ABSTRACT

MENG, TING. Effect of Storage on Bacteriological and Immunological Qualities in Fresh, Pasteurized and Leftover Human Milk. (Under the direction of Dr. April D. Fogleman and Dr. Jonathan C. Allen).

Human milk is an optimal feeding source for infants. However, it is difficult for mothers to implement breastfeeding with working demand, where a reported 85% of breastfeeding mothers in the U.S. have ever expressed milk for continuous human milk feeding. In neonatal intensive care unit, when mother’s own milk is not available for hospitalized infants, pasteurized donor milk is recommended as a feeding alternative. Both situations require milk storage. Most guidelines on fresh human milk storage are based on milk bacteriological quality, while the immunological quality was neglected. Although the immunological status of human milk was proven stable during storage, very few studies reached the length that the guidelines recommended. Furthermore, the qualities of stored pasteurized milk or leftover milk were not researched.

Mother-baby dyads were recruited. Each baby was fed 1–2 ounces of mother’s fresh and pasteurized milk from a bottle and the remaining milk was collected as the leftover milk. Each mother’s fresh milk (FM, unpasteurized frozen milk), leftover milk (LM, leftover of unpasteurized frozen milk), pasteurized milk (PM, pasteurized frozen milk) and pasteurized leftover milk (PLM, leftover of pasteurized frozen milk) were divided and stored at both room temperature (24°C) and refrigerated temperature (4°C). At each designed time point, bacteriological qualities (aerobic bacteria, coliform/Escherichia coli and Staphylococcus aureus) and immunological qualities (protein content, lysozyme and SIgA activity) were analyzed.
Firstly, bacteriological property measurements found that, at room temperature storage, FM and LM had a significant increase in bacteria after 9 hours (P<.01) and 6 hours (aerobic bacteria, P<.05; coliform, P<.001), respectively. For refrigerated storage, bacteria growth in FM and LM was minimal throughout 7 days. PLM had a significant increase (P<.01) in aerobic bacteria after 9-hour room temperature storage, while a significant decrease (P<.001) was found during 7-day refrigerated storage. No positive coliform was found in PLM.

Secondly, the immunological quality of FM and LM during storage was investigated. Lysozyme activity and SIgA activity remained stable for both FM and LM under all storage conditions examined. No significant change in protein content was observed for both FM and LM during room temperature storage. A significant increase (P< .05) in FM protein content was found after 5 days refrigerated storage. This increase may be caused by the presence of di or tripeptides that could produce a greater amount of color in the BCA assay. LM, on the other hand, had a slower protein increase than fresh milk but was not significant in refrigerated storage. In addition, LM had a significant decrease in SIgA activity (P<.05) compared to FM.

Thirdly, we determined the immunological quality of PM and PLM during storage. No significant change was found in protein content, lysozyme activity or SIgA activity for either PM or PLM under all the storage conditions examined. No significant difference was also shown in the examined components after baby’s partial consumption.

Last, the effect of Holder pasteurization on immune active components in human milk was studied. Protein content in most samples was stable after pasteurization except for three samples (3/13) that showed a decrease. Lysozyme activity was significantly reduced
(P<.001) by Holder pasteurization in all samples whereas a significant increase (P<.05) in SIgA activity was found.

These findings suggested that guidelines for mothers’ storage of their milk can be extended for longer periods of time and still maintain the nutritional elements in the milk critical to the healthy growth of babies. Furthermore, the leftover human milk can be stored for a next feeding with a bacteriologically safe and immunologically stable status.
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Effect of Storage on Bacteriological and Immunological Qualities in Fresh, Pasteurized and Leftover Human Milk

by
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A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Master of Science

Nutrition
Food Science

Raleigh, North Carolina

2014

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DEDICATION

I dedicate this thesis and degree to my beloved parents who have always given me unconditional support on my dreams. Their affirmation and encouragements make me who I am. I love them more than anything else in the world. I also dedicate the thesis to my significant other, Rubin, who has always been with me through the happiest and hardest time. Without him, I would not gain so much. Thank you my dearest papa and mama, and thank you Rubin.
BIOGRAPHY

Ting Meng was born in 1988, a year of dragon in Hebei Province, China. Her father is from a family of scholar in Northern part of China, and her mother is from the Southern. The cultures divergence in North and South offered her a special family environment where she can think independently and be open to new matters, which lays foundations to her future learning.

Though she was talented in music in her young age, she decided to transfer the focus to science learning. Luckily, she was outstanding in school work and was accepted by Zhejiang University, a top 3 university in China. There she met her most important half Runlai Luo, and with his encouragements, Ting decided to explore the world outside and pursue a degree she was always interested in abroad.

When she came to North Carolina State University, she found herself especially interested in human milk. With the help of Dr. April Fogleman, she started working in the lab with the subject she was fascinated with. At the same time, she found the interest in Food Science and began actively involved in the learning. After her study in NCSU, she would like to work as a food scientist where her imagination and love on nutrition and food science can be realized.
ACKNOWLEDGMENTS

I would like to give my special thanks to my dear advisor, Dr. April D. Fogleman, for offering such a valuable opportunity so that I can learn more about human milk. Her support and help was absolutely important: it not only makes me enjoy my time in the lab but also provides me valuable opportunities for internship and conferences. She is an awesome advisor and I know she will have more excellent students in the future.

I want to thank Dr. Jonathan C. Allen, not only as a committee co-chair, but also the director of graduate program. He was the first professor I knew in the department. I cannot count how much help he has given me. He was so generous to provide me helps during my research and other course work.

I am also thankful to Dr. Sophia Kathariou, for serving on my committee. Dr. Kathariou was always very helpful in my learning on Microbiology as well as on my research. She gave me valuable advice and helped me figure out problems. Her smiling encouragements and knowledge are always appreciated.

Special thanks to Ruth Watkins for all her time in the lab answering my questions and helping me solve problems. I am also grateful to Jason Osborne, Mara Massel, Judy Cooper, Sihang Chen for their support and help during my research.

At last, I would like to say thank you to all the mothers and babies that participated in my study which would not be possible without their support.
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CHAPTER 1

1. Literature Review

1.1 Benefits of Human Milk

Human milk not only provides essential nutrients for infants during growth and development, but also offers unique immunological compounds to help defend the baby from diseases. According to epidemiologic studies and related surveys, breastfeeding of infants can significantly decrease the risks of acute respiratory infection\textsuperscript{1,2} and diarrhea\textsuperscript{1-3}, which are the leading causes of morbidity and mortality in infants, especially in developing countries\textsuperscript{4}. Necrotizing enterocolitis (NEC), a serious gastrointestinal disease most commonly occurring in preterm infants, may be prevented by feeding human milk\textsuperscript{5,6}. Breastfed infants are also associated with lower incidence of developing acute otitis media, leukemia, sudden infant death syndrome and other diseases\textsuperscript{7-9}. Infants who were not breastfed were found to have a higher incidence of infectious morbidity, as well as more possibility to develop childhood obesity and diabetes in the babies’ later lives\textsuperscript{10}. It is reported that partial or no breastfeeding was associated with 2.40 and 3.94 folds higher risk of infant deaths caused by respiratory infections and diarrhea, respectively\textsuperscript{1}. Studies suggested that in order to confer a protective impact on infants’ health, breastfeeding shall be exclusively continued for at least three months\textsuperscript{11,12}.

Furthermore, the mothers who had breastfed may have lower risks in developing breast cancer and ovarian cancer at later life stages. Numerous studies\textsuperscript{13-16} have been done showing that breastfeeding can lower the incidence of breast cancer, especially when
continued for at least six months\textsuperscript{13,14}. The relationship between breastfeeding and ovarian cancer is also revealed by several studies. One study\textsuperscript{17} suggested that prolonged lactation had a lower risk than short-term lactation. Similarly, Gwinn\textsuperscript{18} found the decreased trend in developing ovarian cancer correlated with a longer duration of breastfeeding. A meta-analysis\textsuperscript{19} based on 5 prospective and 30 case-control studies was able to conclude that breastfeeding and prolonged breastfeeding can both reduce the risks of ovarian cancer.

Benefits of breastfeeding is widely recognized and endorsed by the following organizations, while long breastfeeding duration is especially recommended to maximize the benefits for both the babies and mothers.

\begin{itemize}
  \item CDC\textsuperscript{20} (United States Centers for Disease Control and Prevention) “Breastfeeding is the most effective preventive measures a mother can take to protect the health of her infant”. It is necessary for everyone to recognize the value of breastfeeding and start to support breastfeeding as “the success rate among mothers who want to breastfeed can be greatly improved through active support from their families, friends, communities, clinicians, health care leaders, employers, and policymakers.”
  \item WHO\textsuperscript{21} (World Health Organization) & AAP\textsuperscript{8} (American Academy of Pediatrics) Mothers should exclusively breastfeed for the first six months of life and breastfeeding shall be continued for up to two years and beyond as long as mutually desired by mother and child.
  \item National Health Service\textsuperscript{22} (United Kingdom) “The longer you breastfeed, the longer the protection lasts and the greater the benefits”.
\end{itemize}
1.2 Factors that Impact Breastfeeding

Efforts were being made to promote breastfeeding and the benefits of breastfeeding are usually well understood by parents. The desire of mothers to breastfeed is compromised shortly after a baby is delivered\textsuperscript{23}. According to the CDC report in 2011\textsuperscript{24}, the breastfeeding rate of new-born in the United States was 75%, but only 15% remained exclusively breastfeeding 6 months later. Scott\textsuperscript{25} found that if it would be easy and convenient, 92.9% of the mothers would agree to choose breastfeeding.

One of the major constraints in breastfeeding is the public attitude. A survey\textsuperscript{26} focused on the public opinion about breastfeeding in the U.S. showed that though the benefits of breastfeeding are generally understood by the public, most of the population (63.9% to 79.6% based on region) consider it inappropriate to show a woman breastfeeding on television, and about half of the population think it is not right to breastfeed in public. On the other hand, it was found that women are more likely to feel it is embarrassing or unacceptable to breastfeed in public compared to men\textsuperscript{27}, especially those from low-income families\textsuperscript{28}. Public attitude and approval on breastfeeding is of importance to provide an environment that is easy for moms to breastfeed. Support from family members and friends is critical when mothers make breastfeeding decisions\textsuperscript{26,29}.

Professional peer support was an effective way of promoting breastfeeding. Well-designed professional peer support in both prenatal and postpartum periods was evaluated in a study\textsuperscript{30} to examine its effectiveness on breastfeeding practice. Fifty-five mothers participated in that study and nineteen received professionally mediated peer support (others in the control group), by which a deliberative plan was carried out to educate the
mothers about breastfeeding benefits throughout the pregnancy period as well as how to successfully breastfeed. After six weeks, mothers who received professionally mediated peer support were found to have longer breastfeeding duration, higher exclusive breastfeeding rates as well as better satisfaction towards breastfeeding. Mothers who did not receive professional support were more likely to encounter physical conditions such as sore nipples, engorgement, and mastitis, which led to unsuccessful breastfeeding.

It is especially hard for working mothers to breastfeed. According to surveys\textsuperscript{31–34}, work demands were found as one of the top reasons why mothers stop breastfeeding. Most of the new moms in the U.S. have to return to work within three months after delivery\textsuperscript{35}, and this may cause a sharp decline in exclusive breastfeeding rate, which was observed in Li’s study\textsuperscript{34}. Mothers described inconveniences associated with work, such as fatigue, breast engorgement and leaking milk, and suggested the necessity of establishing a breastfeeding friendly working environment\textsuperscript{36}. On the other hand, supportive state laws on workplace breastfeeding were found to be associated with higher rates of breastfeeding\textsuperscript{33}.

Above all, breastfeeding is not just a matter related to the mothers. It should be advocated by the whole society to create a healthier environment, where breastfeeding is widely approved by the public and protected by state laws, professional support is easily accessible, family members are obligated to support breastfeeding and public facilities are established to provide convenience for the nursing mothers.
1.3 Human Milk Expression

To ensure human milk supply and avoid inconvenience, more and more mothers choose to express and store human milk for later feeding\textsuperscript{37}. A U.S. based study\textsuperscript{38} reported that 85\% of breastfeeding mothers between 1.5 and 4.5 months postpartum had ever expressed milk since their infant was born, with 43\% having done so occasionally and 25\% on a regular basis. The human milk expression rate is even higher in Australia. Based on a survey\textsuperscript{39} of 903 mothers, 98\% of the survey takers indicated that they have ever expressed milk before. In Singapore, an increased trend in human milk expression was also observed, while direct human milk feeding had declined over the same 8 years\textsuperscript{40}.

As mentioned above, a lot of constraints to direct breastfeeding exist. Concerns about public attitudes\textsuperscript{41,42} as well as physical conditions (including breast engorgement, mastitis and nipple pain)\textsuperscript{39,43} were found as the most common reasons why mothers express milk. Working mothers contributed another large group to milk expression\textsuperscript{38,39}. Human milk expression allows someone else to feed the baby when the mother is not available, which offers mothers a more flexible schedule. Besides, the increased commercial availability of breast pumps and infant feeding bottles have facilitated the convenience on human milk expression\textsuperscript{37}.

The outcomes of human milk expression on breastfeeding are under debate. Some studies found that human milk expression may help extend the lactation duration for up to 12 weeks\textsuperscript{44} or 6 months\textsuperscript{45} due to the fact that breastfeeding was more likely to be discontinued if mothers couldn’t produce enough milk or had to return to work\textsuperscript{44}, and human milk expression was able to solve these problems and thus encouraged mothers to have a longer
lactation period. A survey\textsuperscript{46} found that mothers who received an electronic pump in WIC during early lactation were 5.5 times less likely to request formula after 6 months compared with the ones didn’t receive an electronic pump. Since human milk expression makes it easier for mothers to feed infants human milk, mothers are able to continue breastfeeding longer than the ones who encountered difficulties during breastfeeding.

However, the impact of milk expression on mothers’ milk producing ability (lactogenesis) may be a different case. In a clinical trial\textsuperscript{47}, sixty women were asked to use electric pumps for 10 to 15 minutes from 24 to 72 hours postpartum. Milk transfer and breastfeeding behavior were evaluated daily. It was found that mothers who used a breast pump earlier had shorter lactation duration than the control group (mothers held the pump to their breasts without suction), which suggested a negative impact of milk expression on lactogenesis. However, there are limitations in the study. Firstly, only electric pumps were evaluated and the result may be different with hand pumps. Secondly, a negative impact on lactogenesis was only found in primiparous women, and was not significant, so might be a random error. More studies could be done to further investigate the relationship between breast pumping and lactogenesis.

1.4 Human Milk Banking

When mother’s own milk is not available for the hospitalized newborn, pasteurized human donor milk as the recommended feeding alternative to human milk, rather than commercial formula\textsuperscript{48}, is the position of the American Academy of Pediatrics\textsuperscript{49}, the Academy of Breastfeeding Medicine\textsuperscript{50} and WHO\textsuperscript{21}. It is found that 30.8\% of maternity hospitals
routinely provide pasteurized human donor milk in NICU. The pasteurized human donor milk is processed under standard procedures, by the human milk banks.

1.4.1. History of Human Milk Banks

The United States is one of first countries to have donor milk banks. The Boston Wet Nurse Directory officially became a donor milk bank in the year of 1919.

Massive efforts have been made to better preserve the donor milk. In the early years when refrigeration was not available, it was extremely difficult to store the milk. The very first attempts, which were the additions of hydrogen peroxide and calcium peroxide to the milk, were not ideal. Afterwards, different kinds of heat treatments including autoclaving or boiling were utilized, which resulted in a large nutritional loss in the milk. In some other milk banks, milk was dispensed unprocessed.

In the middle of 1980s, concerns on donor milk safety arose with the advent of AIDS and resulted in the closure of many milk banks. By the 1990’s, the benefits of donor milk to the premature infants were being realized. Research was done to examine the methods and effectiveness of donor milk processing in order to develop a safe donor milk standard operating procedure.

At the same time, the Human Milk Banking Association of North America (HMBANA) was established in the year of 1985 to standardize the protocols for human milk banks and to ensure a safe delivery of donor milk, in the regions of Canada, Mexico and the United States. Since then, guidelines on donor milk operation have been established, reviewed and updated annually to support the increased demand on donor human milk.
1.4.2. How Human Milk Banks Operate

In human milk banks, donors shall be screened by blood testing and other medical risk factors to ensure the donor is in healthy condition with no medication or drug usage\textsuperscript{56}. Once the human milk from a healthy donor is received, it is kept frozen under a temperature no higher than -20°C to inhibit bacteria growth during storage. Milk from several donors (usually 3 to 5 donors) is pooled to ensure even distribution of milk components. Afterwards, milk is Holder pasteurized (62.5°C for 30 minutes) to eliminate the microorganisms in the milk. A pasteurized milk sample is checked for bacteria growth after processing each batch. Once no bacteria growth is confirmed, the pasteurized milk batch is shipped frozen overnight to the recipients. Otherwise, the milk will be discarded immediately. All donor milk is processed based on the standard HMBANA guidelines\textsuperscript{57–59} to ensure the human milk safety while delivering the optimal nutritional and immunological quality.

