ABSTRACT

FOGLEMAN, APRIL DANIELLE. The Effect of Storage Time and Temperature on Nutritional Components in Human Breast Milk. (Under the direction of Dr. Jonathan C. Allen).

Breastfeeding is the optimal nutrition for infants and is recommended for the first year of life by the American Academy of Pediatrics. However, mothers who return to work within this time period cannot always breastfeed on demand and often pump their milk and send it with the baby to a day care setting. Infants in the neonatal intensive care unit rely heavily on donor breast milk or their mothers' own breast milk, all of which is expressed, stored, transported to the hospital, and stored again. There are few guidelines on the length of time that this milk can be kept and the optimum and tolerable storage temperatures for human milk. Guidelines that are sometimes used are based on very few scientific studies of the impact of storage on milk quality and stability. There is a need for science-based standard protocols for milk handling and storage in hospitals and milk banks as well as a need for evidence-based recommendations for safely handling milk at home and in child care facilities.

The objective of the first study was to investigate the effects of storage temperature and time on components in freshly expressed, human breast milk. Eleven breast milk samples were collected from twelve women who volunteered with informed consent. The samples were stored either at -20°C, 4°C, or 24°C. All samples were stored for three weeks and analyzed for total protein and free amino ends, total fat and non-esterified fatty acids (NEFA), bacterial growth, secretory immunoglobulin A (SIgA) activity, and lysozyme

activity. Samples stored at -20°C only showed a significant increase in NEFA ($p < 0.0001$). Samples stored at 4°C showed a significant increase in NEFA ($p < 0.0001$), free amino ends ($p < 0.0001$), and bacterial growth ($p < 0.01$). Samples stored at 24°C showed a significant increase in NEFA ($p < 0.0001$), free amino ends ($p < 0.0001$), bacterial growth ($p < 0.0001$), and a decrease in total protein concentration ($p < 0.0001$). All samples showed a rapid increase in NEFA ($p < 0.0001$). Total fat, lysozyme, and SIgA remained stable throughout the storage conditions.

The objective of the second study was to observe the difference in bacterial growth in breast milk contaminated by an infant in mother's own milk and donor milk and determine if it is safe for infants to drink breast milk from a bottle, store it for a period of time, and then drink from it again. Five women volunteered with informed consent and provided parental consent for their infant to participate. Each infant was fed 1 – 2 ounces of breast milk from a bottle prepared with the mother's pasteurized milk and the mother's unpasteurized milk. At least one ounce of leftover milk was collected in sterile containers and analyzed for bacterial growth at specific time points during storage. Leftover samples were stored at 4°C for 0 hours, 2, 4, and 6 days, and at 24°C for 0, 3, 6, 9, and 24 hours.

Based on the data, it appears that unpasteurized breast milk can be stored up to 6 – 9 hours and pasteurized breast milk between 9 – 24 hours at 24°C. Unpasteurized breast milk can be stored between 4 – 6 days and pasteurized breast milk can be stored longer than 6 days at 4°C.

Results of both studies indicate that breast milk may be stored longer than suggested by the current recommendations. Understanding safety and quality of expressed, stored,

breast milk will allow mothers to confidently extend breast feeding duration when the infant is left in a child care setting or when the infant is in a neonatal intensive care unit.

Effect of Storage Time and Temperature on Components in Human Breast Milk

by
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DEDICATION

This research, document, and degree are dedicated to my family – my parents, my brother, and my husband. I love them with all of my heart and am so thankful for their love and support.

BIOGRAPHY

April was born and raised in Georgia and moved to North Carolina in 1999. While attending college at North Carolina State University with hopes of becoming a medical doctor, she became fascinated with Human Nutrition and the science involved. Her career path changed and she enrolled in the graduate program in Nutrition at NC State, under the direction of Dr. Jonathan C. Allen.

Upon completion of her Master's degree, she plans to stay at NC State to pursue a Doctorate degree in Nutrition, become a registered dietitian, and teach people the fascinating relationship between Human Nutrition and Biochemistry.

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INTRODUCTION

Breastfeeding is the optimal nutrition for infants and is recommended for the first year of life by the American Academy of Pediatrics (American Academy of Pediatrics 1997). However, mothers who return to work within this year cannot always breastfeed on demand and often pump their milk and send it with the baby to a day care setting. There are few guidelines on how long this milk can be kept and the optimum and tolerable storage temperatures for human milk. Guidelines that are sometimes used are based on very few scientific studies of the impact of storage on milk quality and safety. There is a need for evidence-based standard protocols for milk handling and storage in hospitals and milk banks as well as a need for evidence-based recommendations for safely handling milk at home and in child care facilities.

Currently, there is a policy within the North Carolina Division of Child Development licensing guidelines that when human breast milk is brought into child care facilities, any remaining milk must be taken home at the end of each day. There are no data comparing the risks for temperature abuse in the short term with the risks of leaving the milk refrigerated at the child care facility for multiple days. This problem presents a need for evidence-based research in order to evaluate this policy and to see if allowing milk to be stored in the child care facility longer than one day is warranted.

The project addresses a concern for premature as well as healthy babies. The issue of breast milk storage is a common topic regularly encountered by clinicians that has received

sporadic research attention over the past several decades. No research has been done to determine how long breast milk can be saved after a baby has suckled from and contaminated a bottle of pumped breast milk with oral bacteria and salivary enzymes.

The concern for premature and healthy babies presents a need for evidence-based standard protocols for milk handling and storage in hospitals, milk banks, in the home, and in child care facilities. The research will provide current evidence on which to base recommendations for the proper storage time and temperatures of milk.

The research also investigates common questions as milk storage times and temperatures. Is it safe for infants to drink breast milk from a bottle, store it for a period of time, and then drink from it again? The findings will be applicable in childcare facilities, milk banks, neonatal intensive care units, as well as in the home.

The effect of storage time and temperature of human breast milk on the total fat, NEFA, total protein, free amino acids, activity of immunological factors such as secretory immunoglobulin A and lysozyme, and bacterial growth was determined. Normally, human milk anti-bacterial factors will reduce the bacterial count after its secretion. With a high bacterial load or after prolonged storage, the antibacterial factors could be used up, bacterial counts could again increase, or the milk could be less effective at attacking bacterial pathogens in the infant's gut.

CHAPTER 1

Literature Review

1.1. Human Milk Nutrition

Human milk supplies infants with the most superior form of nutrition, tailored to their specific needs. It is a remarkable and dynamic life-sustaining fluid that protects the infant from disease and efficiently transfers nutrients from the mother to the infant.

There are three aspects of lactation efficiency (Newburg 2001). The first aspect of lactation efficiency is the way in which nutrients in the maternal diet are brought into the milk. Constant milk composition and synthesis are based on the needs of the infant rather than the nutritional status of the mother. De novo milk synthesis is balanced with the availability of preformed precursors of milk components from the mothers diet as well as body reserves. The second aspect of efficiency is that the rate of milk production matches the rate of milk expression, without limiting nutrition for the infant. The third aspect of efficiency is that milk components contribute to nutritional support of the infant as well as to immune system development and support (Newburg 2001).

Human milk provides many methods of protection against pathogens, including support and development of the innate and acquired immune system. Acquired immunity is immunity that develops with exposure to various antigens. Secretory Immunoglobulin A (SIgA), serum antibodies, and leukocytes are part of the acquired immune system and are provided to the infant through human milk. The concentration of these components depends

on the mother's prior exposure to their target pathogens. Components of the innate immune system include constitutive components of human milk such as lysozyme, lactoferrin, and NEFA (Newburg 2001). Table 1.1 illustrates the immune benefits of breast milk (Newman 1995).

Table 1.1 Immune Benefits of Breast Milk at a Glance (Newman 1995)

Component	*Action*
White Blood Cells	
B lymphocytes	Give rise to antibodies targeted against specific microbes.
Macrophages	Kill microbes outright in the baby's gut, produce lysozyme and activate other components of the immune system.
Neutrophils	May act as phagocytes, ingesting bacteria in baby's digestive system.
T-lymphocytes	Kill infected cells directly or send out chemical messages to mobilize other defenses. They proliferate in the presence of organisms that cause serious illness in infants. They also manufacture compounds that can strengthen a child's own immune response.
Molecules	
Antibodies of secretory IgA class	Bind to microbes in baby's digestive tract preventing them from passing through walls of the gut into body's tissues.
B12 binding protein	Reduces amount of vitamin B12, which bacteria need in order to grow.
Bifidus factor	Promotes growth of *Lactobacillus bifidus*, a harmless bacterium, in baby's gut. Growth helps crowd out dangerous varieties.

Table 1.1 Continued

Fatty acids	Disrupt membranes surrounding certain viruses and destroy them.
Fibronectin	Increases antimicrobial activity of macrophages; helps repair tissues that have been damaged by immune reactions in baby's gut.
Gamma-interferon	Increases antimicrobial activity of immune cells.
Hormones and growth factors	Stimulate baby's digestive tract to mature more quickly. Once initially "leaky" membranes lining gut mature, infants become less vulnerable to microorganisms.
Lactoferrin	Binds to iron, a mineral many bacteria need to survive. By reducing available iron, lactoferrin thwarts growth of pathogens.
Lysozyme	Kills bacteria and viruses by disrupting their cell walls.
Mucins	Adhere to bacteria and viruses, keeping them from attaching to mucosal surfaces.
Oligosaccharides	Bind to microorganisms and keep them from attaching to mucosal surfaces.

Human milk protects infants from illnesses during and after breast feeding. Illnesses prevented during breast feeding are mainly acute infections including upper respiratory tract infections, diarrheal diseases, ear infections, urinary tract infections, meningitis, and neonatal sepsis (Cleary 2004). Premature infants are protected from acute necrotizing colitis (Cleary 2004). Long term protection includes protection from Chrohn's disease, ulcerative colitis, type I diabetes mellitus, multiple sclerosis, rheumatoid arthritis, obesity, hypertension, and celiac disease (Cleary 2004).

Many studies have shown a protective effect of breast milk against pathogens, including *Escherichia coli* (Hayani and others 1992), *Vibrio cholerae* (Glass and others 1983), rotavirus (Newburg and others 1998), enterotoxigenic *E. coli* (Cruz and others 1988), *Camphylobacter* (Ruiz-Palacios and others 1990), and *Guardia duodenalis* (Walterspiel and others 1994).

The clinical significance of these immune factors has been demonstrated in reports that compared the morbidity and mortality of breast-fed and formula-fed infants (France and others 1980; Jason and others 1984). The availability of these immune factors depends largely on the method of milk storage and processing (Ford and others 1977; Liebhaber and others 1977; Pittard and Bill 1981).

1.2. Breastfeeding among Working Mothers

The American Dietetic Association released their position statement regarding breastfeeding in 2001 (ADA). It is as follows:

> *"It is the position of the American Dietetic Association (ADA) that broad-based efforts are needed to break the barriers to breastfeeding initiation and duration. Exclusive breastfeeding for 6 months and breastfeeding with complementary foods for at least 12 months is the ideal feeding pattern for infants. Increases in initiation and duration are needed to realize the health, nutritional, immunological, psychological, economical, and environmental benefits of breastfeeding."*

Although the rate of breastfeeding initiation in the United States is near the national goal of 75%, at 6 and 12 months postpartum the rates of breastfeeding duration are still below the national goals of 50% and 25%, respectively (Li and others 2005).

Li and others (2005) used data from the 2002 National Immunization Survey regarding breastfeeding initiation and duration to estimate breastfeeding rates in the United States by characteristics of the child, mother, and family. They found a sharp decline in exclusive breastfeeding between 3 and 5 months, which is often the time in which most mothers return to work or school (Li and others 2005).

Although there are many barriers to continuing breastfeeding, a major barrier is the increasing numbers of mothers around the world have to work soon after delivering their baby. They may still want to continue breast feeding exclusively, but women have a relatively short maternity leave from work (Fein and Roe 1998), inflexible work hours when returning to work, and the lack of paid breastfeeding or pumping breaks in the workplace (Anonymous 2001). Also, lack of support for lactation in the workplace has been shown to be a major barrier to continuing breastfeeding (Fein and Roe 1998; Lindberg 1996).

1.3. Human Milk Banking

The need to store breast milk is unavoidable in a neonatal intensive care unit taking care of sick and pre-term infants. Maternal milk may be inadequate for pre-term infants because they may have a weak or inadequate suckling ability for milk letdown stimulation. Pre-term infants may need to be given additional milk, some infants may be excessively hungry, and others may have mothers who are not well enough after delivery to be able to breast feed, all of which are reasons a baby may need to be given donor milk (Ogundele 2000).

Modern milk banks may be located in large hospitals, while milk donated from the smaller hospitals and voluntary donors often needs to be temporarily stored before being transported to the milk bank. Upon arrival to the milk bank, milk needs to be stored during microbial testing. Therefore, milk donated to milk banks often needs to be stored (Ogundele 2000).

1.4. Current Recommendations for Breast Milk Storage

The Centers for Disease Control recommends that breast milk should be stored according to the following guidelines (CDC 2008).

Table 1.2 Breastmilk Storage Guidelines (CDC 2008)

Location	Temperature	Duration	Comments
Countertop, table	Room temperature (up to 77°F or 25°C)	6–8 hours	Containers should be covered and kept as cool as possible; covering the container with a cool towel may keep milk cooler.
Insulated cooler bag	5-39°F or -15-4°C	24 hours	Keep ice packs in contact with milk containers at all times, limit opening cooler bag.

Table 1.2 Continued

Refrigerator	39°F or 4°C	5 days	Store milk in the back of the main body of the refrigerator.
Freezer			Store milk toward the back of the freezer, where temperature is most constant. Milk stored for longer durations in the ranges listed is safe, but some of the lipids in the milk undergo degradation resulting in lower quality.
Freezer compartment of a refrigerator	5°F or -15°C	2 weeks	
Freezer compartment of refrigerator with separate doors	0°F or -18°C	3–6 months	
Chest or upright deep freezer	-4°F or -20°C	6–12 months	

However, the publication does not cite research-based evidence supporting the fact that breast milk should be stored at these temperatures and times. The book *Best Practices for Expressing, Storing and Handling Human Milk in Hospitals, Homes and Child Care Settings* (Jones and Tully 2006) is intended to provide the clinician with the best practices based upon the most current peer-reviewed research or with expert opinion if the research is lacking. Research is lacking on the topic of breast milk storage, thus the authors chose to provide expert opinions from the Centers of Disease Control and the United States Department of Agriculture.

There is limited current research that examines the effects of storage time and temperature on the nutritional components of breast milk such as protein quality and lipid oxidation. No research has been done to answer a question regularly encountered by clinicians – how long can milk be saved after a baby has drunk from the bottle? Many studies that have been done on human milk storage have limited applicability due to the short time frames of the studies themselves or lack of practical applicability. The time frames of the studies usually run from 0 – 10 days, with 2 days being the longest period of time that refrigerated milk has been stored. Many studies also freeze the milk at -70°C, which is not applicable to freezers in homes, child care facilities, and hospitals.

Miranda and others (2004) examined changes in glutathione peroxidase (GPx) activity and in the concentration of malondialdehyde (MDA) when milk was kept refrigerated at 4-6°C or frozen at -20°C. Glutathione peroxidase is a key enzyme in an antioxidant pathway. Malondialdehyde is a final product of lipid peroxidation, so it is used as a marker of the degree of oxidation. The first analysis was done 6 – 8 hours after the extraction. The second analysis was done on milk stored at refrigeration conditions during 48 hours and the third analysis was done on milk stored under freezing conditions during 10 days. The researchers concluded that storage at -20°C is better than refrigeration to preserve the quality of the milk. However, the analysis on refrigerated milk was done after only 48 hours and the analysis of the frozen milk was done after only 10 days, so the kinetics of degradation could not be determined (Miranda and others 2004).

Monera-Pons and others (1998) evaluated the effect of different storage methods on the stability of the triacylglycerides in human milk. The researchers evaluated the lipolytic activity in human milk during storage at -20°C for four months. Storage at -20°C without previous heat treatment led to the hydrolysis of triacylglycerides and the appearance of NEFA. Although the researchers stored the milk at -20°C for four months, they only studied triacylglycerides and they did not study protein quality or degradation (Pons and others 1998).

Other research done on the storage of human breast milk studied the effects of storage time and temperature on proteolysis and lipolysis over a time period of 3 hours to 15 days (Eteng and others 2001; Hamosh and others 1996; Miranda and others 2004; Silvestre and others 2006). The data generally show an increase in proteolysis and lipolysis over time at every storage temperature. However, only milk stored at -20°C was stored for 15 days. The longest amount of time refrigerated breast milk has been stored in these studies is 48 hours.

Silvestre and others (2006) examined the available lysine content in human milk and the variations in lysine resulting from storage at refrigeration temperature (4°C) for 48 hours and at -20°C for 15 days. The amount of available lysine content in both refrigerated and frozen milk significantly decreased from the available lysine content in fresh milk. However, the difference between the available lysine content in the refrigerated milk and the frozen milk after storage was not significant (Silvestre and others 2006). Although available lysine decreased within the milk samples, the total protein content was not affected by these manipulations.

According to the Dietary Reference Intakes (USDA 2004) people of all ages require 51 mg of lysine per gram of protein. The AI for infants 0-6 months of age is 9.1 g of protein/day and the RDA for infants 7-12 months of age is 11 g of protein/day (USDA 2004). Based upon these recommendations, infants 0-6 months of age require 464 mg lysine/day and infants 7-12 months of age require 561 mg lysine/day. These recommendations are in agreement with a review by Tome and Bos (2007). According to Silvestre and others (2006), fresh breast milk contains 161 mg lysine/100 ml milk, the refrigerated breast milk contains 99 mg lysine/100 ml milk, and the frozen breast milk contains 95 mg lysine/100 ml milk. Infants drink between 570-900 ml of breast milk per day, therefore if they drink 570 ml refrigerated breast milk stored for 48 hours, they will still meet the requirements for lysine at 561 mg.

Although studies may find a statistically significant decrease in protein and lipids after cold storage for 48 hours to 10 days, these results may not be clinically significant. In the previous example, although available lysine decreased during cold storage, the remaining lysine was still sufficient to meet the requirements of infants 0 – 12 months of age.

Many studies have evaluated the effects of storage time and temperature on the ability of breast milk to suppress the growth of bacteria within the milk (Eteng and others 2001; Hamosh and others 1996; Ogundele 2002; Olowe and others 1987; Sosa and Barness 1987). The results of these studies are not in agreement with one another. For example, Ogundele and others (2002) found that the bactericidal activity of breast milk decreased after 14 days of storage at 4 °C, but the decrease in bactericidal activity is compensated for by enhanced

bacteria sequestration activity in the breast milk. Sosa and Barness (1987) also found that storage at refrigeration temperatures for five days decreases the bacterial content in the breast milk (Sosa and Barness 1987). Olowe and others (1987) found that storage of breast milk at refrigeration temperatures for 24 hours resulted in an increase in bacterial growth in 61% of the breast milk samples and inhibition of bacterial growth in 39% of the samples (Olowe and others 1987), most likely due to initial levels of contamination in the breast milk. Other studies examining the effect of storage time and temperature on the bactericidal activity of breast milk either only stored the milk for 24 hours and at ambient temperatures (Eteng and others 2001; Hamosh and others 1996) or they stored the milk at -70 °C, which would not be applicable in child care facilities, hospitals, or in the home. In addition, none of the research addresses the question – how long can breast milk be safely stored after a baby has suckled from a bottle?

1.5. Effect of Storage Temperature and Time on Compounds in Human Milk

A limited amount of research has examined the effect of storage temperature and time on protein, lipids, and bacteria in human milk. The research that has investigated these factors has looked at the effects of human milk storage for a limited number of days. Although a few studies utilized typical storage temperatures, some studies store samples either at ambient temperatures or at -70°C, which is not available in most homes, hospitals, or child care centers.

A study by Miranda and others (2004) assessed changes in glutathione peroxidase activity and in the concentration of malondialdehyde when milk was kept refrigerated or frozen. Glutathione peroxidase (GPx) is a seleno-enzyme, so it is a source of selenium for the newborn, but it is also an enzyme that protects against free-radical attack. Malondialdehyde (MDA) is a final product of lipid peroxidation, so it is used as a marker of the degree of oxidation. They stored breast milk at 4-6^{o}C for 48 hours and at -20^{o}C for 10 days. The milk was analyzed at 4-6 hours after extraction, at 48 hours, and at 20 days. GPx activity decreased significantly from the fresh samples in the refrigerated and frozen samples, but activity was greater in the frozen samples than refrigerated samples. Fresh samples show a lower MDA concentration than those kept refrigerated or frozen, but refrigeration storage shows the greatest MDA concentration. The researchers concluded that storage at -20^{o}C is better than refrigeration to preserve the quality of the milk; however, they did not look at refrigeration storage longer than 48 hours (Miranda and others 2004).

Hamosh and others (1996) assessed microbial growth and stability of milk protein and lipid at 15^{o}C, 25^{o}C, and 38^{o}C for up to 24 hours. These temperatures were chosen because they mimic suboptimal storage conditions that may used for human milk storage in developing countries as well as in work situations in industrialized countries. Sixteen healthy women who breastfeed exclusively donated milk. Dissimilar from the present study, the milk was stored at 15^{o}C, 25^{o}C, and 38^{o}C for 1 – 24 hours to determine pH, proteolysis, and lipolysis. Bacterial growth was determined at 0, 4, 8, and 24 hours of storage.

Proteolysis was minimal during milk storage at 15°C or 25°C for 24 hours and was only noticeable after 24 hours of storage at 38°C. However, the differences were not significant. The greatest rise in proteolysis products was a 40% increase above baseline after 24 hours of storage at 38°C. Lipolysis began during the first hours of storage and increased to 8% at 24 hours of storage. After the milk had been stored for 24 hours at 38°C, the concentration of NEFA were 440% to 710% higher than in freshly expressed milk. Lipolysis was higher at 24 hours' storage at 25°C than 15°C or 38°C, possibly due to inactivation of lipoprotein lipase at 38°C. Bacterial growth was mainly limited to nonpathogens. Bacterial growth was minimal at 15°C within 24 hours, was low at 25°C for the first 4 – 8 hours, and was higher at 38°C at 4 hours (Hamosh and others 1996).

Eteng and others (2001) tested the hypothesis that storage does not affect the biochemical and nutritional quality of human breast milk. The components studied were lactose level, protein level, pH, and microbial content. Milk samples were divided into three portions and analyzed after 3, 6, and 24 hours for lactose, protein, pH levels, and microbial content at a temperature of 29°C. They found that there were significant decreases in protein, lactose, and pH upon storage for 6 and 24 hours, as compared with storage for 3 hours. The microbial content increased with an increase in storage time from 3 to 24 hours. They concluded that breast milk is stable at an ambient temperature for 3 hours and that beyond three hours, significant changes occur in biochemical composition and nutritional quality (Eteng and others 2001).

