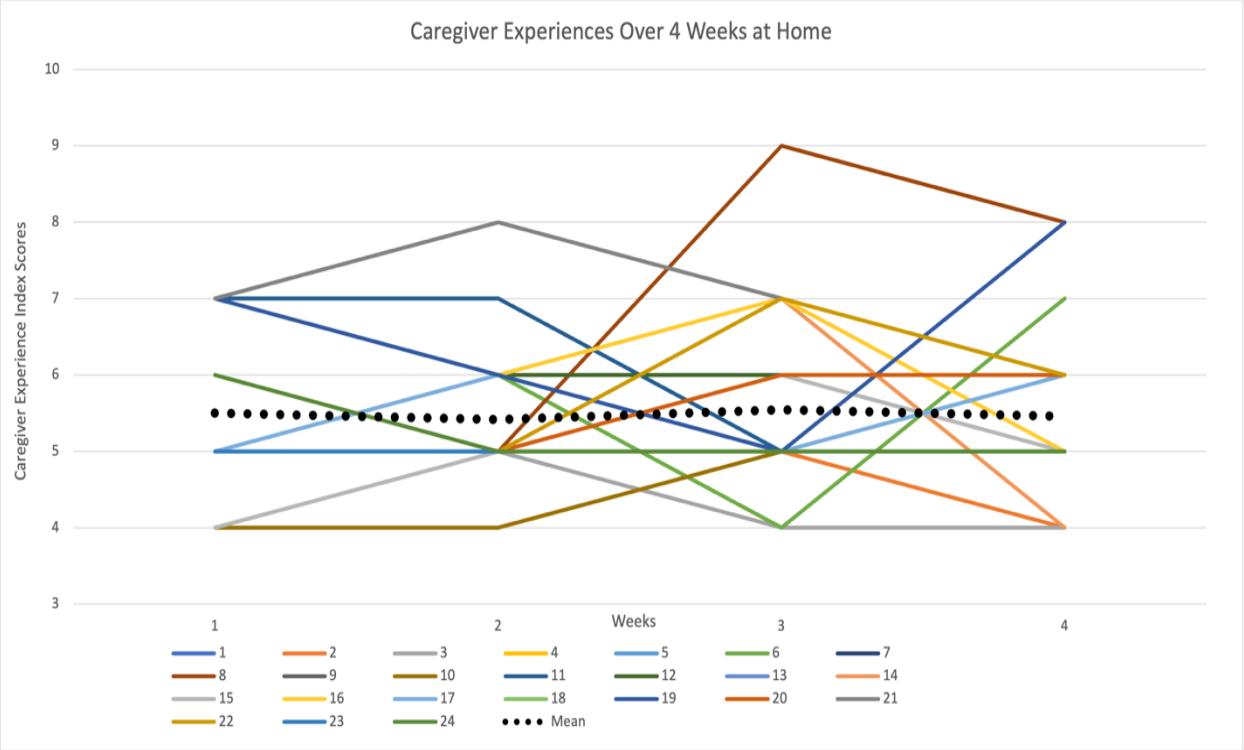
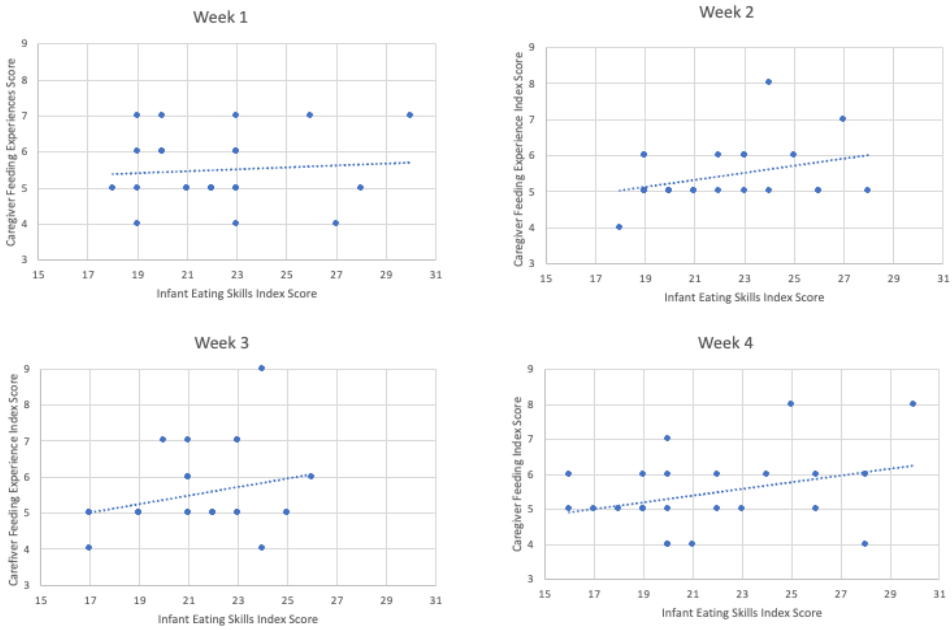


Figure 8. Correlation of Infant eating skills and caregiver experiences by week. Each dot represents a unique participant.