The demand for human donor milk is huge and increasing year by year. Currently there are 17 human milk banks in North America with 3 more banks in development\textsuperscript{60}. It is reported that HMBANA milk banks process more than one million ounces of human milk every year\textsuperscript{48,59} and in the year of 2011, about 2.18 million ounces of human milk were distributed by HMBANA milk banks\textsuperscript{61}. Among those recipients, the population that comprised the greatest demand for donor milk was hospitalized infants, including the preterm infants who are critically ill or premature\textsuperscript{62}. It is also HMBANA’s mission to ensure that the processed milk is firstly served to the preterm babies as a priority\textsuperscript{63}. 
1.4.3. Benefits of Human Donor Milk

To hospitalized infants, human donor milk has superior and obvious advantages over commercial formula. Milk of the human origin is not only species specific, thus fitting the infant’s nutritional need, but also provides precious immune active compounds that protect sick babies from diseases. Though some of the bioactive compounds were affected by pasteurization, most of the key nutritional and biological compounds remained stable after pasteurization. A reduction in IgA and SIgA concentration was observed\(^{64-66}\) after pasteurization, but the decrease did not diminish milk’s activity against enteropathogenic \textit{E.coli}\(^ {67}\). Besides, immunoglobulins\(^ {68}\), carbohydrates\(^ {48,69}\), fat content\(^ {70}\) and lysozyme activity\(^ {66}\) were found unchanged after pasteurization. Furthermore, clinical studies\(^ {71,72}\) have found that human donor milk is able to prevent NEC among preterm infants. Two meta-analyses based on previous studies (one based on three studies and the other based on five) found that infants fed with human donor milk had a lower risk of necrotizing enterocolitis (NEC) compared with infants being fed infant formula\(^ {73,74}\).

Another fact worth mentioning is the cost effectiveness of human donor milk. Compared with the cost of NEC treatment, the dollars spent on donor milk is minimal. If the use of donor human milk does reduce the infants’ length of stay in NICU for NEC, every $1 spent for human donor milk could save about $11 on health care and medication expenses, based on Wight’s calculation\(^ {75}\).

1.5 Storage of Human Milk

Along with the increased human milk expression rate, human milk storage becomes more and more common. At the same time, concerns arise on how storage may impact the
precious nutrients and bioactive compounds in human milk. Numerous studies have been performed to examine the effect of storage.

1.5.1. Protein during Storage

Though protein constitutes only 0.9% of the human milk contents, it plays critical roles in human growth, development and metabolism. Proteins in human milk include casein, serum albumin, immunoglobulins, lysozyme as well as many enzymes and other minor proteins\(^76\). It provides essential amino acids to supply infants’ growth and development, and at the same time delivers immune defense compounds to protect infants from diseases. Several proteins in human milk inhibit pathogenic bacteria as well as viruses\(^77\)–\(^79\) including HIV\(^80\). Furthermore, immune active proteins in human milk were also reported to develop the baby’s immune defense system faster than artificial feedings\(^81,82\).

The stability of protein during room temperature and refrigerated storage was confirmed in several studies\(^83\)–\(^85\). Particularly, Garza measured the storage effect on human milk protein in both room temperature and refrigerated temperature\(^85\). The protein content during each storage temperature did not show significant change, but a significant difference was found between room temperature and refrigerated temperature, where protein content at refrigerated storage was slightly lower than that of room temperature. Most other studies\(^83,84,86\) only focused on the effect of a single storage temperature and thus neglected the effect of different storage temperatures on protein content. The protein loss indicated a possible effect of refrigeration temperature on human milk protein content. This can be examined in future research.
Molinari\textsuperscript{84} examined some specific protein components during room temperature storage. He found the immunological proteins including secretory IgA (SIgA), lysozyme and lactoferrin were resistant to breakdown during room temperature storage. The stability of immune active compounds was also confirmed in refrigerated storage for 96 hours and frozen storage for 3 months in studies\textsuperscript{83,87,88}.

1.5.2. Lipids during Storage

At least half of the energy content in human milk is attributed to the milk lipids. Human milk lipids not only serve as a primary energy supplier, but also perform critical functions in the body development of infants. It is rich in both omega-6 and omega-3 polyunsaturated fatty acids (PUFA) including linoleic (18:2n6) and linolenic acid (18:3n3). These PUFA can be converted to long chain PUFA and further facilitate infant development of cardiovascular, immune and nervous systems\textsuperscript{89}.

Lavine and Clark\textsuperscript{90} measured the lipolysis in human milk at various storage temperatures including 25, 4, -11 and -70°C. Milk lipolysis was detected in all storage temperatures except for -70°C. In temperatures other than -70°C, free fatty acid levels in human milk increased with the length of storage and with increased temperature. The increase in free fatty acids during storage at room temperature\textsuperscript{91}, refrigerated temperature\textsuperscript{86} and frozen temperature (-20°C)\textsuperscript{92,93} was also observed in other studies. However, the lipolysis process especially increased the free 18:2 and 20:4 PUFA which are required for body growth and development of the infants, and these free fatty acids might possess anti-microbial effects against bacteria, viruses and protozoa\textsuperscript{94–96}. 
The inhibition of lipolysis at -70°C was again reported by Berkow and his colleagues\(^93\). He examined the lipid status in human milk during frozen storage for both -20 and -70°C where significant lipid degradation was only observed at -20°C storage. However, a recent study\(^97\) stated significantly lowered lipid content in human milk after storage at -80°C within 1 to 10 weeks. The opposing opinions on lipid content at deep frozen storage encourage more research on this subject.

1.5.3. **Carbohydrates during Storage**

Mature human milk contains 6.9% - 7.2% carbohydrate\(^76\), with about 6.8% from lactose and 1% from oligosaccharides\(^98\). Lactose has been specifically linked to newborn growth by enhancing calcium absorption and preventing rickets\(^99\). Oligosaccharides, on the other hand, may function to inhibit pathogens and promote the growth of intestinal micro flora\(^98\).

Limited studies have been done related to the carbohydrate status during storage. Nevertheless, decrease in carbohydrates have been found at different storage conditions including ambient temperature storage (29°C) after 3 hours\(^100\), refrigerated storage (4°C) over 96 hours\(^83\), frozen storage (-20°C) over 180 days\(^101\) and deep frozen storage (-80°C) over 83 days\(^7\). The decline might have been caused by the activity of bacteria during storage, which converts the main sugar, lactose, to lactic acid by anaerobic glycolysis\(^83,100\).

1.5.4. **Vitamins during Storage**

Vitamins are critical nutrients in metabolism. With vitamins, numerous biological reactions can occur and various components get regulated.
Significant losses of vitamin C in human milk were reported in studies. Buss\textsuperscript{102} reported loss of one-third of vitamin C after refrigeration (4-6°C) for 24 hours cold temperature storage and a two-thirds decrease after freezing(-16 to -17°C) for 2 months. The loss of vitamin C content in cold storage was also confirmed by Romeu-Nadal\textsuperscript{103} after 96 hours of refrigerated storage at 4°C, and 12 months of frozen storage at −20°C or −80°C.

Unlike vitamin C, vitamins B and E were preserved during storage. Moffatt\textsuperscript{104} examined the vitamin E content at extensive storage conditions including 24 hours at 4°C, 72 hours at -11°C, -20°C and -70°C. In none of these conditions were observed significant differences. A similar result was also found in another study\textsuperscript{103}. Vitamin B was also reported stable during 24 hours of refrigerated storage\textsuperscript{105} and 3 months frozen storage at -25°C\textsuperscript{106}. Little research has focused on human milk vitamin content during room temperature storage.

1.5.5. \textit{Bacteria Activity during Storage}

Human milk is easily contaminated by bacteria from maternal skin and the surrounding environment. Inadequate sanitation and improper storage can result in bacterial contamination and threaten infant health. Studies found that pathogenic bacteria can cause severe infant infections including diarrhea\textsuperscript{107}, meningitis\textsuperscript{108} and bloodstream infections\textsuperscript{109,110}, to name a few. Therefore, it is of great importance to understand the bacteriological status of human milk during storage and thus ensure proper milk handling procedures.

It is found that most bacterial species isolated from expressed human milk were normal skin flora including staphylococci, streptococci and some lactic acid bacteria\textsuperscript{111}. Moreover, bacteria activity was suppressed in human milk, especially during cold storage. In
studies on human milk refrigerated storage, no increase in bacteria level was observed in any of the examined storage durations including 24 hours or 48 hours\textsuperscript{112}, 4 days\textsuperscript{86,113} or 5 days\textsuperscript{114}. Human milk was also found to be bacteriologically stable for up to 6 weeks at -20°C\textsuperscript{111–113}.

In contrast to the findings in refrigerated storage, the understanding of human milk microbiological status in room temperature is under debate. Several studies\textsuperscript{91,115} suggested little change in bacteria levels after 25°C storage for 4 to 8 hours. However, Yasuko\textsuperscript{112} reported a significant increase in bacteria after 3 or 6 hours of room temperature storage (20°C). Similar to Yasuko, a significant bacterial increase in human milk was found by Eteng after 3 hours of room temperature (29°C) storage\textsuperscript{100}. The difference may be caused by the variance in temperatures studied. Nevertheless, more research is encouraged to further investigate the bacteriological status during room temperature storage.

1.6 Human Milk Storage Guidelines

Human milk storage guidelines have been established to support the increased prevalence of human milk expression as well as to ensure proper milk handling guidelines. Based on the proposal of The Academy of Breastfeeding Medicine\textsuperscript{116}, Centers for Disease Control and Prevention(CDC)\textsuperscript{117} recommend that human milk can be stored for 6-8 hours at room temperature (up to 25°C), 5 days in refrigerator (4°C) and 2 weeks, 3-6 months or 6-12 months frozen depending on different freezers used.

National Guideline Clearinghouse (NGC, belongs to U.S. Department of Health & Human Service) made a more elaborated recommendation on human milk storage\textsuperscript{118}, where the cleanliness of the expression equipment and various room temperature ranges were taken
into consideration. They recommended that human milk can be stored for 3 to 4 hours at room temperature (27 to 32°C), and for very clean milk, 6 to 8 hours may be reasonable with lower room temperature. The suggested storage durations in refrigerated and frozen temperatures were similar with that of CDC recommendation.

La Leche League\textsuperscript{119}, an international nonprofit breastfeeding organization has also provided recommendations on milk storage. Longer storage durations were proposed for room temperature and refrigerated storage: human milk can be stored for up to 10 hours at room temperature (19-22°C) and up to 8 days in a refrigerator (0-4°C).

The definition of room temperature is somewhat dim and may vary by geography. It is preferred to use digital temperature when making guidelines for clarification.

1.7 Future Work

Massive efforts have been made to improve the understanding of human milk during storage, and with the endeavor of both policy makers and researchers, more progress can be carried forward in the future.

1.7.1. For Policy Makers

The human milk storage protocol\textsuperscript{116} written by Academy of Breastfeeding Medicine (ABM) Protocol Committee has been widely used to establish guidelines by CDC and other social media\textsuperscript{117,118,120}. In the protocol, the storage time recommended is mostly based on the bacteriological quality of human milk during storage, which serves as “a marker for milk quality”. It is a priority to consider the bacteriological status of milk during storage to ensure it is safe to offer to a baby, but the quality changes of other nutritional and immunological
components are also important to consider. Only frozen milk was discussed in the protocol for its fragile components including the immune active compounds during storage. The same problem was found in other storage guidelines\textsuperscript{118,120,121}. The storage length of human milk shall be considered in an integrated standpoint so that the milk quality could be preserved for the infants.

On the other hand, research was being done examining different components of human milk during storage to provide scientific evidence for both policy makers and family members. However, the suggestions on storage duration vary. Regarding refrigerated storage, studies recommended a maximal storage length anywhere ranging from 2 days up to 8 days\textsuperscript{122}. This is mainly due to different criteria used to determine the expiration time such as the antioxidant activity\textsuperscript{123}, immunological activity\textsuperscript{124,125}, bacteriological properties\textsuperscript{126,127}, or the degree of component breakdown during the storage\textsuperscript{91}.

Criteria or baselines should be established to standardize the minimal acceptances of human milk during storage, with regard to the important nutritional, immune and bacterial milk properties. For example, as carbohydrates decrease during storage, how much reduction is allowed and how long the milk will reach such a reduction? Moreover, for bacteria, is it necessary to prohibit any growth during storage? Or can we set a number or concentration of bacteria that is a safe level, like the dairy industry does? It is urgent to answer the above question and clarify how quality controls can be facilitated in human milk applications. Especially for the infants who are premature or critically ill in NICU, the preservation of human milk nutritional, immune and bacteriological qualities is all of great importance.
1.7.2. For Researchers

The length of human milk storage studies can be extended. The stability of immune active proteins during refrigerated storage has been confirmed in several studies, but most are limited to a storage duration no longer than 96 hours\textsuperscript{83,86,88}. According to CDC\textsuperscript{117} and ABM\textsuperscript{116} human milk storage guidelines, human milk can be stored for much longer than 96 hours in refrigerator. Therefore, it is necessary to extend the study length and understand the immunological status of human milk throughout the recommended storage period.

On the other hand, more attention should be paid to the immune active compounds during room temperature storage. Emanuele\textsuperscript{128} studied SIgA, lysozyme and lactoferrin content during room temperature storage and reported no significant change after 4 hours at 25°C. Similar to the studies on refrigerated storage, the research did not reach the CDC recommended storage time of 6-8 hours in room temperature storage. Though Molinari\textsuperscript{129} extended the storage length to 72 hours and found immunological proteins were stable, more research could be done to validate the findings.

Human milk bacteria growth was found to be restricted during refrigerated storage in various storage duration studies, including 24 hours or 48 hours\textsuperscript{112}, 4 days\textsuperscript{86,113} or 5 days\textsuperscript{114}; in none of these studies was bacteria growth observed. This demonstrates the bacteriological safety of human milk longer than 5 days in refrigerator temperature. Therefore, an extended refrigerated storage length with bacteriologically safe human milk might be possible.

Additional research on milk component stability is needed. Firstly, as mentioned earlier, Garza\textsuperscript{85} found that protein content did not change significantly in either room temperature or refrigerated storage, but a significant difference was observed between the
two different temperatures, where protein content at refrigerated storage was slightly lower than that of room temperature. Though most studies\textsuperscript{83,84,86} claimed no change in protein content during storage, they only focused on the effect of a single storage temperature and failed to examine the effect of temperature on protein content.

Secondly, lipolysis in human milk was believed to be inhibited at deep frozen temperature(-70°C)\textsuperscript{93,97}. However, a recent study\textsuperscript{97} observed significantly lowered lipid content in human milk after storage at -80°C within 1 to 10 weeks. The opposing opinions on lipid content at deep frozen storage encourage more research on this subject.

Furthermore, conflicts exist on bacteria growth at room temperature storage. Several studies\textsuperscript{91,115} suggested little change in bacteria level after 25°C storage for 4 to 8 hours. On the contrary, Yasuko\textsuperscript{112} claimed a significant bacterial increase after 3 or 6 hours of lowered room temperature storage (20°C). Significant bacteria increase was also found by Eteng after 3 hours at room temperature (29°C)\textsuperscript{100}. Though variance in studied temperatures might have caused some difference, more research can further investigate the bacteriological status of human milk during room temperature storage.

In addition, a lot of questions regarding human milk storage remain unanswered. Holder pasteurization is utilized in human milk banks to eliminate pathogenic bacteria in human milk. The pasteurization process has been found to negatively impact some immunoglobulins\textsuperscript{64–66}. However, it may also inactivate degradation enzymes and help “stabilize” the milk content. No attention was paid to the nutritional and immunological quality of pasteurized human milk during storage. This topic is of importance since it helps ensure sufficient beneficial compounds in human milk are delivered to a hospitalized baby.
Little attention has been given to the storage of leftover human milk. There are situations in which infants do not finish the feeding, resulting in a portion of human milk left in the bottle. Current guidelines recommend discarding the leftover human milk 1-2 hours after finishing feeding\textsuperscript{116} because of the possibility of milk contamination by baby’s saliva. On the other hand, if leftover milk may be saved for the next feeding, it not only means less waste of the milk, but may also spare mothers with more milk to donate. Currently, no study has been done on leftover human milk and scientific support is needed for the guidelines regarding the leftover human milk storage, for both fresh milk in home settings as well as pasteurized donor milk in NICU settings. If the bacteriological, nutritional and immune qualities are acceptable after partial consumption, leftover human milk could be reusable and tons of “golden milk” can be saved.