Pons and others (1998) evaluated the effect of various storage methods on the stability of the triacylglyceride fraction of human milk. The purpose was to evaluate the lipolytic activity in human milk during storage at -20°C and to examine the effect of freezing and thawing on samples stored at this same temperature. Thirty milk samples were from six women. The samples were divided into five groups:

1. Storage at -20°C for four months.
2. Rapidly heated at 80°C and held for 1.5 minutes to inactivate bile salt stimulated lipase (BSSL). Samples were then stored at -20°C for 4 months.
3. Storage at -80°C for 4 months. Before analyzing the samples, they were thawed in a 25°C bath and rapidly extracted.
4. Storage at -80°C for 2 months. They were then thawed for an hour in a 25°C bath and stored at -80°C for another 2 months. At the moment of analysis, samples were thawed in the same way and rapidly extracted.
5. Storage at -20°C for 2 months. Then the samples were placed in a refrigerator and thawed slowly by being held for one week at 5°C. Then the samples were stored at -20°C for another month before lipid analysis.

Group two and three did not yield any products of hydrolysis, indicating that these storage procedures were satisfactory. Group one, storage at -20°C without previous heat treatment, led to the hydrolysis of triacylglycerides and the appearance of NEFA. Freezing and thawing activated lipolysis and increased the production of NEFA, monoacylglycerides, and diacylglycerides (Pons and others 1998).

1.6. Effect of Storage Temperature and Time on Bacterial Growth in Human Milk

Bacterial growth in human milk actually decreases during storage for five days due to the antibacterial factors contained within the milk (Sosa and Barness 1987). Storage beyond a couple of days will result in an increase in bacterial growth.

A study by Sosa and Barness (1987) was done to assess the potential risks of using unprocessed breast milk to feed premature infants. The researchers studied the bacterial growth in 41 samples of unprocessed human milk for a period of five days under refrigeration. No bacteria were cultured in eight samples of milk and the bacteria that were cultured in the other 33 samples were similar to bacteria found on the nipple and skin of the breast. However, two samples contained *Klebsiella* and one sample contained *Pseudomonas*. The initial concentration of bacteria was low, with a mean of 10,000 colonies. Bacterial colony counts continued to decrease during the five days of refrigeration. It can be assumed that when breast milk is collected under appropriate conditions, the bacteria represent contamination from normal skin flora. They found that antibacterial factors in breast milk can control bacterial growth when the milk is refrigerated for at least five days (Sosa and Barness 1987).

In contrast, Olowe and others (1987) determined the bacteriological quality of expressed breast milk, but examined the quality over 24 hours rather than 5 days. The researchers also evaluated the effectiveness of a domestic refrigerator to preserve the quality of breast milk when stored for 24 hours. Eighty-seven breast milk samples were obtained and immediately cultured for bacteria after collection and then stored in a refrigerator for 24

hours. Cultures were taken at 6 hour intervals. At baseline, 69% were either sterile or contained only normal skin flora and 31% grew potential pathogens. During the 24 hours of storage in the refrigerator, bacteria multiplied in 50 samples and bacterial growth was inhibited in 32 samples. The mean bacterial count at any time during the 24 hours was not significantly different from that at the beginning of the storage in the refrigerator. The researchers concluded that when breast milk is collected under appropriate conditions, the bacteria represent contamination from normal skin flora, and the milk can be safely given to infants within 24 hours of refrigeration (Olowe and others 1987).

Although Olowe and others (1987) and Sosa and Barness (1987) determined that breast milk may be safely stored for 24 hours and 5 days, respectively, Oqundele and others (2002) provided evidence that breast milk may be stored longer than 5 days. They examined the effects of different storage methods on the pH and antibacterial activities of human milk, specifically, the bactericidal activities and the adhesion of milk fat globule membranes to suspended bacteria. Milk and colostrum samples from healthy, lactating mothers were stored at 4^{o}C and -20^{o}C for periods ranging from one day to 12 weeks. The samples were analyzed for pH, bactericidal, and bacteria sequestration activities against a serum-sensitive *E. coli*, and compared with freshly collected samples, with and without EDTA. The pH decreased during storage. Bactericidal activity of the refrigerated samples diminished rapidly. However, up to two-thirds of the bactericidal activity was preserved by freezing for up to three months. The ability of the milk fat globules to adhere to suspended bacteria decreased in the frozen milk samples, but it greatly increased during the first few days in the refrigerated samples

and then it sharply declined. The results indicate that loss of bactericidal activity in refrigerated milk is compensated for by enhanced bacteria sequestration activity, thus stored human milk is safe for infant consumption.

Hernandez and others (1979) evaluated the effects of freezing and pasteurization on the ability of breast milk to suppress the growth of a bacterial inoculum in vitro. Breast milk was obtained from nursing mothers during the second week of postnatal life. Aliquots of the fresh milk were allocated to each of the four study groups.

1. Fresh: untreated and maintained at room temperature.
2. Pasteurized: immersed in boiling water for five minutes, and then cooled to room temperature.
3. Fresh frozen: frozen at -70^{o}C for two minutes, and then thawed to room temperature.
4. Frozen 21 days: frozen at -70^{o}C for three weeks, then thawed, at which time one aliquot was inoculated directly while another was pasteurized before inoculation.

The samples were inoculated within 30 minutes of obtaining the sample. Commercial formula and nutrient broth served as controls. Samples were inoculated 10 to 50 cfu/ml of *E. coli* or group B *streptococcus*, and incubated at 37^{o}C. Quantitative growth was measured at eight and 24 hours. Bacterial inhibitory activity was not demonstrated in the control broth, commercial formula, and pasteurized breast milk. Fresh breast milk, fresh frozen breast milk, and breast milk frozen and stored for 21 days demonstrated a significant inhibition of bacterial growth. There was a trend toward gradual loss of inhibitory activity with prolonged freezing. Therefore, freezing provides a way to store breast milk without reducing bacterial inhibitory capacity as opposed to pasteurization. The results support the recommendations

from the American Academy of Pediatrics that frozen breast milk is a good alternative for feeding premature infants when fresh milk is not available.

1.7. Effect of Storage Temperature and Time on IgA Concentration

Jocson and others (1997) tested the hypothesis that total bacterial colony counts and immunoglobulin A (IgA) concentration were not affected by the addition of fortifier even when tested under extreme storage conditions and that osmolality of fortified human milk does not increase with storage. Osmolality was used as an indicator of milk lipolysis and protein degradation, which may be signs of potential rancidity. Osmolality is a count of the number of particles in a solution, so as the particles in the breast milk decrease, the authors hypothesize that the amounts of protein and fat are decreasing. Ten frozen milk samples and ten fresh milk samples were divided into fortified and unfortified milk and stored for 72 hours at either refrigeration or room temperature. Aliquots were taken at 0 and 72 hours and tested for total bacterial colony count (TBCC), osmolality, and total IgA. Fortified milk was stored at refrigeration temperature for 20 hours, warmed in a 40^{o}C laboratory incubator for 20 minutes, and placed in a 34^{o}C infant incubator for 4 hours. At 0, 20, and 24 hours TBCC increased in both types of milk with storage. Osmolality increased in both types of milk by 4% with storage, indicating that nutrient degradation does occur during storage. IgA concentration was not affected by fortification or by storage. The results of this study indicate that nutrient fortification and storage alters some of the host defense properties of human milk, but not IgA.

1.8. Protein in Human Breast Milk

In addition to providing adequate amounts of essential amino acids, human breast milk contains proteins that provide antimicrobial and immunostimulatory effects, such as SIgA, lactoperoxidase, κ-casein, haptocorrin, casein phosphopeptides, and lactadherin. Human breast milk also contains proteins that aid in nutrient absorption in the infant's gut, such as lactoferrin, bile-salt stimulated lipase, haptocorrin, folate-binding protein, and κ-casein (Bernt and Walker 2001).

Milk proteins can be classified into three groups: mucins, casein, and whey proteins. Mucins are milk fat globule membrane proteins that surround the lipid globules in milk (Patton and Huston 1986). Colostrum has a high protein concentration, consisting primarily of whey protein due to the low synthesis of casein during the first few days of lactation (Atkinson and Lonnerdal 1989). The protein concentration of breast milk decreases rapidly during the first month of lactation but it decreases slowly throughout the remainder of lactation. The whey to casein ratio in early lactation is about 80:20 and in late lactation it is about 50:50 (Atkinson and Lonnerdal 1989).

1.8.1. Protein Concentration in Human Breast Milk

The nutritional value of protein in human milk is appreciated for many reasons, including caloric content, constituent amino acids used for growth and development, and immunological compounds.

The protein concentration of human breast milk is 14 to 16 g/L during early lactation, 8 to 10 g/L at 3 to 4 months of lactation, and 7 to 8 g/L at 6 months and later (Newburg 2001). However, these protein concentrations were determined by measuring nitrogen with the Kjeldahl method, subtracting non-protein nitrogen from total nitrogen, and multiplying by the conventional Kjeldahl factor of 6.25. Non-protein nitrogen is 20-25% of the total nitrogen in human milk; therefore this method of measuring protein in human breast milk may underestimate protein concentration if too large a value is subtracted for non-protein nitrogen. Protein concentrations may also be higher in breast milk of women with higher protein content in their diet.

1.8.2. Casein in Human Breast Milk

The casein micelle in human milk contains β-casein, κ-casein, and α_{s1}-casein. The α_{s1}-casein concentration contributes about 0.06% of total protein (Newburg 2001). The main subunit of human milk casein is β-casein, which contains clusters of phosphorylated serine and threonine residues close to the N-terminal end, which are able to complex with calcium ions (Sato and others 1991). Therefore, when β-casein is digested, it may aid in calcium absorption by keeping calcium soluble, leading to the high bioavailability of calcium in human milk (Sato and others 1986).

As opposed to β-casein, which is highly phosphorylated, κ-casein is highly glycosylated and contains several N-acetylneuraminic acid residues (Brignon, 1985). The

carbohydrate component of κ-casein may prevent the adhesion of *Helicobacter pylori* to the infant's gastric mucosa (Stromqvist and others 1995).

1.8.3. Whey Proteins in Human Breast Milk

The major whey protein in human milk is α-lactalbumin, which consists of 123 amino acids (Phillips and Jenness 1971). The amino acid composition of α-lactalbumin is listed in table 1.3, along with the amino acid composition of lactoferrin and serum albumin. It is able to bind calcium in a 1:1 molar ratio and calcium may have a structural role for α-lactalbumin (Lonnerdal and Glazier 1985). The main role of α-lactalbumin is to act as a part of lactose synthase, which is the enzyme responsible for lactose synthesis in the mammary gland. Lactose synthase is composed of α-lactalbumin and galactosyltransferase and together they catalyze the binding of glucose to UDP-galactose to form lactose (Brew and Hill 1975). As a part of this enzyme, α-lactalbumin modifies the catalytic site of galactosyltransferase and promotes the binding of glucose to the enzyme (Atkinson and Lonnerdal 1989).

In addition to aiding in lactose synthesis, α-lactalbumin has a high nutritional value for the infant. Table 1.3 illustrates the amino acid composition of major human whey proteins (Atkinson and Lonnerdal 1989).

The whey protein α-lactalbumin may inhibit growth of potential cancer cells. Preparations of α-lactalbumin from human, camel, cow, and goat milk have all been found to be growth inhibitory for cultured mammary epithelial cells (Thompson and others 1992).

Bovine α-lactalbumin also has antiproliferative effects in human colon cancer cell lines (Sternhagen and Allen 2001).

Hakansson and others (2000) reported an antimicrobial activity resulting from the folding of α-lactalbumin into an active complex with oleic acid (C18:1). They found that native α-lactalbumin exerted no bactericidal effects but that it could be converted into an active form by ion exchange chromatography in the presence of human milk casein fractions containing oleic acid. The α-lactalbumin – oleic acid complex is selective for streptococcal strains, including strains of *Streptococcus pneumoniae*, *Staphylococci* (gram-positive) and *Enterococci* (gram-negative) were resistant to this agent (Hakansson and others 2000).

Table 1.3 Amino Acid Composition of Major Human Whey Proteins (Lonnerdal 1989)

	Amino Acid Residues		
	Lactoferrin	α-lactalbumin	Serum albumin
Asp	71	17	53
Thr	31	6	28
Ser	50	7	24
Glu	70	15	82
Pro	35	2	24
Gly	56	6	12
Ala	63	6	62
Val	49	2	41
Met	6	2	6
Cys	32	8	35
Ile	16	12	8

Table 1.3 Continued

Leu	61	14	61
Tyr	20	4	18
Phe	31	4	31
Lys	46	12	59
His	9	2	16
Arg	46	1	24
Trp	11	3	1
Total	703	123	585

1.8.3.1. Lysozyme in Human Breast Milk

Lysozyme is another major whey protein in human milk, representing 1-4% of whey protein nitrogen (Atkinson and Lonnerdal 1989). Lysozyme is an enzyme found in many secretions, such as human milk, saliva, tears, nasal mucus, and pancreatic juice (Fleming 1922). It is capable of breaking down the outer cell wall of gram-positive bacteria and some gram-negative bacteria by hydrolyzing the β1,4 linkages of N-acetylmuramic acid and 2-acetylamino-2-deoxy-D-glucose residues (Chipman and Sharon 1969).

Human milk contains about 400 μg/ml of lysozyme, which is about 3,000 times more than the amount of lysozyme in bovine milk (Floris and others 2003). Lysozyme is secreted in concentrations between 2 and 6 mg/dL into human milk, which makes it the highest observed concentration among bodily secretions (Bernt and Walker 2001). Lysozyme concentrations stay relatively constant over the course of lactation, and levels may rise at 4

months of lactation (Bernt and Walker 2001). It is also very resistant to break down by acid in the stomach as well as trypsin, suggesting that adequate amounts of lysozyme reach the intestinal tract (Bernt and Walker 2001).

Lysozyme is active against gram-positive and gram-negative bacteria. Lysozyme has been shown to kill gram-negative bacteria and inhibit the growth of gram-positive bacteria (Shah 2000). Lysozyme may also work together with lactoferrin, which is an iron-binding protein capable of binding two ferric ions (Lonnerdal and Iyer 1995), to kill gram-positive bacteria. Lactoferrin binds lipopolysaccharide (LPS) and removes it from the outer cell membrane of gram-positive bacteria so that lysozyme can degrade the outer membrane and kill the bacteria (Ellison and Giehl 1991).

1.8.4. Immunoglobulins in Human Breast Milk

Paul Ehrlich was the first to show the transfer of maternal antibodies to the neonate by human milk (Bernt and Walker 2001). Immunoglobulins are beneficial because they provide protection to the infant's immature immune system. Immunoglobulins, or antibodies, that are present in human milk include immunoglobulins A, G, M, D and E, with SIgA being the most abundant (Newman 1995).

Immunoglobulin A (IgA) comprises over 90% of antibody molecules in human milk, but only 15% of immunoglobulins in human serum (Goldman and Goldblum 1989). The forms of IgA differ in serum and in milk. In serum, IgA is a four-chain structure consisting of two heavy, identical polypeptide chains and two identical light polypeptide chains linked by

disulfide bonds (Goldman and Goldblum 1989). In milk, IgA is in a polymeric form called SIgA, consisting of two four-chained units united by a 15-kD polypeptide called the joining chain and complexed to a 75-kD glycopeptide called the secretory component (Brandtzaeg 1974; Wilde and Koshland 1973).

1.8.4.1. Secretory Immunoglobulin A

SIgA is made from proteins produced by plasma cells and epithelial cells of the mammary gland. The plasma cells that produce dimeric IgA are native to the intestinal and respiratory tract. The dimeric IgA binds to receptor secretory component (SC) molecules on the mammary gland epithelium. The extracellular part of SC attached to an IgA dimer is transported across the cell and secreted as SIgA (Atkinson and Lonnerdal 1989).

Levels of SIgA vary greatly among individuals and the pattern of SIgA levels in milk is consistent over the course of lactation, as the following chart demonstrates (Atkinson and Lonnerdal 1989). During the first days postpartum, IgA secretion reaches 4 grams per day, at which point its concentration is higher in milk than in any other bodily fluid. IgA levels drop to about 1g/d at day 10 and levels are detectable for the next 4 months (Bernt and Walker 2001). Table 1.4 outlines the concentrations of immunoglobulins during different stages of lactation.

Table 1.4 Concentrations of Immunoglobulins (mg/ml; mean ± SD) in Human Breast Milk

Immunoglobulin Concentrations	Duration of Lactation				
	2-4 days	1 month	6 month	12 month	24 month
Total IgA	2.1 ± 2.3	1.0 ± 0.2	0.6 ± 0.1	1.0 ± 0.5	1.1 ± 0.3
SIgA	2.0 ± 2.5	1.0 ± 0.3	0.5 ± 0.1	1.0 ± 0.3	1.1 ± 0.2
IgM	0.12 ± 0.03	0.02	0.02	0.01	b
IgG	0.34 ± 0.01	0.05 ± 0.03	0.03	0.04	b

[a] Data complied by Goldman and Goldblum from previous studies
[b] Insufficient data

SIgA is able to survive in the hostile environment of the gastrointestinal tract because it is more resistant to degradation from proteases than other immunoglobulins (Atkinson and Lonnerdal 1989). SIgA antibodies against bacterial IgA proteases have been found, including proteases produced by *H. influenzae, Streptococcus sanguis, S. pneumoniae, Neisseria gonorrhoeae, Proteus mirabilis, E. coli, and Neisseria meningitidis* (Gilbert and others 1983).

SIgA antibodies neutralize bacterial toxins and prevent binding of intestinal bacterial pathogens to epithelial cells by binding to their pili (Newberg 2001). The antibodies delivered to the infant ignore the useful bacterial flora native to the gut, and only act against the pathogenic bacteria (Newman 1995). As opposed to other immunoglobulins, such as IgG and IgM, complexes of antigens with IgA do not activate the inflammation pathway and may actually prevent inflammation by competing with IgG and IgM antibodies for antigens (Atkinson and Lonnerdal 1989). Many studies suggest that the principle function of SIgA in

human milk involves blocking the adhesion of microorganisms to the intestinal epithelium and neutralization of microbial toxins (Bernt and Walker 2001).

1.8.5. Proteolysis

Casein is a source of biologically active peptides (Clare and Swaisgood 2000; Meisel and Bockelmann 1999; Schanbacher and others 1997). Table 1.5 outlines the effects of peptides in human milk (Schanbacher and others 1997). The whole antibacterial effect in milk adds up to be more than the total of the individual contributions of immunoglobulin and nonimmunoglobulin defense proteins due to the fact that the defense proteins work together synergistically (Clare and Swaisgood 2000). Table 1.6 outlines the antimicrobial peptides in bovine milk (Clare and Swaisgood 2000).

Table 1.5 Categories of Bioactivities in Bovine Milk and their General Effects and Major Effecters (Schanbacher and others 1997)

GASTROINTESTINAL FUNCTION AND DIGESTION	
Altered rate of gastric emptying	Opioid agonist peptides (casomorphins, lactorphins)
Decreased intestinal motility	Opioid antagonist peptides (casoxins, lactoferroxins)
Decreased rate of passage of digesta	Casein phosphopeptides
Increased nutrient absorption rate	
Enhanced absorption of divalent mineral ions	
Hemodynamic modulation	
Antihypertension	Casokinins (ACE-I; angiotensin I-converting enzyme inhibitor peptides)

Table 1.5 Continued

Increased blood flow	Casoplatenin (K-casein peptides) Lactoferrin and peptides
Probiotic support of intestinal microflora	
Enhanced growth of *Bifidobacteria* in GI tract	K-Casein glycomacropeptide Milk oligosaccharides Lactoferrin and lactoferrin peptides Casomorphins (indirect)
Non-immune disease protection	
Inhibition of coliform and pathogen growth in gut or mammary gland	Lactoferrin, lysozyme, lactoperoxidase
Killing of coliform or pathogenic bacteria in gut or mammary gland	Lactoferrin bactericidal peptides (lactoferricin)
Inhibition of viral infection	Glycolipids, sphingolipids
Passive immunity	
Transfer of immunoglobulins (IgG, IgA; IgM) to blood of neonate	Immunoglobulins IgG, IgA, IgM
Continual presence of Ig in lumen of gut	Casein immunoregulatory peptides
Enhanced phagocytic activity	Cytokines Growth factors
Immunoregulation	
Modulation of lymphocyte function	Casein immunoregulatory peptides
Modulation of lymphocyte differentiation	Lactoferrin and lactoferrin peptides
Modulation of lymphocyte and granulocyte traffic	Cytokines
Enhanced killer cell activity	Growth factors
Anti-inflammation	
Modulation of lymphocyte function	Casein immunoregulatory peptides
Reduced release of cytokines by lymphocytes and macrophages	Lactoferrin and lactoferrin peptides
Modulation of polymorphonuclear leukocyte response	Cytokines

Table 1.5 Continued

Binding of bacterial lipopolysaccharide (endotoxin)	Growth factors
Growth and development	
Enhanced development of intestinal tract	Hormones: prolactin, plus many others in milk
Enhanced gastric development and function	Growth factors: IGF, EGF, TGF-a, TGF-P
Enhanced neuroendocrine development	Cytokines
Enhanced development of immune system	Casein immunoregulatory peptides Lactoferrin

Table 1.6 Antimicrobial Proteins in Milk (Clare and Swaisgood 2000

Milk Peptide Fragment	Release Protease	Gram (+) Activity	Gram (-) Activity	Yeast and Fungi*
Casecidin	Chymosin and chymotrypsin	*Staphylococcus* *Bacillus subtilis* *Diplococcus pneumoniae* *Streptococcus pyogenes*		
Casocidin-I	Synthetic peptide	*Staphylococcus carnosus*	*Escherichia coli*	
Isracidin	Chymosin and chymotrypsin	*Staphylococcus aureus*		*Candida albicans*
Lactoferricin B Lactoferrin	Pepsin	*Bacillus* *Listeria* *Streptococci* *Staphylococci*	*E. coli 0111* *E. coli* 0157H:7 *Klebsiella* *Proteus* *Pseudomonas* *Salmonella*	*Candida albicans* *Dermatophytes:* **Cryptococcus uniguttulatus* **Penicillum pinophilum* **Trichophyton mentagrophytes*

Other physiologically active peptides in milk include antihypertensive peptides, antithrombotic peptides, caseinophosphopeptides, immunomodulatory peptides, opoid milk peptides, casomorphins, caseinomacropeptide, and atriopeptin (Clare and Swaisgood 2000). As mentioned previously, peptides from κ-casein exert antimicrobial (Stromqvist and others 1995) and antithrombotic effects, in particular the κ-caseinglycopeptide (hCGP) (Chabance and others 1995).