Follow Up Visit

Follow up data was obtained for 19 of the infants. Eleven infants (58%) were growing adequately (at least 30 g/d), 6/19 (32%) had moderate growth failure (20–29 g/d), 2/19 (11%) infants had severe growth failure (<20 g/d). One infant had lost weight since hospital discharge. Feeding changes were recommended for 4/19 (21%) of infants at their first follow-up appointment: two infants were recommended to decrease fortification of feeds, 2 were recommended to increase fortification of feeds, 3 were recommended to thicken feeds with oatmeal, and 15 were recommended to maintain their feeding regimen. All infants were recommended to return to the clinic for a second follow up appointment. The IES index and percent fortified feeds over the first 4 weeks at home were not correlated to growth (weight gain, head circumference, or length) at follow-up visit. Any fortification of feeds at time of follow-up

visit was moderately correlated with average weight gain per day since hospital discharge (r=0.491, p=0.033).

Discussion

To our knowledge, there have been no studies that have investigated the feeding behaviors and experiences of caregivers with feeding their preterm infants in this first weeks after hospital discharge. This study aimed to longitudinally investigate the feeding regimen, infant feeding behaviors, and the caregiver perceptions and experiences with infant feeding in the first 4 weeks following hospital discharge of the preterm infant. We found that infant feeding behaviors improved over the first 4 weeks at home, poor infant feeding behaviors were weakly associated with higher caregiver experience discomfort, and that feeding regimen changes were common during this period. Switching formula brands accounted for 40% of the reasons for changing feeds during the first 4 weeks at home. Despite that this study was conducted during an infant formula shortage in the US, only one of the caregivers reported switching formula due to their formula being out of stock.

In this study, we found that the infants weighing less than 1000 g at birth experienced more feeding difficulties. This is in agreement with the findings of DeMauro et al.[108] In their prospective cohort study, DeMauro et al. found higher rates of oromotor dysfunction or difficulties at 3 months in early preterm infants as compared to late preterm infants (29% vs 17%), suggesting that the smaller infants experienced more feeding difficulties.[108] This outcome is expected as the smaller preterm infants were born earlier as compared to the larger and late preterm infants, thus they had less time to mature and develop in utero.

In this study, caregiver experiences were weakly correlated with infant eating skills, however, this association was not statistically significant. DeMauro et al. found that parental

discomfort was significantly associated with feeding dysfunction, suggesting that parents experienced more discomfort as their infants experienced more feeding difficulties.[108] In contrast, Howe et al. conducted an exploratory cross-sectional study that found no significant association between perceived feeding issues and caregiver distress while receiving only HM or formula as their only nutrient source.[110] Of important note, in our study almost half (46%) of caregivers contacted a healthcare provider during the first 4 weeks after hospital discharge with questions related to feeding their infant, suggesting that many caregivers experienced challenges related to feeding their infant. These disparate findings highlight the need to further explore the relationship between caregiver feeding experiences and infant eating skills.

While most infants were growing appropriately at their follow up visit, 21% were growing inappropriately and required adjustments to their feeding regimens. Zhang et al. found that 51% of preterm infants required modifications to the caloric density of their feedings at their NICU follow up visit.[25] Rates of infants receiving HM at discharge was less in Zhang et al. where 52% of infants were receiving HM as part of their feeding regimen at discharge compared to 70% of infants in this study. Additionally, a higher percentage (71%) of infants in this study were receiving feeds with a caloric density of 24 kcals/oz compared to 61% in Zhang et al. Moreover, Zhang et al. reported a high rate (42%) of fortification non-compliance issues within their population.[25] These differences in feedings may have contributed to differences in the proportion of infants requiring interventions of their feeding regimens.

Additionally, many of the infants in our study had likely been followed by a pediatrician, however, some still required nutritional interventions at their NICU follow-up visit to ensure adequate growth. The rates of nutritional interventions at NICU follow-up clinics highlights the importance of close monitoring of the post-discharge preterm population. Appropriate growth is

critical for proper development of preterm infants and reducing risk of future morbidities. Because HM is nutritionally inadequate for the preterm infant, it is often fortified which has been shown to improve growth and neurodevelopmental outcomes.[111] Increased nutritional demands extend beyond the NICU, therefore, many preterm infants are discharged with feedings that are fortified with preterm formula. The majority (18/24, 75%) of infants in this study were discharged with fortified feeds and 75% of HM feeds were fortified. This is in line with Zhang et al. who found that 79% of infants in their study were discharged with fortified feeds.[25] Average weight gain (g/day) per day at NICU follow-up visit was moderately correlated (r=0.49) with any fortification at follow-up visit, suggesting that infants who were receiving fortified feeds at follow-up visit were growing better than those on unfortified feeds. A recent follow-up of a randomized-controlled trial found that there was no improvement in cognitive outcomes in infants who received fortified HM feeds as compared to unfortified feeds, however, HM fed infants had significantly higher cognitive outcomes as compared to formula fed infants.[112] This study by Klamer et al. suggests that fortification of HM may not improve cognitive outcomes in preterm infants, however, nutritional adequacy for growth should still be considered. In our study, we found that 63-71% of infants received fortified feeds over the first 4 weeks at home and only 11% (2/19) of infants were growing too quickly and recommended to decrease fortification at their follow-up visit.

