ABSTRACT

LIMA, HOPE KATHERINE. Optimizing Medical Nutrition for Exclusively Human Milk Fed Infants (Under the direction of April D. Fogleman and Jonathan C. Allen).

Breastfeeding is the optimal source of nutrition for infants during the first six months of life. Unfortunately, medically challenging circumstances can arise that separate mother and baby and cause challenges with initiating direct breastfeeding. During this time, mothers may experience delayed onset of lactogenesis II and/or difficulty in establishing an adequate milk supply. Maintaining an exclusively human milk diet during this time is essential, as it protects the infant from developing life altering complications including necrotizing enterocolitis (NEC), sepsis, and retinopathy of prematurity, while decreasing patient care costs and readmittance rates.

To maintain an exclusively human milk diet during incidents of separation, occurrences of low maternal milk supply, or absence of maternal breast milk, donor human milk (DHM) can be provided to the infant. Historically, DHM in a hospital setting has been pasteurized using Holder pasteurization (62.5 °C for 30 minutes) due to the extensive research demonstrating the pathogen-eliminating ability and minimal impact on nutritional and bioactive components. Recently a retort processed, shelf-stable (121 °C for 5 minutes) human milk product has become available and little is published about the effect on the nutritional and bioactive components of the milk. Some neonatal intensive care units (NICUs) began using this product to maintain an exclusively human milk diet for their patients, despite the absence of evidence-based research ensuring the nutritional composition and safety of the product. In study 1 and 2, the bioactive and nutritional profiles of retort processed (shelf-stable) human milk (SS) are compared to Holder pasteurized (HP) and raw (RAW) human milk from the same pool. Our results showed retention of macronutrients (fat, total protein, lactose) and destruction
of pathogenic bacteria, but destruction of bioactive proteins (sIgA, lysozyme), lysine, and thiamine in SS samples. The destruction of bioactive proteins in SS samples may be of concern when clinicians are treating premature infants, as their immune system is not yet developed. Loss of lysine is a concern, as lysine is an essential amino acid and loss may limit lean tissue accrual in infants. Lastly, loss of thiamine content may be of concern as thiamine deficiency can cause beriberi and peripheral neuropathy. Due to the losses of key bioactive components, lysine, and thiamine, it is my recommendation that Holder pasteurized human milk be utilized as a feeding option when it is the only nutrition the infant is receiving. Shelf-stable human milk may be considered a secondary feeding option if the mother is providing the infant with some of her own milk or is using it temporarily while she establishes her milk supply.

Beyond pasteurization methods, there are additional concerns with retaining the nutritional content in human milk, including light exposure. Currently, there are no standards for collection containers for at home milk collection and storage. Thus, milk banks receive donations in a variety of containers, many of which do not offer any protection from light exposure. Additionally, Human Milk Banking Association of North America (HMBANA) milk banks do not require any protective measures to be taken within the milk banks to shield the donated milk from additional light exposure. In study 3, human milk is collected using either standard or light-shielding methods and containers and processed using either standard or light-shielding methods, to determine if light exposure significantly impacts the levels of light sensitive vitamins in human milk. Total riboflavin was significantly (p < 0.05) decreased by light exposure in both raw and pasteurized samples (62.1 µg/L and 73.7 µg/L, respectively) when compared to light protected raw samples (99.68 µg/L). Retinol, tocopherols, and beta-carotene were not affected by the level of light exposure experienced in this trial. Thiamine
was significantly affected by light exposure; however, levels were still within expected ranges (0.23-0.26 mg/L) to provide infants with adequate thiamine if they were taking in between 750-850 mL human milk/day. Due to the protective nature of the light shielding on riboflavin, it is my recommendation that human milk being processed in a commercial setting should be shielded from light during the intake and pasteurization process.

It is my hope that the research presented in this dissertation is used to optimize collection and processing of human milk for use in premature and fragile infants when their mother’s own milk is unavailable. Additionally, I hope to highlight the differences between different donor human milk products to allow clinicians to make an informed decision when choosing a feeding option for their patients.

**Funding Sources:** North Carolina State University Department of Food, Bioprocessing, and Nutrition Sciences; WakeMed Mothers’ Milk Bank, Cary, NC.
DEDICATION

First and foremost, all the goals I have accomplished are dedicated to God, for never leaving my side and always providing me with the mental fortitude to continue to push through every difficult situation I have stumbled upon. Also to my mentor, colleague, and friend, April Fogleman. You have guided me, encouraged me, held my hand while I cried, celebrated with me, and been a source of unwavering love and support. God has shown me, through you, that His plans for me are brighter than I could have ever imagined. Thank you.
I earned my BS in biology from West Virginia Wesleyan College in 2012, my dual MS in animal science and nutrition from North Carolina State University in 2014, my BS in human and consumer sciences with a focus in dietetics from North Carolina Central University in 2016, and became an International Board Certified Lactation Consultant in December 2017. I plan to complete my dietetic internship through the individualized supervised practice pathway (ISPP) by 2019 to become a Registered Dietitian (RD). Completing my PhD at North Carolina State University will mark the endpoint of my formal classroom-based education, and I will be accepting a position at Oregon State University to continue to study bioactivity of human milk. My hope is that my formal education has provided me the framework and experience to offer equitable, accessible education in infant feeding, human milk, and lactation, while continuing to support and empower mothers in their journeys.
ACKNOWLEDGMENTS

I would like to acknowledge my committee (April Fogleman, Jonathan Allen, MaryAnne Drake, and Joan Eisemann) for their encouragement and guidance over the past few years. I would like to acknowledge Montana Wagner-Gillespie, Destiny Davis, and Courtney Hubble for their help with all the lab work that I conducted throughout my PhD. I would like to acknowledge Ken Vogel for teaching me the fine art of HPLC/UPLC. I would like to thank Nicola Singletary for being a constant source of encouragement and support as we completed our PhDs together. I would like to thank Hunter Freeman for putting up with all my late nights as I completed my dissertation and his support as I move across the country to further my career. Lastly, thank you to all my family and friends who supported me through this difficult endeavor.
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CHAPTER 1: Introduction – The Importance of Human Milk for Premature Infants

The Role of Breastfeeding

Over the past thirty years, research has started to reveal the complex structure and function of human milk, providing a baseline understanding of how human milk delivers individualized nutrition, immune support, and psychological benefits for the infant [1-3]. Current health recommendations from the World Health Organization state that infants should be exclusively breastfed until 6 months of age, and continue to partially breastfeed until 2 years of age and beyond [4]. The American Academy of Pediatrics recommends exclusive breastfeeding until 6 months of age, with the introduction of complementary foods until at least 12 months of age, and the continuation of breastfeeding for as long as mutually desired by mother and baby [5]. Despite these recommendations, the Center for Disease Control (CDC) reported in 2014 that only 18.8% of infants in the United States were still being exclusively breastfed at 6 months of age [6] and worldwide estimates of exclusive breastfeeding rates are around 43% [7] with breastfeeding defined as an infant receiving human milk directly from their mother or a wet nurse, or expressed human milk, and no other liquids or solids.

For the developing infant, human milk plays a critical role in providing optimal nutrition and hydration for growth and development. Water makes up 87.5% of human milk and this meets all the water requirements of a developing infant. The adequate intake (AI) recommendations from the Food and Nutrition Board, Institute of Medicine, and National Academies in the United States for infants age 0-6 months are based on estimates of macro- and micronutrients in human milk [8]. A healthy, term infant will consume an average of 710-803 mL per day [9], but should be allowed to self-regulate feedings.
Lactose is the primary carbohydrate contained in human milk. Mature human milk contains an average of 7.2 g/100 mL of lactose [10] and this will provide approximately 40% of an infant’s energy needs. Average total protein in mature human milk is 1.8 g/100 mL and plays a large role in immune protection and body muscle accretion [11]. Mature human milk contains an average of 4.1 g/100 mL of fat and will provide up to 50% of the infant’s energy needs. Fat content in human milk is the most variable macronutrient, and fat content is directly related to the relative fullness or emptiness of the breast [10]. As the breast empties during a feeding or pumping session, the fat content of the milk contained in the breast goes up.

Levels of micronutrients in human milk have been researched and a summary is provided in Table 1.1. It is important to note that human milk is considered low in vitamin D and it is recommended by the American Academy of Pediatrics to provide all (including formula-fed) infants with a vitamin D supplement to prevent rickets [12]. Similarly, vitamin K levels in human milk are of concern, and infants in the United States are required to have a vitamin K prophylactic dose at birth to prevent hemorrhagic disease of the newborn [13].

Human milk also provides necessary immune protection specific to the local environment as the child’s own immune system develops over the first two years of their life [14]. Many of these immune components are bioactive proteins, including but not limited to alpha-lactalbumin, lactoferrin, secretory immunoglobulin A (sIgA), enzymes, hormones and hormone-like substances, and growth factors. Alpha-lactalbumin helps to regulate milk synthesis, produces mucins that bind pathogens, and can kill cancer cells [10,15]. Lactoferrin plays a role in iron transport and absorption, functions as an antibacterial, and promotes growth of lactobacilli in the infant gastrointestinal tract [16]. Secretory IgA is produced by the mother to target specific pathogens that are present in the infant’s environment. It is then secreted in
Table 1.1. Micronutrient concentrations in raw human milk.

<table>
<thead>
<tr>
<th>Micronutrient</th>
<th>Concentration</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin A</td>
<td>1.18-2.71 µmol/L</td>
<td>[17,18]</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>30-158 mg/L</td>
<td>[19]</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>33-68 IU/L</td>
<td>[20]</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>2.13-10.13 TE mg/L</td>
<td>[21]</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>0.25-0.5 µg/dL</td>
<td>[13,22]</td>
</tr>
<tr>
<td>Thiamin</td>
<td>0.21 mg/L</td>
<td>[23,24]</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.18-0.80 mg/L</td>
<td>[23,24]</td>
</tr>
<tr>
<td>Niacin</td>
<td>0.64-0.89 mg/L</td>
<td>[25]</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;6&lt;/sub&gt;</td>
<td>129-314 µg/L</td>
<td>[26]</td>
</tr>
<tr>
<td>Folate</td>
<td>141.4 ng/mL</td>
<td>[27]</td>
</tr>
<tr>
<td>B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>0.43-1.2 µg/L</td>
<td>[24]</td>
</tr>
<tr>
<td>Pantothenic Acid</td>
<td>2.48-2.64 µg/mL</td>
<td>[28]</td>
</tr>
<tr>
<td>Biotin</td>
<td>0-27 µg/L</td>
<td>[29]</td>
</tr>
<tr>
<td>Choline</td>
<td>144-170 mg/L</td>
<td>[24]</td>
</tr>
<tr>
<td><strong>Minerals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>150-257 µg/mL</td>
<td>[30]</td>
</tr>
<tr>
<td>Chloride</td>
<td>0.36-13.90 mEq/L</td>
<td>[2]</td>
</tr>
<tr>
<td>Chromium</td>
<td>0.23-0.34 ng/mL</td>
<td>[31]</td>
</tr>
<tr>
<td>Copper</td>
<td>0.24-0.43 µg/mL</td>
<td>[30]</td>
</tr>
<tr>
<td>Fluoride</td>
<td>0.28-0.37 µmol/L</td>
<td>[32]</td>
</tr>
<tr>
<td>Iodine</td>
<td>33-348 µg/L</td>
<td>[33]</td>
</tr>
<tr>
<td>Iron</td>
<td>0.38-0.49 µg/mL</td>
<td>[30]</td>
</tr>
<tr>
<td>Magnesium</td>
<td>26-37 µg/mL</td>
<td>[30]</td>
</tr>
<tr>
<td>Manganese</td>
<td>1.41-2.53 µg/100 mL</td>
<td>[30]</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>1.9-10.2 µg/L</td>
<td>[34]</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>130.78-169.30 mg/L</td>
<td>[2]</td>
</tr>
<tr>
<td>Potassium</td>
<td>13.00-16.86 mEq/L</td>
<td>[2]</td>
</tr>
<tr>
<td>Selenium</td>
<td>5.3-23.8 µg/L</td>
<td>[35]</td>
</tr>
<tr>
<td>Sodium</td>
<td>6.83-9.54 mEq/L</td>
<td>[2]</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.60-1.60 µg/mL</td>
<td>[30]</td>
</tr>
</tbody>
</table>

The milk and coats the mucosal surfaces of the gastrointestinal tract, preventing adherence and penetration of pathogens, protecting the infant from infection [16]. There have been over 400 enzymes identified that are secreted in human milk. These enzymes aid in digestion of the nutrients once delivered to the infant, including lipases, amylases, and proteases. Some of these enzymes, such as bile-salt stimulated lipase and lysozyme, also function as antimicrobials and protect the infant from illness [36-38].
Feeding an infant directly at the breast allows for bonding and development of a secure attachment between the mother and the infant [39], which can aid in maternal sensitivity to needs of the infant. In current Breastfeeding Friendly Hospital Initiative (BFHI) guidelines, infants are to go skin-to-skin directly after birth and allowed to self-initiate breastfeeding. When leaving the mother-infant dyad undisturbed, breastfeeding can occur in less than 1 hour. This early skin-to-skin contact and feeding interaction may result in a more positive feeding experience for the infant, and produce an increased maternal sensitivity to infant needs [40]. This early interaction may also lead to beneficial outcomes in later childhood, with one study finding lowest risks of internalizing behavior problems at age 6 in breastfed children [41].

The World Health Organization breastfeeding recommendations state that breastfeeding should continue to two years of age and beyond, but as a whole, society does not seem to approve of an older child breastfeeding. Prior to the creation of Western artificial infant feeding methods, human weaning appeared to occur between 3 and 4 years of age [42]. Anthropological research estimates that if weaning occurred based only on physiological indicators, it would occur in humans at 3 to 5 years of age [43]. The recommendation to continue breastfeeding for two years or beyond becomes especially important in developing nations. In areas where food is limited, or clean drinking water is scarce, breastfeeding plays an important role in providing food security and disease prevention for children under age 5. Human milk has a predictable nutritional composition and allows infants to be fed without needing a clean water source, which can improve infant mortality rates [44].

Breastfeeding can also have significant public health and economic impacts for developed nations. A 2010 study published in Pediatrics [45] looked specifically at the projected savings if 80% and 90% of families in the United States could exclusively breastfeed
all children for 6 months. The savings are estimated based on a cost analysis for pediatric diseases that could be decreased if the child were breastfed, including gastroenteritis, necrotizing enterocolitis, atopic dermatitis, sudden infant death syndrome, asthma, leukemia, obesity, and Type 1 diabetes. The study found that if 90% of families could breastfeed exclusively for 6 months, the United States would save an estimated $13 billion per year and prevent up to 911 deaths – nearly all of which would be infants.

In 2016, 9.85% of live births in the United States were preterm births [46]. For preterm infants, human milk is especially important as it protects against development of necrotizing enterocolitis, retinopathy of prematurity, sepsis, and lowers the length of hospital stay and readmittance rates [47-51]. Unfortunately, feeding premature infants is far more complicated and requires close monitoring and support.

**Considerations for Premature Infants**

The etiology of prematurity is extremely varied. Any infant born prior to 37 weeks gestation is considered premature. Causes of preterm birth include intra-uterine growth restriction, maternal gestational diabetes mellitus, multiple births, or may be of unknown causes [52]. Additionally, pregnant women who have previously delivered a preterm infant, are African-American, suffer from periodontal disease, or have a low BMI are at increased risk for delivering prematurely [52]. Premature infants can also be classified as low birthweight (LBW), very low birthweight (VLBW) or extremely low birthweight (ELBW) if they are born weighing less than 2500 g, 1500 g, or 1000 g, respectively [53].

Infants born prematurely are at high nutritional risk for several reasons. First, premature infants have poor nutrient stores at birth [53]. In utero nutrient accretion rates are high, and
these rates can be difficult or impossible to match after delivery [54]. Nutrients of specific concern include macronutrients (glucose, lipids, protein), iron, calcium, and phosphorous. In utero placental transfer rates of these nutrients are summarized in Table 1.2. If a premature infant experiences inadequate nutrient accretion rates it can result in energy, protein, or vitamin and mineral deficiencies. These deficiencies can cause major growth and development issues including stunting, wasting, neural and motor deficits, among others [8].

Table 1.2. Placental transfer of key nutrients during the third trimester of pregnancy.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Placental transfer during third trimester</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.22 mmol/L</td>
<td>[55]</td>
</tr>
<tr>
<td>Fatty Acids</td>
<td>552 mg/d omega-6</td>
<td>[56]</td>
</tr>
<tr>
<td></td>
<td>67 mg/d omega-3</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>1.8-2.2 g/d</td>
<td>[55]</td>
</tr>
<tr>
<td>Iron</td>
<td>1.6-2.0 mg/kg/d</td>
<td>[57,58]</td>
</tr>
<tr>
<td>Calcium</td>
<td>92-120 mg/kg/d</td>
<td>[59]</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>69-75 mg/kg/d</td>
<td>[59]</td>
</tr>
</tbody>
</table>

Second, premature infants are experiencing physical and metabolic stress because some of their organ systems are underdeveloped [53]. There is specific concern for the gastrointestinal tract and respiratory system, as these two systems undergo rapid development during the final weeks of gestation. The underdeveloped gastrointestinal tract causes specific issues for the premature infant, including feeding intolerance and subsequent development of hyperbilirubinemia, microbiome dysbiosis, and risk for development of necrotizing enterocolitis (NEC). Depending on how premature the infant is delivered, it is possible that they require feeding by total parenteral nutrition (TPN) and initially may only be able to tolerate trophic feeds to promote development of the gastrointestinal tract [60].
Lastly, premature infants have high nutrient demands for continuation of rapid growth and development [53]. In order to track growth postnatally, infants will be monitored for weight gain and growth in length and head circumference. Eventually, premature infants will be able to tolerate enteral feeds. Indications that an infant is ready to begin or increase their enteral feeds include cardiorespiratory stability, ventilation stability, vital sign stability, gastrointestinal tract function, and knowledge/control of any infections. Depending on the stability of the infant, feeding directly at the mothers’ breast may be attainable, and can be supplemented with human milk feeds from an alternate device.