The objectives of the following research was to investigate the bacteriological and immunological activity of fresh, pasteurized and leftover milk during room temperature and refrigerated storage, and thus to provide scientific support on human milk storage guidelines for both home settings and donor milk settings. In the study, the storage of fresh milk (FM, freshly frozen milk that was not pasteurized) and leftover milk (leftover of FM) mimic the home setting, and the storage of pasteurized milk (PM, pasteurized frozen milk) and pasteurized leftover milk (PLM, leftover of PM) represent the donor milk setting. Chapter 2 focuses on the bacteriological status of stored FM, LM, PM and PLM. In chapter 3 and 4, the immunological quality of FM and LM, and PM and PLM during storage is discussed, respectively. Finally, chapter 5 reveals the effect of Holder pasteurization on the immunological quality of human milk.
1.8 References


60. HMBANA. HMBANA milk bank locations. Available at: https://www.hmbana.org/milk-bank-locations.


Abstract

**Objectives:** The bacteriological properties of leftover human milk were examined to determine how long human milk may be stored in both home and human milk bank settings.

**Methods:** Mother-baby dyads were recruited and each baby was fed 1-2 ounces of mother’s fresh and pasteurized milk from a bottle. Leftover milk, the portion of the bottle not consumed, was collected in sterile containers and analyzed for aerobic plate count, *Escherichia coli* count and *Staphylococcus aureus* immediately after the designed storage times of 0 hours, 3 hours, 6 hours and 9 hours at room temperature (24°C) and 1 day, 3 days, 5 days and 7 days in refrigerator (4°C).

**Results:** At room temperature, leftover milk and fresh milk had a significant increase in bacteria when stored for 6-hours (aerobic bacteria, P<.05; coliform, P<.001) and 9-hours (P<.01), respectively. Bacteria growth in fresh milk and leftover milk was minimal during 7 days of refrigerated storage. Pasteurized, leftover milk had a significant increase in aerobic bacteria when stored for 9-hours (P<.01) and the bacteria growth rate was slower than unpasteurized milk in room temperature. A significant decrease in aerobic bacteria was found in pasteurized, leftover milk during 7-day (P<.001) refrigerated storage. No positive coliform was found in pasteurized, leftover milk.

**Conclusions:** Leftover milk may be stored up to 3 hours at 24°C and at 4°C for 7 days without a negative effect on the bacteriological quality. Fresh milk and pasteurized milk may
be stored at 24°C for at least 12 hours and at 4°C for at least 7 days. Pasteurized leftover milk may be stored for at least 6 hours at 24°C and 7 days at 4°C. Bacteria introduced by the infant may have lower resistance to immune compounds in human milk than the bacteria introduced through milk expression and handling.

2.1 Introduction

Breastfeeding is known as the optimal feeding method for infants. The World Health Organization (WHO) and American Academy of Pediatrics recommend exclusive breastfeeding for the first six months to ensure the best nutritional and immunological needs for infants\textsuperscript{1,2} with breastfeeding continuing as long as mutually desired by mother and baby\textsuperscript{3}. It is the position of the American Academy of Pediatrics\textsuperscript{2}, the Academy of Breastfeeding Medicine\textsuperscript{3} and WHO\textsuperscript{4} that when mother’s own milk is unavailable, donor human milk (DHM) should be used for infant feeding, if possible.

It is reported that among breastfeeding mothers whose infants were younger than 4.5 months, 85% had expressed milk since their infant was born, with 43% expressing milk occasionally and 25% on a regular basis\textsuperscript{5}, mainly due to mothers’ work-place demands\textsuperscript{6} as well as the increasing convenience in the use of human breast pump\textsuperscript{5}. Additionally, mothers may express milk in order to donate their oversupply to human milk banks. While there is diversity among the recipients of DHM in regards to age and health condition, the greatest numbers of recipients of donor milk are premature infants in the hospital Neonatal Intensive Care Unit (NICU)\textsuperscript{7}. There are currently 17 established and 3 developing human milk banks in North America\textsuperscript{8}. In 2011, about 2.18 million ounces of DHM were distributed through the
Human Milk Banking Association of North America\(^9\) (HMBANA). It was also found that 30.8\% of maternity hospitals routinely provide DHM in NICU\(^{10}\).

Both expression of human milk in home settings and DHM processed in human milk banks require the storage of human milk, and thus there is a need for human milk storage guidelines. Though studies have been done to improve the guidelines of human milk storage, the recommendations vary. Various studies recommend that human milk should be stored anywhere ranging from 2 days up to 8 days\(^{11}\). This is mainly due to different criteria used to determine the expiration time, including the antioxidant activity\(^{12}\), immunological activity\(^{13,14}\), bacteriological properties\(^{15,16}\) as well as the component breakdown during the storage\(^{17}\).

Centers for Disease Control and Prevention\(^{18}\) propose that unpasteurized human milk can be stored for 6-8 hours at room temperature (up to 25\(^{\circ}\)C), 24 hours in an insulated cooler bag, 5 days in the refrigerator (4\(^{\circ}\)C) and 2 weeks, 3-6 months or 6-12 months in a freezer, depending on the freezer temperature used (Table 1). These recommendations are based on a clinical protocol written by the Academy of Breastfeeding Medicine Protocol Committee. Storage durations suggested in the protocol are based on studies examining bacterial growth of human milk during storage, which emphasize the importance of considering bacteriological properties when reviewing the human milk storage guidelines in order to prevent spoilage and ensure the bacteriological safety of human milk. Studies examining the bacteria level in human milk report no significant increase in bacteria during 8 hours of storage at room temperature or 5 days in the refrigerator. Eight hours of storage at room
temperature or 5 days in the refrigerator\textsuperscript{19–21}. This suggests that a longer storage time may be examined.

Furthermore, little attention has been given to the storage of leftover human milk. There are situations in which infants do not finish the feeding, resulting in a portion of human milk left in the bottle. Current guidelines recommend discarding the leftover human milk 1-2 hours after finishing feeding\textsuperscript{22} because of the possibility of milk contamination by baby’s saliva. On the other hand, if leftover milk may be saved for the next feeding, it not only means less waste of the milk, but may also spare mothers with more milk to donate. So far no study has been done on leftover human milk and scientific support is needed for the guidelines regarding the leftover human milk storage.

In addition to studying storage of unpasteurized milk, there is a need to investigate the bacteriological status of Holder pasteurized leftover human milk to give insight on the storage of leftover DHM, specifically in the NICU. The heat during pasteurization was found to have adverse effects on antimicrobial properties of human milk\textsuperscript{23,24}, but most immunological proteins were retained after Holder pasteurization\textsuperscript{25}. Most bacteria in human milk are destroyed during pasteurization, leading to lower bacteria in pasteurized leftover milk than in unpasteurized leftover milk. To better understand the above uncertainties, we designed a study to examine the bacteriological properties of leftover unpasteurized and pasteurized human milk, with the goal of improving guidelines for human milk storage in both home and human milk bank settings.
2.1.1. Bacteria Indicator Selection

Proper bacteria indicators should be used to indicate the overall bacteriological condition of human milk during storage. There are currently no regulations established on the microbiological criteria for human milk, which is strongly needed along with the increasing demand on human milk storage. The most widely used bacteria indicators in food and water system are coliforms including total coliforms, fecal coliforms and thermotolerant coliforms. Among those, the total coliform count is an indicator for the non-fecal origin and the others (fecal or thermotolerant coliforms) are more specifically targeted for fecal contamination. Aerobic plate count is also commonly used to indicate the overall bacteria populations on food samples\textsuperscript{26}. Referring to the microbiological criteria for cow’s milk, total coliform count and aerobic plate count are also most widely used indicators in United States, Europe and other countries\textsuperscript{27}.

In addition, to ensure the bacteriological safety of human milk, the bacteria types that highly associated with infant infections shall be considered as a critical factor. \textit{Escherichia coli} (\textit{E. coli}) is the most common micro flora found in human’s lower intestine. Most \textit{E. coli} strains are harmless but some can cause infections or even produce poisons. In a study examining the causative pathogens for diarrhea among hospitalized infants, enteropathogenic \textit{E. coli} was found as the leading pathogen that caused infant diarrhea\textsuperscript{28}. \textit{E. coli} is also the leading culprit for infant meningitis\textsuperscript{29}. Another main pathogenic bacterium that can cause infant meningitis is \textit{Staphylococcus aureus}\textsuperscript{29}. Furthermore, \textit{Staphylococcus aureus} is also one of the most common causes of bloodstream infections, which can lead to serious complications among infants\textsuperscript{30,31}. Thus, in addition to total coliform count and aerobic plate
count, *E. coli* and *Staphylococcus aureus* are also of interest in this study in order to ensure a bacteriological safe condition in human milk.

2.2 Methods

2.2.1. Study Design

♦ Human Milk Sample Collection

*Home Settings.* Eleven mother and baby dyads were recruited in the area of Raleigh, Durham, and Chapel Hill, North Carolina from March to December 2013. Volunteer mothers were all Caucasian women with lactation stages between 2 to 10 months. Mothers were asked to express milk according to the protocol they would usually follow and to freeze the milk immediately after expression. Once 8 ounces of a mother’s milk was saved, the milk was collected and delivered to a laboratory. The milk was transported in a cooler full of ice. The 8 ounces of fresh milk was then divided into two 4-ounce sterile containers and kept frozen at -20°C. On the second meeting, 4 ounces of the mother’s frozen fresh milk was delivered back to the original mother and baby. The mother thawed the milk in a way she usually did and fed her baby 1 to 2 ounces of the milk with their own clean bottle. The feeding process usually took 1 to 2 minutes. The leftover milk was collected in a new sterile container and delivered back to the laboratory in an ice cooler. Each mother’s milk sample was handled separately. The whole sample collection process for each mother was finished within one month. The mothers were asked to express and handle their milk as they normally would in order to mimic the human milk handling procedures at home.

*Human Milk Bank Settings.* Ten mother-baby dyads were recruited in the area of Raleigh, Durham and Chapel Hill, North Carolina. Mothers were asked to express milk...
according to the protocol they would usually follow and to freeze the milk immediately after expression. Once 8 ounces of a mother’s milk was saved, the milk was collected and delivered to a laboratory. The milk was transported in a cooler full of ice. The 8 ounces of fresh milk was then divided into two 4-ounce sterile containers. The 4-ounce containers filled with milk were pasteurized at 62.5°C for 30 minutes, according to the Holder Pasteurization method utilized in all HMBANA milk banks. The pasteurization process was conducted using a shallow-form shaking bath (Thermo Scientific, USA). Three thermo detectors were used to detect the temperature during pasteurization, with one indicated on the screen, one inserted in water bath and the other one inserted in the middle of a milk sample as a control. After all thermometers reached a temperature of 62.5°C, the batch was held for 30 minutes and then cooled immediately in an ice bath. After samples were cooled, the pasteurized milk was stored in a freezer at -20°C. After processing, the 4-ounce container with mother’s pasteurized milk was delivered back to the original mother. The mother was asked to thaw the milk as she usually would and to feed her baby 1-2 ounces of the milk with their own clean bottle. The feeding process usually took 1-2 minutes. The leftover milk was collected in a new sterile container and delivered back to the laboratory in an ice filled cooler. Each mother’s milk sample was handled separately. The whole sample collection process for each mother was finished within one month.
Bullet: Human Milk Sample Storage

Immediately after the leftover milk was collected (both pasteurized and unpasteurized), they were transported back to the laboratory. Both the unfed (fresh) and the leftover milk samples were allocated into pre-marked sterile conical tubes. The leftover samples were stored at room temperature (24°C) for 0 hours, 3 hours, 6 hours and 9 hours and at a refrigerated temperature (4°C) for 1 day, 3 days, 5 days and 7 days, respectively. The unfed fresh milk samples were stored at room temperature (24°C) for 0 hours, 3 hours, 9 hours and 12 hours and at a refrigerated temperature (4°C) for 1 day, 3 days, 5 days and 7 days, respectively. Note that the milk stored at room temperature for 0 hours stands as a control sample because it had not been stored yet.

Bullet: Bacteriological Assays

Milk samples were pulled for bacteriological analysis immediately after storage. Each sample was tested on aerobic plate count media, and all leftover milk samples were also tested for coliform count, *Escherichia coli* and *Staphylococcus aureus* to examine the bacteriological safety of leftover milk. In the study, 3M Petrifilm plates were used to examine the bacteriological level in the human milk. It is a ready-made culture medium system which was found to give results with no significant difference when compared to the standard agar plating method. Autoclaved peptone water was used to dilute the milk samples. One milliliter of milk and diluted milk were plated onto the Petrifilm and incubated at 35°C for 24 hours or 48 hours depending on the bacteria type tested. A blank control (autoclaved peptone water) was set during the tests. For a detailed description of bacteria analysis, please refer to Appendix 1.
2.2.2. **Statistical Analysis**

Linear mixed effects models appropriate to the repeated measures design were fit using the MIXED procedure of the SAS software package. In particular, fixed factorial effects for storage treatment (milk usage by a baby), temperature and time were included along with random effects for sample and sample-by-treatment interaction. The design could be called a complete block split-plot design, with storage treatment as a whole-plot factor, sample as a complete block, and time and temperature as split-plot factors, since each subsample was measured repeatedly over time. The time regime over which these measurements were made was different for the two temperatures, so that time effects are nested within temperature in the following formulation of the model:

$$Y_{ijkt} = \mu + \alpha_i + \beta_j + \tau_{(ij)} + (\alpha\beta)_{ij} + (\alpha\tau)_{it(j)} + S_k + (\alpha S)_{ik} + E_{ijkt}$$

Here $i$, $j$, $k$ and $t$ are indicators for treatment, temperature, sample and time, respectively. Greek symbols ($\alpha$, $\beta$ and $\tau$) denote fixed factorial effects, while capital letters ($S$ and $E$) denote random effects. Selected pairwise comparisons were used to evaluate simple treatment effects in cases where there was evidence of interactions involving the treatment (using the LSMEANS statement and the SLICE option of the MIXED procedure). Separate univariate models were fit to each of the two response variables (aerobic bacteria count and coliform count) for the experiment. Diagnostic plots of residuals did not indicate any violations of the usual assumptions underlying analysis of variance.
2.3 Results

2.3.1. Bacteriological Status – Home Setting

In this study, fresh and leftover human milk are representative of human milk in the home setting. Eleven mother and baby dyads participated in this study.

There was no significant change in aerobic bacteria count for both fresh milk and leftover milk for at least 7 days refrigerated storage (Figure 2). At room temperature storage, significant bacteria growth was observed in both fresh (P<.001) and leftover milk (Figure 1, P<.01).

To further investigate at which time point the aerobic bacteria began to increase significantly, the differences between each time point were analyzed by pairwise comparison (Figure 1). In fresh milk, bacteria significantly increased at 9 hours of room temperature storage. In leftover milk, bacteria significantly increased at 6 hours of room temperature storage. For both fresh and leftover milk, the aerobic bacteria growth was slow or nonexistent during the first 3 hours of room temperature storage. Moreover, in the case of fresh milk, there was no significant change during the first 6 hours of room temperature storage, which indicates an overall slow bacteria growth in human milk (Figure 1).

2.3.2. Bacteriological Status – Donor Milk Setting

In this study, pasteurized human milk and pasteurized leftover human milk are representative of DHM. Ten mother-baby dyads participated in the study.

As is standard procedure for HMBANA, bacteria analysis was performed after Holder pasteurization of the milk. If any bacterium is found in the milk after pasteurization, the milk
will be discarded. Thus, the acceptable aerobic bacteria count is 0 log CFU/mL for pasteurized human milk.

Bacteria in pasteurized leftover milk increased significantly (P<.05) at room temperature storage and decreased significantly (P<.001) in refrigerated storage. Detailed changes between each time point are indicated in Figure 3 and 4.

A significant increase in bacteria in pasteurized leftover milk occurred at 9 hours of room temperature storage. No significant changes in bacteria were observed during the first 6-hour room temperature storage. A significant decrease in bacteria was observed for pasteurized leftover milk continually from days 1 - 7.

Comparing to the aerobic bacteria growth in unpasteurized milk (fresh and leftover milk), it seems that the aerobic bacteria growth was inhibited in pasteurized leftover milk. Due to the fact that there were initially no bacteria after Holder pasteurization, the bacteria in pasteurized leftover milk are all newly introduced from the outside environment, feeding bottle or infant’s mouth. The newly introduced bacteria may have a lower resistance to the immune factors in human milk.

No standards are established to evaluate the bacteriological threshold for human milk. If the initial bacteria level in leftover milk is considered acceptable which is 4.7 log CFU/mL in the study since no significant aerobic bacteria growth was found in leftover milk for the first three hours room temperature storage and seven days refrigerated storage, leftover human milk may be stored for up to 3 hours in room temperature and at least 7 days in refrigerator. According to the aerobic bacteria growth trend shown in Figure 1-4, fresh milk,
pasteurized milk and pasteurized leftover milk always had an aerobic bacteria level under 4.7 log CFU/mL at the examined time points.

On the other hand, if the maximum level of acceptance is set at 5.0 log CFU/mL, which is commonly used for bovine milk, leftover milk may be safely stored at room temperature for up to 6 hours and refrigerated for at least 7 days. Pasteurized leftover milk may be stored for at least 9 hours at room temperature and 7 days in the refrigerator. Fresh milk and pasteurized milk may be stored at room temperature for at least 12 hours and in a refrigerator for at least 7 days.