The bioactive peptides in human milk proteins are dormant until released from the parent protein and activated by enzymatic proteolysis, such as during digestion or food processing (Meisel and Bockelmann 1999). Ferranti and others (2004) found that most peptides identified in human milk originate from β-casein and not the most abundant whey protein, α-lactalbumin. They identified these proteins using mass spectroscopy and outlined the proteolytic steps that β-casein follows (Ferranti and others 2004). They outlined three proteolytic steps in β-casein breakdown. The first step involves the action of a plasmin-like enzyme at Lys_{18}, Lys_{23}, and Lys_{160}. The next step involves the action of endogenous endopeptidases at Ala_{73}, Ala_{180}, and Glu_{186}. Finally, these peptides are substrates for other exopeptidases, including aminopeptidases, carboxypeptidases, and peptidases cleaving after proline residues, which all require short peptides as substrates (Ferranti and others 2004).

Several theories regarding the mechanism of antimicrobial peptides *in vivo* have been proposed and reviewed by Clare and others (2003). Among such key hypotheses are the assumptions that bacterial DNA/protein synthesis might be affected, breakage of single

stranded DNA occurs, autolytic enzymes, such as amidases that degrade the peptidoglycan layer, are stimulated, bactericidal molecules, such as hydrogen peroxide, are produced, and/or binding of cationic peptides to cellular nucleic acids results in antimicrobial effects (Clare and others 2003).

1.8.5.1. Proteases Endogenous to Human Breast Milk

The existence of proteolytic enzymes in human milk provides the infant with significant digestive assistance during the period of adjustment from prenatal nutrition by the placenta to postnatal oral feeding (Storrs and Hull 1956). In human milk there are proteolytic enzymes, a fibrinolytic system, plasmin, and a plasminogen activator (Atkinson and Lonnerdal 1989).

Plasmin is an alkaline serine protease and is the principle endogenous proteolytic enzyme in milk. It is associated with the casein fraction of milk but it can be found in whey and under certain conditions it can shift from the casein to whey fraction. The plasmin system has five elements: plasmin (PL), plasmin inhibitors (PI), the inactive zymogen plasminogen (PG), plasminogen activators (PA), and inhibitors of plasminogen activators (PAI) (Chen and others 2003). Plasminogen is activated through proteolysis by plasminogen activators, which are serine proteases. Plasmin has specificity to hydrolyze bonds on the C-terminal side on lysine and arginine residues, cleaving Lys-X bonds faster than Arg-X bonds. Plasmin preferentially hydrolyzes β-casein at positions Lys_{28}-Lys_{29}, Lys_{105}-His_{106}, and

Lys_{107}-Glu_{108} to give three β-casein fragments γ_1-, γ_2-, and γ_3-caseins as well as proteose-peptones (Chen and others 2003). Plasmin does not hydrolyze α-lactalbumin.

There are also several protease inhibitors in human milk, including α_1-antichymotrypsin and α_1-antitrypsin. Protease inhibiting activity was found in two thirds of milk samples collected during the first 3 days of lactation and in all the samples collected from later stages of lactation, including inhibitors of trypsin, chymotrypsin, and elastase (Lindberg 1979). The same samples had low proteolytic activity and the milk protease inhibitors formed complexes with added trypsin and chymotrypsin, but not pepsin (Lindberg 1979). These results suggest that the proteases were inactive due to the complexes with the inhibitors (Lindberg and others 1982).

1.8.5.2. Bacterial Proteases

In addition to plasmin, which is endogenous to milk, psychrotrophic bacteria produce heat-stable metalloproteases that can increase proteolysis in milk, even at refrigeration temperatures (Frohbieter and others 2005). Psychrotrophic bacteria are cold-loving bacteria with an optimal growth temperature at 15°C - 20°C and a minimal temperature for growth lower than 0°C. Production of proteases by psychrotrophic bacteria occurs mainly at the end of the exponential growth phase (Dupont and others 2007). The nature of the proteases produced in milk by psychrotrophic bacteria depends on the species of bacteria, and within the same species will differ from one strain to another (Dupont and others 2007).

Gram-negative psychrotrophic bacteria include Pseudomonas, *Aeromonas, Serratia, Acinetobacter, Alcaligenes, Achromobacter, Enterobacter,* and *Flavobacterium.* Gram-positive psychrotrophic bacteria include *Bacillus, Clostridium, Corynebacterium, Microbacterium, Micrococcus Streptococcus, Staphylococcus,* and *Lactobacillus.* The organisms most commonly isolated from breast milk, including gram-positive, coagulase-negative staphylococci and gram-negative *Acinetobacter* are also known skin colonizers (Botsford and others 1986) (el-Mohandes and others 1993).

Similar to plasmin, proteases from psychrotrophic bacteria preferentially hydrolyze casein over whey, particularly κ-casein, resulting in the cleavage of the $Phe_{105} - Met_{106}$ peptide bond, leading to the release of the C-terminal caseinomacropeptide (CMP) (Chen and others 2003) (Dupont and others 2007).

1.8.6. Proteolysis and Bioavailability

The pre-term infant may be more dependent upon the immunoprotective actions of lysozyme, lactoferrin, and SIgA than the term infant, due to its immature immune function, but there is no doubt that the term infant is dependent upon these components as well. Proteolysis of proteins during storage of human milk may be of concern because these proteins need to be intact in the GI tract in order to exert their protective functions. As previously mentioned, SIgA is able to endure in the hostile environment of the gastrointestinal tract because it is more resistant to degradation from proteases than other immunoglobulins (Atkinson and Lonnerdal 1989). Also, the immunoprotective components

of human milk are stable when stored at room temperature for 8 hours, when stored at 0 - 4°C for three days, or when frozen at -20°C for 12 months (Lawrence 2001).

Studies have indicated minimal protein digestion in the infant's stomach due to a low pepsin output (Agunod and others 1969; Weisselberg and others 1992; Yahav and others 1987) and high postprandial gastric pH (Hamosh and others 1978; Mason 1962). Because infants have a higher pH in their gastrointestinal tract than do adults, proteins are not as readily denatured. Therefore, proteolysis of milk proteins prior to ingestion may increase bioavailability of protein to the infant because they are already broken-down before ingestion. Table 1.7 summarizes current knowledge on gastric proteolysis in infants (Henderson and others 2001).

Table 1.7 Hydrolysis of Milk Proteins by Gastric Aspirates of Infants (Henderson and others 2001)

- Gastric pH is 5.0 to 6.0 during the 1st hour postprandial
- Pepsin activity and output are significantly lower in infants (especially preterm) than in children and adults
- Peptic activity tested in vitro with specific human or bovine milk proteins is very low to nil at pH > 4.5
- Hydrolysis of human or bovine milk protein by infant duodenal juice in vitro is also low
- Hydrolysis of milk whey proteins, under optimal in vitro conditions, by infant gastric or duodenal juice is lower than that of casein
- Proteins in human milk or formula are hydrolyzed to the same extent (15%) in the stomachs of preterm infants; in contrast, fat in human milk is digested to a greater extent (25%) than fat in formula (13-14%)

1.9. Lipids in Human Breast Milk

Triacylglycerols comprise 98% of total lipids on human milk, phospholipids 0.8%, and cholesterol 0.5% (Jensen 1999). The majority of the fatty acids are medium and long chain fatty acids (C:10 to C:22) (Manson and Weaver 1997). Lipids are present in milk between 3% and 5%, and they exist as an emulsion in the aqueous phase (87%) of human milk (Jensen 1999). The fat globules are surrounded by a membrane called the milk fat globule membrane (MFGM), which is composed of phospholipid proteins, mucopolysaccharides, cholesterol, and enzymes (Jensen 1999). Table 1.8 summarizes the effects of lipids in human milk (Jensen 1999).

Table 1.8 Effects of Lipids in Human Milk (Jensen 1999)

1. Source of 50–60% of the calories, about 70 kcal/dL, in human milk. Not usually responsive to diet.
2. Fatty acids combined into triacylglycerols to maintain a bulk melting point below 38°C.
3. Provide about 15 mg of cholesterol/dL. May predispose the infant to efficiently metabolize dietary cholesterol as an adult. Precursor of steroid hormones and other derivatives in humans. Not affected by changes in maternal diet.
4. Contain the essential polyunsaturated fatty acids 18:2n-6 and 18:3n-3 and their products, 20:4n-6, 20:5n-3, and 22:6n-3. Required in maternal diet.
5. The 20:4n-6, 20:5n-3, and 22:6n-3 in proper balance may be required for maturation and optimal function of the visual process and brain and nervous system.
6. Contain 8:0–14:0, which if absorbed in the stomach, are transported to the liver and oxidized in decreasing amounts as the molecular weight increases. Quantities in milk dependent on amount of carbohydrate in diet.
7. If present, the conjugated fatty acid, *c*9, *t*11-18:2, or rumenic acid, may exert anticarcinogenic, antiatherosclerotic, and other beneficial effects. Responds to ruminant products, particularly dairy products.

Table 1.8 Continued

8. *Trans* unsaturates believed by some to adversely affect infants growth. Some positional isomers of *c*-18:1 may ameliorate atherosclerosis.
9. When produced by lipolysis in the stomach and small intestine, 12:0 and 18:2 and their monoacylglycerols have potent cidal effects against some microorganisms. Soluble and dispersible salts of fatty acids may act against microorganisms.
10. Milk gangliosides inactivate cholera and other enterotoxins.
11. Contain eicosanoids and their precursors, which act as first and second messengers. Precursors respond to diet.
12. Lipolysis of triacylglycerols by gastric lipase, regioselective for *sn*-3/*sn*-1,3/1; produces *sn*-1,2-diacylglycerols, which can be second messengers in stomach and small intestine.
13. Milk lipid globule membrane binds, protects, and releases bioactive compounds as needed. Graded doses of lipid provided. Membrane stabilizes globules in an oil/water emulsion.
14. Increases in lipid content and numbers of globules during a nursing may help develop appetite and its control in the infant by approaching satiety and by the tactile effect of the globules.
15. Lipid content increases as lactation progresses to help provide for growth and development of infant.
16. Carrier of fat-soluble vitamins.
17. Can transport undesirable compounds such as dioxins.
18. When milk is stored in the frozen state, it can be the source of soapy flavor due to free 12:0 and to oxidized flavors (cardboardy) due to polyunsaturated fatty acids.
19. Carotenoids, tocopherols, and conjugated 18:2 may be antioxidants.

Lipids in human milk provide 40-50% of the infant's calorie requirements and they are also integral constituents of neural and retinal tissues (Manson and Weaver 1997). Agostoni and others (2001) found that breast-feeding for 6 months or longer with milk of high fat content was associated with higher developmental scores at one year of age. The researchers hypothesize that the supply of fat contribute energy and/or affect brain composition (Agostoni and others 2001).

The fat content in human milk increases from 2.0g/dL in colostrum to 4.9g/dL in mature milk, which is a reflection of the rising energy requirement of the growing infant (Manson and Weaver 1997). Fat content also varies during feedings and throughout the day. Fat content is about 3.0 g/dL in midday foremilk and 4.0 g/dL in midday hindmilk. Fat content is about 3.0 g/dL in early morning milk and 4.5 g/dL in evening milk (Jensen and others 1978).

1.9.1. Fatty Acids in Human Breast Milk

The infant consumes fat mostly as triacylglycerides, which need to be broken down by enzymes called lipases before absorption. The fatty acids are esterified to a glycerol molecule (or backbone) to form triacylglycerols. In this form, they are hydrophobic and aid in the transport of the fat soluble vitamins, A, D, E, and K. Fatty acids are also esterified to cholesterol and phospholipids, and in this form they are ambiphilic. Table 1.9 shows the relative concentrations of some fatty acids in mature human milk (Manson and Weaver 1997).

Table 1.9 Fatty Acid Composition of Human Milk (Manson and Weaver 1997)

Fatty Acid	Mature Human Milk
10:0	1.4
12:0	5.4
14:0	7.3
16:0	26.5
16:1	4
18:0	9.5
18:1	35.5
18:2	7.2
18:3	0.8
20:0	0.2
20:4	0.3
22:6	1.1
Total fat (g/L)	42

1.9.2. Polyunsaturated Fatty Acids in Human Breast Milk

The lipids in human milk and formula are very important for the growth and development of infants. Lipids are the major energy source for infants and are tolerated better than proteins and carbohydrates. Human milk provides linoleic acid (18:2 n-6, LA), α-linolenic acid (18:3 n-3, ALA), docosahexaenoic acid (22:6 n-3, DHA), arachidonic acid (20:4 n-6, AA), and other long chain polyunsaturated fatty acids (LCPUFA).

DHA and AA are the major LCPUFA components in membranes of the brain and retina. AA is found mainly in phosphatidylcholine (PC) and phosphatidylinositol (PI). DHA is found mainly in phosphatidylethanolamine (PE) and phosphatidylserine (PS). DHA can be synthesized in the body from ALA and AA can be synthesized from LA. However, AA can be synthesized in greater amounts as evidenced by the fact that infants fed formula can

accumulate more AA than DHA, even when there is sufficient ALA for DHA synthesis (Fleith and Clandinin 2005). The reason AA may be synthesized more so than DHA is because AA synthesis is subjected to less regulatory steps than is the synthesis of DHA. DHA synthesis requires a second Δ^6-desaturase step and β-oxidation in the peroxisomes.

Humans can desaturate and elongate ALA into EPA and DHA only when the ratio of LA (n-6) to ALA (n-3) is low. Excess n-6 fatty acids in the diet saturate the enzymes and prevent conversion of ALA into EPA and DHA (Mahan and Escott-Stump 2004). Therefore, the amount of DHA that an infant can synthesize is limited by the ratio of LA (n-6) to ALA (n-3) in dietary lipids. Human infants can synthesize small amounts of LCPUFA from LA and ALA, but the rate of synthesis is not adequate for LCPUFA accumulation in body tissues (Fleith and Clandinin 2005).

During the third trimester, the neonate accumulates about 40 to 60 mg n-3 PUFA per kg body weight per day (Koletzko and others 2008). LCPUFA intake is important for lactating mothers because an estimated 30% of human milk fatty acids are derived directly from the mother's diet (Koletzko and others 2008). LCPUFA have important effects on membrane function, photoreceptor differentiation, activation of rhodopsin, enzyme activity, ion channel function, and the levels and metabolism of neurotransmitters and eicosanoids. In addition, data from clinical studies in term and preterm infants comparing breastfed and formula fed infants show enhanced development of visual function, improved retinal function, and improved visual acuity (Jensen and others 1997; Jensen and others 1978; Koletzko and others 2008). In addition to possibly enhanced visual function, some studies

have found that visual attention, problem-solving, and global development is improved in infants fed LCPUFA (Jensen and others 1978).

Makrides and others (2000) compared fatty acid status, visual evoked potential acuity, and growth of term infants who were fed formula containing LA:ALA of 10:1 or 5:1 to those of exclusively breast-fed infants (Makrides and others 2000). The formula-fed infants were randomly assigned in a double blind manner to formula containing LA:ALA of 10:1 or 5:1. ALA content was increased by replacing soy oil with low-erucic acid canola oil. Growth and fatty acid status in all groups were assessed at 6, 16, and 34 weeks of age, and visual evoked potential acuity was assessed at 16 and 34 weeks. They found that infants fed the 5:1 LA:ALA formula had significantly greater DHA plasma and erythrocyte phospholipid concentrations than did infants fed the 10:1 formula, but concentrations in both formula groups were still significantly lower than those in the breast-fed infants. However, there were no differences seen in visual evoked potential acuity, weight, length, or head circumference within the groups. These results lead to the conclusion that lowering the LA:ALA ratio in infant formula by using low-erucic acid canola oil results in a significant increase in plasma DHA, but the concentrations still do not match those of the breast fed infants (Makrides and others 2000).

Jensen and others (1997) also compared fatty acid pattern, visual function, and growth of term infants who were fed formulas with varying levels of fatty acids as LA and ALA (Jensen and others 1997). Exclusively breast fed infants were utilized as a control for determining visual function, but not for growth or fatty acid profiles. The formulas contained

approximately 16% of total fatty acids as LA and 0.4%, 1.0%, 1.7%, or 3.2% as ALA, resulting in LA:ALA ratios of 44, 18.2, 9.7, and 4.8. The fatty acid profile of plasma phospholipids was determined at 21, 60, and 120 days of age in the formula fed groups. Anthropometric data to determine growth were assessed at 21, 60, 120, and 240 days of age in the formula fed groups. Transient visual evoked responses (VERs) to determine visual function were measured at 120 and 240 days of age in all groups. The results showed that VER latency and amplitude did not differ within any groups. Infants who received formula with an LA:ALA ratio of 4.8 had higher plasma DHA concentrations, but lower AA acid concentrations at 21, 60, and 120 days of age. The average weight of this group at 120 days was 760 grams (1.67 pounds) less than the average weight of the group fed the formula with the LA:ALA ratio of 44. However, this association was not found at any other time points. The authors did correct for birth weight, but they did not report any influence of gender on weight. Although plasma DHA increased as the LA:ALA ratio decreased, weight gain significantly decreased.

A review of the literature by Fleith and Clandinin (2005) concluded that LCPUFA should be supplied to preterm infants in the range provided by feeding human milk in Western countries. The range can be achieved by feeding AA and DHA at a ratio of 1.5 AA to DHA, with DHA at 0.4%. Addition of LCPUFA in infant formulas, in reasonable quantities, for term infants is safe and will enable formula-fed infants to achieve the same blood LCPUFA concentrations as breast-fed infants. Adequate supply of LCPUFA is especially important in preterm infants because they are at much higher risk for inadequate

LCPUFA accumulation due to disruption of the placental supply. Another review by Koletzko and others (2001) reports conclusions of a workshop on the role of LCPUFA in maternal and child health. For healthy infants, the authors support breastfeeding as the preferred method of feeding.

1.9.3. Non-esterified Fatty Acids in Human Breast Milk

It has been demonstrated that NEFA increase in stored breast milk due to the lipolysis of triacylglycerols. Hamosh and others (1996) found that when breast milk is stored at ambient temperatures for 24 hours, there is a 440% - 710% increase in free fatty acid concentration above that in freshly expressed milk (Hamosh and others 1996). The enzymes thought to be responsible for lipolysis of triacylglycerols are the bile-salt stimulated lipase (BSSL), lipoprotein lipase, or both (Atkinson and Lonnerdal 1989; Hamosh and others 1996). However, BSSL, as the name implies, is inactive without added bile salts, which should not be present in human milk in the concentration needed for BSSL activation (Atkinson and Lonnerdal 1989). Therefore, it is more likely that lipoprotein lipase is responsible for the release of non-esterified fatty acids from the triacylglycerols.

An increase in NEFA in stored breast milk causes an increase in titratable acidity, which has been of concern because of the belief that it may be caused by bacterial fermentation of lactose, resulting in lactic acid. However, is has been shown that the increase in titratable acidity is in fact due to an increase in NEFA and not lactic acid (Atkinson and Lonnerdal 1989).

1.9.3.1. Bioavailability of Non-esterified Fatty Acids in Human Breast Milk

The consequences of increased NEFA on human milk quality and digestibility are unknown. Hernell and Blackberg (1982) suggested that NEFA are absorbed better than esterified fatty acids when intraduodenal bile salts are low in the infant (Hernell and Blackberg 1982). Therefore, it is a possibility that an increase in NEFA could increase bioavailability to the infant. On the other hand, Patton and Carey (1979) suggested that NEFA may bind to calcium or other components of the milk and make them unavailable for absorption (Patton and Carey 1979).

Ionized fatty acids (fatty acid soap) may form during storage and bind with other milk components and make them unavailable for absorption. Lavine and Clark (1987) measured ionized fatty acids in breast milk and found trace amounts (<1.0mg/dL) in freshly expressed milk (Lavine and Clark 1987). As storage time and temperature progressed, there was increased evidence of ionized fatty acids but in small quantities. The greatest amount of ionized fatty acids found was in milk stored for 8 weeks at -11°C, containing 2.2 mg/dL, which was only 1.4% of the total fatty acids in the milk (Lavine and Clark 1987).

1.9.4. Antiviral Activity of Non-esterified Fatty Acids

NEFA produced during storage of human milk have been shown to have potent cytolytic effects on normal human blood cells, on intestinal parasites *Giardia lamblia* and *Entamoeba histolytica*, as well as on gram-positive bacteria and yeast (Ogundele 2000).

NEFA have also caused membrane disruption of enveloped viruses in cultured cells. Thormar and others (1987) found that lipids in fresh human milk do not inactivate viruses but they become antiviral after storage at 4°C and 23°C, probably due to the release of NEFA from milk triglycerides. They compared the effect of fatty acids and monoglycerides on enveloped viruses. They found that short chain fatty acids, such as butyric, caproic, and caprylic, as well as long-chain saturated fatty aids, such as palmitic and stearic, had no or very small antiviral effects at the highest concentration tested. However, the medium-chain saturated and long-chain unsaturated fatty acids were all antiviral but at different concentrations. The polyunsaturated fatty acids had the most antiviral activity. The level of lipoprotein lipase in the breast milk was correlated with the antiviral activity of the milk, indicating that it is caused by the release of NEFA or other products of lipid hydrolysis (Thormar and others 1987). They also found that the antiviral effect is caused by disintegration of viral envelopes by fatty acids, and these results were confirmed by Sarkar and others (1973), who treated mouse mammary virus with the cream fraction of human milk and found that the viral envelope disintegrated (Sarkar and others 1973).

1.9.5. Non-esterified Fatty Acids and Breast Milk Jaundice

Lavine and Clark (1987) determined total lipid and free fatty acid concentrations in breast milk samples obtained from eight mothers and stored at 25°C, 4°C, or -11°C for 6, 12, 24, or 48 hours. Additional samples were stored at -11°C or -70°C for 1, 2, 4, 6, or 8 weeks. Free fatty acid concentrations increased with storage temperature and with time. The pattern

of NEFA changed as storage progressed; with free 16:0 decreasing and 18:2 increasing, and long-chain polyunsaturated fatty acids increasing (Lavine and Clark 1987). Hargreaves (1973) demonstrated that unsaturated fatty acids inhibit bilirubin conjugation *in vitro*, with the inhibition increasing with the degree of unsaturation (Hargreaves 1973). Therefore, it is a possibility that high amounts of polyunsaturated fatty acids in breast milk could inhibit bilirubin conjugation, leading to jaundice in the infant.

It has been proposed that mothers with jaundiced infants have increased lipolytic activity, which results in large amounts of NEFA in the milk after storage (Poland and others 1980). One hypothesized cause of jaundice is that NEFA inhibit bilirubin conjugation, causing neonatal unconjugated hyperbilirubinemia (Lavine and Clark 1987). Another hypothesized cause of jaundice is altered intestinal bilirubin absorption (Gartner and others 1983). Gartner and others (1983) observed in rats that normal milk decreased bilirubin absorption in the intestine. However, milk from mothers with jaundiced infants enhanced bilirubin absorption and increased the bilirubin in the liver (Gartner and others 1983).