Human milk is the ideal source for beginning enteral feeds with premature infants. When mothers deliver prematurely, the milk they produce is higher in protein, iron, sodium, chloride, and magnesium [2], helping to meet the specific nutrient requirements of the preterm infant. Additionally, the bioactive components present in the mother’s milk will provide individualized immune protection against pathogens present in the infant’s environment [16,61]. In the event that mother’s own milk does not meet the nutrient requirements of the premature infant, research has shown that there are specific benefits to feeding fortified human milk rather than switching to preterm formula including earlier discharge from the hospital and lower rates of sepsis and NEC [51].

There are specific challenges that come with providing an exclusively human milk diet to a premature infant. Many times, infants who are born prematurely are separated from their mother. Mothers who are separated from their infants can struggle with delayed onset of lactogenesis II and may have difficulty producing a full milk supply [62-64]. Premature infants can also have inadequate muscle strength in their jaw and tongue, leading to disorganized suck if or when they can feed at the breast. When this occurs, mothers may not receive adequate
breast and nipple stimulation from the infant alone, and are advised to pump to encourage and maintain their milk supply [65]. When a mother cannot produce adequate milk volume to meet the feeding requirements of her infant, donor human milk is a necessary secondary feeding option to maintain an exclusively human milk diet.

**Donor Human Milk**

Mothers around the world commonly use breast pumps as a method for removing milk from their breasts while separated from their children. Reasons for pumping milk rather than feeding the infant directly include returning to work, maternal or infant health complications, providing milk for another caretaker to feed their child, creating an emergency supply, relieving engorgement, increasing milk supply, and nipple soreness [66]. While only around 1% of mother’s report pumping their milk specifically for donation [66], the outcome of these pumping situations may be an abundance of stored milk. When mothers have excess milk beyond the requirements of their own child they can choose to donate their milk to other mothers through one of three different avenues: 1) non-profit human milk banks, 2) for-profit human milk banks, or 3) peer-to-peer milk sharing.

In the North America, the non-profit human milk bank model is governed by the Human Milk Banking Association of North America (HMBANA). Currently, HMBANA has 26 running milk bank facilities across the United States and Canada, with 5 milk banks currently in development [67]. Within the HMBANA model, milk banks accept donations of human milk after the inquiring donor has received physician approval for donation to ensure the infant is prioritized in receiving human milk, has undergone verbal screening by the milk bank for any risk factors, and undergone serologic testing to verify the health of the donor [68].
With HMBANA milk banks, mothers receive no monetary compensation for their donation, but costs of blood tests and milk shipment to the milk bank are covered. After a donor has been approved, mothers receive education on safe pumping and handling techniques for human milk and any excess human milk the mother has is shipped to the milk bank.

Upon arrival at the milk bank, milk from 4-5 donors is pooled to normalize any donor variations in nutrient or bioactive content, subject to a preliminary bacterial screening for *Bacillus cereus*, undergoes Holder pasteurization (62.5 °C for 30 minutes) if bacterial screening is negative, and then is screened for remaining bacteria. Any milk containing > 1 colony-forming unit (CFU) is discarded [68]. Precautions are taken surrounding bacterial screening because Holder pasteurization does not create a sterile product, and is known to cause sporulation of *B. cereus* spores [69,70]. After the secondary bacterial screening is cleared, milk is frozen and shipped overnight to recipients.

Benefits of the non-profit model of human milk donation include altruistic motivation for donations [68], provision of a pathogen-free product that has been verified by bacterial testing [69], and retention of biological activity of a number of proteins providing immune protection to the recipient [71-73]. Limitations of the non-profit human milk donation process include cost of human milk for recipients (approximately US $5.00/1 ounce), limited accessibility to infants outside of a hospital setting, loss of biological activity of some proteins compared to raw human milk [73], and costs of starting and maintaining a non-profit model milk bank [74]. Still, many hospitals in the United States use HMBANA milk for their preterm and medically fragile infants.

In the United States, there is a relatively active for-profit model of human milk banking. Companies include the International Milk Bank, Prolacta Bioscience, Ni-Q, and Medolac, with
new companies arising. Globally, there is one other for-profit company and that is the Australian Breast Milk Bank. In this model, individuals receive monetary compensation from the company for providing their milk. In all of these products, the human milk is collected from mothers, screened, pasteurized, and packaged for distribution. The specific pasteurization method is different among companies, with some using Holder pasteurization (62.5 °C for 30 minutes) or a variation called vat pasteurization (62.8 °C for 30 minutes), and some are using retort processing (121 °C for 5 minutes) to create a shelf stable product. The exact methodologies of each company, though, are not released to the public.

Benefits of the for-profit model of human milk sharing depends on the product that is being used. Some of the products available are marketed as having a known concentration of nutrients to make diet formulation within the hospital easier and more consistent. Shelf-stable products may possibly increase access to human milk in rural locations that cannot afford to store frozen human milk on site. Limitations of the for-profit model include monetary incentive for mothers, which may discourage providing adequate volumes of human milk to their own infant. Processing methods using higher temperatures to create a shelf-stable product have been shown to have a significant negative impact on bioactive components [71], and have no peer-reviewed articles providing information on nutritional composition of the resulting product. Human milk products available from for-profit companies are also expensive, restricting the accessibility. Additionally, the lack of transparency about specific heat-processing methodology make it difficult to investigate the efficacy and provide external validation of nutritional composition for the resulting products.

Peer-to-peer milk sharing is on the rise in the United States and there are a number of organizations that are facilitating the process. Companies include Only the Breast, Eats on
Feets, Human Milk 4 Human Babies, and Letche, among others. Additionally, the Academy of Breastfeeding Medicine recently released a position statement on informal breast milk sharing for the term healthy infant that overviews the benefits and risks of peer-to-peer milk sharing. In this model of human milk sharing, mothers are connected to each other directly. Mothers who have an excess supply provide raw, unpasteurized human milk to parents who are having difficulty meeting their child’s human milk requirements. This method can be used for providing milk for biological and adoptive children. In this model, the milk is donated and the mothers providing the milk do not receive compensation.

Benefits of the peer-to-peer milk sharing model include altruistic motivation, exchange of raw human milk, which will provide the highest level of nutrition and immune protection to the recipient [1], possibility for interpersonal relationship formation between donor and recipient if desired, and no cost - as the milk is donated to directly the recipient [75,76]. Limitations of the peer-to-peer milk sharing model include a possible lack of access to past medical history, possible incomplete information concerning risky behaviors (smoking, drinking, recreational drug usage, etc.), and lack of protection from heat processing, which kills pathogenic bacteria and life-threatening viruses such as cytomegalovirus and HIV [76].

With so many options for obtaining human milk, it is important that healthcare providers and mothers understand the differences between the types of milk they are obtaining. Holder pasteurization is currently the most common heat processing method utilized, but many technologies are emerging that provide equivalent bacterial destruction with less exposure to heat, helping to maintain the nutritional composition and bioactivity of the raw human milk.
References


CHAPTER 2: Literature Review – The Effect of Processing Methods and Packaging Materials Utilized for Commercial and Non-commercial Human Milk Products on Nutritional Composition of Human Milk

Lima HK, Perrin MT, Weaver G, Israel-Ballard K, Admunson K, Simunovic J, Fogleman AD. [to be submitted for peer review]

Introduction

Human milk banking is growing in response to public health recommendations to expand access to donor human milk for medically fragile infants when they don’t have access to their mother’s own milk [1-3]. There are currently over 500 human milk banks (HMB) globally including more than 200 in Brazil and the same numbers in Europe. In 2014, the Human Milk Banking Association of North America (HMBANA) dispensed 3.77 million ounces of donor milk and the Brazilian Network of Human Milk Banks dispensed 4.94 million ounces, which represented an increase of 151% and 13% respectively compared to 2009 volumes [4-6]. Very low birth weight (VLBW) infants who receive a human milk diet, including pasteurized donor milk, have lower incidence of necrotizing enterocolitis (NEC) and sepsis and lower health care costs than VLBW infants who receive formula [7-9].

The role of milk banks is to screen potential donors and collect and process human milk to provide a safe product. Globally, six commercially available human milk products exist: Human Milk Banking Association of North America (HMBANA), Australian Breast Milk Bank, International Milk Bank, Prolacta Bioscience, Ni-Q, and Medolac. The products produced by these organizations are all processed and packaged differently (Table 2.1).
<table>
<thead>
<tr>
<th>Organization</th>
<th>Processing Method Utilized</th>
<th>Available Product Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Milk Banking Association of North America (HMBANA)</td>
<td>Holder pasteurization: 62.5 °C for 30 minutes</td>
<td>• Clear glass or polypropylene containers</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 2+ donors per pool of milk</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Product must be frozen until used</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Good for 6 months unopened at -20 °C</td>
</tr>
<tr>
<td>Australian Breast Milk Bank</td>
<td>High pressure processing: specific time, temperature, and pressure not released by company</td>
<td>• Product release announced for February 2018 pending pilot trials</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Undisclosed packaging specs</td>
</tr>
<tr>
<td>International Milk Bank</td>
<td>Information not released by company</td>
<td>• Sterile product</td>
</tr>
<tr>
<td>Prolacta Bioscience</td>
<td>Vat pasteurization: 63 °C for ≥ 30 minutes</td>
<td>• Polypropylene containers</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 100+ donors per pool of milk</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Product must be frozen until used</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Good for 2 years unopened at -20 °C</td>
</tr>
<tr>
<td>Ni-Q</td>
<td>Information not released by company</td>
<td>• Undisclosed packaging specs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Light shielded</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Shelf-stable product</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Sterile product</td>
</tr>
<tr>
<td>Medolac</td>
<td>Retort processing: 121 °C for 5 minutes at 15 PSI</td>
<td>• Undisclosed packaging specs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Light shielded</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 100+ donors per pool of milk</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Shelf-stable product good for 3 years unopened</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Sterile product</td>
</tr>
</tbody>
</table>

Clinically it is important to know the differences between these products, as processing method and packaging materials can impact the nutritional and bioactive properties of human milk. Holder pasteurization (62.5 °C for 30 minutes) has been the standard processing method.
in human milk banking, therefore, it has been extensively reviewed elsewhere [10-13]. Recent reviews on the impact of Holder pasteurization found that there was greater than 25% loss of several nutritional and immunological factors including available lysine, immunoglobulin A (IgA), lactoferrin, lysozyme, lipoprotein lipase, bile salt stimulating lipase, and lymphocytes [10,11,14]. The loss of nutritive and immune factors after pasteurization may contribute to the slower growth that has been observed in infants fed pasteurized donor milk and may potentially diminish the protective effect of human milk [15].

The purpose of this review is to summarize the nutritional and immunological impacts of commercially and non-commercially utilized pasteurization technologies outside of Holder pasteurization and the impacts of different packaging materials on human milk composition. This overview will provide a resource to clinicians to better understand the differences between the human milk-based feeding options when the mother’s own milk is unavailable.

**Methods**

Pasteurization

PubMed, Food Science and Technology Abstracts, and Web of Science databases were searched using the following Boolean term: ((("human milk" OR "breast milk" OR breastmilk)) AND (processing OR "high pressure" OR "high temperature" OR HTST OR microwave OR flash OR ultraviolet OR UV OR ultrasound OR pulsed electric field OR vat OR retort)) on 23 January 2018 to identify studies that used alternative methods to Holder pasteurization for processing human milk. 1,228, 296, and 1,476 articles were retrieved from PubMed, Food Science and Technology Abstracts, and Web of Science, respectively. Inclusion criteria included original research published between 1980 and 2017 that measured bacteria
levels, nutritive factors, or immunological components in human milk using alternative methods to Holder pasteurization that had full text available through Google Scholar or North Carolina State University Library licenses. All references of included articles were reviewed to identify any additional relevant articles. A total of 51 studies were identified, including 12 studies that used high pressure processing (HPP), 13 studies that used high temperature short time (HTST), 8 studies that used flash heating, 7 studies that used microwave radiation, 5 studies that used ultraviolet-C irradiation (UV-C), 2 studies that used retort processing, 1 study that used vat pasteurization, and 2 studies that used ultrasound. Table 2.2 provides a summary of these studies by processing method and their findings regarding the impact on various components in human milk.
### Table 2.2. Effect of processing method on components in human milk.

<table>
<thead>
<tr>
<th>Component</th>
<th>Finding</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vat Pasteurization</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macronutrients</td>
<td>Significantly less total protein when compared to Holder pasteurized samples; no difference in fat or carbohydrate concentrations when compared to Holder pasteurized samples; significantly more calculated energy when compared to Holder pasteurized samples</td>
<td>Meredith-Dennis 2017</td>
</tr>
<tr>
<td>Antimicrobials</td>
<td>Significantly less IgM, lactoferrin, alpha-lactalbumin, alpha-antitrypsin, alpha-casein, beta-casein, and kappa-casein when compared to Holder pasteurized samples; no difference in IgA, IgG, or osteopontin concentrations when compared to Holder pasteurized samples; greater lysozyme concentration when compared to Holder pasteurized samples</td>
<td>Meredith-Dennis 2017</td>
</tr>
<tr>
<td><strong>High Pressure Processing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microbes</td>
<td>Reduced by 6-8 log at 400 MPa in milk inoculated with five common pathogens. <em>S. aureus</em> took the longest time to reduce (30 minutes compared to 2-10 minutes for other pathogens)</td>
<td>Viazis 2008</td>
</tr>
<tr>
<td></td>
<td>Reduced endogenous bacteria to undetectable levels at 400, 500, and 600 MPa</td>
<td>Permanyer 2010</td>
</tr>
<tr>
<td></td>
<td>Endogenous bacteria reduced to undetectable levels; inoculations of <em>S. aureus</em> reduced by 5 logs at 500 MPa, 4°C for 15 minutes and by 8 logs when heat added (50°C)</td>
<td>Windyga 2015</td>
</tr>
<tr>
<td>PUFAs</td>
<td>No change in PUFAs, at 400, 500, and 600 MPa</td>
<td>Molto-Puigmarti 2011</td>
</tr>
<tr>
<td></td>
<td>Stable or increasing PUFAs at 300 to 500 MPa; PUFAs decreased at 600 MPa</td>
<td>Delgado 2013, 2014</td>
</tr>
<tr>
<td>Antimicrobials</td>
<td>Increase in lysozyme activity and 85% retention of sIgA at 400 MPa</td>
<td>Viazis 2007</td>
</tr>
<tr>
<td></td>
<td>No change in IgA at 400 MPa, 90% retention at 500 MPa, 75% retention at 600 MPa</td>
<td>Permanyer 2010</td>
</tr>
<tr>
<td></td>
<td>No change in IgA at 400 MPa; 85% retention at 500 MPa; 70% retention at 600 MPa</td>
<td>Franch 2010</td>
</tr>
<tr>
<td></td>
<td>50% retention of IgA at 300 MPa and 5% retention at 600 MPa when combined with heat (50°C)</td>
<td>Delgado 2013</td>
</tr>
<tr>
<td></td>
<td>85% retention of IgA at 400 MPa</td>
<td>Contador 2013</td>
</tr>
<tr>
<td></td>
<td>77% and 66% retention of lactoferrin at 400 and 500 MPa; Increase in lysozyme activity between 300 MPa and 600 MPa; IgA retention higher with HPP at 400-500 MPa than Holder</td>
<td>Mayayo 2014, 2015</td>
</tr>
<tr>
<td>Vitamins</td>
<td>No change in vitamin C, ascorbic acid, and tocopherols at 400-600 MPa</td>
<td>Molto-Puigmarti 2011</td>
</tr>
<tr>
<td></td>
<td>No change in alpha tocopherol at 400 MPa or at 300 to 600 MPa with heat (50°C); gamma and delta tocopherols decrease with pressure and temperature</td>
<td>Delgado 2013, 2014</td>
</tr>
</tbody>
</table>
### Table 2.2. (continued)

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Greater retention of IL-6, IL-13, and TNF at 400 MPa, than at 500 MPa, 600 MPa, or Holder pasteurization</th>
<th>Franch 2010</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stable or increasing IL-6 and IL-8 at 300 to 600 MPa with heat (65°C); stable IL-6, IL-8 and TNF at 400 to 600 MPa</td>
<td>Delgado 2013, 2014</td>
</tr>
<tr>
<td>Nucleotides</td>
<td>No change in CMP, AMP and UMP at 400, 500, and 600 MPa</td>
<td>Mateos-Vivas 2015</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>&lt;5% retention with pressure (300MPa to 900MPa) and heat (50°C and 85°C)</td>
<td>Delgado 2013</td>
</tr>
<tr>
<td></td>
<td>10 – 15% retention at 400 MPa</td>
<td>Contador 2013</td>
</tr>
</tbody>
</table>

### Retort Processing

<table>
<thead>
<tr>
<th>Microbes</th>
<th>Endogenous bacteria were undetectable</th>
<th>Lima 2017</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macronutrients</td>
<td>Significantly less total protein when compared to Holder pasteurized samples; no difference in fat, carbohydrate, or energy concentrations when compared to Holder pasteurized samples</td>
<td>Meredith-Dennis 2017</td>
</tr>
<tr>
<td>Antimicrobials</td>
<td>No detectable sIgA activity; 89% reduction in lysozyme activity when compared to raw samples</td>
<td>Lima 2017</td>
</tr>
<tr>
<td></td>
<td>Significantly less IgM, IgG, lactoferrin, alpha-lactalbumin, alpha-antitrypsin, osteopontin, alpha-casein, beta-casein, and kappa-casein when compared to Holder pasteurized samples; no difference in IgA and lysozyme concentrations when compared to Holder pasteurized samples</td>
<td>Meredith-Dennis 2017</td>
</tr>
</tbody>
</table>