2.3.3. Coliform Count of Leftover Milk

Coliform count was measured in both leftover milk and pasteurized leftover milk. No positive coliform was found in any pasteurized leftover milk samples throughout 9 hours of room temperature storage and 7 days of refrigerated storage (Figure 5-6).

A significant coliform level increase (P<.001) was observed in leftover milk at room temperature storage. According to pairwise comparison between the time points in room temperature storage, significant change in coliform level was not observed until 6 hours of room temperature storage and the coliform growth in leftover milk was minimal in the first three hours (Figure 5). Coliform growth was minimal in refrigerated storage. No significant coliform change in leftover milk was observed throughout 7 days of refrigerated storage. The coliform findings are in accordance with the result in aerobic bacteria analysis for leftover milk.
E. coli and Staphylococcus aureus colonies were not observed during the storage conditions examined.

2.4 Discussion

Due to its natural antimicrobial activity, the overall bacteria growth in human milk is slow. In home settings, the bacteria growth at room temperature was minimal in the first three hours of storage. A significant increase in bacteria was not observed until 6 hours of room temperature storage for leftover milk and 9 hours for fresh milk. In refrigerated storage, bacteria level in fresh milk and leftover milk was stable throughout the 7-day storage. The stable bacteria level of fresh milk in refrigerated storage is also confirmed by several other studies\textsuperscript{36–38}. The trend in bacteria growth in fresh milk and leftover milk are very similar in both room temperature storage and refrigerated storage, though leftover milk had a higher initial bacteria level, indicating that partial consumption may not impact the antimicrobial activity of human milk.

Pasteurized leftover milk seems to have a slower increase in bacteria than unpasteurized milk at room temperature. Furthermore, there was even a significant bacteria decrease (P<.001) in pasteurized leftover milk during refrigerated storage. It may be due to a lower resistance of bacteria to the antimicrobial compounds when the bacteria are newly introduced from environment, bottle and infant’s mouth.

If the acceptable initial leftover aerobic bacteria level (bacteria level at 0 hour for leftover milk) is set as the bacteriological safe threshold of 4.7 log CFU/mL, leftover milk may be stored at room temperature for up to 3 hours and in the refrigerator for 7 days. This
means that human milk that has been partially consumed by a baby does not have to be discarded immediately. Fresh milk and pasteurized milk is able to be stored for at least 12 hours at room temperature and 7 days in the refrigerator. Due to a low initial bacteria level as well as the slow bacteria growth rate, pasteurized leftover milk is bacteriologically safe for at least 9-hours at room temperature storage and 7-days at refrigeration temperature. On the other hand, if the bacteriological safety threshold is set at 5.0 log CFU/mL aerobic bacteria, the same conclusion applies to fresh milk, pasteurized milk and pasteurized leftover milk, whereas leftover milk had a longer safe storage time at room temperature, which was 6 hours. The same conclusion would apply if the coliform level of human milk were used as the standard.

However, caution shall be taken when setting the bacteriological standards for pasteurized leftover human milk, as most of the milk is served to vulnerable hospitalized infants. The bacteriological safety threshold could be set even lower. A possible threshold could be the bacteria level at 0 hour for pasteurized leftover milk which averaged 2.8 log CFU/mL in this study. According to the data, no significant bacterial change was observed during the first 6-hour room temperature storage for pasteurized leftover milk, therefore it may be able to be stored at room temperature (24°C) for up to 6 hours and in a refrigerator (4°C) for at least 7 days.

In addition, the situation may be different if the baby has a thrush or yeast/fungus infection. In this case, to avoid the milk that may be infected, it is recommended to discard the leftover milk immediately after consumption39. More research can be done to investigate the safety risks associated with human milk in such cases.
2.5 Future work

Along with the increased rate of human milk storage, bacteriological acceptance standards for human milk should be developed to ensure milk safety for infants in various settings. More research on human milk bacteriological status regarding specific pathogens should be done to further improve the knowledge of this area, thus to provide better data for formulating human milk storage guidelines.

In this study, volunteers were all recruited from the same area (Raleigh, Durham and Chapel Hill, North Carolina). Situations may vary in other locations. Thus, more research should be done in other areas to avoid the geographic limitation.
2.6 References


## Tables

### Table 1: Human milk storage recommendations (CDC & ABM, 2010)

<table>
<thead>
<tr>
<th>Location</th>
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<th>Duration</th>
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</thead>
<tbody>
<tr>
<td>Countertop, table</td>
<td>Room temperature (up to 77°F or 25°C)</td>
<td>6–8 hours</td>
</tr>
<tr>
<td>Insulated cooler bag</td>
<td>5-39°F or -15-4°C</td>
<td>24 hours</td>
</tr>
<tr>
<td>Refrigerator</td>
<td>39°F or 4°C</td>
<td>5 days</td>
</tr>
<tr>
<td>Freezer compartment of a refrigerator</td>
<td>5°F or -15°C</td>
<td>2 weeks</td>
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<tr>
<td>Freezer compartment of refrigerator with separate doors</td>
<td>0°F or -18°C</td>
<td>3–6 months</td>
</tr>
<tr>
<td>Chest or upright deep freezer</td>
<td>-4°F or -20°C</td>
<td>6–12 months</td>
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Figures

Figure 1. Aerobic bacteria growth at room temperature storage, home settings (Fresh Milk vs. Leftover Milk, n=11). Error bar represents the standard error. Letters (a,b et al) indicate the significant difference between two points: if the two points do not have any same letter, they are significantly different. No significant differences in aerobic bacteria count were found in the first three hours of room temperature storage for either fresh milk or leftover milk. Significant increase in aerobic bacteria level occurred at the 6-hour sample time for leftover milk ($P<.05$), and at 9 hours for fresh milk ($P<.01$).
Figure 2. Aerobic bacteria growth in refrigerated storage, home settings (Fresh Milk vs. Leftover Milk, n=11). Error bar represents the standard error. There was no significant aerobic bacteria growth during the 7 days refrigerated storage for either fresh milk or leftover milk. If the human milk is bacteriologically safe right after consumption, it shall be safe to store at refrigerator for 7 days.
Figure 3. Aerobic bacteria growth at room temperature storage, donor milk settings (Pasteurized Milk vs. Pasteurized Leftover Milk, n=10). Error bar represents the standard error. Letters (a, b et al) indicate a significant difference between two points: if the two points do not have any same letter, they are significantly different. The standard error for pasteurized milk is 0 because all samples had an initial bacteria count of 0 CFU/mL. Pasteurized milk had an initial aerobic bacteria level of 0 CFU/mL after pasteurization. After baby’s consumption, there was an average aerobic bacteria increase of 2.7 log CFU/mL. During room temperature storage, the aerobic bacteria growth was inhibited in the first 6 hours. A significant increase (P<.01) occurred at 9 hours of room temperature storage.
Figure 4. Aerobic bacteria growth in refrigerated storage, donor milk settings (Pasteurized Milk vs. Pasteurized Leftover Milk, n=10). Error bar represents the standard error. Letters (a,b et al) indicate the significant difference between two points: if the two points do not have any same letter, they are significantly different. The standard error for pasteurized milk is 0 because all samples had an initial bacteria count of 0 CFU/mL. A significant aerobic bacteria decrease (P<.001) was observed throughout the 7 days refrigerated storage for pasteurized leftover milk. This is not only due to the bacterial inhibition by refrigerated temperature, but may also be caused by a lower bacteria resistance to the antimicrobial activity of human milk, since the bacteria were newly introduced from the outside environment, feeding bottle and infant’s month by consumption.
Figure 5. Leftover milk coliform growth at room temperature storage
Leftover Milk (n=11) vs. Pasteurized Leftover Milk (n=10). Error bar represents the standard error. Letters (a,b etc) indicate the significant difference between two points: if the two points do not have any same letter, they are significantly different. The standard error for pasteurized leftover milk is 0 because all samples had a coliform count of 0 CFU/mL throughout 9 hours of room temperature storage. Leftover milk had an initial average coliform level of 1.9 log CFU/mL. Significant coliform increase in leftover milk was observed during 9 hours of room temperature storage (P<.001).
Figure 6. Leftover milk coliform growth in refrigerator
Leftover Milk (n=11) vs. Pasteurized Leftover Milk (n=10). Error bar represents the standard error. The standard error for pasteurized leftover milk is 0 because all samples had a coliform count of 0 CFU/mL during refrigerated storage. No significant coliform increase was observed in leftover milk throughout 7 days of refrigerated storage.
CHAPTER 3

3. Bioactive Factors in Fresh, Stored, and Leftover Human Milk

Abstract

Objective: The objective of the study was to examine the immunological quality of fresh and leftover milk during storage and to create evidence based protocols for the human milk handling for human milk in home settings.

Methods: Mother-baby dyads were recruited and each baby was fed 1-2 ounces of mother’s fresh milk from a bottle. Milk remaining in the bottle was collected in sterile containers. Each mother’s fresh milk was divided and stored at room temperature (24°C) for 0 hours, 3 hours, 9 hours and 12 hours and in a refrigerator (4°C) for 1 day, 3 day, 5 day and 7 days. Leftover milk was stored at room temperature for 0 hours, 3 hours, 6 hours and 9 hours and in a refrigerator for 1 day, 3 day, 5 day and 7 days. At each time point, the milk was analyzed for total protein content, lysozyme activity and secretory Immunoglobulin A (SIgA) activity.

Results: Lysozyme activity and SIgA activity remained stable throughout room temperature and refrigerated storage for fresh milk. After fresh milk consumed by a baby, lysozyme activity did not decrease in leftover milk but SIgA activity significantly decreased (P<.05) by 18%. No significant change in lysozyme and SIgA activity was observed during storage of the leftover milk. Total protein content had minimal change during room temperature storage for both fresh and leftover milk. A significant increase (P< .05) in fresh milk’s protein content was found after 5 days refrigerated storage. The increase in protein content may be caused by the presence of di or tripeptides that could produce a greater amount of color in
BCA assay than does the protein standard. Leftover milk had a slower but not significant protein increase in refrigerated storage, in contrast to fresh milk.

**Conclusions:** Lysozyme and secretory IgA in both fresh and leftover milk were found to retain activity during the storage times and temperatures investigated. Five days prolonged refrigeration seems to activate the protease that caused an increase in short-chain peptides in human milk. The degree of proteolysis in leftover milk was slower, which may be due to the inactivation of protease by the pre-feeding handling process (mainly heating). A significant reduction in SIgA activity was observed with partial consumption.

3.1 **Introduction**

The benefits of human milk to child, mother and the whole family is widely recognized. Breast milk not only delivers exclusive nutrients to support the growth of infants\(^1,2\), but also provides various immune active compounds to help baby build the defense system\(^2\). On the other hand, it is found that infants not being breastfed have higher risks of infectious morbidity and are more likely to develop childhood obesity and diabetes in later life. Mothers who do not breastfeed also have a higher incidence of developing breast cancer and ovarian cancer\(^3\). Breastfeeding is recommended by many organizations including American Academy of Pediatrics, Centers for Disease Control and Prevention as well as World Health Organization\(^4\). It is recommended that babies be breastfed exclusively for their first 6 months and can be continued as long as the mother and baby mutually wish\(^5\).

Most of the working mothers in the U.S. have to return to work within three months after delivery\(^6\), causing a sharp decline in the exclusive breastfeeding rate\(^7\). Many mothers
choose to express breast milk so that their child can be fed human milk even with mothers’ absence. It is reported that about 85% of the breastfeeding mothers had expressed milk after delivery, with 43% having done so occasionally and 25% on a regular basis.\textsuperscript{8,9}

Guidelines have been established on human milk handling as well as milk storage.\textsuperscript{10–12} Among those, a clinical protocol\textsuperscript{11} written by Academy of Breastfeeding Medicine (ABM) Protocol Committee is widely used by CDC and other social media for human milk storage. In the protocol, the storage time recommended is mostly based on the bacteriological quality of human milk during storage, which serves as “a marker for milk quality”. It is a priority to consider the bacteriological status of milk during storage to ensure it is safe to offer to a baby, but the quality change of other components is also important to consider. Only frozen milk was discussed in the protocol for its fragile components including the immune active compounds during storage. The same problems were found in other storage guidelines.\textsuperscript{10,12,13} As critical ingredients in human milk, immune active compounds shall be preserved to ensure it is fully delivered to the baby.

Most previous studies regarding the immune active compounds in human milk were focused on either the active ingredients of human milk without storage and how these ingredients affect infants,\textsuperscript{14–17} or the heat and pasteurization effect on immune active compounds in human milk.\textsuperscript{18–21} Though a few research studies\textsuperscript{19,22,23} investigated immunological quality of human milk during refrigerated storage, most were limited to a storage duration no longer than 96 hours. According to CDC\textsuperscript{24} and ABM\textsuperscript{11} human milk storage guidelines, human milk can be stored for much longer than 96 hours in a refrigerator. Therefore, it is necessary to extend the study length and understand the immunological
property of human milk throughout the recommended storage period. Furthermore, little attention was paid to the immune active compounds during room temperature storage. Emanuele\textsuperscript{25} examined the immune active compounds at room temperature storage (25°C) and found that SIgA, lysozyme and lactoferrin content were not altered after 4 hours. However, similar to the problem with the findings on refrigerated storage, the study did not reach the CDC recommended storage time of 6-8 hours at room temperature. Though Molinari\textsuperscript{26} examined room temperature storage in a length of 72 hours and found immunological proteins were stable, more research can be done to validate the findings.

There were two goals for the current study. The first goal was to study the immune factors in human milk stored for a longer time period than has previously been studied, at both room temperature and refrigerated temperature. The second goal was to study the impact of storage on immune factors in leftover human milk, which is the milk in a bottle that has been partially consumed by a baby and left in the bottle. It is common for milk to be leftover; however, currently there are no studies examining the immunological properties of leftover milk during storage\textsuperscript{12}. Data from the study will provide evidence to inform human milk storage guidelines, especially in regards to the immunological quality of human milk at various storage conditions for fresh and leftover human milk.

3.2 Methods

3.2.1 Study Design

Ten mother-baby dyads were recruited in the area of Raleigh, Durham and Chapel Hill, North Carolina from March to December 2013 following the criteria below:

✧ Mother is currently breastfeeding an infant who is between 2-11 months old;
Mother had a healthy, full term (>37 weeks gestation) delivery of baby;
Mother expresses milk using a breast pump and she cleans her pump parts regularly;
Baby is able to drink breast milk from a bottle.

Volunteer mothers were all Caucasian women with lactation stages between 2 to 10 months. Mothers were asked to express milk according to the protocol they would usually follow and to freeze the milk immediately after expression. Once 8 ounces of a mother’s milk was saved, milk was collected and delivered to the laboratory. The milk was transported in a cooler full of ice. The 8 ounces of breast milk was then divided into two 4-ounce sterile containers and kept frozen at -20°C. On the second meeting with the mother, a 4-ounce container with that mother’s fresh frozen milk was delivered back to the mother and baby. The mother thawed the milk in a way she usually did and fed her baby 1-2 ounces of the milk with their own clean bottle. The feeding process took 1-2 minutes. The leftover milk was collected in a new sterile container and delivered back to the laboratory in an ice-filled cooler. Each mother’s milk was handled separately. The whole sample collection process for each mother was finished within a month.

Immediately after the leftover milk was collected, it was transported back to the laboratory. Both the unfed (fresh) and leftover milk samples were allocated into pre-marked sterile conical tubes. The leftover samples were stored at room temperature (24°C) for 0 hours, 3 hours, 6 hours and 9 hours and refrigerated temperature (4°C) for 1 day, 3 days, 5 days and 7 days, respectively. The unfed fresh milk samples were stored at room temperature (24°C) for 0 hours, 3 hours, 9 hours and 12 hours and refrigerated temperature (4°C) for 1 day, 3 days, 5 days and 7 days, respectively.
After the designed storage time, each sample was separated into three sterile tubes and stored in a freezer at -80°C, preparing for total protein, lysozyme activity and secretory IgA activity measurement. Total protein content was measured by Pierce® BCA Protein Assay Kit (Prod # 23227, Thermo Scientific) using 96-well plates. Lysozyme activity was measured by a *Micrococcus lysodeikticus* based turbidimetric assay using 96-well plates. Method was adopted from Worthington Biochemical Corporation\textsuperscript{27} and Kenitic 96-Well Turbidimetric Lysozyme Assay Manuel \textsuperscript{28}. Secretory IgA activity was determined by a kinetic indirect enzyme linked immunosorbent assay (ELISA) using heat-killed *E. coli* somatic O antigens, modified by Chen\textsuperscript{29} and Viazis\textsuperscript{30}. All assays were conducted in triplicate. For detailed description of the assay procedure, please refer to Appendix 2 (Total Protein Measurement), 3 (Lysozyme Activity Measurement) and 4 (Secretory IgA Activity Measurement).