However, Constantopoulous and others (1980) measured LPL activity and NEFA from mothers of infants without and with prolonged neonatal jaundice (Constantopoulos and others 1980). The LPL activity and levels of NEFA from mothers of infants without jaundice increased with the duration of breast feeding until 12 days postpartum, and they then fell to original levels. In the group of mothers with jaundiced infants, LPL and NEFA were within normal limits when measured between the 15^{th} and 37^{th} day postpartum. They concluded that LPL and NEFA are not responsible for the development of breast milk jaundice

(Constantopoulos and others 1980). Jalili and others (1985) also could not confirm the observation that increased levels of NEFA are associated with breast milk jaundice (Jalili and others 1985). They analyzed breast milk samples and serum fatty acids in mothers with jaundiced infants and mothers without jaundiced infants. The concentrations of FFA increased after storage of the milk from both groups, but no difference in the composition of milk FFA or serum fatty acids were found (Jalili and others 1985).

More recent studies have focused on the enzyme β-glucuronidase as a contributory factor in neonatal jaundice. This enzyme may lead to production of unconjugated bilirubin by cleaving the ester bond of bilirubin glucuronide in the neonatal intestine (Poland and Odell 1971). Ince and others (1995) analyzed breast milk samples from breast milk of mothers with jaundiced infants and breast milk of mothers with non-jaundiced infants for β-glucuronidase activity (Ince and others 1995). Enzyme activity was slightly higher in the control group, but the difference was not statistically significant. Based on these results, β-glucuronidase may be one of the factors contributing to breast milk jaundice, but it is not the only factor.

1.9.6. Lipases in Human Breast Milk

Triacylglycerols can not be absorbed into the intestinal wall and must first be converted into NEFA and monoacylglycerols. As previously mentioned, lipases catalyze the hydrolysis of long chain fatty acid esters of glycerol (Jensen and others 1982). In the infant, triacylglycerols are hydrolyzed by a triad of lipases, including lingual lipase, bile-salt-stimulated lipase (BSSL), and pancreatic lipase (Jensen and others 1982). The net effect of

the lipolytic triad is to provide caloric density during a period of rapid growth. There are also other lipases such as serum-stimulated lipoprotein lipase (LPL) and a bile-salt-stimulated esterase.

Lingual lipase is secreted from the lingual serous glands in response to the suckling action of the infant (Jensen and others 1982). Fat digestion in the stomach is much more important in the infant than in the adult due to the low concentration of pancreatic lipase in the infant (Jensen and others 1982). Studies have shown that most of the lipolytic activity in the stomach originates from lingual lipase, hydrolyzing 60-70% of ingested fat (Hamosh and others 1979). Studies show that in the newborn infant, the stomach is the major site of fat digestion (Jensen and others 1982) and there are several physiological characteristics of the gastrointestinal tract that aid in gastric fat digestion. As mentioned previously, gastric pH in infants is more basic than in adults being about 5 instead of 2. Stomach emptying is slower and there is a lower bile salt concentration in the intestine of the infant than in the adult. These characteristics allow lingual lipase to remain active after passing with the food through the stomach and into the duodenum (Jensen and others 1982).

Bile-salt-stimulated lipase (BSSL) is a glycoprotein with proline making up about 10% of the amino acids (Blãckberg and Hernell 1981). It is most likely synthesized in the mammary gland and its site of action is the small intestine. Bile salts are required for the enzyme to be active as well as to resist proteolysis. It is able to hydrolyze all three esters into non-esterified fatty acids and glycerol and is required for hydrolysis of long-chain

triacylglycerides. BSSL does not have positional specificity and it hydrolyzes emulsified and micellar and water-soluble substrates (Blãckberg and Hernell 1981).

Pancreatic lipase converts dietary triacylglycerides to products that can be absorbed into the intestinal wall in the form of micelles (Jensen and others 1982). As the name implies, it originates in the pancreas. Its site of action is the duodenum and its hydrolysis of milk fat is facilitated by BSSL. In preterm and term infants, pancreas and liver function are not optimal and there is a reduced availability of pancreatic lipase and bile salts, which could curb the digestion and absorption of dietary fat in the intestine (Jensen and others 1982). Milk fat globules are resistant to pancreatic lipase (Cohen and others 1971), thus making it difficult to digest and absorb fat if the fat globules have not been exposed to lingual lipase and BSSL.

Human milk contains LPL, which is probably the only lipase found in milk of most species (Atkinson and Lonnerdal 1989). LPL is produced by the milk-producing cells in the mammary gland but functions at the plasma membrane of endothelial cells. LPL synthesis is under hormonal control and its activity in the mammary gland increases at parturition. The enzyme diverts blood triacylglycerols to the gland so they can be used for production of milk fat (Hamosh and others 1970).

1.9.6.1. Bacterial Lipases

Bacterial lipases are extracellular and produced during the late log and early stationary phases of growth (Chen and others 2003; Jaeger and others 1994). Many bacteria can produce more than one type of extra-cellular lipases that hydrolyze different chain length

fatty acids (Chen and others 2003). Unlike LPL, bacterial lipases are able to hydrolyze intact milk fat globules, but the mechanism is not known (Chen and others 2003).

1.10. Bacteria in Human Breast Milk

Studies performed under temperate climate conditions have shown that breast milk stored for 48-72 hours in the refrigerator is bacteriologically acceptable (Bjorksten and others 1980; Deodhar and Joshi 1991). The level of bacterial colony counts in breast milk usually declines during refrigeration or freezing at -20°C followed by thawing (Deodhar and Joshi 1991; Hernandez and others 1979).

Studies have shown that the ability of colostrum samples stored in the refrigerator at 4°C to bind pathogenic bacteria in-vitro was enhanced within the first three days of storage, in comparison to freshly expressed milk or frozen milk. The initial increase in levels of sequestered bacteria was followed by a progressive fall, gradually approaching the level cultured from the normal saline control after three weeks (Ogundele 2000).

Other studies have also shown that storing breast milk at refrigeration and freezing temperatures is associated with enhanced bacteriostatic activities and reduced bacterial load (Hernandez and others 1979; Reynolds and others 1982), and that freezing milk for one day destroys cytomegalovirus (Cheeseman and McGraw 1983). Martinez-Costa and others (2007) stored nine samples of mature human milk at refrigeration temperatures for 48 hours and 72 hours and then analyzed the bactericidal activity of the milk. Fresh samples exhibited a bactericidal activity with an average value of 83.5% ± 18.4% of the total bactericidal

activity. Refrigeration for 48 hours did not cause a significant change in bactericidal activity, but storage for 72 hours significantly lowered bactericidal activity to an average value of 9.67% (Martinez-Costa and others 2007).

1.10.1. Safety and Quality of Human Breast Milk

Freshly collected breast milk is usually not sterile. The bacteria originate from the maternal skin and nipple duct micro-flora, but it may contain potential pathogens (Deodhar and Joshi 1991; Olowe and others 1987). The ability of these bacteria to infect the infant is not known, but probably is minimal (Bjorksten and others 1980). However, some of the pathogens could produce lipases, proteases, and decarboxylases that could damage anti-microbial proteins or convert free amino acids into toxic amines (Bjorksten and others 1980).

El-Mohandes and others (1993) collected 108 milk samples from 40 women and tested them for bacterial contamination. Only 12.5% of the samples showed no bacterial growth. Of the contaminated samples, 38% contained more than 30,000 colony-forming units/ml (CFU/ml). The most common organisms found in the samples include *Staphylococcus epidermidis* (82%) and *Acinetobacter* (9%). Table 1.10 shows the other contaminants found in the milk samples (el-Mohandes and others 1993).

Table 1.10 Prevalence of Bacterial Isolates of Frozen Human Breast Milk (el-Mohandes and others 1993)

Organism	Prevalence by Mother	Prevalence by Milk Specimen	Range (CFU/ml)	Mean ± SEM
Staphylococcus epidermidis	38/40	82/108	0-3 x 10^5	32 ± 3.5 x 10^3
Other coagulase-negative staphylococci	9/40	11/108	0-5 x 10^4	1.6 ± 0.8 x 10^3
Staphylococcus aureus	5/40	7/108	0-8 x 10^4	2 ± 1 x 10^3
Enterococcus	5/40	5/108	0-15 x 10^3	0.8 ± 0.6 x 10^3
Acinetobacter species	7/40	8/108	0-10^6	17 ± 13 x 10^3
Klebsiella species	4/40	4/108	0-3 x 10^4	0.5 ± 0.4 x 10^3
Proteus species	1/40	4/108	0-1 x 10^3	0.5 ± 0.3 x 10^2
Diphtheroids	5/40	5/108	0-1 x 10^5	2 ± 1.5 x 10^3

Staphylococcus epidermidis is part of the normal skin microbial flora and has been shown to be safe (Law and others 1989). The organisms most commonly isolated, including gram-positive, coagulase-negative staphylococci and gram-negative *Acinetobacter* are also known skin colonizers (Botsford and others 1986). Despite the growing need for milk storage, especially in the neonatal intensive care units, there are no agreed upon guidelines regarding the acceptable microbiological quality of fresh and frozen human milk (el-Mohandes and others 1993). Recommendations on raw milk have ranged from bacterial counts of 10^3 CFU/ml with no enteropathogens (Sauve and others 1984) to 10^5 CFU/ml excluding pathogens such as *Staphylococcus aureus*, group B streptococci, pneumococci, and coliforms (Tyson and others 1982).

1.10.2. Milk Safety in the Milk Bank and Hospital Settings

Safe limits for bacterial concentration in human milk used to feed premature infants have never been established (Jones and others 2000; Law and others 1989). Many donor milk programs have adopted arbitrary limits based on those used by the dairy industry for bovine milk (Botsford and others 1986; Carroll and others 1979; Ryder and others 1977). Unfortunately, breast milk may be a source of infection of *Salmonella kottbus*, *Serratia marcescens*, and Klebsiella in neonatal intensive care units, as reported by studies from the 1970s and 1980s (Donowitz and others 1981; Gransden and others 1986; Ryder and others 1977).

Law and others (1989) determined the frequency at which infants fed raw human milk were exposed to milk-associated bacteria and compared the bacterial counts of donor and maternal milk. The purpose of the study was to assess the effectiveness of the donor milk screening program in the milk bank at the Health Services Centre in Winnipeg, Manitoba, Canada. Human milk fed to 98 premature infants was cultured for bacterial growth. Samples of each feeding (0.5 – 1.0 ml human milk) were set aside in sterile labeled containers while the infants were feeding. Throat and rectal swabs were obtained from all infants before and several times during the monitored feedings. Within infant participants, 100% were exposed to coagulase-negative staphylococci, 41% were exposed to *S. aureus*, and 64% were exposed to gram-negative bacilli. Within all 10,128 milk samples, there was no detectable bacterial growth in 19.1%, only gram-positive bacteria in 74.3%, only gram-negative bacteria in 1.1%, and mixed gram-negative and gram-positive species in 5.7%. There were not adverse events

due to ingestion of milk-associated bacteria. Donor milk was significantly less likely to contain gram-negative staphylococci, and there was no difference between either source of milk in terms of *S. aureus* and gram-negative bacterial count. Based on the results of this study, the screening program was discontinued because there were no adverse events associated with consumption of bacteria in raw breast milk (Law and others 1989).

The Hazard Analysis Critical Control Point (HACCP) protocol is used regarding the use of expressed milk in a hospital setting, including donor milk or the infant's own mother's milk. The protocol was created by Hunter in 1991(Hunter 1991) and reviewed by Tully in 2006 in the following table (Jones and Tully 2006).

Table 1.11 Summary of HACCP for Handling Expressed Human Milk in the Hospital Setting (Hunter 1991)

Critical control points	*Potential hazards*	*Concern level*	*Proposed control options/ patient staff education*
Milk Collection	Human milk is not sterile. Pathogenic microbes may be present in expressed human milk.	High	Correct labeling and double verification before feeding
	Hands touch many surfaces and are likely to touch the breast and the milk during expression or pumping.	Medium	Careful hand washing by mother and staff before expressing or handling milk
	Pump exterior may be contaminated with pathogens.	High	Cleaning pumps on a regular basis; responsibility designated for pump cleansing; education for patients on the importance of using clean equipment

Table 1.11 Continued

Milk transfer to unit	Transfer of expressed milk to a hospital unit from home or from another unit in the hospital	Low (from another unit) Medium (from home)	Educate parents to refrigerate expressed milk and to refrigerate or freeze as soon as possible. Provide information in the mother's language regarding appropriate storage conditions for transporting milk Educate staff about appropriate handling of expressed milk
Storage in unit	Storage of milk in refrigerator may exceed 48 - 72 hours	Low	Mark each bottle with date and time of expression. Freeze milk that has not been used. Rarely should milk be discarded.
	Defrosted milk is not used within 24 hours of thawing	Low	Hospital labels to indicate when milk is defrosted.
	Temperature of refrigerator is too high	Low	Install refrigerator thermometer; monitor temperature daily.
	Samples or other products in freezer/ refrigerator may contaminate one another	Low	Maintain a freezer and refrigerator exclusively for human milk.
	Milk can be removed by unauthorized person	Low	Storage of milk is in a secure, labeled refrigerator/ freezer.

Table 1.11 Continued

Milk preparation	Bacterial growth may occur if milk is left at room temperature for long periods	Low	Individual feedings should be prepared. Aliquot milk into sterile, capped syringe or other clean container labeled with baby's name and patient number and store in refrigerator before use. Any milk from a partially consumed feeding should be discarded
Feeding	Expressed milk may be given to the wrong infant. There is a loss of milk to the infant for whom it was intended. There is also stress for both families caused by concern for potential disease transmission.	High	Milk must be clearly labeled Before feeding, milk labels and infant's ID bracelet should be checked by two individuals.

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CHAPTER 2

The Effect of Storage Time and Temperature on Total Protein, Proteolysis, Secretory Immunoglobulin A Activity, Lysozyme, Total Lipid, Non-esterified Fatty Acids, and Bacteria in Freshly Expressed Human Breast Milk

2.1. Abstract

Breastfeeding is the optimal nutrition for infants and is recommended for the first year of life by the American Academy of Pediatrics (American Academy of Pediatrics 1997). However, mothers who return to work within this year cannot always breastfeed on demand and often pump their milk and send it with the baby to a day care setting. Infants in the neonatal intensive care unit rely heavily on donor breast milk or their mothers' own breast milk, all of which is expressed, stored, transported to the hospital, and stored again. There are few guidelines on how long this milk can be kept and the optimum and tolerable storage temperatures for human milk. Guidelines that are sometimes used are based on very few scientific studies of the impact of storage on milk quality and stability. There is a need for evidence-based standard protocols for milk handling and storage in hospitals and milk banks as well as a need for evidence-based recommendations for safely handling milk at home and in child care facilities.

The objective of this study was to investigate the effects of storage temperature and time on components in freshly expressed, human breast milk. Eleven breast milk samples were collected from twelve women who volunteered with informed consent. The samples were stored either at -20°C, 4°C, and 24°C. All samples were stored for three weeks and

analyzed for total protein and free amino ends, total fat and non-esterified fatty acid (NEFA), bacterial growth, secretory immunoglobulin A activity, and lysozyme activity. Samples stored at -20°C only showed a significant increase in NEFA ($p < 0.0001$) and the increase was significant at 2 weeks of storage ($p < 0.05$).

During three weeks of storage, samples stored at 4°C showed a significant increase in NEFA ($p < 0.0001$), free amino ends ($p < 0.0001$), and bacterial growth ($p < 0.01$). The increase of NEFA was significant at 48 hours ($p < 0.001$), the increase in free amino ends was significant at 1 week ($p < 0.05$), and the increase in bacterial growth was significant at 2 weeks ($p < 0.05$).

During three weeks of storage, samples stored at 24°C showed a significant increase in NEFA ($p < 0.0001$), free amino ends ($p < 0.0001$), bacterial growth ($p < 0.0001$), and a decrease in total protein concentration ($p < 0.0001$). All samples showed a rapid increase in NEFA ($p < 0.0001$). Total fat, lysozyme, and SIgA remained stable throughout the storage conditions. The decrease in protein was significant at 1 week ($p < 0.01$). The increase of NEFA was significant at 24 hours ($p < 0.001$), the increase in free amino ends was significant at 1 week ($p < 0.001$), and the increase in bacterial growth was significant at 48 hours ($p < 0.01$).

Based on the data, it appears that breast milk may be stored in the freezer at -20°C for longer than three weeks, in the refrigerator at 4°C for up to 2 weeks, and at room temperature (24°C) for up to 48 hours. However, the impacts of increased NEFA are controversial and must be taken into consideration when storing breast milk.

2.2. Introduction

Breastfeeding is the optimal nutrition for infants and is recommended for the first year of life by the American Academy of Pediatrics. However, mothers who return to work within this year cannot always breastfeed on demand and often pump their milk and send it with the baby to a day care setting. There are few guidelines on how long this milk can be kept and the optimum and tolerable storage temperatures for human milk. Guidelines that are sometimes used are based on very few scientific studies of the impact of storage on milk quality and safety. There is a need for evidence-based standard protocols for milk handling and storage in hospitals and milk banks as well as a need for evidence-based recommendations for safely handling milk at home and in child care facilities.

The nutritional value of protein in human milk is appreciated for many reasons, including its caloric content, the constituent amino acids used for growth and development, and the immunological compounds. The major proteins in milk are casein and whey. Casein is a source of biologically active peptides (Clare and Swaisgood 2000; Meisel and Bockelmann 1999; Schanbacher and others 1997). When β-casein is digested, it may aid in calcium absorption by keeping calcium soluble, leading to the high bioavailability of calcium in human milk (Sato and others 1986). The carbohydrate component of κ-casein may prevent the adhesion of *Helicobacter pylori* to the infant's gastric mucosa (Stromqvist and others 1995). The whey protein, α-lactalbumin has a high nutritional value for the infant and aids in lactose synthesis.

Immunological components include lysozyme and SIgA. Lysozyme is an enzyme found in many secretions, such as human milk, saliva, tears, nasal mucus, and pancreatic juice (Fleming 1922). It is capable of breaking down the outer cell wall of gram-positive bacteria and some gram-negative bacteria by hydrolyzing the β1,4 linkages of N-acetylmuramic acid and 2-acetylamino-2-deoxy-D-glucose residues (Chipman and Sharon 1969). Immunoglobulins are beneficial because they provide protection to the infant's immature immune system. Immunoglobulins, or antibodies, that are present in human milk include immunoglobulins A, G, M, D and E, with SIgA being the most abundant (Newman 1995). SIgA is able to endure in the hostile environment of the gastrointestinal tract because it is more resistant to degradation from proteases than other immunoglobulins (Atkinson and Lonnerdal 1989). SIgA antibodies against bacterial IgA proteases have been found, including proteases produced by *H. influenzae, Streptococcus sanguis, S. pneumoniae, Neisseria gonorrhoeae, Proteus mirabilis, E. coli, and Neisseria meningitidis* (Gilbert and others 1983).

Lipids in human milk provide 40-50% of the infant's calorie requirements and they are also integral constituents of neural and retinal tissues (Manson and Weaver 1997). Agostoni and others (2001) found that breast-feeding for 6 months or longer with milk of high fat content was associated with higher developmental scores at one year of age. The researchers hypothesized that the supplies of fat contribute energy and/or affect brain composition (Agostoni and others 2001). Human milk provides linoleic acid (18:2 n-6, LA),

α-linolenic acid (18:3 n-3, ALA), docosahexaenoic acid (22:6 n-3, DHA), arachidonic acid (20:4 n-6, AA), and other long chain polyunsaturated fatty acids (LCPUFA).

NEFA produced during storage of human milk have been shown to have potent cytolytic effects on normal human blood cells, on intestinal parasites *Giardia lamblia* and *Entamoeba histolytica*, as well as gram-positive bacteria and yeast (Ogundele 2000). NEFA have also caused membrane disruption of enveloped viruses in cultured cells (Thormar and others 1987).

Freshly collected breast milk is usually not sterile. The bacteria originate from the maternal skin and nipple duct micro-flora, but it may contain potential pathogens (Deodhar and Joshi 1991; Olowe and others 1987). The ability of these bacteria to infect the infant is not known, but probably minimal (Bjorksten and others 1980). However, some of the organisms could produce lipases, proteases, and decarboxylases that could damage anti-microbial proteins or convert free amino acids into toxic amines (Bjorksten and others 1980). Safe limits for bacterial concentration in human milk used to feed premature infants have never been established (Jones and others 2000; Law and others 1989), but many donor milk programs have adopted arbitrary limits based on those used by the dairy industry for bovine milk (Botsford and others 1986; Carroll and others 1979; Ryder and others 1977).

Our project addresses a concern for premature as well as healthy babies. The issue of breast milk storage is a common topic regularly encountered by clinicians that has received sporadic research attention over the past several decades.

The concern for premature and healthy babies presents a need for evidence-based standard protocols for milk handling and storage in hospitals and milk banks as well as a need for evidence based recommendations for safely handling milk at home and in child care facilities. The research will provide current evidence on which to base recommendations for the proper storage time and temperatures of milk.

The objectives of this study were to determine the effect of storage temperature and time on components in breast milk including total protein, free amino groups, NEFA, lysozyme, SIgA activity, and bacterial growth.

2.3. Materials and Methods

2.3.1. Collection and Preparation of Human Breast Milk Samples

The study was approved by the North Carolina State University Institutional Review Board. Potential participants were recruited by e-mail and telephone. Twelve lactating mothers between the ages of 25 and 40 volunteered with informed consent.

Before the study began, the participants were mailed three individually wrapped, sterile containers as well as specific instructions regarding participation. A specific meeting time and place for breast milk collection was prearranged with the researcher and each participant. On the day of breast milk collection, each participant pumped the entire contents of one or both breasts, as she normally would. The participants transferred the breast milk into the sterile containers that were mailed to them, and placed the milk in a refrigerator for storage until pick-up.

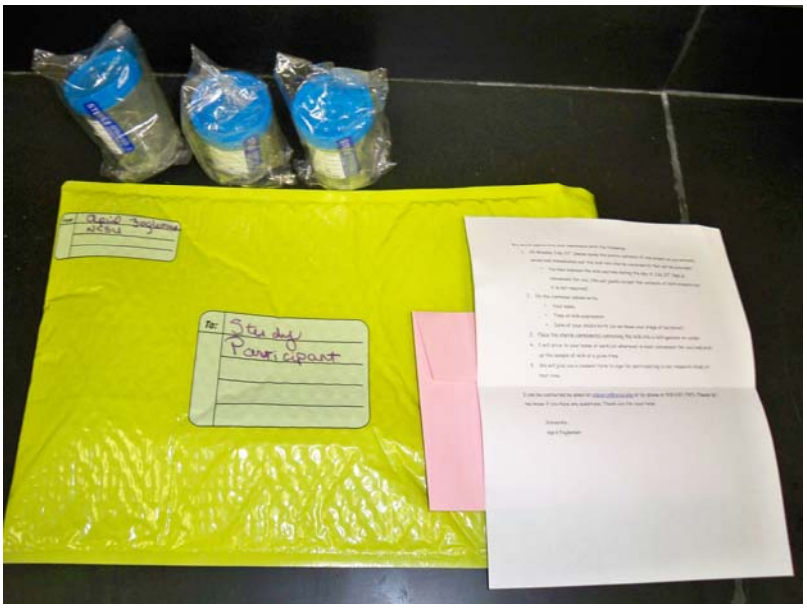


Figure 2.1 Packages mailed to participants before the study began. Contents include sterile containers and specific study instructions.