### High Temperature Short Time

<table>
<thead>
<tr>
<th>Microbes</th>
<th>Infections of CMV and endogenous bacteria were undetectable</th>
<th>Goldblum 1984</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infections of <em>E. coli</em> and <em>S. aureus</em> completely inactivated</td>
<td>Dhar 1996</td>
</tr>
<tr>
<td></td>
<td>Endogenous bacteria were undetectable</td>
<td>Chen 2001</td>
</tr>
<tr>
<td></td>
<td>No detection of infections of <em>E. coli</em>, <em>S. aureus</em>, <em>S. agalactiae</em>, HIV and HepC. HepA virus and human parvovirus still active after HTST.</td>
<td>Terpstra 2007</td>
</tr>
<tr>
<td></td>
<td>Infections of <em>L. monocytogenes</em> and <em>C. sakazakii</em> completely inactivated, infection of <em>S. aureus</em> reduced by 4.5 log</td>
<td>Giribaldi 2016</td>
</tr>
<tr>
<td></td>
<td>No detection of gram-negative bacteria, 4.5 log reduction in coagulase-negative staphylococci, 0.92 log reduction of enterococcus species</td>
<td>Klotz 2017</td>
</tr>
<tr>
<td>Macronutrients</td>
<td>No significant loss of total protein</td>
<td>Silvestre 2006</td>
</tr>
<tr>
<td>Antimicrobials</td>
<td>No significant loss of lactoferrin, lysozyme or IgA</td>
<td>Goldblum 1984</td>
</tr>
<tr>
<td></td>
<td>74% retention of IgA</td>
<td>Dhar 1996</td>
</tr>
<tr>
<td></td>
<td>HTST and Holder milk inoculated with <em>Lysteria</em> showed similar rates of bacteria loss</td>
<td>Chen 2001</td>
</tr>
<tr>
<td></td>
<td>No change in lactoferrin concentration versus significant loss with Holder</td>
<td>Baro 2011</td>
</tr>
<tr>
<td>Enzyme activity</td>
<td>Complete loss of lipase activity</td>
<td>Goldblum 1984</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td></td>
<td>No change in lipase activity versus significant loss with Holder</td>
<td>Baro 2011</td>
</tr>
<tr>
<td></td>
<td>Significantly more bile salt stimulating lipase activity than Holder pasteurized samples (0.26 µmol/min/mL and 0.09 µmol/min/mL respectively)</td>
<td>Giribaldi 2016</td>
</tr>
<tr>
<td></td>
<td>0.8% retention of bile salt-stimulated lipase; 6% retention of alkaline phosphatase</td>
<td>Klotz 2017</td>
</tr>
<tr>
<td>Vitamins</td>
<td>No significant loss of thiamine, riboflavin, B6, folic acid, or vitamin C</td>
<td>Goldblum 1984</td>
</tr>
<tr>
<td>Amino acids</td>
<td>85% retention of available lysine</td>
<td>Silvestre 2006</td>
</tr>
<tr>
<td></td>
<td>No change in available lysine</td>
<td>Baro 2011</td>
</tr>
<tr>
<td>Growth factors</td>
<td>No change in IGF-1, IGF-2, IGFBP3-, and EGF. 5% reduction in IGFBP-2</td>
<td>Goelz 2009</td>
</tr>
<tr>
<td>Antioxidants</td>
<td>No significant loss of antioxidant capacity compared to loss with Holder</td>
<td>Silvestre 2008</td>
</tr>
</tbody>
</table>

**Flash heat**

| Microbes | No detectable reverse transcriptase (RT) in samples inoculated with cell-free HIV-1 | Israel-Ballard 2005 |
|          | No detectable cell-free HIV-1 in naturally infected breast milk | Israel-Ballard 2007 |
|          | No detectable cell-associated HIV-1 in samples inoculated with cell infected HIV-1 | Volk 2010 |
| Antimicrobials | 15% retention of lactoferrin | Israel-Ballard 2005 |
|              | 80% retention of IgA; 67% retention of IgG; binding capacity to influenza increased after flash heating | Chantry 2009 |
|              | No difference in bacteriostatic activity between raw and flash milk spiked with E. coli and S. aureus; antibacterial activity of lactoferrin and lysozyme reduced by 11% and 57% respectively | Chantry 2011 |
|              | 39% retention of lactoferrin, 78% retention of lysozyme, 97% retention of IL-10, 100% retention of IL-8, 25% retention of IgA | Daniels 2017 |
| Vitamins    | No significant loss of vitamin A, C, E, thiamin, riboflavin, folate, B6 or B12 | Israel-Ballard 2005 |
Table 2.2. (continued)

<table>
<thead>
<tr>
<th>Vitamin Retention</th>
<th>Study</th>
<th>Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>No significant loss of vitamin A, C, B12, folate; 59% retention of riboflavin; 96% retention of B6</td>
<td>Israel-Ballard 2008</td>
<td>No significant loss of vitamin A, C, B12, folate; 59% retention of riboflavin; 96% retention of B6</td>
</tr>
<tr>
<td>75% retention of folate, with greater loss in tetrahydrofolate vitamer than in 5-methyl-tetrahydrofolate</td>
<td>Buttner 2014</td>
<td>75% retention of folate, with greater loss in tetrahydrofolate vitamer than in 5-methyl-tetrahydrofolate</td>
</tr>
</tbody>
</table>

**Microwave**

<table>
<thead>
<tr>
<th>Category</th>
<th>Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbes</td>
<td>Milk inoculated with <em>T. cruzi</em> and processed to 63°C did not infect orally treated mice</td>
</tr>
<tr>
<td>Antimicrobials</td>
<td>56% retention of IgA (no endpoint temperature provided)</td>
</tr>
<tr>
<td>Antimicrobials</td>
<td>81% and 4% retention of lysozyme and 67% and 2% retention of IgA after microwaving for 30 seconds on LOW (median 33.5°C) and HIGH (median 90.5°C), respectively</td>
</tr>
<tr>
<td>IgA, IgG and IgM stable until 57°C with complete loss at 77°C; 63% IgA retention at HIGH setting (52°C to 62°C)</td>
<td>Sigman 1989, Ovesen 1996, Carbonare 1996</td>
</tr>
<tr>
<td>Macronutrients</td>
<td>No change in total protein at HIGH setting (52°C to 62°C)</td>
</tr>
<tr>
<td>Macronutrients</td>
<td>No change in triglycerides when heated to 35°C to 40°C</td>
</tr>
<tr>
<td>PUFAs</td>
<td>No change in alpha-linoleic or linolenic acid up to 77°C</td>
</tr>
<tr>
<td>Vitamins</td>
<td>No change in Vitamin E or thiamine up to 77°C</td>
</tr>
<tr>
<td>Vitamins</td>
<td>No change in carotenoids when heated to 35°C to 40°C</td>
</tr>
</tbody>
</table>

**Ultraviolet C Irradiation**

<table>
<thead>
<tr>
<th>Category</th>
<th>Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbes</td>
<td>5-log reduction in 5 common pathogens spiked in human milk achieved between 8 and 27 minutes</td>
</tr>
<tr>
<td>Antimicrobials</td>
<td>89%, 87%, and 75% retention of sIgA, lactoferrin, and lysozyme respectively which was significantly greater than Holder pasteurized samples.</td>
</tr>
<tr>
<td>Lipase activity</td>
<td>No change in lipase activity</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>No change in free fatty acid profile</td>
</tr>
<tr>
<td>Complete destruction of CMV with UV-C irradiation for 10 seconds at 254 nm, 1-5 cm from light</td>
<td>Lloyd 2016</td>
</tr>
<tr>
<td>Lipase activity</td>
<td>No change in lipase activity</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>No change in free fatty acid profile</td>
</tr>
</tbody>
</table>
Table 2.2. (continued)

<table>
<thead>
<tr>
<th>Ultrasound</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microbes</strong></td>
<td>96% and 72% deactivation of <em>E. coli</em> and <em>S. epidermidis</em> spiked in human milk with ultrasound alone; 99% deactivation with the addition of heat (45°C to 50°C) Czank 2010</td>
</tr>
<tr>
<td><strong>Antimicrobials</strong></td>
<td>91%, 80%, and 77% retention of sIgA, lysozyme, and lactoferrin when combined with heat (50°C) Czank 2010</td>
</tr>
<tr>
<td><strong>Lipase</strong></td>
<td>45% lipase retention when combined with heat (50°C) Czank 2010</td>
</tr>
</tbody>
</table>

Packaging

PubMed, Food Science and Technology Abstracts, and Web of Science databases were searched using the following Boolean term: ((("human milk" OR "breast milk" OR breastmilk)) AND (packaging OR plastic OR hdpe OR "high density polyethylene" OR polyethylene OR glass OR "light exposure" OR photodegradation OR "photo degredation")) on 23 January 2018 to identify studies that evaluated the effect of packaging materials on the nutritional and immunological components of human milk. 221, 57, and 207 articles were retrieved from PubMed, Food Science and Technology Abstracts, and Web of Science, respectively. Inclusion criteria included original research looking at the effect of container materials or light exposure on nutrients or immunological components of human milk and had accessible full text articles through Google Scholar search or North Carolina State University Library licenses. All references of included articles were reviewed to identify any additional relevant articles. A total of 11 studies were identified that met all inclusion criteria, including 8 studies looking at glass containers, 5 studies looking at polyethylene containers, 5 studies looking at polypropylene containers, 2 studies looking at stainless steel containers, and 1 study looking at polystyrene containers. Table 2.3 provides a summary of these studies by container types and their findings regarding the impact on various components in human milk.
Table 2.3. Effect of packaging type on components in human milk.

<table>
<thead>
<tr>
<th>Component</th>
<th>Finding</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass (Pyrex)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>54% retention of cells after 8-16 hours of storage when compared to control samples; greater proportion of neutrophil retention than samples stored in polystyrene</td>
<td>Burns 1979</td>
</tr>
<tr>
<td></td>
<td>Greater loss of leukocytes in glass containers compared to plastic (non-specific) containers</td>
<td>Paxson 1979</td>
</tr>
<tr>
<td></td>
<td>Significant increase in macrophages and neutrophils (combined count) and lymphocytes after 24 hours of storage; significant loss of lymphocyte function after 24 hours; greater retention of all cell types when compared to polypropylene containers; no difference in phagocytic capability of cells between glass, polyethylene bags, and polypropylene containers</td>
<td>Goldblum 1981</td>
</tr>
<tr>
<td></td>
<td>No effect on macrophages and neutrophils (combined count) or lymphocytes after 24 hours when storing colostrum; no difference in cell counts between glass and polypropylene containers when storing colostrum</td>
<td>Goldblum 1982</td>
</tr>
<tr>
<td></td>
<td>Greater retention of cells after 7, 24, 48, and 72 hours when compared to samples in stainless steel containers; greater retention of cell viability at 7, 24, 48, and 72 hours when compared to samples in stainless steel containers</td>
<td>Williamson 1996</td>
</tr>
<tr>
<td>Antimicrobials</td>
<td>Significant loss of lysozyme and lactoferrin (40% and 30% respectively; p &lt; 0.01) after 24 hours of storage; no change in IgA or slgA concentrations, or slgA ability to bind E. coli O antigens after 24 hours of storage</td>
<td>Goldblum 1981</td>
</tr>
<tr>
<td></td>
<td>No effect on lysozyme, lactoferrin, IgA, slgA, or slgA E. coli O antigens after 24 hours when storing colostrum; no difference in these antimicrobials between glass, polyethylene, or polypropylene containers when storing colostrum</td>
<td>Goldblum 1982</td>
</tr>
<tr>
<td></td>
<td>Bactericidal activity stable for 24 and 48 hours of storage; significantly more bactericidal activity after 24 and 48 hours of storage when compared to polyethylene containers</td>
<td>Takci 2013</td>
</tr>
<tr>
<td>Vitamins</td>
<td>No effect on Vitamin A concentration</td>
<td>Garza 1982</td>
</tr>
<tr>
<td></td>
<td>No difference in Vitamin A, D, E, B2, B6, B12, or folacin concentrations when compared to samples in polypropylene containers; 29% greater retention of Vitamin C when compared to samples in polypropylene containers</td>
<td>Van Zoeren-Grobben 1987</td>
</tr>
<tr>
<td>Minerals</td>
<td>No effect on sodium, iron, zinc, or copper concentrations</td>
<td>Garza 1982</td>
</tr>
<tr>
<td>Polystyrene</td>
<td></td>
<td></td>
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<tr>
<td>Cells</td>
<td>83% retention of cells after 8-16 hours of storage when compared to control samples; greater proportion of macrophages and lymphocytes than samples stored in glass</td>
<td>Burns 1979</td>
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<td>Polyethylene</td>
<td></td>
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<tr>
<td>Macronutrients</td>
<td>Fat loss of 0.29 g/100 mL during storage; no difference in fat loss compared to polypropylene containers</td>
<td>Janjindamai 2013</td>
</tr>
<tr>
<td><strong>Table 2.3. (continued)</strong></td>
<td></td>
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<td>---------------------------</td>
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<tr>
<td><strong>Cells</strong></td>
<td>Significant increase in macrophages and neutrophils (combined count), and lymphocytes after 24 hours of storage; significant loss of lymphocyte function after 24 hours; greater retention of lymphocytes when compared to glass containers; no difference in phagocytic capability of cells between glass, polyethylene bags, and polypropylene containers</td>
<td>Goldblum 1981</td>
</tr>
<tr>
<td><strong>Antimicrobials</strong></td>
<td>No change in lysozyme, lactoferrin, IgA, or sIgA concentrations after 24 hours of storage; 60% reduction of sIgA ability to bind E. coli O antigens after 24 hours of storage; No effect on lysozyme, lactoferrin, IgA, sIgA, or sIgA E. coli O antigen binding after 24 hours when storing colostrum; no difference in these antimicrobials between glass, polyethylene, or polypropylene containers when storing colostrum</td>
<td>Goldblum 1981, Goldblum 1982</td>
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<tr>
<td><strong>Vitamins</strong></td>
<td>50% of riboflavin and 70% of vitamin A destroyed in translucent polyethylene bottles when exposed to normal daylight for 7 hours; 13% increase in riboflavin retention when bottles covered in foil;</td>
<td>Bates 1985</td>
</tr>
<tr>
<td><strong>Polypropylene</strong></td>
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<tr>
<td><strong>Macronutrients</strong></td>
<td>Fat loss of 0.32 g/100 mL during storage; no difference in fat loss compared to polyethylene containers</td>
<td>Janjindamai 2013</td>
</tr>
<tr>
<td><strong>Cells</strong></td>
<td>Significant increase in macrophages and neutrophils (combined count), and lymphocytes after 24 hours of storage; lower retention of macrophages, neutrophils, and lymphocytes when compared to glass containers; no difference in phagocytic capability of cells between glass, polyethylene bags, and polypropylene containers; No effect on macrophages and neutrophils (combined count) or lymphocytes after 24 hours when storing colostrum; significant loss of lymphocyte function after 24 hours; no difference in cell counts between glass and polypropylene containers when storing colostrum</td>
<td>Goldblum 1981, Goldblum 1982</td>
</tr>
<tr>
<td><strong>Antimicrobials</strong></td>
<td>Significant loss of lysozyme and lactoferrin (40% and 30% respectively; p &lt; 0.01) after 24 hours of storage; no change in IgA or sIgA concentrations after 24 hours of storage; No effect on lysozyme, lactoferrin, IgA, sIgA, or sIgA E. coli O antigens after 24 hours when storing colostrum; no difference in these antimicrobials between glass, polyethylene, or polypropylene containers when storing colostrum</td>
<td>Goldblum 1981, Goldblum 1982</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td>No effect on Vitamin A concentration; No difference in Vitamin A, D, E, B2, B6, B12, or folacin concentrations when compared to samples in glass containers; significant loss (29%) of Vitamin C when compared to samples in glass containers</td>
<td>Garza 1982, 1987 Van Zoeren-Grobben</td>
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<tr>
<td><strong>Minerals</strong></td>
<td>No effect on sodium, iron, zinc, or copper concentrations</td>
<td>Garza 1982</td>
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Table 2.3. (continued)

<table>
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<tr>
<th>Stainless Steel</th>
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<tr>
<td>Cells</td>
</tr>
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<td>Greater loss of cells after 7, 24, 48, and 72 hours when compared to samples in glass containers; significant loss of cell viability at 7, 24, 48, and 72 hours when compared to samples in glass containers</td>
</tr>
<tr>
<td>Williamson 1996</td>
</tr>
<tr>
<td>Significantly greater decrease in both cell count and viability of colostrum samples when stored at 4 °C in stainless steel when compared to samples in plastic (non-specific) containers; significantly greater decrease in cell count of colostrum when stored at 28 °C when compared to samples in plastic containers; no difference in cell viability of colostrum when stored at 28 °C when compared to samples in plastic containers</td>
</tr>
<tr>
<td>Manohar 1997</td>
</tr>
</tbody>
</table>

**Commerically Utilized Pasteurization Technologies**

Vat Pasteurization

Vat pasteurization is a LTLT processing method where the milk is heated to 63 °C and held for 30 minutes. Vat pasteurization differs from Holder pasteurization in that the product is heated in a jacketed stainless-steel vat that has been fitted with pipes to deliver water and steam to the jacket liner [16]. Thermometers monitor and record the product temperatures and an agitation method is used to assure uniformity in temperature distribution [16].

One study was identified that examined the impact of vat pasteurization on nutritional and bioactive components of human milk [17]. In this study, Meredith-Dennis *et al.*, found that vat pasteurized human milk had less total protein, more total fat, and similar carbohydrate concentrations as Holder pasteurized human milk [17]. Concentrations of IgA, IgG, and osteopontin were similar in vat and Holder pasteurized human milk samples, while IgM, lactoferrin, alpha-lactalbumin, alpha-antitrypsin, alpha-casein, beta-casein, and kappa-casein concentrations were significantly lower in vat pasteurized samples when compared to Holder pasteurized samples. Lysozyme concentrations were significantly greater in vat pasteurized samples when compared to Holder pasteurized samples. This study does have significant limitations in that all samples analyzed originated from different pools of human milk, which
makes comparisons between products difficult, as the original raw milk may have started with different levels of each of these components. No research was found that investigated the impact of vat pasteurization on microbes, specific fatty acids, cytokines, nucleotides, leukocytes, lipase activity, or micronutrient retention. Additionally, no studies were identified that investigated the ability of vat pasteurization to deactivate CMV or HIV.

High Pressure Processing

HPP is a non-thermal processing method that can destroy microorganisms at low temperatures using pressures in the range of 100 to 800 megapascal (MPa) [18]. HPP can also be combined with thermal processing. There is a growing body of evidence regarding the impact of HPP processing on human milk. Viazis et al. described a 6-8 log reduction of bacteria in human milk that was inoculated with *S. aureus* and *E. coli* when processed at 400 MPa for 30 minutes [19] and Windyga et al. described a 5 log reduction in *S. aureus* when processed at 500 MPa for 15 minutes [20]. Endogenous bacteria in human milk were reduced to undetectable levels when processed between 400 MPa and 600 MPa for 10 to 15 minutes at temperatures of 4 °C, 12 °C, 20 °C and 50 °C. Minimal reductions were observed at 300 MPa, suggesting that higher pressures are needed to eliminate microorganisms in human milk [20,21].