**3.2.2. Statistical Methods**

Linear mixed effects models appropriate to the repeated measures design were fit using the MIXED procedure of the SAS software package. In particular, fixed factorial effects for storage treatment, temperature and time were included along with random effects for sample and sample-by-treatment interaction. The design could be called a complete block split-plot design, with storage treatment as a whole-plot factor, sample as a complete block, and time and temperature as split-plot factors, since each subsample was measured repeatedly over time. The time regime over which these measurements were made was
different for the two temperatures, so that time effects are nested within temperature in the following formulation of the model:

\[
Y_{ijkt} = \mu + \alpha_i + \beta_j + \tau_{t(j)} + (\alpha\beta)_{ij} + (\alpha\tau)_{it(j)} + S_k + (\alpha S)_{ik} + E_{ijkt}
\]

Here \(i, j, k\) and \(t\) are indicates for treatment, temperature, sample and time, respectively. Greek symbols (\(\alpha, \beta\) and \(\tau\)) denote fixed factorial effects, while capital letters (\(S\) and \(E\)) denote random effects. Selected pairwise comparisons were used to evaluate simple treatment effects in cases where there was evidence of interactions involving the treatment (using the LSMEANS statement and the SLICE option of the MIXED procedure). Separate univariate models were fit to each of the three response variables (protein, lysozyme activity and SIgA activity) for the experiment. Diagnostic plots of residuals did not indicate any violations of the usual assumptions underlying analysis of variance.

3.3 Results

3.3.1. Room Temperature Storage

During room temperature storage (24°C), no significant change in protein content, lysozyme activity and secretory IgA activity was found throughout 12 hours of fresh milk storage (Table 7). Those components also remained stable in leftover human milk for up to 9 hours at room temperature (Table 8).
To understand the effect of partial consumption on the examined components, pairwise comparison was conducted between fresh and leftover milk at each storage time. Lysozyme activity (table 7) and total protein content (table 8) of fresh and leftover milk were similar throughout the room temperature storage. Pairwise comparison also confirmed that there were no significant differences between fresh and leftover milk on protein content and lysozyme activity during 9 hours of room temperature storage. However, a significant decrease in SIgA activity was observed after partial consumption (Figure 9). A reduction of 18.0% (P<.05) was observed in leftover milk at 0 hours compared with that of fresh milk at 0 hours. This reduction may be caused by the extra heating step during partial consumption when mothers prepared milk for infant feeding or adherence to the feeding bottle. Mothers in the study mostly heated milk using a hot water tub and one used electronic bottle warmer system that utilized hot steam to warm the milk. We did not measure the temperature of the reheated milk, and it may vary among individuals, but the degree of denaturation increases with time and temperature treatment. However, as mentioned earlier, though SIgA activity in leftover milk was lower than fresh milk, its concentration remained stable during storage.

3.3.2. Refrigerated Storage

Lysozyme and secretory IgA activity in fresh milk remained stable throughout 7 days of refrigerated storage (4°C) (Table 9).

In fresh milk, a significant increase in total protein content (P<.05) was found during refrigerated storage (Table 9). According to pairwise comparison between each time point, a significant protein increase occurred at day 5 (P<.05) and became more significant at day 7
(P<.01), as is also seen in Figure 10. The increased protein content detected by BCA assay might be a signal of increase in amino acid and di or tripeptides caused by protein degradation. As described in Pierce® Protein Assay Technical Handbook, though BCA assay is a sensitive method to detect protein, the presence of any four single amino acid residue including cysteine, cystine, tyrosine and tryptophan may reduce Cu$^{2+}$ to Cu$^{1+}$, thus, developing the color reaction. Furthermore, studies performed with di and tripeptides indicate a total amount of color produced in BCA greater than can be accounted from the protein standard alone, which causes an abnormally high result. Protein degradation may occur within the seven days of refrigerated storage, which causes an increase in short chain peptides and amino acids. Since no protein change occurred at room temperature storage, refrigerated temperature may have activated the protease in human milk. In previous studies, refrigerated temperature has been confirmed to activate the lipase during human milk storage. A similar mechanism may exist for protease in human milk during refrigeration. Further research is needed to confirm the mechanism.

In leftover milk, though an increase in protein content was also observed (Table 10), it was much slower than that of fresh milk and it was not significant (Figure 10). When mothers prepared milk to feed the babies, the heating step may have destroyed some protease, causing minor protein degradation in leftover milk.

Similar with fresh milk, no significant change was found in the SIgA activity of leftover milk during 7 days of refrigerated storage (Table 10), although SIgA activity in leftover milk was lower than that of fresh milk (Figure 11).
According to the observations in room temperature storage, lysozyme activity was not affected by partial consumption (Figure 7). No significant difference was either found between fresh and leftover milk at day 0 as shown in Figure 12. However, a sharp decline (9.5% reduction, P<.01) in lysozyme activity was observed at day 1 compared with that at day 0. After that, lysozyme activity remained stable throughout 1 to 7 days of refrigerated storage (Table 10). It is hypothesized that since it is not an optimal temperature for the enzyme, refrigerated temperature may have weakened the lysozyme activity in leftover milk which took place immediately when placed in refrigerator, but its activity remained through the storage period.

3.4 Discussion

*Fresh milk.* No changes in total protein content, lysozyme activity and secretory IgA activity were observed during 12 hours of room temperature storage, which is in accordance with the observations of Molinari\(^\text{26}\) and Chandan\(^\text{35}\) and suggest that immune active compounds can hold activity during room temperature storage. Lysozyme activity and SIgA activity remained active throughout the 7 days’ refrigeration which is similar with Giribaldi’s finding\(^\text{23}\). An increase in total protein content (P< .05) was observed at day 5 and day 7 of fresh milk’s refrigerated storage, which may indicate increased amino acids and di or tripeptides content in milk caused by protein degradation. A significant difference in protein content was also observed by Garza between room temperature and refrigerated storage, in which the protein content in refrigerated storage was slightly lower\(^\text{36}\). Though most studies found no change in protein content during 4°C refrigerated storage, the milk had been stored
no longer than 96 hours\textsuperscript{22,23,37}. In our study, protein change was not observed until day 5. This indicates that proteolysis may only occur after prolonged refrigeration. Prolonged refrigerated storage may have activated protease, which is similar to the activation of lipase under refrigerated temperature. More research with extended storage duration is in need to improve the understanding.

*Leftover milk.* This is the first study we are aware of that focuses on leftover human milk. In our study, no significant change in leftover milk was found for any of the examined components (protein, lysozyme and SIgA) in either 9 hours of room temperature storage or 7 days of refrigerated storage. Protein degradation in refrigerated storage was minimized in leftover milk, which is probably due to the extra heating step during partial consumption that destroyed some protease in human milk. The extra heating step was also found to cause a reduction in SIgA activity of leftover milk. However, the activity of SIgA remained stable throughout the storage period. The impact of partial consumption on lysozyme activity was not significant by comparing between fresh and leftover milk at room temperature storage. At the same time, lysozyme activity in leftover milk might be sensitive to the refrigerated temperature, which caused a reduction observed at first day of refrigerated storage.

Volunteers were all recruited from Raleigh, Durham and Chapel Hill of North Carolina. Sample variance may exist in different regions. Additional research should be done in other areas to avoid the geographic limitation.
3.5 Conclusion

Lysozyme in fresh and leftover human milk maintained its activity throughout the room temperature and refrigerated storage times examined, and was not affected by partial consumption. However, lysozyme activity in leftover milk stored at the refrigerated temperature was somewhat lower than that at room temperature, which is probably due to the enzyme sensitivity to low temperature. Protein content remained stable during room temperature storage in both fresh milk and leftover milk. Prolonged refrigeration may activate the protease in human milk thus causing protein degradation in refrigerated storage. An extra heating step during human milk consumption may destroy some protease and slow down the protein degradation in leftover milk during refrigerated storage. The extra heating step also caused a reduction in SIgA activity, but SIgA in both fresh milk and leftover milk resisted breakdown during the storage conditions examined. More research can be done to investigate the effect of prolonged refrigeration on protease and the effect of partial consumption on SIgA activity.
3.6 References


### Tables

#### Table 2 Fresh milk protein content, lysozyme and SIgA activity during room temperature storage

<table>
<thead>
<tr>
<th>Storage Time (h)</th>
<th>Total protein (mg/ml)</th>
<th>Lysozyme activity (unit/l)</th>
<th>SIgA activity (ug/ml)</th>
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<tr>
<td>0</td>
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<td>86.16</td>
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</table>

*P value<sup>a</sup> 0.7351 0.9221 0.1851

Data represent means ±MSE (mean square error) obtained by milk samples from 10 individual volunteers.

<sup>a</sup>Source of variance: time (Model specifies sources of variance for the ANOVA), indicating the effect of time on the above milk components.

#### Table 3 Leftover milk protein content, lysozyme and SIgA activity during room temperature storage

<table>
<thead>
<tr>
<th>Storage Time (h)</th>
<th>Total protein (mg/ml)</th>
<th>Lysozyme activity (unit/l)</th>
<th>SIgA activity (ug/ml)</th>
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<td>MSE</td>
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*P value<sup>a</sup> 0.7356 0.9812 0.4087

Data represent means ±MSE (mean square error) obtained by milk samples from 10 individual volunteers.

<sup>a</sup>Source of variance: time (Model specifies sources of variance for the ANOVA), indicating the effect of time on the above milk components.
Table 4 Fresh milk total protein, lysozyme and SIgA activity during refrigerated storage

<table>
<thead>
<tr>
<th>Storage Time (d)</th>
<th>Total protein (mg/ml)</th>
<th>Lysozyme activity (unit/l)</th>
<th>SIgA activity (ug/ml)</th>
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<td>13.47</td>
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\( P \text{ value}^{a} \) < .05 0.4025 0.3694

Data represent means ±MSE (mean square error) obtained by milk samples from 10 individual volunteers. 
\(^a\)Source of variance: time (Model specifies sources of variance for the ANOVA), indicating the effect of time on the above milk components.

Table 5 Leftover milk total protein, lysozyme and SIgA activity during refrigerated storage

<table>
<thead>
<tr>
<th>Storage Time (d)</th>
<th>Total protein (mg/ml)</th>
<th>Lysozyme activity (unit/l)</th>
<th>SIgA activity (ug/ml)</th>
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\( P \text{ value}^{a} \) 0.1123 0.6652 0.5224

Data represent means ±MSE (mean square error) obtained by milk samples from 10 individual volunteers. 
\(^a\)Source of variance: time (Model specifies sources of variance for the ANOVA), indicating the effect of time on the above milk components.
Figure 7. Lysozyme activity of fresh and leftover milk at room temperature storage (n=10). Storage time of fresh milk: 0 hours, 3 hours, 9 hours and 12 hours; storage time of leftover milk: 0 hours, 3 hours, 6 hours and 9 hours. There was no significant change of lysozyme activity over time for either fresh or leftover milk. The lysozyme activity difference between fresh milk and leftover milk was minimal according to the pairwise comparison on each storage time point.
Figure 8. Protein content of fresh and leftover milk at room temperature storage (n=10). Storage time of fresh milk: 0 hours, 3 hours, 9 hours and 12 hours; storage time of leftover milk: 0 hours, 3 hours, 6 hours and 9 hours. There was no significant change of total protein content over time for either fresh or leftover milk. The difference of protein content between fresh milk and leftover milk was minimal according to the pairwise comparison at each storage time point.
Figure 9. Secretory IgA activity of fresh and leftover milk at room temperature storage (n=10). Storage time of fresh milk: 0 hours, 3 hours, 9 hours and 12 hours; storage time of leftover milk: 0 hours, 3 hours, 6 hours and 9 hours. There was no significant change of SIgA activity over time for either fresh or leftover milk. A significant reduction of SIgA activity (P<.05) was observed after partial consumption in leftover milk.
Figure 10. Protein content of fresh and leftover milk at refrigerated storage (n=10). Storage time of fresh milk and leftover milk: 0 days, 1 days, 3 days, 5 days and 7 days. Letters (a, b et al) indicate the significant difference between two points: if the two points do not have any same letter, they are significantly different. Fresh milk had a significant protein increase starting from day 5, which might indicate the protein degradation by protease after prolonged refrigerated storage. On the other hand, the protein increase in leftover milk was not significant, where the protease may be inhibited by an extra heating step during partial consumption. According to pairwise comparison, no significant difference was found at any time point before day 5.
Figure 11. Secretory IgA activity of fresh and leftover milk at refrigerated storage (n=10). Storage time of fresh milk and leftover milk: 0 days, 1 days, 3 days, 5 days and 7 days. According to pairwise comparison, SIgA remained active in fresh and leftover milk throughout 7 days of refrigerated storage. A significant reduction of SIgA activity (P<.05) was observed after partial consumption in leftover milk.
Figure 12. Lysozyme activity of fresh and leftover milk at refrigerated storage (n=10). Storage time of fresh milk and leftover milk: 0 days, 1 days, 3 days, 5 days and 7 days. According to pairwise comparison, no significant change was observed throughout the 7 days of refrigerated storage in either fresh milk or leftover milk. The difference between fresh and leftover milk at each time point was not significant either.
CHAPTER 4

4. Bioactive Factors in Fresh, Stored, and Leftover Pasteurized Human Milk

Abstract

Objective: To determine the immunological quality of fresh and leftover pasteurized human milk during storage and to provide scientific evidence for the handling and storage of pasteurized leftover human milk.

Methods: Mother-baby dyads were recruited and expressed milk was pasteurized in our lab. Each baby was fed 1-2 ounces of her own mother’s pasteurized milk from a bottle. Milk that was left in the bottle (pasteurized leftover milk) was collected in sterile containers. Each mother’s pasteurized leftover milk was assayed for total protein content, lysozyme activity and secretory IgA activity after the designed storage times of 0 hours, 3 hours, 6 hours and 9 hours at room temperature (24°C) and 1 day, 3 days, 5 days and 7 days in refrigerator (4°C).

Results: There was no significant change (P>.05) in total protein content, lysozyme activity and secretory IgA activity for 12 hours of room temperature storage and 7 days of refrigerated storage for pasteurized milk, or in pasteurized leftover milk during 9 hours of room temperature storage and 7 days of refrigerated storage. No significant differences were found in the components examined after baby’s partial consumption.

Conclusions: Prolonged storage at room temperature and refrigerator (12 hours at room temperature and 7 days in refrigerator) had little impact on overall immunological quality of pasteurized donor milk. Pasteurized leftover milk that was partially consumed by a baby had no significant difference in immunological quality compared with pasteurized milk.
Pasteurized leftover milk was able to maintain its immunological quality for at least 9 hours at room temperature storage and at least 7 days in refrigerated storage.

4.1 Introduction

Human milk as the optimal source for infant feeding is widely agreed upon by several professional organizations\textsuperscript{1-4}. It is recommended that mothers breastfeed their babies exclusively for at least the first six months\textsuperscript{5,6} to best meet the nutritional and immunological needs for the infants. However, there are situations in which mother’s own milk may not be available for the hospitalized newborn. During these situations, DHM is a recommended feeding alternative to mother’s own milk, rather than feeding commercial formula\textsuperscript{7}.

4.1.1. How Human Milk Banks Operate

In human milk banks, donors go through verbal and serum screening to ensure the donor is in a healthy condition with no medication or drug usage\textsuperscript{8}. Once the breast milk from a healthy donor is received in the milk bank, it is frozen at -20\degree C to inhibit bacterial growth during storage. Milk from several donors (usually 3 to 5 donors) is pooled to ensure an even distribution of milk components. Afterwards, milk is pasteurized using the Holder method, in which milk is heated to 62.5\degree C and held for 30 minutes in order to eliminate the microorganisms in the milk. A milk control is checked for bacteria growth after pasteurization. The pasteurized milk is shipped frozen overnight to the recipients. Milk banks follow standard HMBANA guidelines\textsuperscript{9-11} to ensure safety of the milk.

There is an increasing demand for DHM. Currently there are 17 human milk banks in North America, with 3 more banks in development\textsuperscript{12}. It is reported that the Human Milk
Banking Association of North America (HMBANA) process more than one million ounces of human milk every year\textsuperscript{7,11}, and in the year of 2011, about 2.18 million ounces of breast milk were distributed through HMBANA\textsuperscript{13}. Among those recipients, the population that comprised the greatest demand of human milk is the hospitalized infants, including the preterm infants who are critically ill or premature\textsuperscript{14}. It is also HMBANA’s mission to ensure that the processed milk is served to the preterm babies as a priority\textsuperscript{15}.

4.1.2. Benefits of Human Donor Milk

To the hospitalized baby, the advantages of human donor milk over commercial formula are superior and obvious. Milk of human origin not only offers species specific nutrients which best fit an infant’s need, but also provides precious immune active compounds that protect sick babies from diseases. Though some of the bioactive compounds were reduced by pasteurization, most of the key nutritional and biological compounds remained stable after pasteurization: reduction in IgA and SIgA concentration were observed\textsuperscript{16–18} after pasteurization, but the decrease did not diminish milk’s activity against enteropathogenic \textit{E.coli}\textsuperscript{19}. Additionally, other components including immunoglobulins\textsuperscript{20}, carbohydrates\textsuperscript{7,21} and lipids\textsuperscript{22} were found unchanged by pasteurization.