When milk was collected from each woman, the sterile containers of breast milk were placed individually into a Ziploc bag and then in a cooler of ice for transportation to the laboratory.

Upon arrival to the laboratory, breast milk samples were aliquotted into sterile, cryogenic vials. The vials were pre-labeled with the following information:

- Sample number (corresponding to one participant)
- Experiment
- Storage time and temperature

Figure 2.2 Labeled vials for breast milk storage in appropriate environments

Each color on the side of the vials represented storage temperature. Light purple, aquamarine, and maroon represented -20°C, 4°C, and 24°C respectively. Each color on the top of the containers represented the storage time. Dark blue, maroon, orange, dark purple, green, and bright blue represented 0 hours, 24 hours, 48 hours, 1 week, 2 weeks, and 3 weeks, respectively.

Samples were placed into boxes, such as the box shown in figure 2.2, and placed into the appropriate storage environment. The temperature of the environment was labeled on the box. At the appropriate time points, samples were removed from the storage environments

and placed into a -80°C freezer to stop changes in the breast milk until biochemical analyses were performed.

2.3.2. Storage Environments of Human Breast Milk Samples

Milk was stored at the following temperatures: -20°C, 4°C, and 24°C, which is the freezer, refrigerator, and room-temperature, respectively.

Twelve women participated in the study, but each woman was able to pump different amounts of milk. Therefore, milk was pooled so that there was enough milk to perform each biochemical analysis, resulting in 11 samples total. Milk was only pooled from mothers in the same stage of lactation. There was not enough milk from each woman to be able to store each sample at every temperature and time point. As a result, each sample was stored at one temperature for all of the time points.

Samples 1 – 4 were stored at -20°C for three weeks, samples 5 – 8 were stored at 4°C for three weeks, and samples 9 – 11 were stored at 24°C for three weeks.

2.3.3. Biochemical Assays

2.3.3.1. Total Protein

The BCA protein assay (BCA Protein Assay KitTM #23227, Pierce Biotechnology Inc., Rockford, IL) is based on the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium, using bicinchoninic acid for the colorimetric detection and quantification of total protein present in a sample (Smith and others 1985). Peptide bonds in protein reduce Cu^{+2}

(from cupric sulfate pentahydrate included in the reagent) to Cu^{+1}. The amount of Cu^{+2} reduced to Cu^{+1} is proportional to the amount of protein in solution. Two molecules of bicinchoninic acid chelate with each Cu^{+1} ion and form a purple complex that absorbs light at 562 nm. The amount of protein in solution is then quantified by measuring the absorbance at 562 nm and comparing it with absorbencies of solutions containing known concentrations of protein.

The assay was performed after storage at the appropriate time and temperature. A 2600 Gilford spectrophotometer (Oberlin, OH) was used to measure absorbance at 562 nm. A standard curve was created by plotting standard bovine serum albumin absorbance at 562 nm against a known concentration (mg/ml). The standard curve was used to determine the protein concentration of each unknown sample.

The standard curve samples are as follows:

	Water	Standard	Concentration (mg/ml)
1	100 µL	0 µL	0
2	75 µL	25 µL	0.5
3	50 µL	50 µL	1.0
4	25 µL	75 µL	1.5
5	0 µL	100 µL	2.0

First, 50 ml reagent A was mixed with 1 ml reagent B to make the working reagent. Human milk samples were diluted 1:10 so that the absorbencies would fall within the range of the standard curve. Next, 100 µl of each sample was pipetted into a test tube. Working reagent (2 ml) was added to each test tube and mixed. The samples were incubated at 37°C

for 30 minutes in a shaking water bath. After the 30 minute incubation period, they were cooled in an ice bath. The absorbance of each sample was measured at 562 nm in triplicate.

The equation resulting from the standard curve was used to determine the protein concentration of each sample. The equation is as follows:

Protein concentration = [(absorbance – intercept) / (slope)] x (DF)

Absorbance: average of the absorbencies of each sample
Intercept: y-intercept from the standard curve graph
Slope: slope from the standard curve graph
DF: dilution factor used to dilute human milk samples (dilution factor used was 10)

2.3.3.2. Proteolysis

The concentration of free amino ends was measured to determine the extent of proteolysis by using o-phthaldialdehyde (OPA) (Church and others 1983). When hydrolysis of proteins occurs, α-amino groups are released and react with OPA and β-mercaptoethanol to form an adduct that absorbs at 340 nm. The absorptivity is 6,000 M^{-1} and is similar for all α-amino groups.

The o-phthaldialdehyde reagent (Pierce #26025) includes: 100 mM sodium tetraborate, 20% (w/w) sodium dodecyl sulfate, OPA (dissolved in 1 ml methanol), and 2-mercaptoethanol. Sodium dodecyl sulfate terminates proteolysis and insures full exposure and complete reaction of amino groups.

The OPA reagent was stored in the refrigerator, but a given amount was warmed to room temperature before use. Cold reagent will cause the crystal cuvette to develop condensation on the outside and lead to an inconsistent and inaccurate absorption reading.

The milk samples were diluted with deionized water 1:5 so that the range of absorption was between 0.1 and 1 Au. The spectrophotometer was set to 340 nm and zeroed with 2 ml of the OPA reagent in a 3-ml quartz cuvette. Each diluted human milk sample (25 µl) was added individually to 2 ml of the OPA reagent. The mixture in the cuvette was briefly mixed by inversion. The absorption at 340 nm was recorded after 2 minutes. Each milk sample was analyzed in triplicate.

The following equation was used to determine the number of amino ends in each sample:

$$\textit{Amino ends (µM)} = [(\textit{Abs} / 6{,}000\ M^{-1}) \times (1{,}000{,}000)] / [(\textit{Protein concentration (g/L)}) \times (0.025)]$$

Abs: absorption at 340 nm
6,000 M^{-1}: molar protein concentration
Protein concentration: protein concentration (mg/ml)
0.025: 25 µl (0.025 ml) of diluted sample was used

2.3.3.3. Preparation of the *Escherichia coli* Somatic O Antigens

The procedure for preparation of the *Escherichia coli (E. coli)* somatic O antigens was modified from Chen (2000) and Viazis and others (2008).

Dry pellets of *E. coli* serotypes: O1, O2, O4, O6, O7, O8, and O18 were provided by Dr. Thomas Whittam from the National Food Safety and Toxicology Center at Michigan State University.

Table 2.1 E. coli Serotypes used in Preparation of the E. coli Somatic O Antigen for ELISA

Accession #	**Strain Name**	**O**	**Class**	**Host**
TW01064	840691	1	UPEC	Cat
TW01906	820846	2	UPEC	Human
TW01045	840383	4	UPEC	Dog
TW01768	830080	6	UPEC	Human
TW03295	ECOR-40	7	UPEC	Human
TW01734	830426	8	UPEC	Human
TW01916	820877	18	UPEC	Human

Once received, the bacterial strains were refrozen in propylene glycol and stored for future use. Before use, they were rehydrated. They were grown individually in tryptic soy broth at 37°C until each culture reached an OD600 reading of 1.0.

The *E. col*i were pooled to a concentration of 10^8 to 10^9 cells/ml. The 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 PBS. The wash procedure was repeated one additional time.

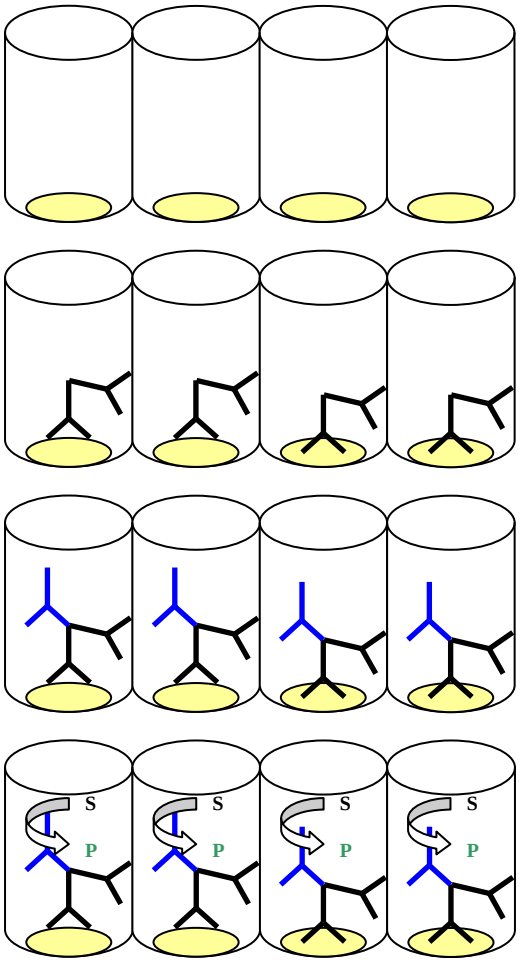
The bacterial concentration was adjusted to $5x10^9$ cells/ml. The *E. coli* suspension was then transferred to a steam bath, heated for 2 hours, and then cooled for 10 – 15 minutes in an ice bath.

The heat-killed bacterial debris was centrifuged at 3000 rpm for 30 minutes at 4°C. The supernatant was saved (~$5x10^{10}$ *E. coli*/ml) for the somatic O antigens for ELISA. The *E. coli* antigen was diluted 1:50 before being used to coat the plates for ELISA.

2.3.3.4. SIgA Activity

A kinetic indirect enzyme linked immunosorbent assay (ELISA) was used to measure the activity of SIgA, as previously modified by Chen (1998) and Viazis (2005). Antigen was added to the wells of a microtiter plate and incubated overnight to allow the antigen to attach to the plate. After rinsing to remove excess, unbound antigen, the milk samples were added that contain antibodies (SIgA) to the antigen (*E. coli* antigen). After the antibodies attach to the antigen, excess antibodies are rinsed out. Horseradish peroxidase (HRP)-labeled anti-human IgA conjugate was added to bind with the constant region of antibodies. HRP reacts with the colorless substrate that is added in the last step, causing it to develop color that can absorb light at 405 nm. The activity of SIgA was determined by measuring the absorbance of the wells at time 0 and every 2 minutes for 20 minutes. The rate of increase in absorbance

(slope) was calculated for each well by linear regression and used to determine concentration in the unknown samples in comparison with a standard curve of known SIgA concentration. Figure 2.3 illustrates the indirect enzyme-linked immunosorbent assay.



E. coli antigen

SIgA

Horseradish peroxidase (HRP)-labeled anti-human IgA conjugate

Substrate is converted to product and produces a color that absorbs light at 405nm

Figure 2.3 Indirect Enzyme-linked Immunosorbent Assay

Appropriate plate designations were predetermined for each sample before the experiment began, including five standards and four blanks analyzed in duplicate. Each human milk sample was diluted 1:100 and analyzed in triplicate. Known quantities of purified human colostrum IgA (Sigma #I-2636) were used as standards in the concentrations of 0.076, 0.12, 0.23, 0.46, 0.92 μg/100 μL.

The diluted *E. coli* antigen (200 μl) was added to the appropriate wells of the assay plate and incubated for 12 to 18 hours at room temperature. Next, 200 μL of the standards and diluted human milk samples were added to the appropriate wells and incubated for 3 hours at room temperature. Horseradish peroxidase (HRP)-labeled anti-human IgA (alpha-chain specific) conjugate (Sigma #A-0295) was diluted 1:5000 with PBST, and 100 μl was then added to the designated wells and incubated for 1 hour at room temperature.

Between each of the above steps, the plate was washed 3 times with PBS with 0.05% Tween-20 (PBST) in order to remove unbound molecules. Before incubation, the wells were covered with parafilm.

After incubation for 1 hour with HRP-labeled antihuman IgA, 100 μL of the substrate solution was added to the wells. The substrate solution consisted of 20 ml 0.05 M citrate buffer, 0.1 ml 3% hydrogen peroxide, and 0.5 ml 40 mM 2,2'-azinodi-3-methylbenzothiazoline-6-sulfonic acid (ABTS). The plate was inserted into the plate reader for absorbance readings at time 0 and every 2 minutes for 20 minutes at 405 nm using a Spectronic Genesys 2 spectrophotometer (Thermo Fisher Scientific).

Absorbance was plotted versus time to create a best-fit line for each well. The average slope for each absorbance vs. time curve was determined for each sample, blank, and standard. The immunoactivity of SIgA corresponded to the average slopes formed by plotting absorbance at 405 nm versus time.

2.3.3.5. Lysozyme

Lysozyme activity was determined using a *Micrococcus lysodeikticus* based turbidimetric assay (Worthington Biochemical Corporation 1972). The activity of lysozyme was measured by continuous readings of the change in turbidity of the bacterial suspension at 450 nm (Shugar, 1952), using a Spectronic Genesys 2 spectrophotometer (Thermo Fisher Scientific). A 0.015% (w/v) *Micrococcus lysodeikticus* cell suspension substrate was prepared by using 66 mM potassium phosphate buffer to mix with *Micrococcus lysodeikticus* ATCC 4698 lyophilized cells (Sigma #M-3770). The 66 mM potassium phosphate buffer was prepared and the pH adjusted to 7.2. The A450 nm of this suspension was always between 0.6 and 0.7. The A450 nm of the substrate suspension will decrease over time. One liter of the substrate solution was prepared at one time and the entire experiment was completed within one week. The substrate solution was refrigerated between uses, but allowed to warm to room temperature before use to prevent condensation from forming on the cuvette.

A standard lysozyme enzyme solution was prepared using the potassium phosphate buffer, pH 7.2, to produce solutions containing 200-600 units/ml. The spectrophotometer was

set to 450 nm, and zeroed using a cuvette with 2.6 ml of 66 mM potassium phosphate buffer. The substrate solution consisting of reconstituted *Micrococcus lysodeikticus* ATCC 4698 cells (2.5 ml) was mixed with 0.10 ml of buffer and the A450 nm was adjusted to fall within 0.6 and 0.7 absorbance units.

Each human milk sample was diluted 1:100. Each diluted sample (0.10 ml) was added to 2.5 ml of the substrate solution and measured every 15 seconds for 2 minutes at 450 nm in duplicate.

Units of lysozyme per volume of enzyme in ml were calculated using the following formula:

$$\text{units/ml enzyme} = (\Delta A450nm) \times (DF) / (0.001) \times (0.1)$$

DF: Dilution factor (100)
0.001: Change in absorbance at 450 nm as per the unit definition
0.1: volume (ml) of human milk used

The units of lysozyme per unit mass protein (specific activity) within human milk was determined as:

$$\text{units/mg protein} = (\text{units / ml enzyme}) / (\text{mg protein / ml enzyme})$$

2.3.3.6. Validation of a Breast Milk Method for the SMART Trac Analyzer (CEM)

Technical representatives from CEM (Matthews, NC) provided assistance with the fat analysis of the breast milk samples using the SMART Trac analyzer. The analyzer employs an AAOC approved method for analyzing the fat in bovine milk. Although the analyzer is

AAOC approved to analyze fat content in bovine milk, it had not been tested to analyze fat content in breast milk.

The fat content in breast milk is comparable to the fat content in bovine milk at approximately 4% fat. In order to determine the reliability of the SMART Trac analyzer to measure the fat content in breast milk, a CEM technician determined the fat content in 6 breast milk samples using the mojonnier fat test, which is an AOAC approved method for fat analysis. The fat content as measured by the mojonnier fat test was also measured in the same breast milk samples using the SMART Trac analyzer. The values were compared and the SMART Trac analyzer was found to be reliable in measuring the fat content in breast milk.

2.3.3.7. Total Fat Measurement using the SMART Trac Analyzer

Total fat in the breast milk samples was measured using the SMART Trac Rapid Moisture/Fat Analyzer (CEM corporation, Matthews, NC) in the NC State dairy plant (Leffler and others 2008). The SMART Trac analyzes the fat content in milk, cheese, ice cream, sour cream, yogurt, butter, margarine, de-boned poultry, beef, condiments, cookies, crackers, snack foods, as well as other foods.

Nuclear magnetic resonance (NMR) is a non-destructive method that does not require the use of solvents, as the Folch method for fat analysis relies on the use of chloroform and methanol (Folch and others 1957). NMR directly measures fat content using a signal-to-mass ratio. NMR has not been used to analyze liquid samples in the past because water protons

interfere with the measurement of fat protons. The SMART Trac combines microwave drying with NMR, to overcome this problem. The sample is dried to remove any hydrogen bound in the sample as water. The NMR sends a pulse of radio-frequency energy through the sample, which causes the remaining hydrogen to generate a signal, known as Free Induction Decay (FID). The intensity of the FID can then be analyzed to determine the amount of fat protons present in the sample. Fat protons decay more slowly than protein and carbohydrates, thus they can be directly measured.

The following pictures illustrate how the SMART Trac analyzer should be operated.

Illustration I

If the sample is in a paste, semi-solid, or crumb form or a raw or skeletal meat product such as fresh pork, ground beef, or chicken, place the sample on the end of a spatula and spread it across one end of the pad. Then spread the sample to a uniform thickness covering approximately 90% of the pad surface area.

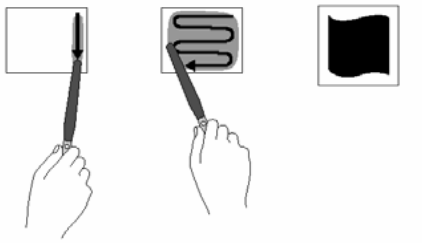


Illustration II

For samples containing bound water such as an all-meat emulsion, cooked all-meat sausage, sausage with extenders, semi-dry sausage, or ham, place the sample on the end of the spatula and apply the sample to the middle of the pad. Then spread the sample around the pad in a circle.

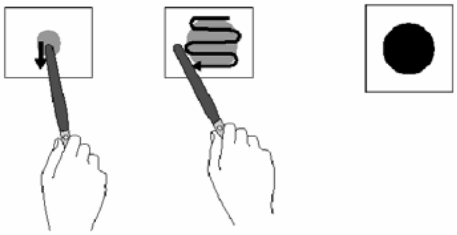


Illustration III

Place the 2 square pads and dried sample in the center of the Trac film. Fold the left corner of the film and pads as illustrated. Fold the right corner. Pull the lower edge of the film and sample pads toward the top and begin to roll them into a tube.

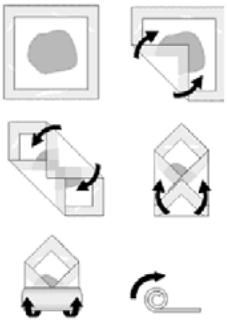


Illustration IV

For samples that are rigid after being dried and more difficult to roll into a cylinder, prepare the pads as illustrated.

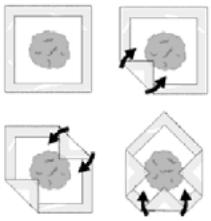


Figure 2.4 Directions for using the SMART Trac Analyzer (Leffler and others 2008)

Figure 2.5 SMART Trac Analyzer (CEM Corporation)

2.3.3.8. Measurement of Non-esterified Fatty Acids

Microtitration was used to determine the amount of NEFA within the breast milk samples. The method was originally used to determine the amount of NEFA in lipid extracted from plasma (Dole 1956).

The Folch technique is commonly used to extract lipid from tissue samples (Folch and others 1957). A modified Folch technique was used to extract the lipid from the breast milk in this study by using an extraction mixture consisting of 40 parts isopropyl alcohol, 10 parts heptane, and 1 part 1 N H_2SO_4. The extraction mixture will cause the lipid to be on the top layer of the sample rather than the bottom layer, as is the case with the Folch method. Having the lipid on the top layer allows for easy removal of the lipid layer. The extraction mixture (15 ml) was added to 3 ml of each breast milk sample in a glass-stoppered tube and shaken vigorously twenty times. After standing 10 minutes or longer, the system was divided into two phases by mixing into it an additional 6 ml of heptane and 9 ml of water. The phases separated rapidly without centrifugation and formed a sharp interface. The top layer was

removed using a 10 ml serological pipette. The contents of the pipette were emptied into three test tubes, resulting in 3 ml of the top layer being transferred into each test tube.

The titration mixture (1 ml) was added to each test tube containing the extracted lipid. The titration mixture consisted of 0.01% thymol blue and 90% ethanol in water, made by dilution of a stock 0.1% thymol blue in water with 9 parts redistilled ethanol. The alkali used for the titration consisted of about 0.020 N NaOH (Fisher # 50-440-0364).

Once a 3 ml aliquot of the upper (lipid) phase was transferred to a test tube containing 1 ml of titration mixture, it was then titrated with the alkali using a pipette. Nitrogen was delivered to the bottom of the tube with a fine glass capillary in order to expel carbon dioxide from the sample. It also keeps the two phases mixed during titration.

As the green-yellow end point was approached, the gas stream was interrupted from time to time for examination of the indicator color in the alcoholic phase. Good lighting was provided by a fluorescent light placed above and in front of the tube.

The percentage of NEFA was calculated using the following formula:

% non-esterified fatty acids = (ml titrant)(N titrant)(266.16) / (ml sample) x 10

ml titrant: ml NaOH used during titration
N titrant: normality titrant (0.02N)
266.16: average (weighted) molecular weight of NEFA in breast milk
ml sample: ml sample used

2.3.3.9. Bacterial Analysis

Breast milk samples stored at -20°C, 4°C, and 24°C were analyzed immediately and without prior freezing at baseline and at each time point for bacterial growth using Petrifilm (3M company, St. Paul, MN). Each sample was analyzed in duplicate, with three dilutions each, resulting in six Petrifilm being used for each sample. Preparing and plating three dilutions for each sample ensured that the amount of bacteria grown on each Petrifilm was between 25 and 250 colony forming units/ml (CFU/ml). Dilutions were adjusted over the course of the study.