There was a high retention of the antimicrobial proteins lysozyme, IgA, and lactoferrin using HPP [21-27]. Viazis et al. and Mayayo et al. reported an increase in lysozyme activity using HPP in the range of 300 MPa to 600 MPa for 30 minutes compared to raw or Holder pasteurized milk [22,27]. IgA retention was higher in HPP processed milk (range of 60% to 90%) compared to Holder pasteurized milk (range of 40% to 70%) at low temperatures and
pressures between 400 MPa and 500 MPa [21-23,25,27]. IgA retention dropped to 50% and 5% when HPP at 300 MPa and 600 MPa respectively was combined with thermal processing at 50 °C [24].

Lactoferrin, which is greatly reduced during Holder pasteurization, had 77% and 66% retention with HPP at 400 MPa and 500 MPa respectively [26]. Other factors that have shown favorable retention with HPP in the 400 to 500 MPa range and low temperatures include polyunsaturated fatty acids (PUFAs) [24,28,29], Vitamin C and E [24,28,29], cytokines [23,24,29], and nucleotides [30]. Leukocytes show high destruction with HPP [24,25]. No research was found regarding the impact of HPP on lipase enzymes, which are destroyed during Holder pasteurization, or on the ability of HPP to deactivate CMV and HIV.

Retort Processing

There were only two studies found in the scientific literature regarding the effects of retort processing on human milk [17,31]. Lima et al. found that retort processing produced a commercially sterile human milk product, but secretory IgA and lysozyme activity were significantly reduced when compared to both raw and Holder pasteurized human milk from the same pool [31]. Meredith-Dennis et al. found that retort processed human milk had significantly less total protein and fat than Holder pasteurized and vat pasteurized samples, respectively, and found significantly less IgA, IgM, IgG, lysozyme, lactoferrin, alpha-lactalbumin, alpha-antitrypsin, osteopontin, alpha-casein, beta-casein, and kappa-casein than vat and Holder pasteurized samples. No studies were found that investigate the effect of retort processing on specific fatty acids, micronutrient retention, cytokines, nucleotides, lipase
enzymes, or immune protective cells. Additionally, no research was identified that investigated the ability of retort processing to deactivate CMV or HIV.

Emerging Pasteurization Technologies – Is There Room for Improvement?

High Temperature Short Time

HTST processing holds human milk at higher temperatures than Holder pasteurization (typically 71 °C to 74 °C) for a shorter time (typically 5 to 20 seconds). This method has been shown to deactivate cytomegalovirus (CMV) [32] and endogenous bacteria in human milk [33,34], along with inoculations of *E. coli*, *S. aureus*, *S. agilactia*, *L. monocytogenes*, *C. sakazakii*, and strains of the HIV and Hepatitis C viruses [35-37]. Inoculations of Hepatitis A and human parvovirus had been reduced but were still present after HTST processing [36]. Research suggests that there is a high retention of the antimicrobial proteins lactoferrin, lysozyme and IgA with HTST processing [32,34,35,37,38]; however, there is conflicting evidence regarding whether lipase enzymes are retained, with one study showing complete destruction of lipase activity [32], and another study showing complete retention [38], and two studies showing partial retention [34,37]. One study has investigated the effect of HTST on alkaline phosphatase and showed 6% retention [34]. Other nutrients that have shown greater than 75% retention with HTST processing include IgA [32,33], lysozyme, thiamine, riboflavin, B6, folic acid, and Vitamin C [32], growth factors [39], and available lysine [14,38]. Chen et al. reported no difference in the antimicrobial capacity of HTST and Holder pasteurized milk when inoculated with *Lysteria innocua* [33].
Flash pasteurization

The basic principal of flash heating is similar to HTST processing, which is to heat milk to 72 °C for 15 seconds; however, it is designed to be done on a small scale using simple equipment and a stovetop heat source so that it can be used in resource-poor or home settings. Flash heating may be a strategy for supporting safe peer-to-peer milk sharing in countries that are experiencing a rise in this practice [40-42]. Briefly, milk is heated in suitable containers in a water bath until the water, not the milk, reaches a boil, and then the containers of milk are removed and cooled. Depending on whether a medium or high flame is used on the stove-top, the internal temperature of the milk may not reach 72 °C at the time that the water bath reaches a boil [43].

To improve the reliability of flash heating for milk banking applications, a monitoring system has been developed that uses a temperature probe and a mobile phone [43]. This system has shown to be safe and effective [44]. Research suggests that flash heating is effective at inactivating cell-free and cell-associated HIV-1 in human milk [45-47]. Retention of lactoferrin and IgA has been reported as 15-39% and 25-80% respectively in flash heated milk [45,48,49]. Other immune components that were shown to be retained at greater than 75% and include lysozyme (78% retention), IL-10 (97% retention), and IL-8 (100% retention) [49]. There was no difference in the ability of flash-heated milk to inhibit the growth of inoculations of *S. aureus* and *E. coli* compared to unheated milk, suggesting that overall bactericidal capacity is retained [50]. A pilot study reported no loss of vitamin A, C, E, thiamine, riboflavin, folate, B₆, and B₁₂ with flash pasteurization [45]; however, larger studies have described 40% loss of riboflavin [51] and 25% loss of folate [52]. No research was found on the impact of flash pasteurization on the lipase activity of human milk.
Microwave

Research into the effects of microwave processing on human milk has primarily been done in the context of warming milk using batch processing in a clinical or home setting, and not in the context of food preservation. Only one study described using an internal probe to measure the temperature during processing [53]; therefore, temperature data from non-probe studies may not reflect maximum values. Ferreira et al. found that human milk inoculated with *T. cruzi* and then microwaved to an internal temperature of 63 °C did not infect orally fed mice [54]. Sigman et al. found that 90% of milk samples contained no colony forming units (CFUs) after microwaving; however, endpoint temperatures were not reported [55]. One study was identified that showed complete neutralization of CMV in human milk samples treated with high-power microwave irradiation [56]. No research was found regarding the impact of microwave processing HIV, or on the retention of lactoferrin and lipase enzymes. IgA retention in the range of 60% to 100% has been reported when microwaving to a temperature up to 62 °C [53,55,57,58], with complete destruction observed at 77 °C [53]. At low temperatures (35 °C to 40 °C), Tacken et al. reported no change in triglycerides or carotenoids [59]. At higher temperatures (77 °C), which would likely be necessary to reduce pathogens, Ovesen et al. reported no loss of PUFAs, vitamin E, and thiamine [53].

UV-C

UV-C pasteurization is a non-thermal processing method that uses short ultraviolet wavelength to eliminate pathogens. A pilot study of 10 human milk samples inoculated with *E. coli, S. epidermidis, E. cloacae, B. cereus,* and *S. aureus* achieved a 5-log reduction in bacteria between 8.3 and 26.5 minutes, with samples containing a higher solid content taking
longer time [60]. Lipase activity and free fatty acid profiles were retained with UV-C processing [60]. Lactoferrin, IgA, and lysozyme retention were reported as 87%, 89%, and 75% respectively with UV-C processing, and were significantly greater than retention after Holder pasteurization [61]. One study showed complete destruction of CMV with UV-C irradiation [62]. No research was found on the ability of UV-C to deactivate HIV, or on the impact of UV-C processing on micronutrient retention in human milk.

**Ultrasound**

Ultrasound is a non-thermal processing method that uses acoustic waves to deactivate pathogenic bacteria. In a pilot study of 6 human milk samples inoculated with *E. coli* and *S. epidermidis*, ultrasound alone was able to reduce the bacteria by 96% and 72% respectively [63]. When combined with heat, 99% of the bacteria were reduced, while lactoferrin, lipase, IgA, and lysozyme were retained at 77%, 45%, 91%, and 80% respectively. No research was found on the ability of ultrasound to deactivate viruses that are of concern for the preterm neonate, including CMV and HIV, or on the impact of ultraviolet processing on micronutrient retention in human milk.

**Packaging Technologies – Does Storage Container Make a Difference?**

Storing human milk is a necessary part of the human milk banking process, however each type of commercially available human milk is packaged differently. Glass and polypropylene containers are currently the most common commercially utilized packaging materials, while polyethylene bags are preferred for a home setting. Additional materials that have been utilized for milk storage include stainless steel, and an undisclosed packaging
material that offers protection from light (see Medolac products). Surprisingly, not much research has been published comparing the effects of different packaging materials on the nutritional and/or bioactive components of human milk. Only 11 studies were identified during the literature review, and 9 of these studies were conducted before the year 2000. A summary of the literature is available in Table 2.4.

Glass

Glass containers are utilized for human milk storage by some HMBANA milk banks functioning in the United States. Before the 1980’s, storage in glass containers was considered undesirable based on two studies investigating retention of cells in human milk. These studies showed only 54% retention of total cells after 8-16 hours of storage in glass containers [64], and greater loss of leukocytes when compared to samples stored in plastic containers [65]. The only positive result concerning glass storage containers was a greater proportion of neutrophil retention than samples stored in polystyrene [64], which is not commonly used for human milk storage. However, glass containers retained 29% less total cells and less macrophages and lymphocytes than when samples were stored in polystyrene containers [64].

Research emerging in the 1980’s and 1990’s showed a greater retention of macrophages and neutrophils (combined count) and lymphocytes when compared to polypropylene containers [66] and greater retention of total cell count and cell viability when compared to stainless-steel containers [67]. Additionally, there was no difference in phagocytic capability of cells when comparing samples stored in glass, polyethylene, and polypropylene containers [66], and significantly more bactericidal activity after 24 and 48 hours of storage when compared to samples stored in polyethylene containers [68]. Storage in glass containers
did show a significant loss of lysozyme and lactoferrin (40% and 30% respectively), but there was no change in IgA or slgA concentrations, and no change in the ability of slgA to bind *E. coli* O antigens after 24 hours [66]. There seems to be no effect of glass storage on vitamin A, sodium, iron, zinc, or copper concentrations [69]. Additionally, when compared to samples stored in polypropylene containers there was no difference in vitamin A, D, E, B2, B6, B12, or folacin concentrations, and 29% greater retention of vitamin C [70]. No research was identified that investigated macronutrients during storage in glass containers.

Colostrum seems to be more stable during storage, with no effect on macrophages and neutrophils (combined count), lymphocytes, lysozyme, lactoferrin, IgA, slgA, or slgA *E. coli* O antigen binding capacity after 24 hours of storage [71]. This stability did not differ, however, from retention of said components when stored in polypropylene or polyethylene containers [71].

**Polystyrene**

Polystyrene is not currently utilized by any companies providing commercial human milk. There was one study that investigated polystyrene as a storage material for human milk, and this study found 83% retention of cells after 8-16 hours of storage when compared to control samples, as well as a greater proportion of macrophages and lymphocytes when compared to samples stored in glass containers [64]. When compared to glass, polystyrene has greater loss of neutrophils after storage of 8-16 hours [64]. No information was identified that looked at macronutrient content, antimicrobials, vitamins, or other micronutrients during storage in polystyrene containers.
Polyethylene

Polyethylene containers are frequently used for at-home storage of human milk. Consequently, most milk that is being distributed commercially will have been stored in a polyethylene container at some point in time – even if it is not distributed in this packaging. As such, it is important to understand the effect of storage in polyethylene containers on human milk composition.

Fat loss was measured to be 0.29 g/100 mL during storage in polyethylene containers, however this did not differ from fat loss observed in polypropylene containers [72]. Human milk stored in polyethylene containers had a greater retention of lymphocytes when compared to glass containers, however there was still a significant loss of lymphocyte function after 24 hours of storage [66]. There was no difference in phagocytic capability of cells between samples stored in glass, polyethylene, and polypropylene containers [66], however bactericidal activity was decreased significantly at 24 and 48 hours of storage, and there was significantly less bactericidal activity at 24 and 48 hours compared to samples stored in glass containers [68]. Additionally, specific bactericidal activity against *E. coli* was reduced at 24 hours [68]. There was no change in lysozyme, lactoferrin, IgA, or sIgA concentrations after 24 hours of storage [66], however there was a 60% reduction of sIgA ability to bind to *E. coli* O antigens after 24 hours of storage [66]. Riboflavin and vitamin A destruction were investigated in translucent polyethylene bottles and found to have a 50% and 70% reduction, respectively, when exposed to normal daylight for 7 hours [73]. When bottles were covered in foil, a 13% increase in riboflavin retention was observed [73]. Similar to glass containers, there was no effect on lysozyme, lactoferrin, IgA, sIgA, or sIgA *E. coli* O antigen binding capacity after 24
hours when storing colostrum. No research was found that looked at cell viability or mineral retention in human milk stored in polyethylene containers.

Polypropylene

Polypropylene containers are the most common way for milk banks to store and distribute processed human milk. Fat loss of 0.32 g/100 mL was observed when human milk was stored in polypropylene containers, but this fat loss was not significantly different than the fat loss observed when polyethylene containers were used for storage [72]. Storage in polypropylene containers had no effect on vitamin A, sodium, iron, zinc, or copper concentrations [69], and there was no difference in vitamin A, D, E, B₂, B₆, B₁₂, or folacin concentrations when compared to samples in glass containers [70], but did retain 29% less vitamin C than samples stored in glass containers [70]. Human milk stored in polypropylene containers had lower retention of macrophages and neutrophils (combined count) and lymphocytes when compared to samples stored in glass containers [66], but there was no difference in phagocytic capability of cells from milk stored in polypropylene containers when compared to glass and polyethylene containers. There is a significant (p < 0.01) effect of storage in polypropylene containers on lysozyme and lactoferrin, with 40% and 30% loss observed after 24 hours of storage, respectively [66]. There was no change observed in IgA or sIgA concentrations in samples stored in polypropylene containers after 24 hours of storage [66]. Colostrum is much more stable during storage in polypropylene containers than human milk, with no effect on lysozyme, lactoferrin, IgA, sIgA, sIgA E. coli O antigen binding, macrophages, or neutrophils after storage for 24 hours [71], however a significant loss of lymphocyte function was observed after storage for 24 hours [71]. No research was found that
investigated the cell viability or bactericidal capacity of human milk stored in polypropylene containers.

**Stainless Steel**

Storage of human milk in stainless steel containers may occur during vat processing, depending on the materials the vat is constructed from. Only one study was identified that looked at storage in stainless steel containers, and found that there was a great loss of cells at 7, 24, 48, and 72 hours when compared to samples in glass containers, as well as a significant loss in cell viability about 7, 24, 48, and 72 hours when compared to samples in glass containers [67]. Additionally, one study investigated the effect of storage in stainless steel containers on colostrum [74] and found a greater decrease in cell count and cell viability compared to samples stored in plastic containers at 4 °C and a greater decreased in cell count when compared to samples stored in plastic containers at 28 °C. No research was found that investigated the macronutrient retention, specific cell viability, phagocytic capacity, bioactive component retention, vitamin retention, or mineral retention of human milk stored in stainless steel containers.
<table>
<thead>
<tr>
<th>Component</th>
<th>Glass (G)</th>
<th>Polystyrene (PS)</th>
<th>Polyethylene (PE)</th>
<th>Polypropylene (PP)</th>
<th>Stainless Steel (SS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macronutrients</td>
<td>no research</td>
<td>no research</td>
<td>fat loss of 0.29 g/100 mL; no difference in fat loss compared to PP</td>
<td>fat loss of 0.32 g/100 mL; no difference in fat loss compared to PE</td>
<td>no research</td>
</tr>
<tr>
<td>Cells</td>
<td>54% retention after 8-16 hours; greater retention of cells than PP and SS</td>
<td>83% retention after 8-16 hours</td>
<td></td>
<td></td>
<td>greater loss of cells after 7, 24, 48, and 72 hours than G</td>
</tr>
<tr>
<td>Cell Viability</td>
<td>greater cell viability at 7, 24, 48, and 72 hours than SS</td>
<td>no research</td>
<td>no research</td>
<td>no research</td>
<td>significant loss at 7, 24, 48, and 72 hours compared to G</td>
</tr>
<tr>
<td>Macrophages</td>
<td>greater retention than PP and PE; lower retention than PS</td>
<td>greater proportion than G</td>
<td>lower retention than G</td>
<td>lower retention than G</td>
<td>no research</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>greater retention than PP and PE</td>
<td>no research</td>
<td>lower retention than G</td>
<td>lower retention than G</td>
<td>no research</td>
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<tr>
<td>Leukocytes</td>
<td>lower retention than PP</td>
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<td>no research</td>
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<td>Lymphocytes</td>
<td>significant loss of function after 24 hours; greater retention than PP</td>
<td>greater proportion than G</td>
<td>significant loss of function after 24 hours; lower retention than G</td>
<td>lower retention than G</td>
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<td>Phagocytic Capacity</td>
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<td>Lysozyme</td>
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### Table 2.4. (continued)

<table>
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<tr>
<th>Lactoferrin</th>
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<th>no change in concentration after 24 hours</th>
<th>30% loss after 24 hours</th>
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<tbody>
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<td>IgA</td>
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<td>no research</td>
<td>no change in concentration after 24 hours</td>
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<tr>
<td>sIgA</td>
<td>no change in concentration after 24 hours</td>
<td>no research</td>
<td>no change in concentration after 24 hours</td>
<td>no change in concentration after 24 hours</td>
<td>no research</td>
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<tr>
<td>sIgA binding to E. coli O antigens</td>
<td>no change after 24 hours</td>
<td>no research</td>
<td>60% reduction after 24 hours</td>
<td>no research</td>
<td>no research</td>
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<td>Bactericidal activity</td>
<td>stable for 24 and 48 hours when compared to PE</td>
<td>no research</td>
<td>decreased significantly at 24 and 48 hours of storage; significantly less at 24 and 48 hours when compared to PE</td>
<td>no research</td>
<td>no research</td>
</tr>
<tr>
<td>Vitamins</td>
<td>no effect on vitamin A concentration; no difference in vitamin A, D, E, B₂, B₆, B₁₂, or folacin concentrations when compared to PP; greater retention of vitamin C compared to PP</td>
<td>no research</td>
<td>50% of riboflavin and 70% of vitamin A destroyed in translucent bottles after exposure to 7 hours daylight; 13% increase in riboflavin retention when bottles covered in foil</td>
<td>no difference in vitamin A, D, E, B₂, B₆, B₁₂, or folacin concentrations when compared to G; greater loss of vitamin C compared to G</td>
<td>no research</td>
</tr>
<tr>
<td>Minerals</td>
<td>no effect on sodium, iron, zinc, or copper concentrations</td>
<td>no research</td>
<td>no research</td>
<td>no effect on sodium, iron, zinc, or copper concentrations</td>
<td>no research</td>
</tr>
</tbody>
</table>

### Discussion

When a mother is unable to provide her own milk, donor human milk is commonly used to maintain an exclusively human milk diet until the mother can provide her own milk or
the infant is medically stable. The primary population of donor human milk recipients within a hospital setting are premature infants. There are a growing number of options for providing human milk to an infant, yet many practitioners do not understand the differences between commercial products that are available. There is a growing body of evidence emerging that shows not all processing and packaging methods result in the same human milk product.