There is clinical evidence that DHM is critical for preterm infants. Studies\textsuperscript{23,24} have found that human donor milk is able to prevent or reduce the incidence of NEC among preterm infants\textsuperscript{23–26}. Two meta-analyses found that infants fed with human donor milk had a lower risk of necrotizing enterocolitis (NEC) compared with infants being fed with artificial milk\textsuperscript{27,28}.
DHM can decrease costs of health care. Compared to the cost of NEC treatment, the cost of DHM is minimal. Feeding of DHM has been shown to reduce hospital stay in the NICU\(^{29}\). Therefore, every $1 spent on human donor milk can save approximately $11 to $37 for the health care and medication expenses, based on Wight’s calculation\(^{30}\).

As one of the most important attributes in breast milk, immune active compounds should be preserved during storage and handling. Several studies have been done examining the impact of Holder pasteurization on the immunological quality of human milk\(^{14,15,24–26}\). However, little attention was paid to the immunological activity of human milk during storage, after the pasteurization process. The impact of storage temperature and duration on fresh and leftover pasteurized human milk are not known. The objective of this study was to examine the immunological quality of pasteurized and pasteurized leftover milk during storage, thus providing scientific support for human donor milk storage in milk bank and hospital settings.

### 4.2 Methods

#### 4.2.1 Study Design

Thirteen mother-baby dyads were recruited in the area of Raleigh, Durham and Chapel Hill, North Carolina from March to December 2013 following the criteria below:

- Mother is currently breastfeeding an infant who is between 2-11 months old;
- Mother had a healthy, full term (>37 weeks gestation) delivery of baby;
- Mother expresses milk using a breast pump and clean pump regularly;
- Baby is able to drink breast milk from a bottle.
Volunteer mothers were all Caucasian women with lactation stages between 2 to 10 months. Mothers were asked to express milk according to the protocol they would usually follow and to freeze the milk immediately after expression. Once 8 ounces of a mother’s milk was saved, milk was collected and delivered to the laboratory. The milk was transported in a cooler full of ice. The 8 ounces of fresh milk was then divided into two 4-ounce sterile containers. The 4-ounce containers filled with milk were pasteurized at 62.5°C for 30 min, according to the Holder Pasteurization method utilized in all HMBANA milk banks. The pasteurization process was conducted using a shallow-form shaking bath (Thermo Scientific, U.S.A.). Three thermo detectors were used to detect the temperature during pasteurization, with one indicated on the screen, one inserted in water bath and the other one inserted in the middle of a controlled milk bottle. After all thermometers reached a temperature of 62.5°C, the batch was held for 30 minutes at the temperature and then cooled immediately in an ice bath. After samples were cooled, the pasteurized milk was stored in a freezer at -20°C. After processing, the 4-ounce container with mother’s pasteurized milk was delivered back to the original mother. The mother was asked to thaw the milk as she usually did at home and to feed her baby 1-2 ounces of the milk with their own clean bottle. The feeding process took 1-2 minutes. The leftover milk was collected in a new sterile container and delivered back to the laboratory in an ice-filled cooler. Each mother’s milk was handled separately. The whole sample collection process for each mother was finished within a month.

Immediately after the pasteurized leftover milk sample was collected, it was transported back to the laboratory. Both the fresh pasteurized milk (PM) sample and the pasteurized leftover milk (PLM) sample were allocated into pre-marked sterile conical tubes.
The PLM samples were stored at room temperature (24°C) for 0 hours, 3 hours, 6 hours and 9 hours and refrigerated temperature (4°C) for 1 day, 3 days, 5 days and 7 days, respectively. The PM samples were stored at room temperature (24°C) for 0 hour, 3 hours, 9 hours and 12 hours and refrigerated temperature (4°C) for 1 day, 3 days, 5 days and 7 days, respectively.

After the designed storage time, each sample was separated into three sterile tubes and stored in a freezer at -80°C, in preparation for total protein, lysozyme activity and secretory IgA activity measurement.

Total protein content was measured by Pierce® BCA Protein Assay Kit (Prod # 23227, Thermo Scientific) using 96-well plates. Lysozyme activity was measured by a Micrococcus lysodeikticus based turbidimetric assay using 96-well plates. Method was adopted from Worthington Biochemical Corporation (1972) and Kenitic 96-Well Turbidimetric Lysozyme Assay Manual. Secretory IgA activity was determined by a kinetic indirect enzyme linked immunosorbent assay (ELISA), modified by Chen and Viazis. For detailed assay procedure, please refer to Appendix 2 (Total Protein Measurement), Appendix 3 (Lysozyme Activity Measurement) and Appendix 4 (Secretory IgA Activity Measurement).

4.2.2. Statistical Methods

Linear mixed effects models appropriate to the repeated measures design were fit using the MIXED procedure of the SAS software package. In particular, fixed factorial effects for storage treatment, temperature and time were included along with random effects for sample and sample-by-treatment interaction. The design could be called a complete block split-plot design, with storage treatment as a whole-plot factor, sample as a complete block,
and time and temperature as split-plot factors, since each subsample was measured repeatedly over time. The time regime over which these measurements were made was different for the two temperatures, so that time effects are nested within temperature in the following formulation of the model:

$$Y_{ijkt} = \mu + \alpha_i + \beta_j + \tau_t(j) + (\alpha \beta)_{ij} + (\alpha \tau)_{tj} + S_k + (\alpha S)_{tk} + E_{ijkt}$$

Here $i$, $j$, $k$ and $t$ are indicates for treatment, temperature, sample and time, respectively. Greek symbols ($\alpha$, $\beta$ and $\tau$) denote fixed factorial effects, while capital letters ($S$ and $E$) denote random effects. Selected pairwise comparisons were used to evaluate simple treatment effects in cases where there was evidence of interactions involving the treatment (using the LSMEANS statement and the SLICE option of the MIXED procedure). Separate univariate models were fit to each of the three response variables (protein, lysozyme activity and SIgA activity) for the experiment. Diagnostic plots of residuals did not indicate any violations of the usual assumptions underlying analysis of variance.

4.3 Results

4.3.1. Total Protein Content

During 12 hours room temperature storage of PM, there was a slight decrease in total protein content from 12.51 to 11.71 mg/ml at 0 hours and 12 hours respectively. But the decrease was not significant (Table 11). In PLM, no significant protein content change occurred during 9 hours of room temperature storage (Table 12).
Similar to the results of room temperature storage, no significant change in protein content was found throughout 7 days of refrigerated storage for either PM (Table 13) or PLM (Table 14).

Protein content in human milk was not significantly affected by partial consumption either. According to Figure 13-14, data at each time point of PM and PLM are all very close to each other. By further comparing the differences between PM and PLM’s protein content by pairwise comparison, no significant differences were found at any time point in both room temperature storage (Table 15) and refrigerated storage (Table 16).

4.3.2. Lysozyme Activity

Lysozyme activity of both PM and PLM remained stable throughout the storage conditions examined (Table 11-14, Figure 15). According to pairwise comparison between PM and PLM (Table 17-18), partial consumption by a baby did not significantly affected the lysozyme activity of pasteurized milk.

4.3.3. Secretory IgA Activity

Secretory IgA activity in PM and PLM was maintained throughout 9 hours of room temperature storage and 7 days of refrigerated storage (Figure 17, 18). No significant change occurred throughout the storage conditions examined (Table 11-14).

The effect of partial consumption on human milk SIgA activity was minimal. In room temperature storage, no significant differences in SIgA activity were found between PM and PLM at 0 hours, 3 hours and 9 hours (Table 19). In refrigerated storage, only at day 7 was there a significant difference in SIgA between PM and PLM (Table 20). Combined with the
fact that the change of SIgA activity over time was not significant for PM as well as PLM, this significant difference at day 7 might due to a random variation of a higher SIgA activity value at PM’s day 7.

4.4 Discussion

Several studies have investigated the immunological properties of human milk during storage\textsuperscript{39–42}, but none have examined the effect of storage on pasteurized human donor milk. Since Holder pasteurization can negatively affect some immune active components including SIgA\textsuperscript{16,18} and other immunological proteins in human milk\textsuperscript{18,43–45}, the immune stability of pasteurized milk during storage should also be considered.

In our study, total protein content, lysozyme and SIgA activity of both PM and PLM remained stable throughout the storage conditions examined. Compared with PM, all the components examined in PLM were unaffected by partial consumption of the milk sample by the baby.

According to the findings above, pasteurization may have inactivated the degradation enzymes such as protease in human milk as was also observed by Friend\textsuperscript{46}. The decreased enzyme activity in pasteurized human milk thus provided a stable environment for immune components during storage.

In this study, volunteers were all recruited from the same area (Raleigh, Durham and Chapel Hill, North Carolina). Milk from populations may vary in different regions. More research should be done in other areas to avoid the geographic limitation.
4.5 Conclusion

In our study, prolonged storage at room temperature and refrigerator were found to have little impact on overall immunological components of pasteurized donor milk. Total protein content, lysozyme activity and secretory IgA of pasteurized donor milk remained stable for at least 12 hours of room temperature storage and 7 days of refrigerated temperature storage. Little effect has been associated with partial consumption on overall immunological quality of pasteurized donor milk. Pasteurized leftover milk was able to maintain its immunological activity for at least 9 hours at room temperature storage and at least 7 days in refrigerated storage. More research can be done on the immunological property of fresh and leftover pasteurized milk during storage to make more valid conclusions.
4.6 References


12. HMBANA. HMBANA milk bank locations. Available at: https://www.hmbana.org/milk-bank-locations.


Tables

Table 6 Pasteurized milk protein content, lysozyme and SIgA activity during room temperature storage

<table>
<thead>
<tr>
<th>Storage Time (h)</th>
<th>Total protein (mg/ml)</th>
<th>Lysozyme activity (unit/l)</th>
<th>SIgA activity (µg/ml)</th>
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<tbody>
<tr>
<td>0</td>
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<td>42.00</td>
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<td>3</td>
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<td>716.71</td>
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<td>11.89</td>
<td>41.94</td>
<td>688.15</td>
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<tr>
<td>12</td>
<td>11.71</td>
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<td>702.96</td>
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<tr>
<td>MSE</td>
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<td><strong>P value</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0688</td>
<td>0.4866</td>
<td>0.166</td>
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Data represent means ±MSE (mean square error) obtained by milk samples from 10 individual volunteers.  
<sup>a</sup>Source of variance: time (Model specifies sources of variance for the ANOVA), indicating the effect of time on the above milk components.

Table 7 Pasteurized leftover milk protein content, lysozyme and SIgA activity during room temperature storage

<table>
<thead>
<tr>
<th>Storage Time (h)</th>
<th>Total protein (mg/ml)</th>
<th>Lysozyme activity (unit/l)</th>
<th>SIgA activity (µg/ml)</th>
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<tr>
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<td>70.99</td>
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<td><strong>P value</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
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Data represent means ±MSE (mean square error) obtained by milk samples from 10 individual volunteers.  
<sup>a</sup>Source of variance: time (Model specifies sources of variance for the ANOVA), indicating the effect of time on the above milk components.
Table 8 Pasteurized milk protein content, lysozyme and SIgA activity during refrigerated storage

<table>
<thead>
<tr>
<th>Storage Time (h)</th>
<th>Total protein (mg/ml)</th>
<th>Lysozyme activity (unit/l)</th>
<th>SIgA activity (µg/ml)</th>
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<tr>
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<td>40.01</td>
<td>704.06</td>
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<td>11.93</td>
<td>41.25</td>
<td>707.15</td>
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<tr>
<td>MSE</td>
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<td>6.11</td>
<td>69.91</td>
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$P value^a$ 0.4267 0.6081 0.673

Data represent means ±MSE (mean square error) obtained by milk samples from 10 individual volunteers.

$^a$Source of variance: time (Model specifies sources of variance for the ANOVA), indicating the effect of time on the above milk components.

Table 9 Pasteurized leftover milk protein content, lysozyme and SIgA activity during refrigerated storage

<table>
<thead>
<tr>
<th>Storage Time (h)</th>
<th>Total protein (mg/ml)</th>
<th>Lysozyme activity (unit/l)</th>
<th>SIgA activity (µg/ml)</th>
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<td>694.31</td>
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<td>12.18</td>
<td>39.71</td>
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<td>MSE</td>
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$P value^a$ 0.9039 0.6883 0.4961

Data represent means ±MSE (mean square error) obtained by milk samples from 10 individual volunteers.

$^a$Source of variance: time (Model specifies sources of variance for the ANOVA), indicating the effect of time on the above milk components.
Table 10 Differences in protein content between pasteurized and pasteurized leftover milk during room temperature storage

<table>
<thead>
<tr>
<th>Effect</th>
<th>Time</th>
<th>Difference (mg/ml)</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>PM vs. PLM</td>
<td>0 h</td>
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</tr>
<tr>
<td></td>
<td>3 h</td>
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</tr>
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<td></td>
<td>9 h</td>
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<td>0.6986</td>
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Table 11 Differences in protein content between pasteurized and pasteurized leftover milk during refrigerated storage

<table>
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<th>Time</th>
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</tr>
</thead>
<tbody>
<tr>
<td>PM vs. PLM</td>
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<td>0.51</td>
<td>0.2571</td>
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<td></td>
<td>3 d</td>
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<td></td>
<td>5 d</td>
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<td></td>
<td>7 d</td>
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Table 12 Differences in lysozyme activity between pasteurized and pasteurized leftover milk during room temperature storage

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<th>Effect</th>
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<th>P value</th>
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<td>PM vs. PLM</td>
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Table 13 Differences in lysozyme activity between pasteurized and pasteurized leftover milk during refrigerated storage

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<td>PM vs. PLM</td>
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<td>7 d</td>
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Table 14 Differences in secretary IgA activity between pasteurized and pasteurized leftover milk during room temperature storage

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<th>P value</th>
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</thead>
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<td>PM vs. PLM</td>
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<tr>
<td></td>
<td>3 h</td>
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</tr>
<tr>
<td></td>
<td>9 h</td>
<td>-10.33</td>
<td>0.5355</td>
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Table 15 Differences in secretary IgA activity between pasteurized and pasteurized leftover milk during refrigerated storage

<table>
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<tr>
<th>Effect</th>
<th>Time</th>
<th>Difference (ug/ml)</th>
<th>P value</th>
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</thead>
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<td>PM vs. PLM</td>
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<td>7 d</td>
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Figure 13. Protein content of pasteurized and pasteurized leftover milk at room temperature storage (n=13). Storage time of fresh milk: 0 hours, 3 hours, 9 hours and 12 hours; storage time of leftover milk: 0 hours, 3 hours, 6 hours and 9 hours.
Figure 14. Protein content of pasteurized and pasteurized leftover milk at refrigerated storage (n=13). Storage time of pasteurized and pasteurized leftover milk: 0 days, 1 days, 3 days, 5 days and 7 days.
Figure 15. Lysozyme activity of pasteurized and pasteurized leftover milk at room temperature storage (n=13). Storage time of fresh milk: 0 hours, 3 hours, 9 hours and 12 hours; storage time of leftover milk: 0 hours, 3 hours, 6 hours and 9 hours.
Figure 16. Lysozyme activity of pasteurized and pasteurized leftover milk at refrigerated storage (n=13). Storage time of pasteurized and pasteurized leftover milk: 0 days, 1 days, 3 days, 5 days and 7 days.
Figure 17. Secretory IgA activity of pasteurized and pasteurized leftover milk at room temperature storage (n=13). Storage time of fresh milk: 0 hours, 3 hours, 9 hours and 12 hours; storage time of leftover milk: 0 hours, 3 hours, 6 hours and 9 hours.
Figure 18. Secretory IgA activity of pasteurized and pasteurized leftover milk at refrigerated storage (n=13). Storage time of pasteurized and pasteurized leftover milk: 0 days, 1 days, 3 days, 5 days and 7 days.
CHAPTER 5

5. Impact of Holder Pasteurization on Immunological Activity of Human Milk

Abstract

Objectives. To investigate the effect of Holder pasteurization on the immune active components in human milk.

Methods. Each of the expressed milk from individual mothers was divided into two equal portions. One portion was Holder pasteurized (62.5°C for 30 minutes), and the other portion was set as a control (unpasteurized milk). Milk samples were measured for total protein content, lysozyme activity and secretary IgA (SIgA) activity.

Results. Protein content was stable after pasteurization except for three samples (3/13) which showed a decrease. Lysozyme activity was significantly decreased (P<.001) by Holder pasteurization in all samples. Last, a significant increase (P<.05) in SIgA activity was also found after pasteurization.

Conclusions. Protein content was stable during Holder pasteurization, but for some individuals, a decrease in protein content may still occur due to higher protein sensitivity to heat. Lysozyme activity levels vary widely by individuals. Pasteurization was found to significantly reduce the lysozyme activity in all human milk samples, and it seems that the greater the lysozyme activity in the unpasteurized milk, the greater the decrease caused by pasteurization. SIgA activity in human milk also varies from mother to mother. The increase in SIgA activity after pasteurization may be due to a release of immune active elements such
as secretory component in SIgA caused by Holder pasteurization, rendering the IgA more likely to bind the fixed antigen in the ELISA assay.