Peptone water was prepared by weighing 1 g of peptone (Sigma #77185) and adding it to 1 L of deionized water. Subsequent dilutions were made by adding 1 ml of the breast milk sample into 9 ml of peptone water, followed by a subsequent 1 ml transfer into another test tube containing 9 ml of peptone water, resulting in a 10^{-1} dilution. The following picture diagrams how dilutions were prepared:

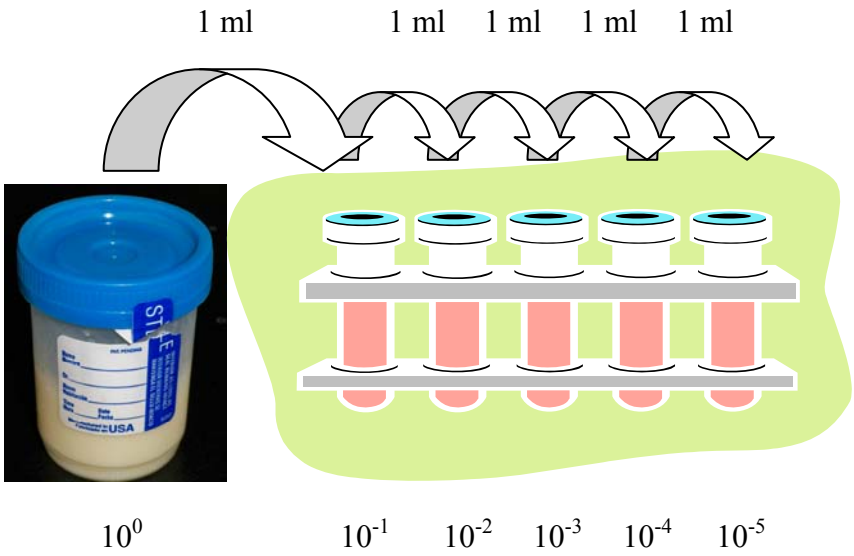


Figure 2.6 Subsequent dilutions

The appropriate dilutions were plated onto Petrifilm, placed in a 37°C incubator, and incubated for 48 hours. After 48 hours of incubation, the colonies were counted. Marking each colony with a permanent marker as it was counted prevented counting the same colony twice. Of the six Petrifilm used for each breast milk sample, the Petrifilm containing 25 – 250 CFU were used for reporting CFU/ml. The number of colonies counted on the plates performed in duplicate were averaged and then divided by the dilution used to plate the sample. For example, if 52 colonies were counted on plate 1 and 64 on plate 2, the average is 58 CFU.

$$\frac{58 \text{ CFU}}{\text{ml}} \times \frac{1}{10^{-4} \text{ dilution}} = 58 \times 10^4 = 5.8 \times 10^5 \text{ CFU/ml}$$

2.4. Statistical Analysis

Independent storage treatments were performed in triplicate for analysis of total protein, free amino ends, NEFA, and SIgA activity. Independent storage treatments were only performed once for total fat analysis due to the consistency of the SMART Trac analyzer. Independent storage treatments were performed in duplicate for lysozyme and bacteria analysis.

Statistical analysis was performed by multiple regression using JMP (SAS, Inc., Cary, NC). Statistical comparison of lysozyme activity retention, SIgA activity retention, total protein, free amino ends, NEFA, total fat, and bacteria were done by one-way analysis of variance (ANOVA).

2.5. Results and Discussion

2.5.1. Total Protein

Individual samples were stored in the appropriate experimental storage environments throughout the course of the study. Samples were analyzed individually for total protein. Results are represented as the average of the total protein concentration at each time point for samples stored at -20°C (samples 1-4), 4°C (samples 5-8), and 24°C (samples 9-11).

No change of total protein was apparent for human breast milk stored for three weeks at -20 and 4°C. There was a statistically significant decrease in total protein in milk samples stored at 24°C ($p < 0.0001$) and the decrease in protein was evident at 24 hours, but it was

significant at 1 week of storage ($p < 0.01$). Data are displayed in table 2.2 and graphically in figure 2.7.

Table 2.2 Change in Protein Concentration (g/L) during 3 Weeks of Storage

Time	-20°C	4°C	24°C
0 hours	13.40	14.88	15.50
24 hours	15.26	15.64	14.64
48 hours	14.85	14.95	14.60
1 week	14.13	14.83	13.65
2 weeks	13.72	14.16	13.28
3 weeks	14.12	15.26	12.96
Intercept	NS	NS	14.933**
Slope vs. time	NS	NS	-0.0048**

NS = Not statistically significant
*** = P < 0.001*
** = P < 0.01*

The stability of breast milk proteins has been confirmed by other studies during storage for 48 hours at 4°C and 15 days at -20°C (Silvestre and others 2006), during storage for 1 – 24 hours at 15°C and 25°C (Hamosh and others 1996) and at 4°C (Garza and others 1982).

Protein break down was apparent in breast milk samples stored for 24 hours at 38°C (Hamosh and others 1996) and significant in samples stored for 6 hours and 24 hours at 29°C (Eteng and others 2001). Therefore it is evident that storage of breast milk at ambient temperatures will result in a decrease in total protein concentration at 24 hours of storage.

However, it appears that no study has examined protein breakdown in breast milk stored longer than 48 hours at 4°C or 15 days at -20°C. Results of the present study indicate that protein is stable at -20°C and 4°C for three weeks. However, protein breakdown did occur at 24°C when stored for three weeks, which was apparent at 24 hours of storage. The degradation of protein in milk to generate amino acids, which are used by bacteria for protein synthesis, may explain the decrease in protein observed.

The stability of breast milk proteins may be due to their extensive glycosylation (Hamosh and others 1996). Another factor that may contribute to the stability of milk proteins is the differences in tertiary structure that result in greater organization and hydrophobicity of milk proteins as compared with that of identical proteins from other sources, such as lysozyme from hen egg white (Dubois and others 1982).

Based on these results, it appears that breast milk can be stored at 4°C and -20°C for three weeks without decreasing the total protein concentration. Breast milk stored at 24°C will have a decrease in total protein concentration.

2.5.2. Proteolysis

Individual samples were stored in the appropriate experimental storage environments throughout the course of the study. Samples were analyzed individually and in triplicate for free amino ends. Results are represented as the average of the free amino ends at each time point for samples stored at -20°C (samples 1-4), 4°C (samples 5-8), and 24°C (samples 9-11).

There was a non-significant decrease in free amino ends in the breast milk samples stored at -20°C. Proteolysis may have been occurring in the breast milk samples before being placed in the -80°C freezer for storage until analysis. Once frozen, proteolysis diminished and stayed constant for the remainder of the study.

Proteolysis was unchanged in breast milk samples stored at -20°C. Proteolysis significantly increased in breast milk samples stored at 4°C ($p < 0.0001$) and in breast milk samples stored at 24°C ($p < 0.0001$) during three weeks of storage. In samples stored at 4°C the increase was significant at 1 week ($p < 0.05$), and in samples stored at 24°C the increase was also significant at 1 week ($p < 0.001$). Data are displayed in table 2.3 and graphically in figure 2.8.

Table 2.3 Change in Free Amino Ends (μM) during 3 Weeks of Storage

Time	-20°C	4°C	24°C
0 hours	143.78	139.36	141.68
24 hours	129.15	140.03	137.64
48 hours	120.19	146.42	153.38
1 week	130.16	154.86	184.65
2 weeks	132.73	172.18	265.32
3 weeks	130.97	138.67	283.00
Intercept	NS	150.77**	135.72**
Slope vs. time	NS	0.0740**	0.3404**

NS = Not statistically significant
*** = P < 0.001*
** = P < 0.01*

Free amino ends increase in breast milk due to the break down of protein into peptides and amino acids. Although free amino ends are increasing in the breast milk samples stored at 4°C, total protein was found to remain stable. Therefore, if the infant consumes breast milk stored at 4°C (up to three weeks) total protein intake will remain the same, but consumption of peptides and amino acids will increase. On the other hand, proteolysis is greater in breast milk samples stored at 24°C for three weeks and there is also a significant loss of protein within these samples as well.

Proteolysis is most likely occurring in breast milk samples stored at 4°C and 24°C due to the action of plasmin as well as bacterial proteases. Plasmin is an alkaline serine protease and is the principle endogenous proteolytic enzyme in milk. In addition to plasmin, which is endogenous to milk, psychrotrophic bacteria produce heat-stable metalloproteases that can increase proteolysis in milk, even at refrigeration temperatures (Frohbieter and others 2005). Production of proteases by psychrotrophic bacteria occurs mainly at the end of the exponential growth phase (Dupont and others 2007). The decrease in free amino ends between 2 and 3 weeks in samples stored at 4°C may be due to bacterial consumption, as there is a high initial level of bacteria in these samples.

Consumption of stored breast milk that contains greater amounts of peptides and amino acids may not be harmful for the infant. In fact, there are many physiologically active peptides in milk including antihypertensive, antithrombotic, immunomodulatory, and opoid milk peptides, as well as caseinophosphopeptides, casomorphins, caseinomacropeptide, and atriopeptin (Clare and Swaisgood 2000). Also, peptides from κ-casein apply antimicrobial

(Stromqvist and others 1995) and antithrombotic effects, in particular the κ-caseinglycopeptide (hCGP) (Chabance and others 1995).

These bioactive peptides are inactive until they are released from the parent protein and activated by enzymatic proteolysis, such as during digestion or food processing (Meisel and Bockelmann 1999). Studies have indicated minimal protein digestion in the infant's stomach due to a low pepsin output (Agunod and others 1969; Weisselberg and others 1992; Yahav and others 1987) and high postprandial gastric pH (Hamosh and others 1978; Mason 1962). Because infants have a higher pH in their gastrointestinal tract than do adults, proteins are not as readily denatured. Therefore, proteolysis of milk proteins prior to ingestion may increase bioavailability of protein to the infant as well as increase bioavailability of the bioactive peptides.

2.5.3. SIgA Activity

Individual samples were stored in the appropriate experimental storage environments throughout the course of the study. Samples were analyzed individually and in triplicate for SIgA activity. Results are represented as the average of the SIgA activity at each time point for samples stored at -20°C (samples 1-4), 4°C (samples 5-8), and 24°C (samples 9-11).

Retention of SIgA activity was apparent for human breast milk stored for three weeks at -20, and 4, and 24°C. There was a non-significant decrease in SIgA activity in breast milk samples stored for two – three weeks at room temperature. Data are displayed in table 2.4 and graphically in figure 2.9.

Table 2.4 SIgA Activity (µg/µL) during 3 Weeks of Storage

Time	-20°C	4°C	24°C
0 hours	0.00288	0.00338	0.00724
24 hours	0.00318	0.00320	0.00667
48 hours	0.00307	0.00312	0.00677
1 week	0.00286	0.00320	0.00685
2 weeks	0.00284	0.00301	0.00659
3 weeks	0.00289	0.00283	0.00537
Intercept	NS	NS	NS
Slope vs. time	NS	NS	NS

NS = Not statistically significant
*** = P < 0.001*
** = P < 0.01*

SIgA concentrations varied considerably at baseline between groups. The average SIgA concentration at baseline was 0.00288 µg/µl in samples 1-4, 0.00338 µg/µl in samples 5-8, and 0.00724 µg/µl in samples 9-11. However, the differences in SIgA levels between groups can be explained by the fact that levels of SIgA vary greatly among individuals (Atkinson and Lonnerdal 1989).

There was no significant change in SIgA activity in all experimental temperatures and time points. It has been shown that SIgA activity is stable during storage for up to 7 hours at 4°C and 24°C (Williamson and Murti 1996) and during 4 and 24 hours at 4°C and -20°C (Lawrence 1999). Also, in the present study, there were significant increases in proteolysis products in samples stored at 4°C and 24°C over three weeks of storage, which is consistent with findings from Hamosh (Hamosh and others 1996). Therefore, although proteolysis

increases at refrigeration and room temperatures, SIgA is not breaking down even though it is a protein.

There was a slight, non-significant decrease in SIgA between two and three weeks in breast milk samples stored at room temperature. SIgA concentration at two weeks was 0.00659 µg/µl and it decreased to 0.00537 µg/µl at three weeks. Although the decrease is not significant, it is still worth noting and it is probably due to the action of the proteolytic enzyme plasmin as well as bacterial proteases. Also, as bacteria increases in the samples, SIgA may be binding the bacteria and thus unable to bind the *E. coli* antigen used in the ELISA assay to measure SIgA.

There was no significant change in SIgA or lysozyme concentration in all experimental temperatures and time points, which is consistent with the findings by Lawrence (2001). Also, in the present study, there were significant increases in proteolysis products in samples stored at 4°C and 24°C over three weeks of storage, which is consistent with findings from Hamosh (Hamosh and others 1996). These results suggest that the SIgA may be resistant to proteolysis during storage. SIgA is able to endure in the hostile environment of the gastrointestinal tract because it is more resistant to degradation from proteases than other immunoglobulins (Atkinson and Lonnerdal 1989). Therefore it is not unexpected that SIgA activity remains stable during storage for three weeks.

Based on these results, it appears that breast milk can be stored at -20°C, 4°C, and 24°C for three weeks without decreasing the SIgA activity.

2.5.4. Lysozyme Activity

Individual samples were stored in the appropriate experimental storage environments throughout the course of the study. Samples were analyzed individually and in duplicate for lysozyme. Results are represented as the average of the lysozyme activity at each time point for samples stored at -20°C (samples 1-4), 4°C (samples 5-8), and 24°C (samples 9-11).

Retention of lysozyme activity was apparent for human breast milk stored for three weeks at -20, 4, and 24°C. There was no change in lysozyme activity in any breast milk samples over the three week storage time. Data are displayed in table 2.5 and graphically in figure 2.10.

Table 2.5 Lysozyme Activity (units enzyme/ mg protein) during 3 Weeks of Storage

Time	-20°C	4°C	24°C
0 hours	1617	1074	707
24 hours	1799	1200	724
48 hours	1273	879	590
1 week	1638	916	610
2 weeks	1944	919	661
3 weeks	2077	869	715
Intercept	NS	NS	NS
Slope of lysozyme vs. time	NS	NS	NS

NS = Not statistically significant
*** = P < 0.001*
** = P < 0.01*

There was no significant change in lysozyme activity in all experimental temperatures and time points. It has been shown that lysozyme activity is stable during storage for up to 7 hours at 4°C and 24°C (Williamson and Murti 1996) and during 4 and 24 hours at 4°C and -20°C (Lawrence 1999). Also, in the present study, there were significant increases in proteolysis products in samples stored at 4°C and 24°C over three weeks of storage, which is consistent with findings from Hamosh (Hamosh and others 1996). Therefore, although proteolysis increases at refrigeration and room temperatures, lysozyme is not breaking down.

Similar to SIgA, these results suggest that the lysozyme may be resistant to proteolysis during storage. Lysozyme is also able to endure in the hostile environment of the gastrointestinal tract because it is more resistant to degradation from proteases than are immunoglobulins (Atkinson and Lonnerdal 1989). Therefore it is not surprising that lysozyme activity remained stable during storage for three weeks.

Although non-significant, an interesting observation is that lysozyme activity increased in all breast milk samples during storage for 24 hours and decreased close to original levels during storage for 48 hours. Milk macrophages can present an antigen to lymphocytes and produce cytokines and antibacterial substances such as lysozyme, lactoferrin, and the complement factors C3 and C4 (Riedel-Caspari 2001). The increase in lysozyme activity may be a result of the increased production of lysozyme from the lymphocytes. The decrease in lysozyme during 48 hours of storage may be due to the noncompetitive inhibition by lipopolysaccharide from bacteria (Ohno and Morrison 1989).

These data suggest that breast milk can be stored at -20°C, 4°C, and 24°C for three weeks without changing lysozyme activity.

2.5.5. Validation of a Breast Milk Method for the SMART Trac Analyzer

In order to determine the ability of the SMART Trac analyzer (CEM Corporation) to analyze the fat content in breast milk samples, analyzer parameters were based on the fat content of six breast milk samples measured using the mojonnier fat test. The fat percentage determined for each sample by the mojonnier fat test was compared to the fat percentage obtained for each sample using the SMART Trac analyzer.

Table 2.6 Comparison of Fat Percentages Obtained from Two Methods

	Fat percentage		
Sample	Mojonnier fat test	SMART Trac analysis	Difference
1	5.18	5.02	0.16
2	2.83	2.98	- 0.15
3	3.46	3.04	0.42
4	3.03	2.98	0.05
5	3.30	3.70	- 0.40
6	4.82	5.17	- 0.35
Average Difference			0.25

Fat percentages given by the SMART Trac analyzer were similar to those given by the mojonnier fat test ($p = 0.94$), which is the official AAOC method to analyze fat in milk. The difference in fat percentages resulting from the two analyses ranged from 0.05 – 0.42, with the average difference being 0.25.

Based on these results, the SMART Trac analyzer provides a reliable way to analyze the fat content in human milk, as well as in bovine milk.

2.5.6. Total Fat

Individual samples were stored in the appropriate experimental storage environments throughout the course of the study. Samples were analyzed individually for total fat. Results are represented as the average of the total fat percentage at each time point for samples stored at -20°C (samples 1-4), 4°C (samples 5-8), and 24°C (samples 9-11).

Retention of total fat was apparent for human breast milk stored for three weeks at -20, 4, and 24°C. There was a slight, non-significant decrease in total fat in milk samples stored at 24°C. Data are displayed in table 2.7 and graphically in figure 2.11.

Table 2.7 Change in Total Fat Percentage during 3 Weeks of Storage

Time	-20°C	4°C	24°C
0 hours	3.67	3.94	3.39
24 hours	3.79	4.26	3.74
48 hours	3.90	4.50	4.10
1 week	3.70	4.48	4.00
2 weeks	3.81	4.46	3.84
3 weeks	3.76	4.72	3.59
Intercept	NS	NS	NS
Slope vs. time	NS	NS	NS

NS = Not statistically significant
*** = P < 0.001*
** = P < 0.01*

There was no significant change in total fat in the breast milk samples stored in all experimental temperatures and time points. Previous studies have confirmed the retention of total fat in breast milk stored at 0 – 4°C for 8 days (Pardou and others 1994). The creamatocrit measurement of fat content decreases within hours at room temperature but is stable for 14 days at 4°C (Clark and others 1984).

Based upon these results, it appears that consumption of breast milk stored at -20°C and 4°C for 3 weeks will not affect the total fat intake of an infant. Consumption of breast milk stored at 24°C for 3 weeks may decrease slightly, but will not significantly decrease the total fat intake of an infant.

2.5.7. Non-esterified Fatty Acids

Individual samples were stored in the appropriate experimental storage environments throughout the course of the study. Samples were analyzed individually and in triplicate for NEFA. Results are represented as the average of the percent NEFA at each time point for samples stored at -20°C (samples 1-4), 4°C (samples 5-8), and 24°C (samples 9-11).

Lipolysis significantly increased in all breast milk samples, as evidenced by the increase in NEFA in all samples stored at -20°C ($p < 0.0001$), 4°C ($p < 0.0001$) and at 24°C ($p < 0.0001$). In samples stored at -20°C the increase in NEFA was significant at 2 weeks of storage ($p < 0.05$), in samples stored at 4°C the increase in NEFA was significant at 48 hours of storage ($p < 0.0001$), and in samples stored at 24°C the increase in NEFA was significant

at 24 hours of storage ($p < 0.0001$). Data are displayed in table 2.8 and graphically in figure 2.12.

Table 2.8 Change in NEFA Percentage during 3 Weeks of Storage

Time	-20°C	4°C	24°C
0 hours	33.05	55.23	50.57
24 hours	35.49	118.2	339.5
48 hours	53.23	349	515.8
1 week	43.03	667.6	969
2 weeks	85.83	907.1	1217
3 weeks	126.4	1030.0	1446
Intercept	31.02**	216.6**	279.7**
Slope vs. time	0.19**	2.58**	2.77**

NS = Not statistically significant
*** = P < 0.001*
** = P < 0.01*

The data show that storage of breast milk at any temperature results in a rapid increase of NEFA due to the lipolysis of triacylglycerols by lipoprotein lipase. Hamosh and others (1996) found that when breast milk is stored at ambient temperatures for 24 hours, there is a 440% - 710% increase in free fatty acid concentration above that in freshly expressed milk (Hamosh and others 1996). The consequences of increased NEFA on human milk quality and digestibility are unknown. Hernell and Blackberg (1982) suggested that NEFA are absorbed better than esterified fatty acids when intraduodenal bile salts are low in the infant (Hernell and Blackberg 1982). Patton and Carey (1979) suggested that NEFA may

bind to calcium or other components of the milk and make them unavailable for absorption (Patton and Carey 1979).

Alternatively, NEFA produced during storage of human milk have been shown to have powerful cytolytic effects on normal human blood cells, on intestinal parasites *Giardia lamblia* and *Entamoeba histolytica*, as well as gram-positive bacteria and yeast (Ogundele 2000). Also, an increase in NEFA could increase bioavailability of triacylglycerols to the infant.

Rancid milk may be a concern of increased NEFA and is a result of increased short to medium chain fatty acids ($C_4 - C_{12}$) produced mostly by bacterial lipases on milk triacylglycerides (Lawrence 1999) and oxidation of unsaturated fatty acids. However, it has been shown that lipolysis increases in breast milk during storage at 25°C, 4°C, or -11°C for 6, 12, 24, or 48 hours and -11°C or -70°C for 1, 2, 4, 6, or 8 weeks. NEFA increased with storage temperature and with time and the pattern of NEFA changed as storage progressed; with free 16:0 decreasing and 18:2 increasing, and long-chain polyunsaturated fatty acids increasing (Lavine and Clark 1987). Therefore it is possible that rancidity may not occur during breast milk storage due to the increase in long-chain fatty acids.

Based upon results of this study, consumption of breast milk stored at -20°C, 4°C, and 24°C for 3 weeks will increase NEFA intake by the infant.

2.5.8. Bacterial Growth

Individual samples were stored in the appropriate experimental storage environments throughout the course of the study. Samples were analyzed individually and in duplicate for bacterial growth. Results are represented as the average of colony forming units per milliliter at each time point for samples stored at -20°C (samples 1-4), 4°C (samples 5-8), and 24°C (samples 9-11).

Bacterial growth was not significant in breast milk samples stored at -20°C during the three week storage time. However, there was statistically significant bacterial growth in breast milk samples stored at 4°C ($p < 0.01$) and 24°C ($p < 0.0001$) during 3 weeks of storage. In samples stored at 4°C the increase in bacterial growth was significant at 2 weeks of storage ($p < 0.05$), and in samples stored at 24°C the increase in bacterial growth was significant at 48 hours ($p < 0.01$). Data are displayed in table 2.9 and graphically in figure 2.13.

Table 2.9 Change in Bacterial Growth (CFU/ml) during 3 Weeks of Storage

Time	-20°C	4°C	24°C
0 hours	2.25E+03	3.20E+06	6.27E+03
24 hours	8.00E+02	3.23E+06	5.03E+03
48 hours	1.81E+03	1.41E+06	3.67E+05
1 week	2.15E+03	1.93E+07	4.68E+07
2 weeks	1.61E+03	4.36E+07	1.74E+08
3 weeks	1.66E+03	3.01E+07	2.35E+08
Intercept	NS	NS	NS
Slope vs. time (hours)	NS	83607*	545409**

NS = Not statistically significant
*** = P < 0.001*
** = P < 0.01*

The bacteria content was initially higher in the samples stored in the refrigerator than the samples stored in the freezer or at room temperature because two women had higher initial amounts of bacteria in their breast milk. However, the change in bacterial growth over the three week storage period was not as great as the change in bacterial growth in the samples stored at room temperature.

Breast milk samples stored at all times and temperatures had an initial decrease in bacterial growth at 24 hours of storage. These results are confirmed by other studies that have shown a decrease in bacterial growth over 24 hours at 4°C (Pardou and others 1994; Sosa and Barness 1987).