With the recipient population being at such a high risk for development of complications, the number one concern of organizations providing commercial human milk should be pathogen elimination. Specific pathogens of interest include the viruses HIV-1 and CMV, and the bacteria *B. cereus*, which has been shown to survive Holder pasteurization [75]. Current HMBANA guidelines stipulate that no bacterial growth should be observed in donor milk (0 CFU/mL) after pasteurization [76], which is a more stringent requirement than the 4.3-log CFU/mL limit defined for Grade A pasteurized bovine milk [77]. Secondarily, maintenance of bioactive components and nutritional value should be of concern to ensure the highest level of immune protection possible while encouraging proper growth and development of the patient.

Pathogen Elimination

Holder pasteurization is the primary processing method utilized by commercial human milk organizations. With the emergence of shelf-stable products, retort processing is beginning to be utilized to some extent. Other processing methods currently being utilized include vat pasteurization and high-pressure processing. Retort processing [31] and high-pressure pasteurization [20,21] have been shown to facilitate a 5-log or greater reduction of bacteria in human milk. Holder pasteurization is known to allow the survival of *B. cereus* [31,78], and no
studies were identified that investigated the effect of vat pasteurization on bacterial elimination. In addition to the currently utilized processing methods, evidence exists regarding the ability of HTST [32,33], and UV-C [60] to facilitate a 5-log or greater reduction of bacteria in human milk, with specific evidence to show \textit{B. cereus} destruction with UV-C processing [60].

Holder pasteurization is the only commercially utilized pasteurization technology that has been shown to deactivate HIV-1 and CMV [79,80]. There were no peer reviewed articles identified that investigated the ability of high-pressure processing, vat pasteurization, or retort processing to deactivate HIV-1 or CMV. Existing research supports HIV deactivation with flash pasteurization [45-47], and HIV and CMV deactivation with HTST [32,36]. Many of these studies were pilot studies with a small number of samples; therefore, larger studies are needed to validate these findings. While microwave processing has been identified as a top emerging food preservation technology in both the United States and Europe, limited research has been done regarding its effectiveness for eliminating pathogens in human milk.

When considering bacterial reduction, HIV-1, and CMV deactivation capabilities, HTST is the only method that has been shown to both destroy bacteria and inactivate HIV-1 and CMV. This technology is not currently utilized by any commercial human milk organizations. Holder pasteurization does deactivate HIV-1 and CMV but needs to be screened for \textit{B. cereus} after pasteurization. Organizations in the United States that follow HMBANA guidelines currently screen for \textit{B. cereus} after pasteurization, ensuring efficacy of the distributed products. Of the currently available human milk products, Holder pasteurized human milk has the most evidence for providing a safe human milk product if screened for \textit{B. cereus} after pasteurization. Vat pasteurization, high-pressure processing, and retort processing
are in immediate need of peer-reviewed studies investigating their capability to deactivate HIV-1 and CMV and destroy *B. cereus* to ensure efficacy of the resulting products.

**Nutritional and Immunological Retention in Processing**

Currently, retort processing and vat pasteurization have very few peer-reviewed articles investigating the retention of nutritional and bioactive components in human milk after processing. The two studies that investigated vat pasteurization and/or retort processing [17,31] were pilot studies, and more research is needed to confirm and expand upon the findings. Preliminary findings show that both processing methods have a significant impact on total protein when compared to Holder pasteurized samples [17,31]. Additionally, retort processing eliminated all sIgA activity, 89% of lysozyme activity, and had significantly less immune components when compared to Holder pasteurized samples [17,31]. Similarly, vat pasteurized samples had significantly less immune components when compared to Holder pasteurized samples [17]. Based on this evidence, products produced using retort processing and vat pasteurization may not be the ideal donor human milk source for infants who are premature or medically fragile, as the destruction of immune components may put them at increased risk for development of sepsis, retinopathy, or necrotizing enterocolitis, among other infections. These products may be helpful for mothers needed a temporary human milk source for their healthy, term infant if they are struggling to establish their own milk supply.

Both Holder pasteurization and high-pressure processing have substantial peer-reviewed evidence supporting nutritional and bioactive retention. However, Holder pasteurization has been shown to have high losses of lactoferrin and lipase activity [10,11]. High-pressure processing improves upon this, with evidence of high retention of lactoferrin
There was no information on the impact of high-pressure processing on lipase activity, which will be an important area for future research.

Other markers for evaluating donor milk processing technologies include total protein, antimicrobial proteins (lysozyme and IgA), PUFAs, and heat-labile nutrients (lysine, thiamine, and vitamin C) that may contribute to poor outcomes in the preterm neonate. There is evidence that high-pressure processing retains lysozyme [22,27], IgA [21,23,25,27], PUFAs [24,28,29], vitamin C and E [28,29], and some cytokines [22,23,30], though no information is available regarding its impact on total protein, lysine, or thiamine. When the high-pressure processed human milk product becomes available, it may be preferential to Holder pasteurized donor human milk for feeding premature infants as it retains important immunological components that are destroyed during Holder pasteurization. This may help to improve outcomes of infants that are fed an exclusively donor human milk diet.

Outside of currently utilized technologies, HTST [32,38], UV-C [61], and ultrasound 58 [63] provide high retention of lactoferrin. Lipase retention has been reported as high with UV-C [61] and moderate with ultrasound when combined with heat [63]. There is conflicting evidence regarding the impact of HTST on lipase retention [32,38]. HTST has shown a high retention of total protein [14], available lysine [14,38], lysozyme and IgA [32,35], B vitamins [32], and growth factors [39] in human milk. There is evidence that microwave processing retains total protein [58], PUFAs, vitamin E, and thiamine [53], though there are conflicting findings on the impact on lysozyme and IgA [53,55,57], likely due to the wide variation in internal temperatures achieved during microwave processing. Many of the studies on microwave processing were done in the 1980s and 1990s with minimal temperature control and monitoring; therefore, technological advances may facilitate improved nutrient retention
with microwave processing. Flash heating has shown greater than 70% retention of IgA, vitamins A, C, E, thiamine, and folate, and moderate retention of riboflavin [48,51,52]. There are minimal studies regarding the impact of UV-C and ultrasound on human milk, though emerging evidence suggests greater than 75% retention of lactoferrin, lysozyme and IgA [61,63].

With each of the processing methods, retention of nutrients and bioactive components is different. Disregarding the pathogen eliminating capacities, it seems that high-pressure processing, HTST, and UV-C show promise as emerging technologies that may improve upon Holder pasteurization.

Nutritional Retention in Packaging

Current milk storage practices do not standardize the packaging materials with which human milk is stored. Unfortunately, this may compound the nutritional and immunological losses that are occurring during pasteurization. While there is minimal research on this topic, a majority of the research available investigates the effect of storage in glass, polyethylene, and polypropylene containers, with a few investigating polystyrene and stainless steel as options for human milk storage.

Retention of macronutrients is largely unexplored. One paper investigated fat loss in polyethylene and polypropylene containers [72], and found no difference in fat loss between containers. Similarly, only 3 studies investigated impact of container type (glass, polypropylene, polyethylene) on micronutrients [69,70,73]. Micronutrient loss was not different between glass and polypropylene containers, except for vitamin C, which was better retained in glass containers [69,70]. Riboflavin and vitamin A concentrations were
significantly reduced (50% and 70% respectively) when exposed to light in polyethylene containers [73]. Most of the research available investigated retention of cells and antimicrobial components.

Where cells are concerned, it seems that storage in glass containers is superior than storage in polyethylene or polypropylene containers for retention of macrophages, neutrophils and lymphocytes; however, there was no difference in phagocytic capacity of the cells when these three storage types were compared [64,66]. Glass containers did have a greater loss of leukocytes when compared to plastic containers [65], but were superior to stainless steel containers for cell survival and cell viability [67]. Surprisingly, polystyrene containers had a greater retention of cells (83%) than glass containers (54%) after 8-16 hours of storage [64], though polystyrene is not commonly used for human milk storage.

For antimicrobial components, polyethylene containers showed no change in concentration of lysozyme, lactoferrin, IgA, or sIgA after 24 hours, while glass and polypropylene containers had a 40% loss in lysozyme and 30% loss in lactoferrin, but no change in IgA or sIgA concentrations [66]. Surprisingly, samples stored in polyethylene did see a 60% reduction of sIgA binding to E. coli O antigens after 24 hours, while samples in glass and polypropylene containers did not see any reduction [66]. Glass containers retained more of the human milk bactericidal activity after 24 and 48 hours than polyethylene containers [68].

Conclusion

Packaging and processing human milk are an important and inevitable part of commercial human milk distribution. The available peer-reviewed literature supports the use
of light-shielding glass containers for storage of human milk, though there is still much to be explored where this topic is concerned. Processing methods, however, are not as cut and dry. Of the products available commercially, Holder pasteurization is the only processing technology that has peer reviewed evidence to show deactivation of HIV-1 and CMV. Holder pasteurization, however, is known to allow survival of *B. cereus*, forcing milk banks utilizing this technology to screen after pasteurization. In order to provide the safest donor human milk product, Holder pasteurization should be utilized and screening for *B. cereus* should be a mandatory post-processing step. High-pressure processing offers an improvement to Holder pasteurization for destruction of endogenous bacterial and maintenance of immunological components but should be thoroughly tested for the ability to deactivate HIV-1 and CMV prior to being utilized to create a donor human milk product.

Of the non-commercially utilized technologies, HTST has been shown to eliminate all endogenous pathogens, deactivate HIV-1 and CMV. HTST currently has the best evidence for a milk banking technology where this standard is concerned. Additionally, HTST offers an improvement to Holder pasteurization regarding retention of immunological components. Unfortunately, there is no commercial product available that utilized HTST for pasteurization. Flash pasteurization also shows promise as an improvement to Holder pasteurization, but more evidence is needed before drawing a conclusion.

With the recipient population of donor human milk being premature and medically fragile infants, it would be important to ensure pathogen elimination above nutrient retention *prior* to changing technologies. Currently, HTST has the best evidence for pathogen elimination and also improves upon nutrient and immunological component retention.
Additionally, the available evidence does suggest that human milk storage should be in light-shielded, glass containers to maximize nutrient retention.
References


CHAPTER 3: Comment and Response: Supplemental Feedings for High-Risk Preterm Infants

Lima HK, Cohen RS, Young TE. Comment and Response: Supplemental Feedings for High-Risk Preterm Infants. [JAMA Peds 2016]

To the Editor: Current literature shows that the use of a predominately (≥98%) human milk diet in very low-birth-weight infants supports appropriate growth and development, decreases rates of necrotizing enterocolitis (NEC), decreases overall patient care costs, allows for earlier discharge, reduces patient risk of re-admittance, and increases human milk consumption post-discharge\(^1\)-\(^4\). In their recent paper, Corpeleijn, et al.\(^5\) conclude that feeding premature infants a diet of pasteurized donor milk during the first few days of life had no significant impact on preventing adverse health events. When considering how this study will impact clinical practice, we would like to encourage health care providers to pay close attention to the following aspects: participant characteristics, duration of study, dose of donor milk or formula, and high rates of NEC observed.

Participant Characteristics. Infants at highest risk for developing complications were excluded from the study, and these infants may be the ones to benefit most from an exclusive human milk diet. Duration of the Study. The 10-day duration of the intervention is too short to assess effect on NEC rates. Large studies have shown that for very low birth weight babies, NEC most commonly presents much later than the first 10 days of life\(^2\). Colaizy, et al.\(^1\) extended their analysis out to 36-weeks postmenstrual age to simulate the highest risk period for NEC. Sullivan, et al.\(^3\) used either the entirety of the hospital stay, or a minimum observation period of 13 weeks in order to accurately assess outcomes stemming from diet differences.

Dose. In both treatment groups, the participants received approximately 90% of their diet as
mother’s own milk, which minimizes the impact of the assigned treatment diet on NEC outcomes. Also, the use of cow milk protein HMF mitigates the protective effect of donor milk\textsuperscript{4}. *Observed Rates of NEC.* Incidence of NEC was approximately 9\% in both treatment groups, indicating that perhaps something other than diet was involved in the development of NEC. Studies have shown that when an infant receives an exclusive human milk diet, NEC rates range from 1.3-7\%\textsuperscript{1-3}.

The above stated limitations are significant and severely restrict any conclusions that can be drawn about the safety and efficacy of donor human milk in VLBW infants. These conclusions only apply when approximately 90\% of the infant’s diet is mother’s own milk, when the intervention is administered for 10 days, and when the most fragile infants are not considered in the study.


CHAPTER 4: Bacteria and bioactivity in Holder pasteurized and shelf-stable human milk products

Lima HK, Wagner-Gillespie M, Perrin MT, Fogleman AD. Bacteria and bioactivity in Holder pasteurized and shelf-stable human milk products. [Curr Dev Nutr 2017]

Abstract

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

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

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

**Results:** One raw sample and 3 HP samples contained *B. cereus* at time of culture. There were no detectable bacteria in SS samples at time of culture. Lysozyme and sIgA activity were significantly greater in raw samples when compared to HP and SS samples (p < 0.0001). HP samples retained significantly more lysozyme and sIgA activity (54% and 87%, respectively) than SS samples (0% and 11% respectively).
Conclusions: Human milk processed using Holder pasteurization should continue to be screened for presence of *B. cereus*. Clinicians should be aware of the differences in the retention of lysozyme and sIgA activity in HP and SS products when making feeding decisions for medically fragile or immunocompromised infants to ensure patients are receiving the maximum immune protection.

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

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

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

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

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

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

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

**Methods**

The study received ethical approval from the North Carolina State University Institutional Review Board. Raw human milk was obtained from 60 approved donors through WakeMed Mothers’ Milk Bank in Cary, NC. When donating to WakeMed Mothers’ Milk Bank, donors consent that if their milk is unable to be used for medical purposes, their milk may be used for research studies. One sample from each mother was obtained and used to create a pool of raw human milk totaling 260 ounces. The samples were pooled in the WakeMed Mothers’ Milk Bank by a trained technician using a standard pooling protocol. Each individual sample was thawed in the refrigerator prior to pooling. Proper personal protective equipment was worn during handling of the milk per HMBANA guidelines. When samples were adequately thawed, they were moved under a sanitized laminar flow hood to prevent contamination during the pooling process. Samples were removed from original milk storage containers and transferred into 4000 mL beakers prior to complete thawing to minimize fat separation and ensure maximum transfer of contents. During thawing, temperatures were maintained at or below 4 °C to discourage additional bacterial growth. When all individual samples were completely thawed, they were combined into multiple wide mouth Erlenmeyer flasks and mixed using a pour down method six times (Figure 4.1a). Once mixed, samples were swirled gently to homogenize and they were strained prior to bottling (Figure 4.1b).
Twenty-four three-ounce samples were taken from the pooled milk and stored in Orthofix Axifeed 100 mL bottles (Product Number 022001010, Nolato Jaycare Limited, Portsmouth, United Kingdom). Twelve samples received no further treatment (RAW) and 12 samples were processed using Holder pasteurization (HP; 62.5 °C for 30 minutes) in an Ace Intermed Special Feed Pasteuriser (Model HMP2070-40HCUL, Handover, Hampshire, England) at the WakeMed Mothers’ Milk Bank, Cary, NC.

All milk was kept refrigerated whenever not being pooled or processed. The remaining pooled milk was transferred into 2000 ml Erlenmeyer flasks and put on ice for transport to North Carolina State University [less than 30 minutes] for bottling and retort processing to create a shelf-stable product. Upon arrival, the remaining pooled milk was gently swirled to

**Figure 4.1 (a)** WakeMed Mothers’ Milk Bank, Cary, NC standard mixing protocol for combination of four flasks of human milk (b) WakeMed Mothers’ Milk Bank, Cary, NC standard homogenization, straining, and bottling protocol for human milk.
homogenize (Figure 2.1b), and then poured into 12 ten-ounce aluminum cans, leaving 3-6 mm of air space at the top. Cans were sealed using a can sealer (Dixie Canner Company, Athens, GA) and retort processed to create a shelf-stable product (SS; 121 °C, 20 PSI for 5 minutes) using a Stock America, Inc. full water immersion retort processor (PR-I900, Raleigh, NC). After all processing was completed, all samples were aliquoted and stored at -80 °C until analysis. Note that all milk only underwent one freeze/thaw cycle to mimic the freeze/thaw cycle that occurs in a HMBANA milk bank. For analysis, each sample was analyzed for bacterial content per standard protocols at WakeMed Pathology Lab, sIgA activity, and lysozyme activity, as described below.

Bacterial Screening

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

Secretory Immunoglobulin A (sIgA) Activity

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

Lysozyme Activity

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

\[
\text{lysozyme (units/mL)} = \left[ \frac{\text{Average Change of Absorption}}{0.001 \times \text{volume of sample in mL}} \right] \times \text{dilution factor of milk samples}.
\]

Statistical Analysis

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

Results

Bacteria

Raw milk samples (RAW) were screened for the presence of *Bacillus cereus*, *Escherichia coli*, general appearance of *Enterococcus*, gram-negative rods, yeast, *Staphylococcus aureus*, and *Pseudomonas* sp. All RAW samples contained *Enterococcus* sp., gram-negative rods, yeast, and *Pseudomonas* sp. One RAW sample was found to contain *B. cereus*. These results were typical of raw human milk and served as a control for Holder pasteurized (HP) samples and shelf-stable (SS) samples (Table 4.1).
HP samples and SS samples went through a complete post-processing screen to characterize any bacteria present. Three samples of HP milk had growth of *B. cereus*. No other growth was observed in HP milk. Shelf-stable samples had no bacterial growth (Table 4.1).