An important limitation of this study was that it was an exploratory study and was not powered, therefore, it was not intended to determine statistical significance of the results. Future studies should be powered to determine if there are any significant associations between infant feeding behaviors and caregiver experiences.

Conclusion

This study found that feeding behaviors improve over the first four weeks at home and that poor caregiver experiences were weakly associated with higher infant feeding behavior difficulty. Additionally, changes in feeding regimens are common in the first 4 weeks after hospital discharge. Many caregivers reported contacting a healthcare provider related to feeding issues, highlighting the importance of post-discharge feeding support for the families of preterm infants. Finally, fortification of feeds at hospital discharge was significantly associated with average daily weight gain at NICU follow up visit. Future studies should further investigate the period after hospital discharge to better establish how preterm infant feeding behaviors and caregiver experiences change over time.

CHAPTER VI: EPILOGUE

Conclusion

We observed that human milk oligosaccharides (HMOs) interfered with BioVision and Miris, but not Ultra-High Pressure Liquid Chromatography Mass Spectrometry (UPLC-MS) or Megazyme. Donor human milk (DHM) with limited bacterial presence post-pasteurization maintains its bacterial and nutrient content over 4 days of refrigerated storage, with 81% of the DHM samples containing less aerobic bacteria as compared to the infant formula. Preterm infant feeding skills improve over the first four weeks at home and that poor caregiver experiences are weakly associated with higher infant feeding behavior difficulty. These findings provide valuable knowledge for DHM and infant nutrition. The following suggestions are based on the findings of this research. First, given that HMOs are non-digestible to the infant, recommended methods for measuring lactose content should be those that are not influenced by HMOs in HM. Second, DHM with limited bacterial presence may be an option as a supplement for healthy term infants to remain on an exclusively human milk diet. Finally, infant feeding issues and caregiver discomfort is common in the first weeks following hospital discharge, with many contacting a healthcare provider regarding feeding concerns, therefore, more guidance and recommendations should be provided to caregivers before hospital discharge to help mitigate this discomfort.

Challenges

In Chapter III, we experienced difficulty developing and running the UPLC-MS method at The Triad Mass Spectrometry Lab. This was due to the machine being older and less sensitive than what Fusch et al. used in their study.[84] These challenges resulted in delayed data analysis, and eventually, we chose to measure our samples using another machine available in the lab.

In Chapter IV, we had trouble with the IgA assay. We were experiencing low activity in our initial plates. Upon investigating, we found that the *E. coli* antigen, IgA standard, and HRP-labeled anti-IgA antibody were old. In addition to needing new products for our assay, we had to troubleshoot to determine optimal concentrations of each.

In Chapter V, we discovered that our initial hospital partner required review through 3 separate Institutional Review Boards (IRB), and the timeline for study approval was 4-6 months. Because of this, we had to seek out a second partner that did not have such a lengthy approval period. Second, we found that our initial inclusion criteria included infants who were not eligible for the NICU follow-up clinic, and therefore, we would not have growth data on. Because of this, we had to update our study protocol and consent form and submit an amendment to the parent IRB. Luckily, we had not yet recruited any infants who were not eligible for the NICU follow-up visit and the IRB quickly approved our amendment. A final challenge with this study was recruitment. The NICU we partnered with had recently reduced its census to roughly a third of its patient population. This occurred because a neighboring hospital opened their own birthing unit, taking many of the expecting mothers in the surrounding area. Because of this, enrollment for the study was slow, and we did not get to our goal of have 30 participants complete the study.

Future Research Implications

In Chapter III, our data highlight the importance of analytical methods for measuring lactose in HM. This is especially important when estimating energy content in HM for preterm infants who require additional nutrients for adequate growth.

In Chapter IV, we found that DHM with limited bacterial presence after pasteurization had no significant changes in the content of bacteria, protein, IgA, or lactose over 4 days of refrigerated storage. The majority of DHM samples contained less than 10^2 CFUs of aerobic

bacteria, while the powdered formula control contained too many to count at all time points. An important limitation of this study was that it did not investigate the types of bacteria in the samples. Future studies should investigate the strains of bacteria present in this milk to determine if any are pathogenic.

In Chapter V, we found that feeding behaviors improve over the first four weeks at home and that poor caregiver experiences were weakly associated with higher infant feeding behavior difficulty. Additionally, changes in feeding regimens are common in the first 4 weeks after hospital discharge. Many caregivers reported contacting a healthcare provider related to feeding issues, highlighting the importance of post-discharge feeding support for the families of preterm infants. Finally, fortification of feeds at hospital discharge was significantly associated with growth at NICU follow up visit. Future studies should further investigate the period after hospital discharge to better establish how preterm infant feeding behaviors and caregiver experiences change over time.

Closing Remarks

The data presented in this research provides new knowledge regarding reliability of analytical methods for measuring lactose in HM, DHM with limited bacterial presence after pasteurization, and preterm infant feeding regimens and behaviors and caregiver feeding experiences in the first weeks after hospital discharge.

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