5.1 Introduction

The significance of immune active components in human milk is widely recognized. Human milk not only contains numerous nutritional components that are tailored to the infant's needs, but also contains a variety of immune active compounds that help protect infants against the potential hazards in the environment. Clinically, infants fed human milk have lower risks of infectious morbidity and diabetes in later life compared with those who are not fed human milk. It is recommended that mothers breastfeed their babies exclusively for at least the first six months of life to ensure the best nutritional and immunological needs for the infants. However, when mother's own milk is not available for the hospitalized newborn, pasteurized human donor milk should be used as a feeding alternative to mother’s own milk as recommended by American Academy of Pediatrics, the Academy of Breastfeeding Medicine and WHO.

The demand for human donor milk in North America is greater than supply and is continuously increasing year by year. The most critical needs are from the hospitalized infants, including the preterm infants who are critically ill or premature, where human milk banks should be made available. Currently (2014) there are 17 human milk banks in North America with 3 more banks in development. It was reported that the Human Milk Banking Association of North America (HMBANA) process more than one million ounces of human
milk every year\textsuperscript{12,13}, and in 2011, about 2.18 million ounces of human milk were distributed through HMBANA\textsuperscript{14}.

In HMBANA milk banks, the milk is processed according to their guidelines\textsuperscript{12,15,16}. To ensure a microbiologically safe delivery of donor milk, the milk is pasteurized using the Holder method (62.5°C for 30 minutes) in order to eliminate all possible pathogens and viruses in milk.

5.1.1. Holder Pasteurization Procedure\textsuperscript{15,17,18}

Thawed human milk from 3 to 5 donors is pooled to ensure an even distribution of milk components. Milk is then bottled in clean containers with a consistent volume. Containers of milk are held in a shaking water bath with a temperature at 62.5°C for 30 minutes to eliminate the microorganisms in the milk. After Holder pasteurization, all containers are immediately cooled in a water-ice bath to reach a temperature of 4°C and then kept frozen at -20°C. A control milk sample from each batch is assayed for bacterial growth after pasteurization. Once no bacterial growth is confirmed, the pasteurized milk batch is shipped frozen overnight to the recipients.

Studies confirmed that Holder pasteurization is effective in eliminating the threat of pathogenic and viral contaminants\textsuperscript{19}, but some concerns also arise on the impact of Holder pasteurization on human milk components, especially the immune active compounds which are of great importance to vulnerable hospitalized infants. Studies have been done to examine the effect of pasteurization on various immune active compounds, but discrepancies among
results exist, particularly on the protein content, lysozyme activity and secretory IgA activity in human milk.

Proteins in human milk were found to be highly associated with the immunological property\(^\text{20}\). Several proteins in human milk provide activity against pathogenic bacteria and viruses\(^\text{21–23}\). A HIV-neutralizing protein was recently detected in human milk, which helps to protect babies from maternal HIV transmission\(^\text{24}\). Proteins in human milk were also reported to stimulate the infant’s immune defense system faster than artificial feedings\(^\text{25,26}\). A debate on the effect of Holder pasteurization on total protein content still remains unsettled. A study by Koenig found significant decrease in protein after Holder pasteurization\(^\text{27}\), whereas Góes and Braga observed no changed in protein content after the process\(^\text{28,29}\).

Lysozyme is a very important antimicrobial component that targets all gram-positive bacteria by degrading their outer cell wall\(^\text{30}\). In some vitro studies, lysozyme in human milk was also found to kill gram-negative bacteria as well as HIV virus\(^\text{31}\). Lysozyme concentration in human milk is higher than in milk from other species\(^\text{32}\) and is 3000 times greater than the level in cow’s milk\(^\text{33}\). Similar to protein, findings on the effect of Holder pasteurization on lysozyme vary: Evans observed a minor loss of 23.7% in lysozyme after Holder pasteurization\(^\text{34}\), Czank reported a tremendous loss of 60% in lysozyme\(^\text{35}\), while Ford found it remained stable\(^\text{36}\).

SIgA is the most critical immunoglobulin protein in human milk, not only in concentration (>90%), but also in biological activity. It acts by binding those microbes in the baby’s digestive tract to prevent their invasion to other tissues\(^\text{26}\) and is found to be associated with the defense of various pathogens including Escherichia coli, Salmonella, Streptococcus
pneumonia and the defense of virus including rotavirus, HIV, etc\textsuperscript{37}. Studies found that SIgA concentration was significantly reduced by Holder pasteurization\textsuperscript{35,36,38}. However, Carbonare and his colleagues reported that SIgA activity towards enteropathogenic \textit{E. coli} was not altered after Holder pasteurization treatment\textsuperscript{39}. These observations suggest that Holder pasteurization may decrease the concentration of SIgA but do no harm to its activity level.

The understanding of Holder pasteurization’s effect on protein, lysozyme and SIgA still demands more clarification and investigation. The divergence on the effect of Holder pasteurization may be due to milk sample variance from different individuals. To examine this possibility, we did not pool the milk samples. Instead, each mother’s sample was studied individually.

5.2 Methods

5.2.1. Study Design

Mothers who regularly express breast milk were recruited in the area of Raleigh, Durham and Chapel Hill, North Carolina from March to December 2013. Thirteen mothers volunteered in the study. Mothers were all Caucasian women with lactation stages between 2 to 10 months.

Mothers were asked to express milk according to the protocol they would usually follow and to freeze the milk immediately after expression. Once 8 ounces of a mother’s milk was saved, milk was collected and delivered to the laboratory. The milk was transported in a cooler full of ice. The 8 ounces of fresh milk was then divided into two 4-ounce sterile containers. One container filled with fresh milk was separated into three sterile tubes for future component analysis and kept in a freezer at -80°C. The other container filled with milk
was pasteurized at 62.5°C for 30 min, according to the Holder Pasteurization method utilized in all HMBANA milk banks\textsuperscript{40}. The pasteurization process was conducted using a shallow-form shaking bath (Thermo Scientific, USA). Three thermo detectors were used to detect the temperature during pasteurization, with one temperature indicated on the equipment screen, one thermometer inserted in the water bath and another one inserted in the middle of a control milk sample. After all thermometers reached a temperature of 62.5°C, the batch was held for 30 minutes at the temperature and then cooled immediately in an ice bath. After samples were cooled, the pasteurized milk was separated into three sterile tubes for future component analysis and stored in a freezer at -80°C. Mothers’ milk samples were not pooled and each mother’s milk was handled separately.

Each sample was measured for total protein content, lysozyme activity and secretory IgA activity. Measurements were conducted in triplicate.

Total protein content was examined using Pierce\textsuperscript{®} BCA Protein Assay Kit (Prod # 23227, Thermo Scientific) in 96-well plates. Lysozyme activity was measured by a Micrococcus lysodeikticus based turbidimetric assay using 96-well plates. The turbidimetric assay was adopted from Worthington Biochemical Corporation\textsuperscript{41} and Kenitic 96-Well Turbidimetric Lysozyme Assay Manuel\textsuperscript{42}. Secretary IgA activity was determined by a kinetic indirect enzyme linked immunosorbent assay (ELISA), modified by Chen\textsuperscript{43} and Viazis\textsuperscript{44}. For detailed assay procedure, please refer to Appendix 2 (Total Protein Measurement), Appendix 3 (Lysozyme Activity Measurement) and Appendix 4 (Secretary IgA Activity Measurement).
5.2.2. **Statistical Methods**

Linear mixed effects models appropriate to the repeated measures design were fit using the MIXED procedure of the SAS software package. In particular, fixed factorial effect for pasteurization treatment was included along with random effects for sample and sample-by-treatment interaction. Selected pairwise comparisons were used to evaluate simple treatment effects. Separate univariate models were fit to each of the three response variables (protein, lysozyme activity and SIgA activity) for the experiment. Diagnostic plots of residuals did not indicate any violations of the usual assumptions underlying analysis of variance.

5.3 **Results**

5.3.1. **Total protein content**

Radar plot was utilized to show the protein content of each milk sample before and after pasteurization (Figure 19). Each point in the radar plot represents the protein content of a milk sample, and the value is the distance of each point to the center. By connecting each data point, the shape of the closed loop illustrates the divergence of protein contents over different mothers’ milk samples. As shown in Figure 19, the closed loop linked by unpasteurized fresh milk data points almost forms a circle, indicating that human milk protein content is quite consistent over individuals.

Comparing the protein content before and after pasteurization, most milk samples did not demonstrate significant change after Holder pasteurization. However, some samples seemed to be more sensitive to Holder pasteurization and had a relatively large decrease in protein content after pasteurization (S3: 25%, S7: 20% and S13: 23%). Overall, the protein
content difference before and after pasteurization was not significant according to statistical analysis, suggesting minimal protein change was caused by Holder pasteurization.

5.3.2. Lysozyme activity

In contrast to the protein content in human milk, the closed loop linked by lysozyme activity of unpasteurized milk samples has irregular shape (Figure 20), showing large variation between milk samples over individuals. Interestingly, the closed loop formed by pasteurized milk sample has a similar irregular shape but with a smaller size, which suggests that lysozyme activity in all milk samples was decreased by Holder pasteurization. A significant lysozyme activity decrease (P<.001) after Holder pasteurization was also detected by regression analysis.

The degree of reduction in lysozyme activity reduction did vary sample by sample, ranging from 16% (S9) to 45% (S2). To further investigate the effect of Holder pasteurization, the relationship between unpasteurized and pasteurized milk in lysozyme activity was examined. We plotted data in a way so that the x-axis represents lysozyme activity in unpasteurized milk, the y-axis represents lysozyme activity in pasteurized milk, and the diagonal line is where lysozyme activity is the same before and after pasteurization. As shown in Figure 21, all data points are below the diagonal line, which means a decrease of lysozyme activity after pasteurization. A linear fit was then executed on the existing data points, and an adequate linear line can be generated with R square at 0.9023. The linear equation could provide a preliminary prediction on the effect of Holder pasteurization on lysozyme activity in human milk. It infers that the larger the lysozyme activity is in
unpasteurized milk, the larger the reduction will be after pasteurization. The slope of the regression line implies that 29% of the activity was lost due to pasteurization. However, the equation and the relationship of lysozyme activity before and after pasteurization still require further examination.

5.3.3. *SIgA activity*

Similar to lysozyme activity, closed loop linked by SIgA activity forms an irregular shape in radar plot (Figure 22), showing a variation in milk sample SIgA activity from mother to mother.

One unexpected effect of Holder pasteurization on SIgA activity was observed: most milk samples showed an increase in SIgA activity after pasteurization (Figure 22), with the highest increase at 249% (S5). Although SIgA activity change in some milk samples was small, the overall activity increase was significant (p<.05). A similar plot demonstrating the relationship before and after pasteurization was generated for SIgA activity (Figure 23) but no obvious relationship was found in SIgA activity before and after pasteurization.

5.4 Conclusions

Protein content in human milk was stable during Holder pasteurization, and no significant difference was observed before and after pasteurization. However, minor reduction did occur in some samples, indicating the protein in some samples may be more sensitive to heat. These results are supported by most previous studies suggesting no significant change in protein content after pasteurization\textsuperscript{28,29}, while some studies reported protein reduction\textsuperscript{27}.
Lysozyme activity in human milk was significantly altered by pasteurization (P<.001), with activity reduction found in all examined samples. Milk samples from different mothers have a wide range in lysozyme activity and the reduction after pasteurization was not uniform over milk samples. This explains why disagreement exists regarding the lysozyme activity reduction after pasteurization among studies\textsuperscript{34–36}, which has been addressed earlier. Since most studies pooled milk to examine the effect of Holder pasteurization to milk lysozyme activity, the variance among milk samples may be neglected and thus causing a different reduction result in different study group set. Furthermore, in the present study, it seems that the larger the lysozyme activity was in unpasteurized milk sample, the larger the reduction occurred after pasteurization. A preliminary prediction of a linear relationship between lysozyme activity before and after pasteurization was introduced. However, more research is needed to confirm the findings.

An increase in SIgA activity was observed after Holder pasteurization. One previous study suggested that SIgA reactivity towards enteropathogenic \textit{E. coli} was not altered after Holder pasteurization\textsuperscript{39}. More information is required to understand why the increase observed in our research occurred. It is possible that Holder pasteurization caused a release in immune active elements in SIgA, such as IgA being released from SIgA, or unbinding from endogenous bacteria, rendering it more able to bind the immobilized antigen in the ELISA assay. SIgA in human milk originally consists of two joined IgA molecules and a secretary component that protects active IgA molecules from being degraded\textsuperscript{26}. Crottet\textsuperscript{45} found that secretary component can delay the conversion of SIgA into antigen-binding element. Holder pasteurization process might have separated secretary component from IgA, which could
speed up the binding to antigen and cause increased SIgA activity in the ELISA assay. More research can be done to clarify this finding.

In this study, volunteers were all recruited from the same area (Raleigh, Durham and Chapel Hill, North Carolina). Milk from populations may vary in different regions. Thus, more research should be done in other areas to avoid the geographic limitation.
5.5 References


Figure 19. Human milk protein content before and after pasteurization, n=13
Milk samples were shown as S1-S13. Each blue point represents the protein content in unpasteurized milk of the corresponding sample, and each red point represents the protein content in pasteurized milk of the corresponding sample. Protein content value is indicated by the distance of each point to the center. By connecting each data point, the shape of the closed loop illustrates the divergence of protein contents over different mothers’ milk samples. The round circle linked by protein content of all samples demonstrates that human milk has consistent protein content over individuals. Most samples had very close protein content before and after pasteurization.
Figure 20. Human milk lysozyme activity before and after pasteurization, n=13
Milk samples were shown as S1-S13. Each blue point represents the lysozyme activity in unpasteurized milk of the corresponding sample, and each red point represents the lysozyme activity in pasteurized milk of the corresponding sample. Lysozyme activity value is indicated by the distance of each point to the center. By connecting each data point, the shape of the closed loop illustrates the divergence of lysozyme activity over different mothers’ milk samples. The irregular shape formed demonstrates a large variation in lysozyme activity between mothers. Lysozyme activity decrease was observed in all samples after Holder pasteurization.
Figure 21. Relationship in human milk lysozyme activity before and after pasteurization, n=13
In figure, x axis represents lysozyme activity in milk samples before pasteurization, y axis represents lysozyme activity in milk samples after pasteurization. The Diagonal Line indicates where lysozyme activity is the same before and after pasteurization. The closer the point is to the diagonal, the smaller the lysozyme activity change is after pasteurization. All data points are below the diagonal line which means the reduction in lysozyme activity occurred in all samples after pasteurization. A preliminary equation was generated predicting the relationship in lysozyme activity before and after pasteurization, with $R^2$ at 0.9023.
Figure 22. Human milk secretary IgA activity before and after pasteurization, n=13
Milk samples were shown as S1-S13. Each blue point represents the secretary IgA activity in unpasteurized milk of the corresponding sample, and each red point represents the secretary IgA activity in pasteurized milk of the corresponding sample. SIgA activity value is indicated by the distance of each point to the center. By connecting each data point, the shape of the closed loop illustrates the divergence of SIgA activity over different mothers’ milk samples. The irregular shape demonstrates that SIgA activity in human milk varies between individuals. SIgA increase was found in most milk samples after pasteurization.
Figure 23. Relationship in human milk secretary IgA activity before and after pasteurization, n=13

In figure, x axis represents SlgA activity in milk samples before pasteurization, y axis represents SlgA activity in milk samples after pasteurization. The Diagonal Line indicates where SlgA activity is the same before and after pasteurization. The closer the point is to the diagonal, the smaller the SlgA activity change is after pasteurization. Most data points are above the diagonal line which means that most samples had an increase in SlgA activity after Holder pasteurization.
APPENDICES
Appendix 1. Bacterial Analysis

After the designed storage time was reached, human milk was tested for bacteria level using 3M Petrifilm plate.

Each human milk sample was diluted using autoclaved peptone water to the following factors: $10^1, 10^2, 10^3$ and $10^4$. Peptone water was prepared by dissolve 1 g of peptone (Sigma # 77185) into 1 L of deionized water.

After the milk was diluted with autoclaved peptone water, it was mixed thoroughly to ensure evenly dispersed bacteria. 1 mL of the human milk or diluted human milk was plated onto the Petrifilm and covered by the upside film. After the Petrifilm was covered, a gentle pressure was applied onto the Petrifilm with even strength on the press. In the study, fresh milk and pasteurized milk were tested by aerobic plate. Leftover milk and pasteurized leftover milk were tested by aerobic plate, *E. coli*/*coli*form count plate, and *Staph* Express Count Plate. Aerobic plate count was measured in duplicate. Aerobic plate was incubated at 35°C for 48 hours. *E. coli*/*coli*form count plate was incubated at 35°C for 24 hours to count the coliform level and incubated at 35°C for further 24 hours (total 48 hours) to count the *E. coli* level. StaphExpress Count Plate was incubated at 35°C for 24+2 hours to count the *Staphylococcus aureus* level. 3M Petrifilm Staph Express Disk was used to identify the suspected *Staphylococcus aureus* from Staphylococci species.