**Table 4.1.** Results of bacterial analysis in raw human milk, Holder pasteurized human milk, and shelf-stable human milk

<table>
<thead>
<tr>
<th></th>
<th><em>Pseudomonas</em> species</th>
<th>Gram negative rods</th>
<th><em>Enterococcus</em> species</th>
<th>Yeast</th>
<th><em>Bacillus</em> cereus</th>
<th><em>Escherichia coli</em></th>
<th><em>Staphylococcus aureus</em></th>
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<tbody>
<tr>
<td>RAW⁴</td>
<td>12 (100)</td>
<td>12 (100)</td>
<td>12 (100)</td>
<td>12 (100)</td>
<td>1 (8.3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>HP⁵</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3 (25)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>SS⁶</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
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</table>

⁴Raw samples (unpasteurized)
⁵Holder pasteurized samples (62.5 °C for 30 minutes)
⁶Shelf-stable, retort processed samples (121 °C, 20 PSI for 5 minutes)

Secretory Immunoglobulin A Activity

Secretory IgA was measured in all samples, using RAW samples as a control. The analysis showed an average of 1.04 ± 0.09 mg active sIgA/mL in RAW samples, and was significantly more than HP and SS human milk from the same pool (p < 0.0001; 0.90 ± 0.03 and 0.11 ± 0.07 mg active sIgA/mL respectively; Figure 4.2).
Figure 4.2. Secretory immunoglobulin A (sIgA) activity (± SD) in raw (RAW), Holder pasteurized (HP) and shelf-stable (SS) human milk. Bars with different letters were significantly different (p < 0.001).

Lysozyme Activity

Lysozyme activity was measured in all samples using RAW samples as the control. RAW samples had an average lysozyme activity of 7969 ± 1394 units/mL and was significantly greater (p < 0.001) than both HP lysozyme activity (4269 ± 963 units/mL), and SS lysozyme activity (no activity detectible; Figure 4.3).
Figure 4.3. Lysozyme activity (± SD) in raw (RAW), Holder pasteurized (HP), and shelf-stable (SS) human milk. Bars with different letters were significantly different (p < 0.001).

**Discussion**

In a neonatal intensive care unit (NICU) it is imperative to scrutinize nutritional interventions to avoid expensive, life threatening complications [6,25]. With emerging options for human milk based feeding, evidence is needed on the full spectrum of feeding choices in order to inform best practice. Mother’s own milk is unequivocally the best option for premature infants [1,2]. Whenever possible, breastfeeding should be supported and encouraged if the mother’s goal is to breastfeed. When the mother faces obstacles establishing an adequate milk supply for her infant(s), donor human milk (DHM) provides a way for the medically fragile infant to maintain exclusively human milk feedings. The effects of various human milk processing methods on nutrient and bioactive retention may impact health outcomes and is an important area of future research.
Compared to raw human milk, our results show that human milk processed via Holder pasteurization retains more sIgA activity and lysozyme activity (HP; 90%, 54%) than shelf-stable human milk (SS; 10%, 0%). When compared to raw human milk, the reduction of activity observed in HP human milk is consistent with ranges reported in the literature [13,14]. As our study looked specifically at biological activity rather than concentration of lysozyme and sIgA, there was no published literature to use as a reference for expected values or ranges. Meredith-Dennis et al. found lower concentrations of IgA and no difference in lysozyme concentrations when comparing Holder and retort processed milk. These results may or may not be in agreement with our findings, as measured protein concentration can remain the same even when there is a loss of biological activity due to partial denaturation. In addition, this was a cross-sectional study with different donor pools represented in each treatment group; therefore, differences in milk composition cannot be specifically attributed to processing effects.

In our study, Holder pasteurization eliminated all bacteria except *Bacillus cereus*. It is understood that Holder pasteurization does not kill *B. cereus* and causes *B. cereus* spores to sporulate during heating [12,26]. HMBANA milk banks have chosen to continue using this method, and screen for and discard any batches that are positive for *B. cereus* post-processing to preserve nutritional value of the milk. Retort processing used to create the shelf-stable product eliminated all bacteria. Our study provides evidence that retort processing is effective at eliminating all bacteria from human milk, while Holder pasteurized DHM must continue to be screened pre- or post-processing for *B. cereus* to ensure its safety for consumption by medically fragile infants. The results for SS samples confirm that retort processed DHM is a sterile product [27].
The small sample size was a limitation to our study. However, clear patterns emerged regarding bioactivity retention during Holder pasteurization and retort processing. Additionally, this study only looked at two of many possible heat-sensitive bioactive components. Additional research is needed on other components in human milk to provide a more complete understanding of the impact of retort processing.

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


CHAPTER 5: Nutritional composition of Holder pasteurized and shelf-stable human milk products


Abstract

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

Objective: We aim to assess the nutritional composition of retort processed human milk and compare the nutritional profile to Holder pasteurized and raw human milk from the same pool of milk donors.

Study Design: Milk samples from 60 mothers were pooled. From this pool, 36 samples were taken; 12 samples were kept raw, 12 samples were Holder pasteurized (HP), and 12 samples were retort processed to create a shelf stable product (SS). Samples were analyzed for percent fat, percent solids, total protein, lactose, amino acid composition, and total thiamine.

Results: Percent fat, percent solids, and lactose were similar between raw, HP, and SS samples. Total protein was statistically increased in SS samples when compared to raw (p = 0.005) and HP (p < 0.001) samples, but protein differences are not clinically relevant (raw = 15.1, HP = 14.8 and RP = 15.8 mg/mL). Lysine was the only amino acid impacted by processing, and destruction increased as heat increased (raw = 0.85 mg/100 mL, HP = 0.77 mg/100 mL, SS = 0.68 mg/100 mL). Total thiamine was significantly decreased in SS samples (0.14 mg/L; p < 0.01) when compared to raw samples (0.24 mg/L) and HP samples (0.26 mg/L).
**Conclusion:** Macronutrient content is relatively unaffected by Holder pasteurization and retort processing. Both methods maintain similar fat, lactose, and total protein levels. Lysine was significantly impacted by retort processing, but not Holder pasteurization. Thiamine content was significantly impacted by retort processing, but not Holder pasteurization.

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

Human milk is considered the only necessary and complete source of nutrition for newborn infants through six months of age (1). When mothers deliver prematurely, the milk they produce is higher in protein, iron, sodium, chloride, and magnesium, offering specific nutritional benefits for the premature infant (2). Mothers who deliver prematurely can be at an increased risk for delayed lactogenesis II and/or low milk volume (3-5). In the event that a mother is experiencing delayed lactogenesis II or low milk volume, donor human milk (DHM) is a necessary secondary feeding option. Maintaining an exclusively human milk diet reduces the risk of the premature infants developing necrotizing enterocolitis, retinopathy of prematurity, and sepsis (6-8) and provides essential human-based immune components until the mother’s milk is available (9-11).

Until recently, all DHM available in a medical setting was provided by the Human Milk Banking Association of North America (HMBANA). Human milk that is distributed by HMBANA is screened and processed using Holder pasteurization (62.5 °C for 30 minutes). A majority of the human milk distributed by HMBANA is fed to medically fragile or premature infants and choosing a processing method that maintains the nutritional content is imperative for maintaining the total energy in the milk. Current literature shows that Holder pasteurization has no effect on the fat, saccharides (including oligosaccharides), or free amino acid content of human milk (12-14).

Currently, a retort-processed (121 °C, 15 PSI for 5 minutes) human milk product is available for use within hospital settings in the United States as a secondary feeding source when the mother’s milk is unavailable. The resulting product is shelf-stable, which provides specific benefits such as an increased shelf-life and lower cost of storage on site. To our
knowledge, the current literature contains only one study that compares the nutritional composition of retort processed, shelf-stable human milk to Holder pasteurized human milk (15). Meredith-Dennis et al. found that shelf-stable products had significantly less total protein than Holder pasteurized samples, but no difference in fat, carbohydrate, or energy concentrations. While this information is helpful, the study was small (n = 3 for each treatment) and all samples analyzed in this study originated from different pools of human milk.

The aim of this study was to quantitatively assess the effect Holder pasteurization and retort processing has on macronutrients and vitamin retention as assessed by thiamine concentration in human milk originating from the same pool of mothers.

**Materials and Methods**

The study received ethical approval from the North Carolina State University Institutional Review Board. Raw human milk was obtained from 60 approved donors through WakeMed Mothers’ Milk Bank in Cary, NC. One sample from each mother was obtained and used to create a pool of raw human milk totaling 260 ounces. The samples were pooled in the WakeMed Mothers’ Milk Bank by a trained technician using a standard pooling protocol (16).

Twenty-four 3 ounce samples were taken from the pooled milk and stored in Orthofix Axifeed 100 mL bottles (Product Number 022001010, Nolato Jaycare Limited, Portsmouth, United Kingdom). Twelve samples received no further treatment (raw) 12 samples were processed using Holder pasteurization (HP; 62.5 °C for 30 minutes) in an Ace Intermed Special Feed Pasteuriser (Model HMP2070-40HCUL, Handover, Hampshire, England) at the WakeMed Mothers’ Milk Bank, Cary, NC. All samples were stored at 0 °C until transported for analysis the same day.
The remaining pooled milk was put on ice for transport to North Carolina State University [less than 30 minutes] for bottling and retort processing to create a shelf-stable (SS) product. All methods are outlined in more detail in a separate manuscript (16). After all processing was completed, all samples were aliquoted and stored in the -80 °C freezer until analysis. Note that all milk only underwent one freeze/thaw cycle to mimic the freeze/thaw cycle that occurs in a HMBANA milk bank. Each sample was analyzed for percent fat, percent solids, total protein, lactose, amino acid content, and total thiamine.

Percent Fat and Percent Solids

Percent fat and percent solids were measured using a NMR-based Smart Trac (CEM, Matthews, NC) analyzer at the North Carolina State University Dairy Processing plant. Four grams of whole donor milk was loaded into the NMR chamber of the Smart Trac, which microwaves the sample for approximately 4 minutes. After this time, percent solids and percent fat are reported.

Total Protein

Total protein was measured using bicinchoninic acid (BCA) assay (Thermo-fisher, Fairlawn, NJ). Briefly, the working reagent was prepared per manufacturer instructions. Standards were prepared using bovine serum albumin (Sigma-Aldrich, St. Louis, MO) and standard curve ranged from 0-2.0 mg/mL. Human milk samples were diluted 1:10 with deionized water. To a 96 well plate (Costar 3590 Flat Bottom, High Binding 96-Well Plate, Corning Incorporated, Corning, NY) 25 μL of standard or sample was added, in triplicate, followed by 200 μL of working reagent. The plate was then covered with Parafilm and gently
agitated. The 96 well plate was incubated at 35 ºC for 30 minutes, cooled on ice for 10 minutes, and read on a spectrophotometer (Multiscan MCC, Fisher Scientific) at 562 nm.

Lactose

Lactose was measured using high pressure liquid chromatography (HPLC; Breeze 2, Waters Corporation, Milford, MA). All chemicals were purchased from Sigma-Aldrich, St. Louis, MO, unless otherwise noted. To a 250 µL Eppendorf tube, 0.9 mL of 0.0045 N H2SO4 was added to 0.1 mL of sample and centrifuged at 6,000 x G for 10 minutes. Supernatant was filtered through a 0.45 µg nylon membrane filter into a 2 mL HPLC vial. 20 µL of sample was injected onto an Aminex HPX-87H ion exclusion column (7.8 mm x 300 mm) with an isocratic flow of 0.0045N H2SO4 at 0.7 mL/min for a run time of 20 minutes. Lactose was measured using a Waters 2414 Refractive Index Detector. The final concentration was determined by standard curve development using pure lactose.

Total Amino Acids

All chemicals and reagents for amino acid content were purchased from Thermo-Fisher Scientific (Fairlawn, NJ) unless otherwise noted. All solvents and reagents were ACS grade or better. The samples were hydrolyzed to reduce all the protein present to amino acids using 6 N HCl containing 1% phenol (17). Aliquots (100 µL) were pipetted into glass hydrolyzer tubes and 4 mL of acid was added. Samples were digested in a Discover reaction system (CEM Corp., Matthews, NC) using a temperature gradient up to 145 ºC. After hydrolysis, samples were cooled in a stream of nitrogen. Digests were transferred to volumetric flasks and diluted to 25 mL with 0.02 N HCl. An aliquot of the dilution (400 µL) was transferred to a HPLC
autosampler vial and diluted to 2000 µL with additional 0.02 N HCl. The samples were injected for analysis onto a Hitachi L8900 amino acid analyzer (Hitachi High Technologies, Dallas, TX). A standard curve was created using Pierce H Amino Acid Standard mix from 0.2 µg/L to 20 µg/L of each amino acid by dilution with 0.02 N HCl and analyzed with the samples.

Total Thiamine

Thiamine was analyzed by UPLC (Acquity H-Class, Waters Corporation, Milford, MA) using a method modified from Fellman, et al. (18). All chemicals were purchased from Sigma-Aldrich, St. Louis, MO, unless otherwise noted. To a 250 µL Eppendorf tube, 46 µL of 60% perchloric acid was added to 500 µL of sample, vortexed for 1 minute, and centrifuged for 10 minutes at 12,000 RPM. Supernatant (400 µL) was transferred to a fresh tube, 140 µL of 1% potassium ferricyanide in 5M NaOH as added to the sample (prepared fresh on day of extraction) and the reaction was immediately quenched with 102 µL of phosphoric acid. Samples were then filtered through a 0.2 µm nylon filter into a sample vial. An Acquity HSS T3 column was used for separation (1.8 µm particle size, 2.1x100 mm). The column was maintained at 35 °C and was run at a flow rate of 0.4 mL/min. A gradient flow was used (Table 5.1). Mobile phase A consisted of 0.2 M monopotassium phosphate with 0.3 mM tetrabutylammonium hydrogen sulfate in H2O and was adjusted to a pH of 7 using potassium hydroxide. Mobile phases B and C were H2O and methanol respectively. Samples were detected using an Acquity FLR detector (Waters Corporation, Milford, MA). The detector was set to λex 365 nm and λem 435 nm. The standard curves were prepared using thiamine hydrochloride for free thiamine, and thiamine monophosphate chloride dihydrate for thiamine monophosphate. Total thiamine was calculated as the sum of thiamine monophosphate and
free thiamine. Concentrations prepared are shown in Table 5.2 and were adjusted to account for purity with a corrected concentration used for calculating the standard curve. The equation used and an example are provided in Figure 5.1 and Figure 5.2.

Table 5.1. Mobile phase gradient for the analysis of total thiamine

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% A</th>
<th>% B</th>
<th>% C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>50</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>1.00</td>
<td>20</td>
<td>70</td>
<td>10</td>
</tr>
<tr>
<td>3.00</td>
<td>15</td>
<td>60</td>
<td>25</td>
</tr>
<tr>
<td>7.00</td>
<td>15</td>
<td>60</td>
<td>25</td>
</tr>
<tr>
<td>8.00</td>
<td>50</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>13.00</td>
<td>50</td>
<td>40</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 5.2. Standard curve concentrations of thiamine hydrochloride and the corresponding corrected free thiamine concentration.

<table>
<thead>
<tr>
<th>As Thiamine Chloride (mg/L)</th>
<th>Free Thiamine Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.0079</td>
</tr>
<tr>
<td>0.02</td>
<td>0.0157</td>
</tr>
<tr>
<td>0.05</td>
<td>0.0393</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0786</td>
</tr>
<tr>
<td>0.2</td>
<td>0.1572</td>
</tr>
<tr>
<td>0.4</td>
<td>0.3144</td>
</tr>
<tr>
<td>0.6</td>
<td>0.4716</td>
</tr>
<tr>
<td>0.8</td>
<td>0.6288</td>
</tr>
</tbody>
</table>

\[ \text{Concentration of salt} \times \frac{1 \text{ mole}}{\text{molar mass of salt}} \times \frac{\text{molar mass of free base form}}{1 \text{ mole}} \times \% \text{ purity of standard} \]

Figure 5.1. Salt to free base correction equation

\[ \text{0.1 mg/L thiamine HCl} \times \frac{1 \text{ mole Thiamine HCl}}{337.27 \text{ g Thiamine HCl}} \times \frac{265.35 \text{ g Thiamine}}{1 \text{ mole Thiamine}} \times 99.9\% = 0.0786 \text{ mg/L Free Thiamine} \]

Figure 5.2. Salt to Free base correction example for Thiamine HCl.
Statistical Analysis

A statistical comparison of percent fat, percent solids, total protein, lactose, total amino acids, total available lysine, and thiamine concentration between raw milk, Holder pasteurized milk, and shelf-stable milk was done by one-way analysis of variance (ANOVA). Differences between means were tested for significance (α = 0.05) by the Tukey HSD test.

Results

Percent Fat and Percent Solids

Percent fat and percent solids were measured in all samples, using RAW samples as a control. The analysis showed an average of 12.37% solids and 3.94% fat (Figure 5.3) in RAW samples. Holder pasteurized were found to have 12.36% solids and 3.95% fat (Figure 5.3). Shelf-stable samples were found to have 12.29% solids 3.88% fat (Figure 5.3). There was not a statistically significant difference between treatments.

Total Protein

Total protein was measured in all samples, using RAW samples as a control. The analysis showed an average of 15.1 ± 0.16, 14.8 ± 0.08, and 15.8 ± 0.18 mg protein/mL human milk in RAW, HP, and SS samples, respectively (Figure 5.4). Statistically, SS samples had higher total protein than RAW and HP samples (p < 0.01). Values are all within 1 mg/mL of each other, and so do not differ clinically.
Figure 5.3. Percent solids and percent fat in raw (RAW), Holder pasteurized (HP), and shelf-stable (SS) human milk.

Figure 5.4. Average protein in raw (RAW), Holder pasteurized (HP), and shelf-stable (SS) human milk. Bars with different letters were significantly different (p < 0.01).
Lactose

Lactose was measured as a marker of carbohydrate retention, using RAW samples as the control. The analysis showed 0.647 ± 0.02, 0.623 ± 0.09, and 0.650 ± 0.03 g lactose/10 mL human milk for RAW, HP, and SS samples, respectively (Figure 5.5). There was not a statistically significant difference between treatments.