Safe limits for bacterial concentration in human milk used to feed premature infants have never been established (Jones and others 2000; Law and others 1989). Many donor milk programs have adopted arbitrary limits based on those used by the dairy industry for bovine

milk (Botsford and others 1986; Carroll and others 1979; Ryder and others 1977). Recommendations for raw milk have ranged from bacterial counts of 10^3 CFU/ml with no enteropathogens (Sauve and others 1984) to 10^5 CFU/ml excluding pathogens such as *Staphylococcus aureus*, group B *streptococci*, *pneumococci*, and coliforms (Tyson and others 1982).

If bacterial growth of 10^5 CFU/ml is chosen as a limit of acceptable bacterial growth within the experimental breast milk samples the following storage recommendations can be suggested based upon the results. It appears that breast milk may be bacteriologically safe when stored at -20°C for greater than 3 weeks, at 4°C for up to 2 weeks, and 24°C for 24 – 48 hours. It has been shown that storage at 0-4°C for up to 8 days is acceptable when carefully covered (Pardou and others 1994).

2.6. Conclusion

Proteins in breast milk are stable when stored in the freezer and refrigerator for three weeks. Based on the results of this study, it appears that breast milk can be stored at 4°C and at -20°C for three weeks without decreasing the total protein concentration, but total protein concentration does decrease in breast milk stored at 24°C and the decrease is significant at one week. Proteolysis was unchanged in breast milk samples stored at -20°C, but it was significantly increased in breast milk samples stored at 4°C at 1 week, and in breast milk

samples stored at 24°C at 1 week. Breast milk can be stored at -20°C, 4°C, and 24°C for three weeks without changing the activity of the antibacterial factors, SIgA and lysozyme.

The SMART Trac analyzer provided a reliable way to analyze the fat content in breast milk. Consumption of breast milk stored at -20°C and 4°C for 3 weeks will not affect the total fat intake of an infant. Consumption of breast milk stored at 24°C for 3 weeks may decrease slightly, but will not significantly decrease the total fat intake of an infant. Consumption of breast milk stored at -20°C, 4°C, and 24°C for 3 weeks will increase NEFA intake by the infant. Increases in NEFA were significant in samples stored at -20°C at 2 weeks, in samples stored at 4°C at 48 hours, and in samples stored at 24°C at 24 hours.

If bacterial growth of 10^5 CFU/ml is chosen as a limit of acceptable bacterial growth within the experimental breast milk samples, then breast milk may be bacteriologically safe when stored at -20°C for greater than 3 weeks, at 4°C for up to 2 weeks, and 24°C for 24 – 48 hours.

Based upon the data in this study, it appears that breast milk may be stored in the freezer at -20°C for longer than three weeks, in the refrigerator at 4°C for up to 2 weeks, and at room temperature (24°C) for up to 48 hours. However, the impacts of increased NEFA are controversial and must be taken into consideration when storing breast milk. Also, growth of specific strains of pathogenic bacteria should be investigated at the storage times and temperatures.

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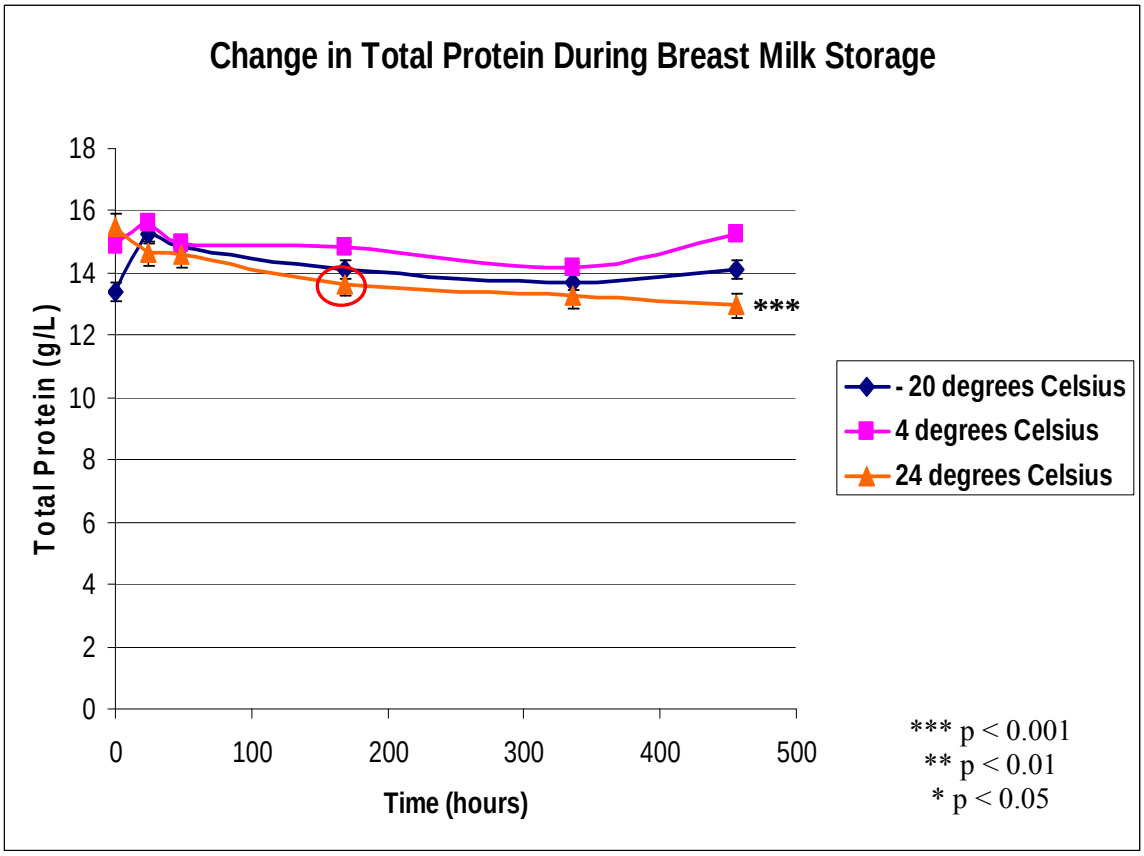

Figure 2.7 Change in total protein during breast milk storage

Vertical bars show the standard error of the mean.

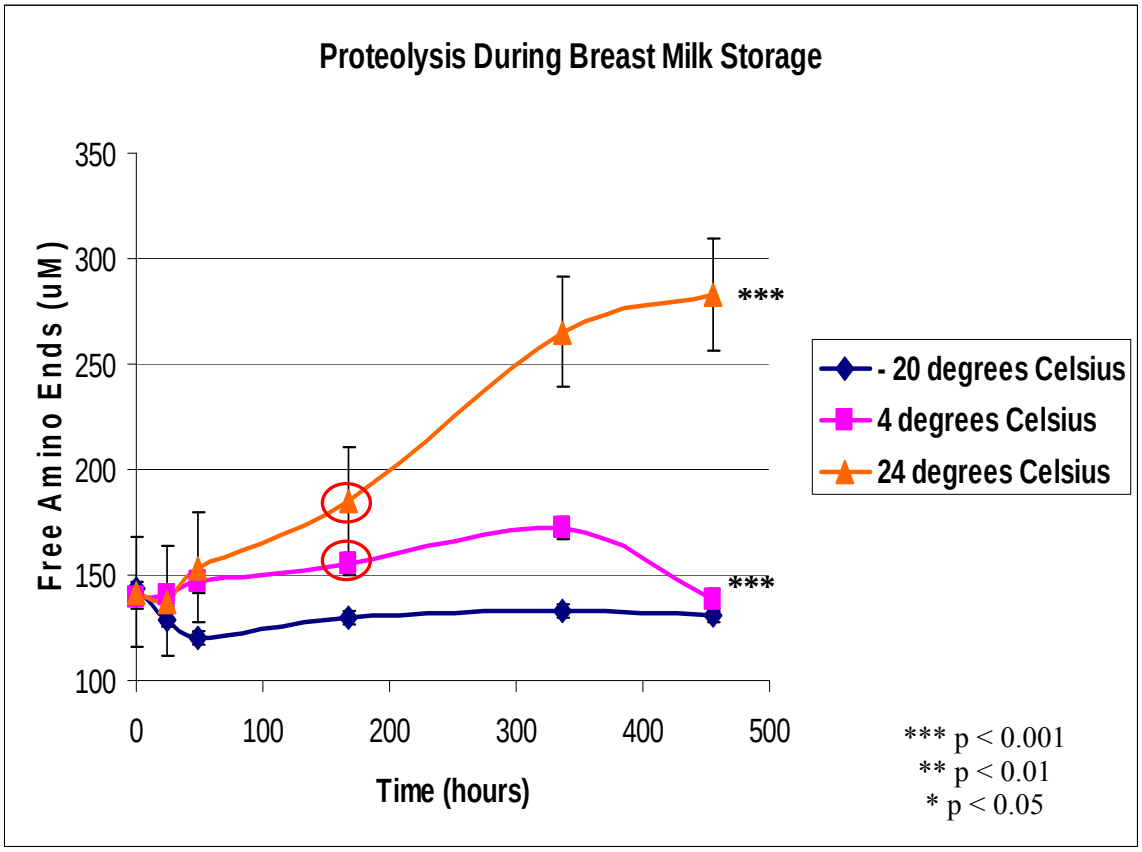

Figure 2.8 Proteolysis during breast milk storage

Red circles indicate the time point at which the change becomes significant.
Vertical bars show the standard error of the mean.

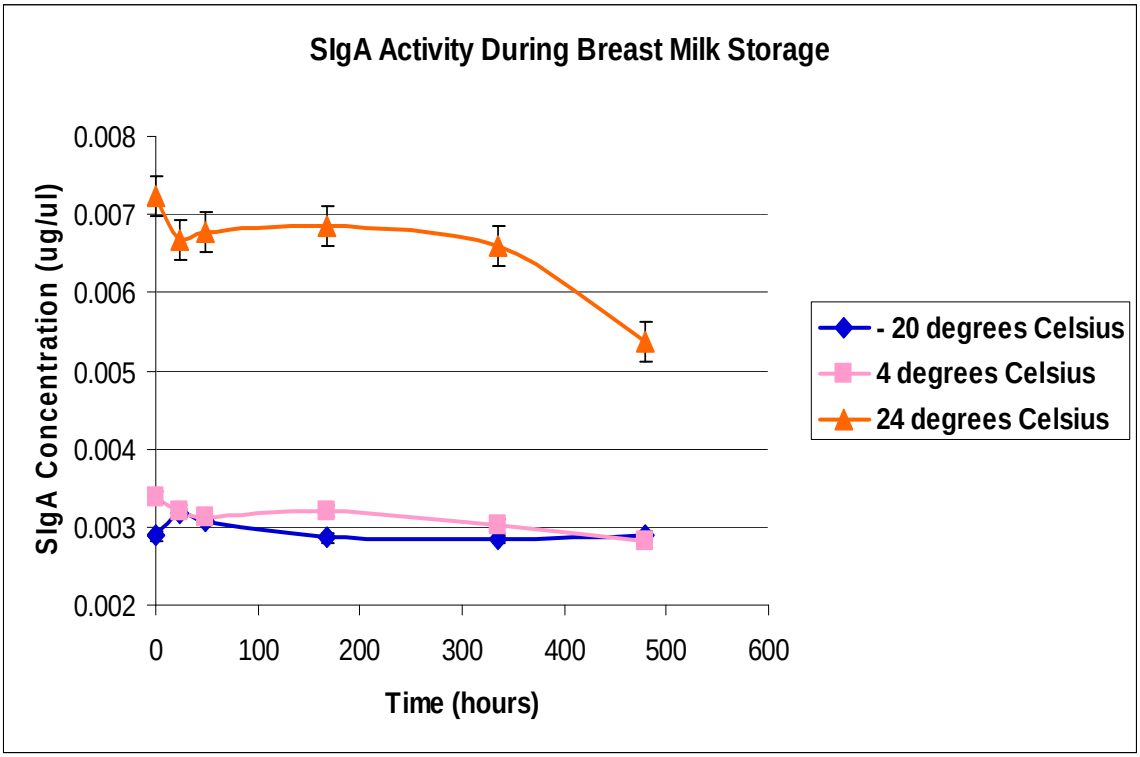

Figure 2.9 Secretory immunoglobulin A activity during breast milk storage

Vertical bars show the standard error of the mean.

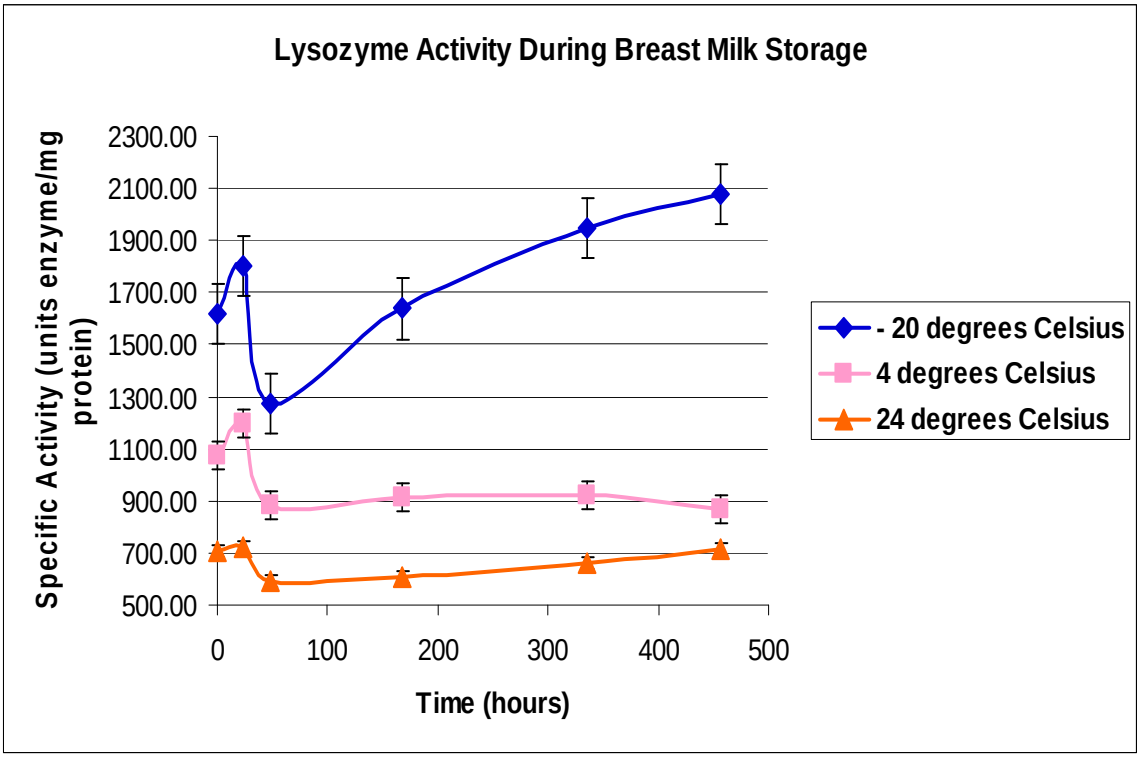

Figure 2.10 Lysozyme specific activity during breast milk storage

Vertical bars show the standard error of the mean.

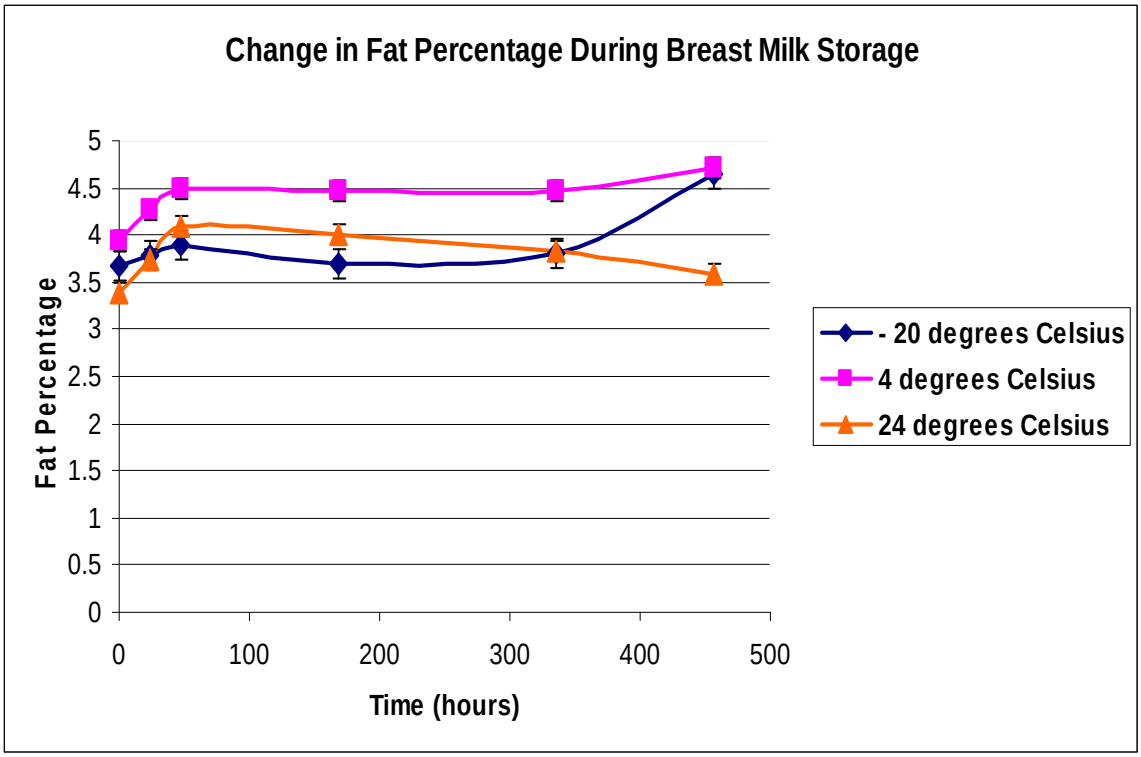

Figure 2.11 Change in total fat during breast milk storage

Vertical bars show the standard error of the mean.

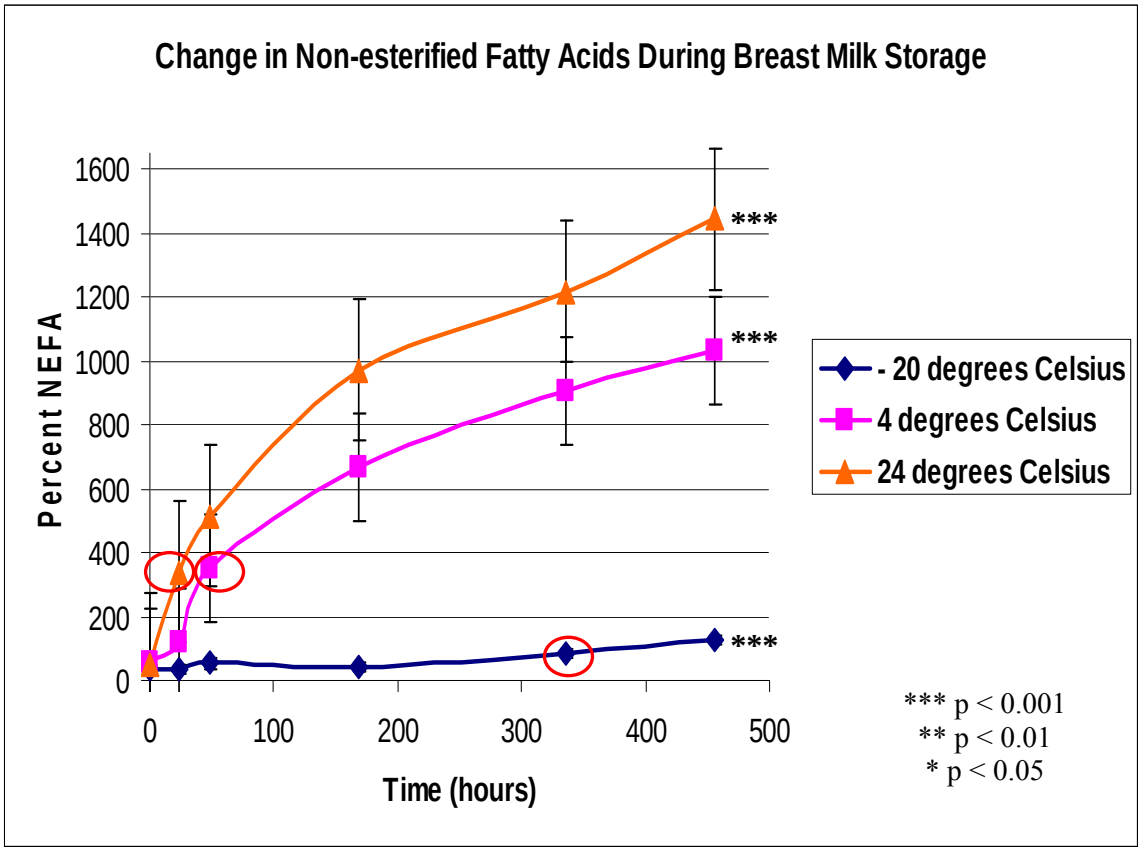

Figure 2.12 Change in non-esterified fatty acids during breast milk storage

Red circles indicate the time point at which the change becomes significant.
Vertical bars show the standard error of the mean.

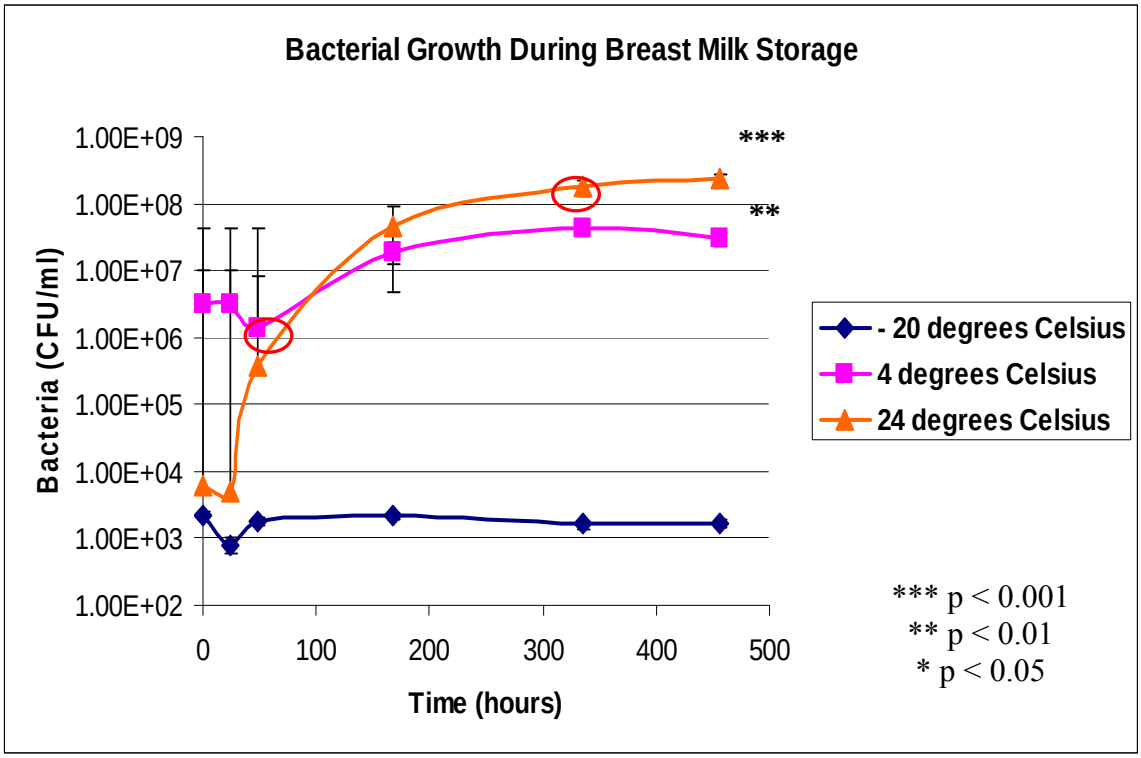

Figure 2.13 Change in bacterial growth during breast milk storage

Red circles indicate the time point at which the change becomes significant.
Vertical bars show the standard error of the mean.