After incubation, the plates were counted for colonies. The plate that contained 25-250 CFU is preferred when counting the colonies, according to the 3M Petrifilm instrument.
Appendix 2. Total Protein Measurement

The protein content in human milk is measured using Pierce® BCA Protein Assay Kit (Prod # 23227, Thermo Scientific). The assay is based on the reduction of Cu$^{2+}$ to Cu$^{+1}$ by protein in an alkaline medium, which Cu$^{+1}$ further reacts with a reagent containing bicinchoninic acid (BCA) to produce a purple-colored product. This water soluble complex exhibits a strong absorbance at wavelengths ranging from 540nm to 590nm. The intensity of the color produced is proportional to the number of peptide bonds participating in the reaction, and it is nearly linear with increasing protein concentrations over a protein content ranging from 0.020-2.0 mg/mL. A set of protein standards made by bovine serum albumin (provided in the kit) was used to estimate the true value of sample protein concentration.

The original bovine serum albumin (BSA) has a total protein content of 2.0 mg/mL.

The protein standards were made in the following sets (Table 16):

<table>
<thead>
<tr>
<th>Standards</th>
<th>Volume of Water (µL)</th>
<th>Volume of BSA (µL)</th>
<th>Final BSA Concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>75</td>
<td>25</td>
<td>0.5</td>
</tr>
<tr>
<td>C</td>
<td>50</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>25</td>
<td>75</td>
<td>1.5</td>
</tr>
<tr>
<td>E</td>
<td>0</td>
<td>100</td>
<td>2</td>
</tr>
</tbody>
</table>

Working reagent was made by combining 50 parts of reagent A and 1 part of reagent B. Human milk samples were diluted 1:10 using deionized water to fall into a protein concentration range of 0.020-2.0 mg/mL.
Each microplate well was plated with 25 µL of each standard or diluted milk sample. Samples and standards were measured in triplicate. To prevent water evaporation and contamination, parafilm was covered on the 96-well plate, except during the pipetting. After all samples were added, 200 µL of the working reagent was added into each well and mixed on a plate shaker for 30 seconds. Next, plate was covered by parafilm and incubated at 37°C for 30 min. After incubation, plate was cooled to room temperature and measured for absorbance at 540nm on a microplate reader (Multiskan Plus Model 355, Fisher Scientific, USA was used in this study).

Protein standards were plotted to calculate out the equation between the known protein concentration and absorbance in a following format:

\[ Y = m \times X + b \]

\( X: 540\text{nm absorbance} \);
\( Y: \text{Protein concentration} \left(\frac{mg}{mL}\right) \);
\( m: \text{Slope} \);
\( b: \text{Intercept} \);

Protein concentration of diluted milk sample was obtained based on the above equation and the original milk sample concentration was multiplied by the dilution factor (dilution factor was 10).
Appendix 3. Lysozyme Activity Measurement

The rate of lysis of *Micrococcus lysodeikticus* can be used to determine the lysozyme activity as mentioned in the manual of Worthington Biochemical Corporation\(^1\). The absorbance decrease at A450 nm of *Micrococcus lysodeikticus* (*M.l.*) suspension was measured over time. One unit of lysozyme activity is equal to a decrease in turbidity of 0.001 per minute at A450 nm. Thus the lysozyme activity in human milk can be calculated by the decrease in absorbance over time at A450nm.

Substrate solution was made by adding a dry freezed *M.l.* to 66 mM potassium phosphate buffer (pH=7.2). The concentration of *M.l.* was adjusted by measuring the absorbance at A450 nm to fall in a range of 0.6-0.7. *M.l.* substrate solution can be stored for up to a week in room temperature and it shall stay at room temperature before the turbidimetric reaction to ensure a proper enzyme working condition.

Human milk samples were diluted 1:100 by deionized water at room temperature. Twenty five µL of diluted milk samples were added into a microplate well in triplicate. To avoid water evaporation or cross contamination, parafilm was used to cover the well during pipetting. After adding all the milk samples, 200 µL of the substrate solution was quickly added into each well using a multi-channel pipette. The plate was immediately placed into the plate reading and rapid mixing. Change in turbidity was measured at A450 nm every 30 seconds for total 11 measurements. The turbidimetric method on microplate reader was adopted from Florida Gulf Coast University (2005).

Lysozyme activity of the measured sample was calculated using the following equation:
\[ \text{Lysozyme activity} \left( \frac{\text{unit}}{\text{mL milk}} \right) \cong (\Delta A_{450nm \text{ per minute}})/(0.001 \times 0.025) \]

0.001: A decrease of 0.001 in turbidity absorbance represents one unit in lysozyme activity
0.025: the amount of sample that added in to the well (25 \( \mu \text{L} \))

The above equation obtains the lysozyme activity of diluted milk sample. Thus, the
lysozyme activity of the original milk sample can be calculated by multiplying the sample
dilution factor (dilution factor was 100 in this case).
Appendix 4. Secretory IgA Activity Measurement

In the study, secretory IgA (SIgA) activity was generally measured by the amount of SIgA antibodies to *Escherichia coli* somatic antigens\(^3\). The entire method is modified based on Chen’s dissertation\(^4\).

*Escherichia coli* Somatic Antigens Preparation

Dry pellets of eight *E. coli* serotypes (shown in Table 17) were rehydrated and grown individually in tryptic soy broth at 37°C until it reached a concentration of 10\(^8\) to 10\(^9\) cells/mL. Individual bacteria were pelleted by centrifugation at 4000 rpm for 10 minutes and resuspended in 50 mL 0.01 M phosphate buffered saline (PBS), pH=7.2. The bacteria were pelleted again at 4000 rpm for 10 minutes and resuspended in 10 ml of the 0.01 M PBS. The above wash step was repeated for one additional time. The bacteria concentration was adjusted to 5×10\(^9\) cells/mL and the samples were then transferred to a steam bath and boiled for 2 hours. After cooling the containers for 10 to 15 minutes, the heat-killed bacteria debris was centrifuged at 3000 rpm for 30 minutes at 4°C. Each supernatant was harvested. All eight were pooled and used as the *E. coli* somatic antigen for ELISA. The antigen was kept frozen before use.

<table>
<thead>
<tr>
<th>Accession #</th>
<th>Strain Name</th>
<th>O</th>
<th>Class</th>
<th>Host</th>
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</thead>
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<td>UPEC</td>
<td>Cat</td>
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<td>Human</td>
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<td>TW01916</td>
<td>820877</td>
<td>18</td>
<td>UPEC</td>
<td>Human</td>
</tr>
</tbody>
</table>
**Solution Preparation**

- **0.01 M Phosphate Buffered Saline (PBS), pH=7.2**

  Dissolve one PBST tablet (phosphate buffered saline tablet, BP2944-100, Fisher Scientific, USA) into 200 mL deionized water. Adjust the pH using 1N hydrochloric acid to 7.2. Store at room temperature for short term and refrigerate for long term.

- **0.01 M Phosphate Buffered Saline with Tween 20, pH=7.2**

  Dissolve 5 PBST tablets into 1000 mL deionized water and add 0.5 g Tween 20. Adjust the pH using 1N hydrochloric acid to 7.2. Store at room temperature for short term and refrigerate for long term.

- **0.05 M Citrate Buffer, pH=5.0**

  Prepare a 0.05 M citric acid solution and a 0.05 sodium citrate solution. Add the sodium citrate solution gradually to the citric acid solution until pH 5.0 is achieved. Store at room temperature for short term and refrigerate for long term.

- **40 mM ATBS, or 2,2’-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)**

  Measure 0.22 g of ABTS powder (11557-1G, Sigma, USA) carefully and mix it with 10 mL deionized water until it completely dissolves. Cover the solution with aluminum foil due to its light sensitivity. The solution can last about 2 to 4 weeks in refrigerated storage.
Standards – Human IgA from Colostrum

IgA human colostrums (I2636, Sigma-Aldrich) were used to prepare ELISA standards. The original IgA concentration is 1.64 µg/µL, and was diluted into a designed concentration sets shown in Table 18.

Table 18 The IgA concentration of ELISA standards*

<table>
<thead>
<tr>
<th>Std #</th>
<th>Std Concentration (µg/µL)</th>
<th>IgA Solution</th>
<th>PBST Amount (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S5</td>
<td>0.0092</td>
<td>100 µL of the original solution from Sigma</td>
<td>17.7</td>
</tr>
<tr>
<td>S4</td>
<td>0.0047</td>
<td>1000 µL of the 0.0092 µg/µL solution</td>
<td>0.957</td>
</tr>
<tr>
<td>S3</td>
<td>0.0024</td>
<td>1000 µL of the 0.0092 µg/µL solution</td>
<td>2.9</td>
</tr>
<tr>
<td>S2</td>
<td>0.0012</td>
<td>1000 µL of the 0.0092 µg/µL solution</td>
<td>6.5</td>
</tr>
<tr>
<td>S1</td>
<td>0.00078</td>
<td>1000 µL of the 0.0092 µg/µL solution</td>
<td>10.8</td>
</tr>
</tbody>
</table>

*Standards shall be kept in refrigerator for short term and freeze for long term.

Anti-Human IgA Solution

Anti-human IgA peroxidase conjugate (A0295, Sigma) was diluted 1:5000 to make the anti-human IgA solution for ELISA. Anti-human IgA shall be kept in refrigerator for short term and freezer for long term.

Substrate Solution

Mix the following ingredient right before usage to avoid degradation:

- 20 mL 0.05 M citrate buffer;
- 0.1 mL 3% hydrogen peroxide (H324-500, Fisher Scientific, USA);
- 0.5 mL 40 mM ABTS.
Assay Procedure

The outside wells of the 96-well plate were not used due to the fact that they reflect and refract light. PBST was placed in the outside wells throughout the assay. Standards were plated in triplicate in each well and there were four blank controls (B1-B4) during the assay.

*Step 1.* E. coli antigen was defrozen and diluted 1:50 using PBS. 200 µL of PBST was added to the outside wells and B1. 200 µL of the diluted antigens was added to each well. Cover the plate with parafilm and incubated at room temperature for 12 to 18 hours so that the enough antigen can attach to the plate.

*Step 2.* After incubation, plate was washed using PBST for three times to remove the unbounded antigen. Human milk samples were diluted 1:100 using PBST. 200 µL of PBST was added to the outside wells and B1 to B4. 200 µL of SIgA standards and 200 µL of the diluted human milk samples were added in the designed wells in triplicate. Cover the plate with parafilm and incubated at room temperature for 3 hours. This step allows SIgA in human milk to attach to the E. coli antigen.

*Step 3.* After incubation, plate was washed using PBST for three times to remove the unbounded SIgA. 100 µL of PBST was added to the outside wells and B3. 100 µL of the anti-human IgA was added to the rest of the wells. The plate was covered by parafilm and incubated for 1 hour. This step allows anti-human IgA to attach to the SIgA.

*Step 4.* After incubation, plate was washed using PBST for three times to remove the unbounded anti-human IgA. 100 µL of PBST was added to the outside wells and B4. 100 µL of the substrate solution was added to the rest of the wells (prepare the substrate solution
right before addition). The addition of substrate solution will cause the bounded anti-human IgA to develop a color that absorbs light 405 nm.

Step 5. Immediately place the plate on a micro plate reader (Safire 2, Tecan, USA) at 405 nm, read every 2 minutes for 20 minutes.

The SIgA activity was determined by the increase rate in absorbance over time and by comparing the absorbance increase rate in the diluted milk samples to that of the human milk standard set.
Appendix 1-4 References


Appendix 5. Informed Consent Form

North Carolina State University
INFORMED CONSENT FORM for RESEARCH

Principal Investigator Ting Meng Faculty Sponsor (if applicable) April Fogleman

What is the purpose of this study?

The purpose of this study is to investigate how long expressed human milk can be stored after it has been offered to a baby. Samples of your donated milk will be tested for nutritional and bioactive components and bacteria counts to compare the difference between fresh milk and leftover milk.

What will happen if you take part in the study?

If you agree to participate in this study, you will be asked to provide approximately 30 oz of your recently pumped milk. We will mail the sterilized, prepackaged containers to you for milk storage. Within 1 – 4 weeks before collection, milk is pumped as you normally would using your own breast pump at home and stored in the containers we provided. On the container, please write your name and date of milk expression. Please keep the milk in freezer immediately after it is expressed.

After collecting your milk, milk will be divided into four sterilized containers provided by Human Milk Bank Association of North America. Two containers will be pasteurized and kept frozen in a food grade refrigerator. The other two containers will be frozen in a food grade refrigerator without treatment. Pasteurization is a standard procedure used by the Human Milk Bank Association of North America, in order to eliminate potential bacteria in the milk.

At a mutually agreeable time, we will meet with you and bring the pasteurized milk for you to feed to your baby.

• Any time before we meet that day, you will be asked to express 1-2 oz of milk and store it in the refrigerator for us to use as a fresh standard for comparison.
• You will be asked to feed your baby a portion of your own unpasteurized milk and a portion of your own pasteurized milk.
• I will record the type of bottle your baby uses, feeding time, duration, and feeding amount. You will also be asked to fill out an information sheet related to our research. The samples will be brought back to the lab for analysis. The process will take no more than 1 hour.

In Summary, you will be asked to provide:

• Approximately 32 ounces of milk
• The time it takes you to pump
• The time it takes your baby to feed

Your baby will be asked to:

• Drink from two bottles with your previously frozen milk, one being pasteurized and one untreated

Benefits

Knowledge will be gained to help others in learning how long expressed milk can be stored after it has been partially consumed by a baby. Guidelines for expressed milk storage will be refined.
Risks

There are minimal risks associated with participating in this study. If pasteurization fails to eliminate bacteria, the milk will remain the same quality as the unpasteurized milk. Milk will be bottled and sealed tightly during pasteurization. When the milk is frozen, it will be frozen in a food grade refrigerator, which will not create quality problems in the milk. If a bottle leaks during pasteurization, causing water to run into the milk, that particular milk sample will be discarded to ensure milk safety.

The Holder pasteurization method is utilized in milk banks around the world to pasteurize milk for fragile, premature infants. It is a safe and effective way to eliminate bacteria in milk. There has never been a case of a baby getting sick from donor human milk.

Confidentiality

The information in the study records will be kept strictly confidential. Data of our analysis will be stored securely and is only available to the researcher of this study. No reference will be made in oral or written reports which could link you to the study.

Compensation

Mothers will obtain a Medela Hand Breast Pump to keep after study. Not necessary to be the same one mom to use for milk expression. Moms may have preference on different milk pumps.

If you do not wish to have a Medela Hand Breast Pump, you may also choose between a new kit for their personal breast pump, a pack of diapers, or an “all-in-one” cloth diaper.

If you chooses to use the school’s hospital grade breast pump (Medela Symphony), then the compensation will be a new kit for the Medela pump.

If you withdraw before all collection or participation is complete then you will not receive the breast pump.

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, Ting Meng, at 204 Schaub Hall, NCSU, Raleigh, North Carolina 27695, by phone (919) 561-8760, or by email: tmeng@ncsu.edu.

What if you have questions about your rights as a research participant?

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).

Consent To Participate

“I have read and understand the above information. I have received a copy of this form. I agree to participate in this study with the understanding that I may choose not to participate or to stop participating at any time without penalty or loss of benefits to which I am otherwise entitled.”

Subject’s signature_______________________________________ Date _________________
Investigator’s signature________________________________________ Date _________________
Appendix 6. Invitation Letters to Participants

*North Carolina State University* is conducting a study, “Effect of Storage Time and Temperature on Leftover Human Milk”.

**Why Conduct the Study?**
How should families handle leftover human milk that has been partially consumed by their baby? Discard it or store it like fresh milk? This study will investigate how long expressed human milk can be stored after it has been offered to a baby.

**Who Can Help?**
If you answer “Yes” to all of the questions below, you meet our criteria for participating:
- Are you located in the Triangle area of North Carolina?
- Are you breastfeeding an infant who is between 2-11 months old?
- Did you have a healthy, full term (>37 weeks gestation) delivery of your baby?
- Do you express milk using a breast pump and clean your pump regularly?
- Will your baby drink breast milk from a bottle?

**What Does the Study Involve?**
You will be asked to provide approximately 16 oz of your recently pumped milk (milk pumped and frozen within the last month-We do the pick-up).
You will be asked to feed your baby 1-2 oz of your pumped milk and your pasteurized milk*. (I’ll come to you)
Pasteurized milk*: A portion of your milk will be pasteurized at NCSU using the Holder Pasteurization technique which is used by North America Milk Banks.

【Mothers will obtain a Medela Hand Breast Pump to keep after study】

If you are interested in the study or want to learn more about the study, please contact Ting Meng at *tmeng@ncsu.edu*.

**Thank You!**