![Figure 5.5. Total lactose in raw (RAW), Holder pasteurized (HP), and shelf-stable (SS) human milk.](image)

Total Amino Acids

Amino acid concentrations were measured in RAW, HP, and SS human milk samples, with RAW samples used as a control. Lysine was the only amino acid that was significantly impacted by heat exposure, with decreasing lysine concentration as level of heat exposure increased. A significant reduction observed in SS samples when compared to RAW samples (Table 5.3).
Table 5.3. Average total amino acid (mg/100 mL ± standard deviation) content of raw, Holder pasteurized, and shelf-stable human milk samples. Samples with different superscripts in the same row are significantly different (p < 0.05).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Raw ± SD</th>
<th>Holder ± SD</th>
<th>Shelf-stable ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>39.0 ± 9.06</td>
<td>35.3 ± 10.7</td>
<td>34.0 ± 7.10</td>
</tr>
<tr>
<td>Arginine</td>
<td>33.8 ± 8.37</td>
<td>32.0 ± 9.73</td>
<td>31.6 ± 6.34</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>103 ± 24.9</td>
<td>97.3 ± 27.1</td>
<td>95.0 ± 18.4</td>
</tr>
<tr>
<td>Cystine</td>
<td>9.76 ± 5.74</td>
<td>13.4 ± 7.36</td>
<td>13.9 ± 10.1</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>220 ± 48.2</td>
<td>201 ± 54.2</td>
<td>197 ± 40.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>23.9 ± 5.77</td>
<td>21.6 ± 6.83</td>
<td>21.1 ± 5.02</td>
</tr>
<tr>
<td>Histidine</td>
<td>33.6 ± 4.31</td>
<td>32.4 ± 7.47</td>
<td>30.5 ± 3.38</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>56.6 ± 14.7</td>
<td>45.9 ± 19.6</td>
<td>48.1 ± 12.4</td>
</tr>
<tr>
<td>Leucine</td>
<td>129 ± 27.3</td>
<td>113 ± 32.7</td>
<td>112 ± 23.2</td>
</tr>
<tr>
<td>Lysine</td>
<td>85.5 ± 12.1 a</td>
<td>76.2 ± 17.8 ab</td>
<td>70.1 ± 9.35 b</td>
</tr>
<tr>
<td>Methionine</td>
<td>21.1 ± 7.49</td>
<td>22.0 ± 12.3</td>
<td>18.1 ± 5.37</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>58.3 ± 11.2</td>
<td>51.5 ± 12.9</td>
<td>52.7 ± 9.94</td>
</tr>
<tr>
<td>Proline</td>
<td>93.7 ± 26.7</td>
<td>83.3 ± 27.8</td>
<td>85.2 ± 20.5</td>
</tr>
<tr>
<td>Serine</td>
<td>21.4 ± 7.91</td>
<td>29.6 ± 16.0</td>
<td>29.2 ± 8.11</td>
</tr>
<tr>
<td>Threonine</td>
<td>31.1 ± 8.54</td>
<td>30.1 ± 10.9</td>
<td>33.3 ± 5.77</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>60.1 ± 9.84</td>
<td>55.2 ± 10.9</td>
<td>55.8 ± 7.80</td>
</tr>
<tr>
<td>Valine</td>
<td>69.5 ± 18.9</td>
<td>58.3 ± 23.0</td>
<td>62.3 ± 16.1</td>
</tr>
</tbody>
</table>

Total Thiamine

Total thiamine concentration, thiamine monophosphate (TMP) concentration, and free thiamine concentrations were measured in RAW, HP, and SS human milk samples, using RAW samples as a control. Total thiamine, TMP, and free thiamine were all significantly reduced in SS samples when compared to RAW and HP samples (p < 0.05; Figure 5.6). HP samples had similar total thiamine, TMP, and free thiamine concentrations as RAW samples.
Figure 5.6. Total thiamine, thiamine monophosphate, and free thiamine concentrations in raw (RAW), Holder pasteurized (HP), and shelf-stable (SS) human milk. Bars with different letters were significantly different (p < 0.05).

Discussion

Donor human milk is a necessary secondary feeding option for premature and medically fragile infants. In 2014 in the United States, a shelf-stable donor human milk product became available. Understanding the effect of each processing method on nutritional components of human milk is important so that clinicians can create an evidence-based feeding plan that best suits their patient. This research shows that shelf-stable human milk processed at 121 °C and 15 PSI for 5 minutes has a similar macronutrient content to human milk processed by Holder pasteurization (62.5 °C for 30 minutes) when lactose, total protein, total fat, and total solids are considered. However, lysine and thiamine loss may be of concern when feeding infants this product.
When protein is analyzed on an amino-acid level, lysine is significantly reduced during heat exposure, with decreasing lysine levels as the temperature of heat used to pasteurize increased. Chemical changes that occur during heating that can affect the bioavailability of lysine in human milk include the Maillard reaction (19-21) and LAL formation (21). The current recommended daily allowance (RDA) for lysine is 640 mg/day (22). Our results show that to meet the needs of a newborn weighing 2.5 to 4.5 kg, the infant would need to consume 0.75, 0.84, and 0.91 liters of raw, Holder pasteurized, and shelf-stable human milk per day, respectively. Average human milk intake ranges from 0.71-0.8 liters per day (23). As the protein requirements are increased for premature infants, and their ability to intake milk volume is decreased, the loss of lysine in Holder pasteurized and shelf-stable products may be an issue for premature or low-birth-weight infants.

Total thiamine, TMP, and free thiamine were all significantly reduced in SS samples when compared to both HP and raw samples. Thiamine is a heat sensitive vitamin, and exposure to heat can result in hydrolytic cleavage to pyrimidine and thiazole derivatives (24) or complete destruction to produce hydrogen sulfide, elemental sulfur, or other minor products (25). When thiamine is exposed to heat and undergoes hydrolytic cleavage or complete destruction, the metabolic value is lost. The adequate intake (AI) of thiamine for infants age 0-6 months is currently set at 0.2 mg/day (26). With the values measured in SS samples, infants would need to consume 1.4 liters of human milk per day to meet the AI. This is concerning, as many infants consume an average of 0.71-0.8 liters per day (23). The loss of thiamine due to retort processing must be considered when using a shelf-stable product as the sole feeding source for an infant.
To our knowledge, this is the first peer-reviewed study conducted that investigates the effect of retort processing on nutritional components in human milk. The study design allows for comparison of nutritional components between treatment groups and uses the raw samples as a baseline for starting values. Our results show that for a healthy, term infants, shelf-stable human milk may be a viable option to maintain exclusive human milk feeds while the mother develops her milk supply. However, loss of lysine and thiamine raise concerns about the efficacy of shelf-stable milk as a feeding option for premature or medically fragile infants.
References


CHAPTER 6: Effect of light exposure during pumping and Holder pasteurization on vitamin concentrations in human milk


Abstract

Background: Donor human milk is a necessary secondary feeding option when mother’s own milk is unavailable. To ensure safety for premature and medically fragile infants, milk banks pasteurize donated milk. During pumping, storage, and pasteurization all milk is exposed to light and currently, milk banks do not regulate light exposure. Riboflavin, thiamine, and vitamin A are vitamins that are highly light labile.

Objective: To determine if light exposure during pumping, storage, and pasteurization in a milk bank setting causes degradation of vitamins in human milk.

Study Design: Thirteen eligible participants donated four milk samples each: 2 light-exposed (E) and 2 light-protected (P) samples. One E and one P sample from each participant were kept raw (R), while one E and one P sample from each participant were subjected to 3 hours of daytime light and Holder pasteurization (H). Subsequently, all samples were analyzed for riboflavin, thiamine, retinol, γ-tocopherol, α-tocopherol, and β-carotene content.

Results: Total riboflavin was significantly (p < 0.05) impacted by light exposure in both raw and pasteurized samples (62.1 µg/L and 73.7 µg/L, respectively) when compared to light protected raw samples (99.68 µg/L). Thiamine content was significantly (p < 0.05) impacted by light exposure and heat exposure, however, concentrations were not reduced to levels that would cause clinical concern. Retinol, γ-tocopherol, α-tocopherol, and β-carotene were not impacted by the level of light exposure utilized in this study.
**Conclusion:** Our results indicate that protecting human milk samples from light exposure during pumping, home storage, and transport to a donor human milk bank may help retain the riboflavin present in human milk. Additionally, milk may have higher concentrations of riboflavin if protected from light exposure during the pasteurization process.

**Keywords:** light exposure; infant nutrition; donor human milk; pumping; human milk storage
Introduction

When a mother is unable to provide her own milk for a premature or medically fragile infant, providing donor human milk to maintain an exclusively human milk diet can reduce rates of necrotizing enterocolitis, sepsis, retinopathy of prematurity, and reduce the length of the hospital stay (1-4). Under the guidelines set by the Human Milk Banking Association of North America (HMBANA), all donated human milk must undergo Holder pasteurization (62.5 °C for 30 minutes) and post-pasteurization screening to ensure destruction of harmful pathogens, including HIV-1, cytomegalovirus, and Bacillus cereus (5-7).

During the collection, storage, and pasteurization process all human milk is exposed to light. Research conducted on bovine milk provides evidence that light exposure can cause degradation of nutrients (8-10). Riboflavin and vitamin A are particularly susceptible to photooxidation, and losses of 40% and 60%, respectively, have been recorded in human milk when stored in plastic bottles that are exposed to light for 7 hours (11). Once riboflavin has undergone photooxidation, it can act as a photosensitizer and cause further degradation of thiamine, among other nutrients (12). Thiamine is sensitive to both heat and light (13, 14), and degradation can be increased once riboflavin has been oxidized (12). Additional losses of light sensitive vitamins may occur if the infant is being fed processed donor human milk by nasogastric tube and/or is being treated for hyperbilirubinemia using phototherapy (11, 15).

Riboflavin plays an important metabolic role as a cofactor in the form of FMN and FAD and inadequate intakes would lead to abnormal intermediary metabolism (16) and riboflavin-responsive anemia in human has been described previously (17, 18). Vitamin A plays an important role in eyesight, with early signs of vitamin A deficiency including night-blindness, and extended deficiency causing blindness (19). As of 2005, vitamin A deficiency was the
leading cause of preventable childhood blindness (20). γ-Tocopherol, α-tocopherol, and β-carotene, are important fat-soluble dietary components as they function as antioxidants and destruction of these components could increase susceptibility of other nutrients to oxidation and further degradation (21); additionally, β-carotene can be converted to vitamin A (22). Thiamine deficiency in humans can cause cardiac and muscular issues and increase the risk for respiratory infections and neurologic issues. (14). Milk banks in the United States and around the world expose human milk to light during pasteurization, therefore it is important to quantify nutrient losses and suggest methods for increasing retention.

Light exposure is possible at any point after milk is removed from the breast, including during pumping, storage (depending on storage container), transport to the milk bank, thawing, pooling, pasteurization, and feeding. Currently, organizations processing donor human milk do not have guidelines to limit light exposure of donated samples or guidelines for limiting light exposure after accepting samples. WakeMed Mothers’ Milk Bank (Cary, NC) reported an average of 3 hours of light exposure for any given sample of human milk from intake to disbursement (23). The purpose of this study was to determine if pumping in a low-light environment and storing and pasteurizing in light-shielding containers helps protect riboflavin, thiamine, and retinol from light oxidation. Conclusions from this study will help determine whether mothers pumping and storing their milk in a home setting or organizations distributing donor human milk should monitor and control exposure to light.

Materials and Methods

The study received ethical approval from the North Carolina State University Institutional Review Board. Human milk samples were collected from 13 eligible participants in the Raleigh area. To be included in the study, individuals had to have no dietary restrictions
and be breastfeeding or pumping their milk for a child born in good health at greater than 38 weeks gestation. The child receiving the milk needed to be greater than 4 weeks of age and pumping needed to be a part of the participants’ average weekly routine. Each participant provided four samples, two pumped in normal light conditions and stored in clear polyethylene containers; two pumped in low light conditions and stored in opaque Purity milk storage bags. One exposed and one protected sample were kept raw and exposed to light only briefly during pumping and storage, while one exposed and one protected sample were subjected to 3 hours of direct daytime light and Holder pasteurization. Samples were exposed to 3 hours of daytime light based on average estimates of light exposure during pasteurization at WakeMed Mothers’ Milk Bank (Cary, NC). This resulted in four treatments of light exposed, raw (ER), light protected raw (PR), light exposed pasteurized (EH), and light protected pasteurized (PH). All samples, including samples that were not pasteurized, were transferred to either clear glass or amber glass bottles prior to pasteurization. Sample collection and processing methods are summarized in Figure 6.1. After processing, all samples were stored in -80 °C until analysis for vitamin A, riboflavin, and thiamine was completed.

**Figure 6.1.** Sample collection and processing overview.
Retinol, γ-tocopherol, α-tocopherol, and β-carotene

Retinol, γ-tocopherol, α-tocopherol, and β-carotene in human milk were analyzed by using a method modified from Turner and Burri (21). Briefly, 100 µL of human milk is added to 1 mL of 0.1% butylated hydroxytoluene (BHT) in ethanol, 100 µL of 10% pyrogallol in ethanol, and 200 µL 30% potassium hydroxide in water. Samples were saponified in darkness at 45 °C for 30 minutes. After cooling the samples in an ice bath, 700 µL water, 3 mL of hexane, and 1 mL of tocol (internal standard, 1 µg/mL in hexane) were added. Samples were mixed for 30 seconds and then centrifuged at 1800 RPM at 4 °C for 2 minutes to achieve phase separation. The upper hexane layer (~3 mL) was transferred into a fresh vial and dried under a gentle nitrogen stream. Dried samples were reconstituted in 50 µL acetonitrile and analyzed. Sample extracts were kept at 5 °C and 20 µL were analyzed using an Agilent 1260 Infinity HPLC coupled with a diode array detector (tocol, tocopherols: 295 nm, retinol: 325 nm, β-carotene: 450 nm) using an Agilent Eclipse Plus C18 column (100x2.1 mm, 3.5 µm) and Thermo Scientific Javelin Guard BDS-Hypersil C18 guard column (20x3 mm, 3 µm) at 15 °C. The mobile phase was isocratic using acetonitrile/methylene chloride/methanol 7/2/1 (v/v/v) at a flow rate of 0.6 mL/min for 6.4 min.

Riboflavin

Riboflavin concentrations in samples were quantified using UPLC with fluorescence detection. All chemicals were purchased from Sigma-Aldrich unless otherwise stated. A 0.5 mL aliquot of human milk was added to 0.75 mL trichloroacetic acid and vortexed for 30 seconds. Samples were then centrifuged for 10 minutes at 14,000 RPM and aqueous layer was filtered into a sample vial through a 0.2 µm nylon filter. Analysis was done using a UPLC
(Acquity H-Class, Waters Corporation, Milford, MA). An Acquity HSS T3 column was used for separation (1.8 µm particle size, 2.1x100 mm). The column was maintained at 35 °C and was run at a flow rate of 0.35 mL/min. A gradient flow was used (Table 6.1). Mobile phase A, consisted of 0.2 M monopotassium phosphate with 0.3 mM tetrabutylammonium hydrogen sulfate in H₂O and was adjusted to a pH of 7 using potassium hydroxide. Mobile phases B and C were H₂O and methanol respectively. Samples were detected using an Acquity FLR detector (Waters Corporation, Milford, MA). The detector was set to λex 280 nm and λem 525 nm. The standard curve was prepared using riboflavin and flavin adenine dinucleotide (FAD).

Table 6.1. Mobile phase gradient for analysis of total riboflavin.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
<th>%C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>50</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>0.50</td>
<td>20</td>
<td>70</td>
<td>10</td>
</tr>
<tr>
<td>1.00</td>
<td>15</td>
<td>60</td>
<td>25</td>
</tr>
<tr>
<td>5.00</td>
<td>15</td>
<td>60</td>
<td>25</td>
</tr>
<tr>
<td>8.00</td>
<td>10</td>
<td>20</td>
<td>70</td>
</tr>
<tr>
<td>10.00</td>
<td>10</td>
<td>20</td>
<td>70</td>
</tr>
<tr>
<td>11.00</td>
<td>50</td>
<td>40</td>
<td>10</td>
</tr>
</tbody>
</table>

Thiamine

Thiamine was analyzed by UPLC (Acquity H-Class, Waters Corporation, Milford, MA) using a method modified from Fellman, et al. (25). All chemicals were purchased from Sigma-Aldrich, St. Louis, MO, unless otherwise noted. Initially, 46 µL of 60% perchloric acid was added to 500 µL of sample, vortexed for 1 minute, and centrifuged for 10 minutes at 12,000 RPM. Subsequently, 400 µL of supernatant was transferred to a fresh tube and 200 µL ethanol was added and briefly mixed. A 140 µL portion of 1% potassium ferricyanide in 5M NaOH as added to the sample and the reaction was immediately quenched with 102 µL of
phosphoric acid. Samples were then filtered through a 0.2 µm nylon filter into a sample vial. An Acquity HSS T3 column was used for separation (1.8 µm particle size, 2.1x100 mm). The column was maintained at 35 °C and was run at a flow rate of 0.4 mL/min. A gradient flow was used (Table 6.2). Mobile phase A consisted of 0.2 M monopotassium phosphate with 0.3 mM tetrabutylammonium hydrogen sulfate in H₂O and was adjusted to a pH of 7 using potassium hydroxide. Mobile phases B and C were H₂O and methanol respectively. Samples were detected using an Acquity FLR detector (Waters Corporation, Milford, MA). The detector was set to λex 365 nm and λem 435 nm. The standard curves were prepared using thiamine hydrochloride for free thiamine, and thiamine monophosphate chloride dihydrate for thiamine monophosphate. Concentrations of thiamine chloride prepared are shown in Table 6.3 and were adjusted to account for purity with a corrected concentration used for calculating the standard curve (Figure 6.2 and 6.3).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% A</th>
<th>% B</th>
<th>% C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>50</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>1.00</td>
<td>20</td>
<td>70</td>
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</tr>
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<td>3.00</td>
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<td>25</td>
</tr>
<tr>
<td>8.00</td>
<td>50</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>13.00</td>
<td>50</td>
<td>40</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 6.3. Standard curve concentrations of thiamine hydrochloride and the corresponding corrected free thiamine concentration.
<table>
<thead>
<tr>
<th>As Thiamine Chloride (mg/L)</th>
<th>Free Thiamine Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.0079</td>
</tr>
<tr>
<td>0.02</td>
<td>0.0157</td>
</tr>
<tr>
<td>0.05</td>
<td>0.0393</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0786</td>
</tr>
<tr>
<td>0.2</td>
<td>0.1572</td>
</tr>
<tr>
<td>0.4</td>
<td>0.3144</td>
</tr>
<tr>
<td>0.6</td>
<td>0.4716</td>
</tr>
<tr>
<td>0.8</td>
<td>0.6288</td>
</tr>
</tbody>
</table>

\[
\text{Concentration of salt} \times \frac{1 \text{ mole}}{\text{molar mass of salt}} \times \frac{\text{molar mass of free base form}}{1 \text{ mole}} \times \% \text{ purity of standard}
\]

**Figure 6.2.** Salt to free base correction

\[
0.1 \text{ mg/L thiamine HCl} \times \frac{1 \text{ mole thiamine HCl}}{337.27 \text{ g thiamine HCl}} \times \frac{265.35 \text{ g thiamine}}{1 \text{ mole thiamine}} \times 99.9\% = 0.0786 \text{ mg/L Free Thiamine}
\]

**Figure 6.3.** Salt to Free base correction example for Thiamine HCl.

**Statistical Analysis**

Power calculations were performed using literature reported values and standard deviations of riboflavin, thiamine, and vitamin A in raw and Holder pasteurized human milk prior to the start of this trial to determine the number of samples that would be necessary to obtain statistical significance if light exposure was to impact riboflavin, thiamine, or vitamin A concentrations in human milk. This power calculation indicated that the effects of light would be detectable with 8-10 mothers. Log transformations were used to normalize data and pairwise comparisons of riboflavin, thiamine, and vitamin A between samples was done by two-factor analysis of variance (ANOVA) accounting for mother-to-mother variation using SAS 9.4 (Cary, NC). Results were considered statistically significant at \( p < 0.05 \).
Results

Retinol

Retinol concentrations were significantly (p < 0.05; data not shown) different between mothers, however concentrations were not affected by light exposure or heat in our samples (Figure 6.4). Average concentrations were 0.66, 0.59, 0.55, and 0.55 mg/L for PR, ER, PH, and EH treatments, respectively.

β-carotene

β-carotene concentrations were significantly (p < 0.05) different between mothers (data not shown), however concentrations were not affected by light exposure or heat in our samples (Figure 6.5). Average concentrations were 0.04, 0.06, 0.04, and 0.06 mg/L for PR, ER, PH, and EH treatments, respectively.

Figure 6.4. Average retinol concentrations in protected, raw (PR), protected, Holder pasteurized (PH), exposed, raw (ER), and exposed, Holder pasteurized (EH) human milk samples. Concentrations were not statistically different between treatments.
γ-tocopherol and α-tocopherol

γ-tocopherol and α-tocopherol concentrations were significantly (p < 0.05) different between mothers (data not shown), however concentrations were not affected by light exposure or heat in our samples (Figure 6.6). Average concentrations of γ-tocopherol were 3.66, 2.69, 2.51, and 2.83 mg/L for PR, ER, PH, and EH treatments, respectively. Average concentrations of α-tocopherol were 5.12, 4.13, 4.37, and 4.51 mg/L for PR, ER, PH, and EH, respectively.

Figure 6.5. Average β-carotene concentrations in protected, raw (PR), protected, Holder pasteurized (PH), exposed, raw (ER), and exposed, Holder pasteurized (EH) human milk samples. Concentrations were not statistically different between treatments.
Figure 6.6. Average γ-tocopherol and α-tocopherol concentrations in protected, raw (PR), protected, Holder pasteurized (PH), exposed, raw (ER), and exposed, Holder pasteurized (EH) human milk samples. Concentrations were not statistically different between treatments.

Riboflavin

Total riboflavin was significantly (p < 0.05) impacted by mother (Chapter 6 Appendix F), as well as light exposure in both raw (ER) and pasteurized (EP) samples (62.1 µg/L and 73.7 µg/L, respectively) when compared to light protected raw samples (PR) (99.68 µg/L; Figure 6.7). While PH samples did not retain significantly more riboflavin than EH samples (p > 0.05), average concentrations were greater in PH samples than in EH samples, and average riboflavin in PH samples was statistically similar to PR samples (Figure 6.7).
Figure 6.7. Average total riboflavin concentrations in protected, raw (PR), protected, holder pasteurized (PH), exposed, raw (ER), and exposed, holder pasteurized (EH) human milk samples. Bars with different letters were significantly different (p < 0.05).

Thiamine

Total thiamine was significantly (p < 0.05) impacted by mother (data not shown) as well as light exposure and heat exposure in our samples (Figure 6.8 and 6.9). Average total thiamine concentrations were 0.25 and 0.24 mg/L for light protected and light exposed samples, respectively. Average total thiamine concentrations were 0.26 and 0.23 mg/L for raw and pasteurized samples, respectively.
Figure 6.8. Average total thiamine concentration in light protected and light exposed human milk samples. Bars with different letters were significantly different (p < 0.05).

Figure 6.9. Average total thiamine concentration in raw and Holder pasteurized human milk samples. Bars with different letters were significantly different (p < 0.05).
**Discussion**

Current human milk banking guidelines do not regulate the exposure of milk to light during the pumping, storage, transport, pasteurization, or distribution process. Unfortunately, there are micronutrients in human milk that are prone to destruction due to light oxidation (8). Literature reported values of riboflavin, thiamine, γ-tocopherol and α-tocopherol, β-carotene, and vitamin A (retinol) were between 90 and 330 µg/L (26, 27), 150-240 µg/L (26, 27), 2.07-2.92 mg/L (28, 29), 2.70-4.41 mg/L (30), 0.01-0.02 mg/L (30), and 650-910 µg/L (31) in human milk, respectively. Studies have shown water-soluble vitamin content of human milk to be influenced by maternal diet (32, 33) and fat-soluble vitamin content can be influenced by the mother’s ability to completely empty her breast of milk during a pumping session, as hindmilk is richer in fat than foremilk (34). Our statistical model accounted for variation between mothers to control for these differences. Our results show that retinol, tocopherols, and beta-carotene were not affected by the level of light exposure experienced in this trial. Thiamine was significantly affected by light exposure, however levels were still within expected ranges (0.23-0.26 mg/L) to provide infants with adequate thiamine if they were taking in between 750-850 mL human milk/day.

Our results indicate that riboflavin concentrations in human milk are reduced to levels that may be of clinical concern due to light exposure during pumping, storage, or current milk bank processing methods. The riboflavin content in the light protected samples ranged from 21 to 280 µg/L and was significantly different between mothers (p < 0.0001; Chapter 6 Appendix F). Raw samples exposed to light and pasteurized samples exposed to light had an average of 38% and 25% less riboflavin compared to raw light protected samples, respectively. A significant reduction in riboflavin concentrations in ER samples provides evidence that light
exposure during the pumping and initial storage period, including when milk is used in a home setting, may cause loss of riboflavin due to light oxidation. These results also indicate that utilizing low-light conditions during pumping and storing pumped milk in light-shielding containers will help to prevent riboflavin loss due to light oxidation. A significant reduction in riboflavin concentrations in EH samples provides evidence that light exposure during the pasteurization process may cause loss of riboflavin due to light oxidation. There was no statistical difference between PH and EH samples. Despite the lack of statistical significance, future research needs to be done to determine if light protection after donated human milk arrives at the milk bank may still help to prevent some loss of riboflavin during processing.

The results of this study provide evidence that light exposure is a concern when mothers are pumping and storing their milk for later use and when milk banks are processing human milk. The adequate intake (AI) for infants 0-6 months of age is 300 µg/day, 200 µg/day, 4 mg/day, and 400 µg RAE OR 400 µg/day for riboflavin, thiamine, vitamin E and β-carotene OR vitamin A, respectively. A healthy, term infant will consume an average of 710-803 mL per day (35), which means that milk provided from a well-nourished mother will need to retain all of the riboflavin in order to be adequate as the sole source of these nutrients for an infant.

Pumping in low light conditions and storing in light-shielded conditions, such as opaque milk storage bags, amber containers, or clear containers wrapped in aluminum foil, will help to protect against degradation of nutrients due to light exposure. The practice of pumping in low-light conditions and storing pumped milk in light-shielding containers is advisable even if mothers are pumping and storing milk just for use at home. While it is challenging to control the conditions in which mothers’ pump and store their milk prior to donation, milk banks can protect from additional light exposure once milk is at the milk bank.
by ensuring that blinds are closed during processing, amber bulbs are used for lighting during processing, and human milk is stored and processed in light-shielding containers. Additionally, milk banks can provide education about best practices for pumping and storing milk for future donations. It is also possible that milk banks could provide replacement milk storage bags to mothers that are light-shielding when feasible.

Clinicians should be aware of the possible micronutrient differences between donor human milk and fresh milk provided by the mother and encourage the mother to express her milk in low-light conditions and provide her milk in light-shielding containers whenever possible. Premature infants who are receiving 100% of their nutrition through unfortified donor human milk may need to consider adding a fortifier to ensure adequate levels of micronutrients are available to the infant for growth and development. This study also raises concerns of micronutrient status of infants receiving 100% of their nutrition through clear, nasogastric tubing, as riboflavin loss may be possible. Future research is needed to determine if light exposure in a home setting followed by light protection during pasteurization helps to decrease the loss of riboflavin compared to light exposure at home and during pasteurization, as this combination of treatments was not tested in this study. The retinol results in this study contradict previous research which showed a steady decrease in vitamin A concentrations during light exposure (11) and additional studies are necessary to determine the true effect of light during milk bank pasteurization. Furthermore, research should be conducted regarding parents’ willingness and ability to protect the milk from light.
References


23. Wagner-Gillespie M. Average amount of time human milk is exposed to light at WakeMed Mothers' Milk Bank in Cary, NC. Personal communication 2017;


APPENDICES
Chapter 6 Appendix A: Eligibility Survey

Eligibility Survey

1. What is your name?

2. Please provide the best contact phone number and email address.

3. Are you currently breastfeeding and/or pumping to provide your breast milk for your own child?
   a. Yes
   b. No

4. Was your child born after 38 weeks gestation?
   a. Yes
   b. No

5. Was your child in good health at birth?
   a. Yes
   b. No

6. Is your child currently in good health?
   a. Yes
   b. No

7. Is your child older than four weeks of age?
   a. Yes
   b. No

8. Do you currently pump as a part of your average weekly routine?
   a. Yes
   b. No

9. List below any dietary restrictions you have.
Chapter 6 Appendix B: Informed Consent

Effect of Light Exposure During Collection, Storage, and Holder Pasteurization on Vitamin Levels in Human Breast Milk – Informed Consent

North Carolina State University
INFORMED CONSENT FORM for RESEARCH

Title of Study: Effect of Light Exposure During Collection, Storage, and Holder Pasteurization on Vitamin Levels in Human Breast Milk
Principle Investigator: Hope Lima, Dr. April Fogleman
Faculty Sponsor: Dr. April Fogleman

Purpose of the study
You are invited to participate in a research study. The purpose of this study is to better understand how light exposure influences the vitamin content of human milk during pumping, storage, and pasteurization at a donor human milk bank. Samples of your donated breast milk will be subjected to experimental procedures to determine levels of light sensitive vitamins when collected in clear and light protected containers. The objective of this study is to provide evidence based research for the type of container used to collect and store human milk, as well as to inform practices for donor human milk pasteurization, to maximize the nutritional content of the stored breast milk.

What will happen if you take part in the study?
You are eligible for this study if you are currently breastfeeding or pump/bottle feeding your child, if you have home access to a breast pump, if your infant was born healthy, at full term, and is currently 4 weeks of age or older, and if you are not currently eating a vegan or vegetarian diet.

If you participate in this study, you will receive a packet via snail mail with collection containers and you will be asked to express the entire content of both breasts for four (4) consecutive mornings. Two (2) of these samples will be collected in low-light conditions (dimmed room with no open windows) and transferred into a light protected container that we will provide. Two (2) of these samples will be collected in your usual pumping conditions and transferred into a clear container that we provide. Each sample will need to be labeled with our provided labels and will have your randomly assigned personal identification number to protect your identity, the time and date the sample was collected, and the approximate number of times you will breastfeed or pump that day. You will store each sample in your personal freezer, and after collecting the fourth sample you will contact Hope Lima to arrange a pick-up of your samples and will be compensated at this point.

Risks
There are minimal risks associated with participating in this study.

Benefits
Currently, breast milk collection containers are clear and do not protect the expressed breast milk from light exposure. In situations of personal use and storage, as well as donor human milk banking protocols, this may change the nutrient content of the breast milk. Knowledge
gained in this study will help to determine if light protected containers are necessary to maintain the nutritional integrity of the breast milk and ensure the optimal nutrition for infants receiving the milk.

Confidentiality
The information in the study records will be kept strictly confidential and destroyed upon completion of the analysis and publication of the results. Results of our analysis will be secured and is available only to the researchers for this study unless you specify otherwise. Data will be stored electronically on the departmental server, the Principal Investigator’s research computer, and an external hard drive. All computers and servers are password protected and available only to authorized personnel. **No reference will be made in oral or written reports which could link you to the study.**

Compensation
Upon collection of the four samples, you will be compensated with a $20 Visa gift card.

What if you have questions about this study?
If you have questions at any time about the study or the procedures, you may contact the researcher, Hope Lima, at (860) 682-0135, at 216 Schaub Food Science Building, NC State University, or hklima@ncsu.edu. If you feel you have not been treated according to the descriptions in this form, or your rights as a participant in research have been violated during the course of this project, you may contact Deb Paxton, Regulatory Compliance Administrator, Box 7514, NCSU Campus (919/515-4514). Your participation in this study is voluntary; you may decline participation without penalty. If you decide to participate, you may withdraw from the study at any time without penalty.

If you have read and understand the above information, received an electronic copy of this form, and agree to participate in this study with the understanding that you may withdraw at any time, please visit [https://ncsu.qualtrics.com/jfe/form/SV_3efYsZ2Np0wgpn](https://ncsu.qualtrics.com/jfe/form/SV_3efYsZ2Np0wgpn) to complete the informed consent document.

Copy of electronic consent text:

1. What is your name?

2. I received an electronic copy of the informed consent document for the "Effect of Light Exposure During Collection, Storage, and Holder Pasteurization on Vitamin Levels in Human Breast Milk" trial at North Carolina State University, I have read and understand the informed consent document, and I wish to participate in the study with the understanding that I may withdraw at any time.

   a. Yes

   b. No
3. If you agree to participate in the study "Effect of Light Exposure During Collection, Storage, and Holder Pasteurization on Vitamin Levels in Human Breast Milk", please provide us with the best mailing address to send your participant package to.
Chapter 6 Appendix C: Participant Email

Dear (insert participant’s name),

Congratulations! You have been selected to participate in the trial at North Carolina State University titled “Effect of Light Exposure During Collection, Storage, and Holder Pasteurization on Vitamin Levels in Human Breast Milk”. You will find attached to this email the additional paperwork that you will need to read, fill out (if applicable), and returned to Hope Lima at your earliest convenience. These documents include:

1. Participant Instructions
   a. This document will give you all the information you will need to understand what will be expected of you if you choose to participate in this trial.

2. Informed Consent
   a. This document will need to be read, signed, and returned to Hope Lima at your earliest convenience if you choose to participate.

When you have read and completed the required paperwork, a package will be sent to you via snail mail containing everything you will need to provide your human milk samples. Once you receive your package, we ask that you begin your 4-day collection process within 2 weeks to help with the timely completion of the experiment.

If you have any questions or concerns, please feel free to contact Hope Lima by call or text at (860) 682-0135 or via email at hklima@ncsu.edu.

Thank you for your participation and we look forward to working with you.
NCSU Fogleman Lab Research Team
Chapter 6 Appendix D: Participant Sample Collection Instructions

Instruction for Participants in NCSU’s Human Milk and Light-Sensitive Vitamins Study
Thank you for agreeing to participate in the North Carolina State University study “The effect of light exposure during collection, storage, and Holder pasteurization on vitamin levels in human breastmilk”. Your participation will help us learn more about the ideal way to collect breastmilk when not feeding directly from the breast. Now that you’ve completed the Informed Consent, below are instructions on how we’d like you to collect your milk samples for this study. We ask that you begin your 4-day collection process within 2 weeks of receiving your collection package to help with the timely completion of the experiment.

How to Collect the Milk
1. You will be collecting breast milk for four (4) consecutive mornings – two mornings will be “control” mornings (using clear collection containers) and two mornings will be “test” mornings (using amber collection containers). You will be provided with extra collection containers in the event that one rips, you do not need to fill them all.

2. Using a pump of your choice, please remove the entire volume of milk available in your breast in one sitting.
   *Note: you can pump from one breast or two breasts. Please ensure complete “emptying” before ending the pumping session to remove hindmilk.

3. If you pumped on both breasts, please combine human milk obtained from both sides, invert gently to mix, and then pour 2 oz of this gently mixed milk into the provided collection container. If you pumped only on one side, invert gently to mix, and then pour 2 oz of this gently mixed milk into the provided collection container.
   *Note: on “control” days, this will be the clear container, on “test” days this will be the amber container.

4. Ideally, we ask that you collect the sample in the morning, as the first expression of the day (after 4 am) and at least 2 hours since the previous feeding. We ask this because human milk composition changes throughout the day, so this will help create consistency across the samples we collect.

5. We will provide labels and collection containers for your sample. Please complete the label at the time of pumping with the following information:
   - Date of collection
   - Time of collection (note AM or PM)
   - Date and time of prior feeding
• Number of anticipated feeding and/or pumping sessions that day. Note: If you feed your child on one side and then pump milk from the other side, this is considered one feeding session. Please include the session to collect milk for this study in your number.

• Your unique identifier

6. Immediately store the sample in your freezer and contact Hope Lima at (860) 682-0135 when you have collected all four samples to arrange pick up.

If you have any questions, please contact Hope Lima at (860) 682-0135 or hklima@ncsu.edu.

Thank you!
SEEKING BREASTFEEDING MOTHERS!

Researchers from North Carolina State University are seeking participation from breastfeeding mothers in the Triangle area to participate in a study of the nutrient composition of expressed human milk.

Inclusion criteria include:

- Breast milk expression as a part of your normal routine
- Access to a breast pump
- Infant was born healthy, at full term, and is currently 4 weeks of age or older
- Unrestricted diet

If you are selected for the study, a researcher will contact you with more information about sample collection and storage. Study participants will receive a $20 Visa gift card.

You may access the eligibility survey here:
https://ncsu.qualtrics.com/jfe/form/SV_ezE5dclFp9xVauN
Chapter 6 Appendix F: Riboflavin Variation Among Mothers

Variation in Individual Subject Riboflavin Measurements

Riboflavin (µg/L)

PR

ER

PH

EH

A B C D E F G H I J K L M N Mean