CHAPTER 3

The Effect of Storage Time and Temperature on Bacterial Growth in Human Breast Milk Contaminated by an Infant

3.1. Abstract

This study is aimed at benefiting premature and sick infants consuming banked breast milk. The findings will be applicable in neonatal intensive care units, as well as childcare facilities, milk banks, and in the home. Because banked milk is expensive and the number of mothers donating is low, it would be extremely beneficial to infants in neonatal intensive care units if the banked milk could be stored for a longer period of time after the infant has already sipped from the bottle, preventing a great deal of banked breast milk from being thrown away when the infants could still safely consume it.

Pasteurized breast milk was compared to unpasteurized breast milk from individual mothers to separate the effects of the different sources of bacterial contaminants, contamination by an infant alone, contamination by natural skin flora, and that from the infant. It is imperative to examine this question in *pasteurized* breast milk because many infants drink donated banked breast milk, which is pasteurized. This is especially true in neonatal intensive care units. Many infants who consume banked breast milk are premature and/or sick, and this is a population of concern. However, findings may also be applied to individual mothers' own *unpasteurized* milk because the infants also consumed milk from a bottle containing their own mother's *unpasteurized* milk.

The objective of this study was to observe the difference in bacterial growth in breast milk contaminated by an infant in mother's own milk and donor milk and determine if it is safe for infants to drink breast milk from a bottle, store it for a period of time, and then drink from it again. Five women volunteered with informed consent and provided parental consent for their infant to participate. Each infant was fed 1 – 2 ounces of breast milk from a bottle prepared with the mother's pasteurized milk and the mother's unpasteurized milk. Leftover milk was collected in sterile containers and analyzed for bacterial growth at specific time points during storage. Leftover samples were stored at 4°C for 0 hours, 2, 4, and 6 days, and at 24°C for 0, 3, 6, 9, and 24 hours.

Acceptable levels of bacteria were chosen as 10^5 colony forming units per milliliter (CFU/ml). Based on the data, it appears that unpasteurized breast milk can be stored up to 6 – 9 hours and pasteurized breast milk between 9 – 24 hours at 24°C. Unpasteurized breast milk can be stored between 4 – 6 days and pasteurized breast milk can be stored longer than 6 days at 4°C. However, more research is needed with a larger number of participants and focusing on the growth of specific strains of pathogenic bacteria in breast milk contaminated by an infant and stored for a certain amount of time and at various temperatures. Also, the data show that bacteria originating from the mother are greater than bacteria originating from contamination by an infant.

3.2. Introduction

The first objective is to answer the question - *is it safe for infants to drink breast milk from a bottle, store it for a period of time, and then drink from it again?* The second objective is to observe the difference in bacterial growth in breast milk contaminated by an infant in mother's own milk and donor milk.

This study is aimed at benefiting premature and sick infants consuming banked breast milk. The findings will be applicable in neonatal intensive care units, as well as childcare facilities, milk banks, and in the home. Banked milk is expensive to collect and process and the number of mothers donating is low, therefore it would be extremely beneficial to infants in neonatal intensive care units if the banked milk could be stored for a longer period of time after the infant has already partially consumed the bottle. This would prevent a great deal of banked breast milk from being thrown away, when the infants could benefit from it.

Pasteurized breast milk was compared to unpasteurized breast milk from individual mothers to study the differences in the bacterial growth in milk with different sources of contamination. It is imperative to study this question in *pasteurized breast milk* because many infants drink banked breast milk, which is pasteurized. This is especially true in neonatal intensive care units. Many infants who consume banked breast milk are premature and/or sick, and this is a population of concern. However, findings may also be applied to individuals' own *unpasteurized milk* because the infants also consumed one - two ounces from a bottle containing their mother's unpasteurized milk. Pasteurizing the milk *decreases*

the antibacterial factors such as IgA and lysozyme (Viazis and others 2008). It was hypothesized that *unpasteurized* milk will be able to be stored in the refrigerator longer than pasteurized milk because it will have greater antibacterial activity.

Mothers used their own expressed milk, pasteurized and unpasteurized, and fed it to their infants. Standardized procedures for pumping milk were not implemented in order to observe what happens when mothers feed the infants as they would under *normal circumstances*. Mothers would not usually sterilize their skin, bottles, and breast pumps.

Microbial stability was evaluated using Petrifilm (3M company) for total aerobic bacterial plate counts. Immediately after expression, total bacterial counts in breast milk decrease from the initial level (Sosa and Barness 1987). After a few days, the bacteria counts begin to rise again to the initial level. We observed the amount of time it takes for the bacteria levels in breast milk to rise above the initial amount of bacteria, after the milk has been contaminated by bacteria from an infant.

3.3. Materials and Methods

3.3.1. Collection and Preparation of Human Breast Milk Samples

Five mothers and five infants were recruited from the first part of the study which consisted of 12 women who donated milk. Volunteers participated with informed consent.

Each mother was required to donate a bag of their own breast milk containing 3 – 4 ounces. The milk was collected at a convenient time for the mothers, on separate days, with

each day referred to as “day one”. The mother’s infant’s names were written on the bag of milk with a permanent marker.

After picking up the milk on day one, the milk was transported to the laboratory at NC State in a cooler filled with ice. At the laboratory on day one, the milk was pasteurized using the protocol from the Wake Med Milk bank, which utilizes the “holder” pasteurization method. Preceding pasteurization, the water bath itself was emptied, cleaned, and sanitized using hot water, soap, and alcohol. Once sanitized, the water bath was filled again with water.

Each milk sample was transferred into a sterile container that had the name of the mother and infant previously written with a permanent marker. The milk in the sterile container was then pasteurized according to the Holder method, heating the milk in a water bath at 66°C for 30 minutes. During pasteurization, the milk was gently agitated by the automatic shaker in the water bath. A control container of breast milk with a thermometer inserted was also placed in the water bath. Timing began when the thermometer in the control sample read 66°C. After 30 minutes of the heat treatment, the sterile containers of pasteurized milk were cooled rapidly in an ice bath. When the thermometer placed in the control container of breast milk indicated the samples had cooled to 10°C, all samples were refrigerated.

On day two, the research assistant drove to each mother’s home at a prearranged time, which was a normal feeding time of the infant, to perform the experiment. The pasteurized milk samples were transported in a cooler full of ice. The unpasteurized milk was prepared as

usual. The mother transferred the pasteurized milk into a clean (not sterile) bottle that the baby would normally use.

The infant drank one – two ounces of the pasteurized milk and the unpasteurized milk. The volume of milk in each bottle was recorded before and after feeding. The amount of milk consumed and the time of consumption of each bottle was recorded so that bacterial growth could be analyzed at the appropriate time point. The remaining milk leftover from the feeding was held on ice in a cooler during transportation to the laboratory.

The milk transported back to the laboratory was transferred into appropriately-labeled tubes and stored as follows: at 4°C for 0 hours, 2, 4, and 6 days and at 24°C for 0, 3, 6, 9, and 24 hours. At the appropriate time point, the samples were immediately plated onto Petrifilm to determine bacterial levels.

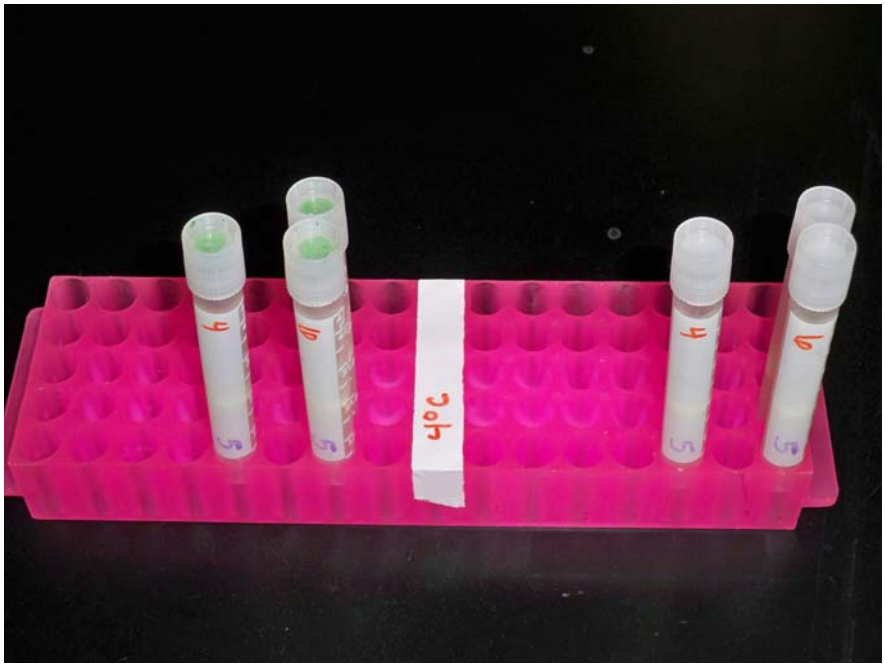

Figure 3.1 Storage containers

There was an initial baseline analysis for each leftover sample of the pasteurized and unpasteurized milk. However, there was not an initial baseline analysis for each sample of milk stored at 24°C and 4°C, because at 0 hours the milk had not been stored yet.

3.3.2. Storage Environments of Human Breast Milk Samples

The milk was stored at 4°C for 0 hours, 2, 4, and 6 days and at 24°C for 0, 3, 6, 9, and 24 hours. At the appropriate time point, the samples were immediately plated onto Petrifilm to determine bacterial levels.

3.3.3. Biochemical Assays

3.3.3.1. Bacterial Analysis

Breast milk samples stored at 4°C and 24°C were analyzed immediately and without prior freezing at baseline and at each time point for bacterial growth using Petrifilm (3M Company, St. Paul, MN). Each sample was analyzed in duplicate, with four dilutions each, resulting in eight Petrifilm being used for each sample. Preparing and plating four dilutions for each sample ensured that the amount of bacteria grown on at least one Petrifilm was between 25 and 250 colony forming units/ml (CFU/ml). Dilutions were adjusted over the course of the study.

Peptone water was prepared by weighing 1 g of peptone (Sigma #77185) and adding it to 1 L of deionized water. Subsequent dilutions were made by adding 1 ml of the breast milk sample into 9 ml of peptone water, followed by a subsequent 1 ml transfer into another test tube containing 9 ml of peptone water, resulting in a 10^{-1} dilution. The following picture diagrams how dilutions were prepared:

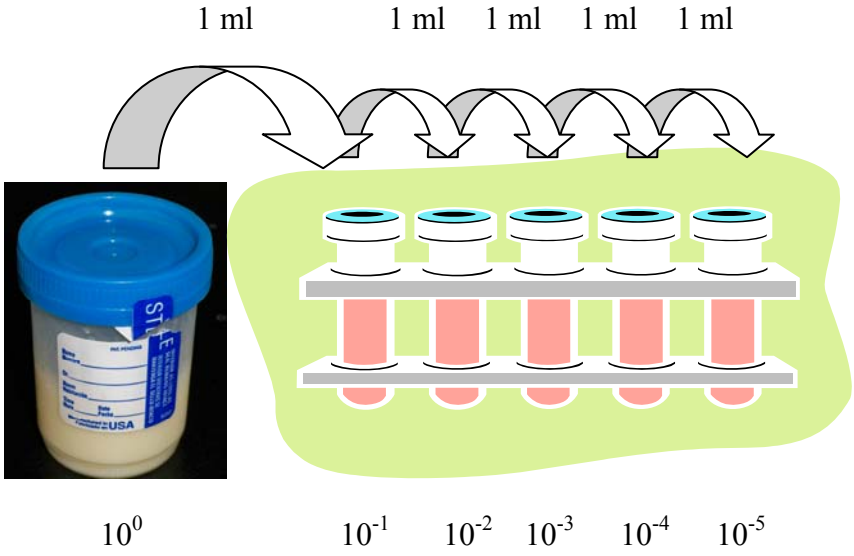

Figure 3.2 Subsequent dilutions

The appropriate dilutions were plated onto Petrifilm, placed in a 37°C incubator, and incubated for 48 hours. After 48 hours of incubation, the colonies were counted. Marking each colony with a permanent marker as it was counted prevented counting the same colony twice. Of the eight Petrifilm used for each breast milk sample, the Petrifilm containing 25 – 250 CFU were used for reporting CFU/ml. The number of colonies counted on the plates performed in duplicate were averaged and then divided by the dilution used to plate the sample. For example, if 52 colonies were counted on plate 1 and 64 on plate 2, the average is 58 CFU.

$$\frac{58 \text{ CFU}}{\text{ml}} \times \frac{1}{10^{-4} \text{ dilution}} = 58 \times 10^4 = 5.8 \times 10^5 \text{ CFU/ml}$$

3.4. Statistical Analysis

Independent storage treatments were performed in duplicate for analysis of bacterial growth. Statistical analysis of the log of bacterial growth was performed by one-way analysis of variance (ANOVA) for each temperature-pasteurization combination, using JMP (SAS, Inc., Cary, NC). Differences between means were tested for significance ($\alpha = 0.05$) by the Tukey HSD test.

3.5. Results and Discussion

Individual samples were stored in the appropriate experimental storage environments throughout the course of the study. Samples were analyzed individually for bacterial growth. Results are represented as the average of the bacterial growth in pasteurized breast milk samples and unpasteurized breast milk samples stored at each time point at 4°C and 24°C.

Safe limits for bacterial concentration in human milk used to feed premature infants have never been established (Jones and others 2000; Law and others 1989). Many donor milk programs have adopted arbitrary limits based on those used by the dairy industry for bovine milk (Botsford and others 1986; Carroll and others 1979; Ryder and others 1977).

Recommendations on raw milk have ranged from bacterial counts of 10^3 CFU/ml with no enteropathogens (Sauve and others 1984) to 10^5 CFU/ml excluding pathogens such as Staphylococcus aureus, group B streptococci, pneumococci, and coliforms (Tyson and others 1982). However, these recommendations are based on freshly expressed breast milk, not breast milk leftover from a bottle which a baby has drunk from.

Table 3.1 Bacterial Growth (CFU/ml) in Pasteurized and Unpasteurized Breast Milk Samples During Storage at 4°C and 24°C

Heat Treatment	Temperature (°C)	Time	Bacterial Count (CFU/ml)
Pasteurized	24	0	1.14E+03 [b]
Pasteurized	24	3	1.47E+03 [b]
Pasteurized	24	6	1.16E+03 [b]
Pasteurized	24	9	1.48E+03 [b]
Pasteurized	24	24	2.09E+06 [a]
Pasteurized	4	0	1.14E+03 [a]
Pasteurized	4	48	7.44E+02 [a]
Pasteurized	4	96	1.25E+04 [a]
Pasteurized	4	144	1.12E+03 [a]
Unpasteurized	24	0	5.24E+04 [b]
Unpasteurized	24	3	6.99E+04 [b]
Unpasteurized	24	6	7.47E+04 [b]
Unpasteurized	24	9	1.32E+05 [b]
Unpasteurized	24	24	1.54E+07 [a]
Unpasteurized	4	0	5.24E+04 [a]
Unpasteurized	4	48	1.25E+05 [a]
Unpasteurized	4	96	6.96E+04 [a]
Unpasteurized	4	144	2.52E+06 [a]

[a, b] Values within the same group that share a superscript letter are not different

Bacterial growth in pasteurized breast milk samples stored at 24°C exceeded 10^5 CFU/ml between 9 and 24 hours. Bacterial growth in unpasteurized breast milk samples stored at 24°C exceeded 10^5 CFU/ml between 6 and 9 hours. Bacterial growth in pasteurized breast milk samples stored at 4°C did not exceed 10^5 CFU/ml before 6 days. Bacterial growth

in unpasteurized breast milk samples stored at 4°C exceeded 10^5 CFU/ml between 4 and 6 days.

No prior publication of the effect of breast milk contamination by an infant on microbial growth was found. Hamosh and others (1997) found that bacterial growth in freshly expressed unpasteurized breast milk was minimal during storage at 15°C for 24 hours, was low at 25°C for 4 – 8 hours, but was considerable at 38°C for 4 hours. Storage at 15°C for 24 hours and 25°C for 4 hours was considered safe and storage at 38°C was not considered safe (Hamosh and others 1997). These results are similar to the present study in that bacterial growth in unpasteurized and pasteurized breast milk stored at room-temperature was minimal for 6 hours. However, bacterial counts actually decreased between 6 and 9 hours, at which point they began to increase.

Sosa and Barness (1987) examined bacterial growth in 41 samples of freshly expressed unpasteurized breast milk stored at 4°C for five days. Similar to the results of the present study, they also found that bacterial colony counts decrease throughout the five day refrigeration period. All bacteria cultured were those normally found on the skin and nipple of the breast with the exception of three samples – two containing *Klebsiella* and one with *Pseudomonas* (Sosa and Barness 1987).

Igumbor and others (2000) also examined bacterial growth in freshly expressed unpasteurized breast milk, but under various storage conditions. Bacterial growth was not evident in stored breast milk after 8 hours at 4 – 10°C and 15 – 27°C. However, they did detect growth at 8 hours at 38°C. After 24 hours of storage, samples stored in the refrigerator

had no bacterial growth, whereas colonies were detected in samples stored at 15 – 27°C and 30 – 38°C. During storage for 48 – 72 hours all samples stored at temperature ranges 4 – 10°C, 15 – 27°C, and 30 – 38°C showed growth after 48 hours, with higher colony counts in the higher temperatures (Igumbor and others 2000).

Several studies have shown that bacteria added to pasteurized milk grow more rapidly than those added to raw human milk. Ford and others (1977) subjected human milk to holder pasteurization and examined its content of immune factors. Holder pasteurization, which was the heat treatment used in the present study, reduced the IgA titer by 20% but lysozyme was stable (Ford and others 1977). Similarly, Gibbs and others (1977) observed a 21% reduction in IgA concentration when breast milk was subjected to the holder pasteurization method. However, a 36% reduction in lysozyme activity as well as a decrease in the ability of the milk to inhibit the growth of *E. coli* was also observed (Gibbs and others 1977). Eyres and others (1978) also subjected human milk to holder pasteurization, and it resulted in a 99.99% decrease in the colony count of the breast milk. Only non-pathogenic bacteria remained, and both *E. coli* and *S. aureus* were destroyed. There was a reduction in SIgA, but lysozyme activity remained stable. The breast milk was still able to inhibit the growth of *E. coli* after pasteurization (Eyres and others 1978). These results indicate that the decrease in SIgA, rather than a decrease in lysozyme, may be the cause of the increased growth of added pathogens in pasteurized milk versus raw milk.

However, these studies did not examine bacterial growth in pasteurized and unpasteurized breast milk after an infant has partially consumed and contaminated the milk.

The present study shows that, based on acceptable bacteria counts of 10^5 CFU/ml, pasteurized breast milk may be stored longer at 24°C and 4°C than unpasteurized breast milk, after a baby has drunk from and contaminated both types of milk. Also, the data show that bacteria originating from the mother are greater than bacteria originating from contamination by an infant.

As evidenced by the previously cited studies, lysozyme is still active in the pasteurized breast milk, which could be the cause of the decrease in bacteria observed during room-temperature storage for 6 – 9 hours and refrigerator storage for 0 – 2 days. In pasteurized breast milk stored in the refrigerator, the bacteria increase to $1.25x10^4$ at 4 days and decrease to $1.12x10^3$ at 6 days of storage. These results are interesting because there is typically a decline in bacteria in breast milk during the first 24 – 48 hours of storage at 4°C. However, the decline in these breast milk samples occurred between 4 – 6 days of storage at 4°C. A possible explanation of these results could be that lysozyme was partially denatured by the heat treatment and experienced a delay in renaturation of its native formation (Dubois and others 1982).

There is always a fear of possible contamination from pathogenic bacteria in stored breast milk. However, the most common organisms found in the 108 freshly expressed, unpasteurized breast milk samples collected from 40 women by El-Mohandes and others (1993) include *Staphylococcus epidermidis* (82%) and *Acinetobacter* (9%). Other bacteria isolated are listed in Table 1.10 (el-Mohandes and others 1993). *Staphylococcus epidermidis* is part of the normal skin microbial flora and has been shown to be safe (Law and others

1989). The organisms most commonly isolated, including gram-positive, coagulase-negative staphylococci and gram-negative *Acinetobacter* are also known skin colonizers (Botsford and others 1986).

Björkstén and others (1980) pooled milk samples from 8 donors and inoculated them with 156 bacterial isolates, including 85 strains of *E. coli*, 15 strains of *Klebsiella pneumoniae*, 10 strains of *Enterobacter* sp, 5 strains of *Citrobacter* sp, *Serratia marcescens*, 5 strains of *Pseudomonas aeruginosa*, 15 strains of *S. aureus*, 10 strains of *S. epidermidis*, and 6 gram-negative non-fermenting psychrotrophic isolates from sewage. All were inoculated at 10^7 CFU/ml. No bacteria grew during incubation at 4°C for 10 days (Bjorksten and others 1980). The authors conclude that breast milk contaminated with various bacteria can be safely stored at 4°C and 6°C for at least 72 hours.

3.6. Conclusion

Acceptable levels of bacteria were chosen as 10^5 colony forming units per milliliter (CFU/ml). Based on the data, after a baby has drunk from and contaminated a bottle of breast milk, it appears that unpasteurized breast milk can be stored up to 6 – 9 hours and pasteurized breast milk between 9 – 24 hours at 24°C. Unpasteurized breast milk can be stored between 4 – 6 days and pasteurized breast milk can be stored longer than 6 days at 4°C. Also, the data show that bacteria originating from the mother are greater than bacteria originating from contamination by an infant. However, more research is needed with a larger number of participants and focusing on the growth of specific strains of pathogenic bacteria in breast

milk contaminated by an infant and stored for a certain amount of time and at various temperatures.

Although studies have shown that pasteurized breast milk has decreased SIgA compared to unpasteurized breast milk (Eyres and others 1978; Ford and others 1977; Gibbs and others 1977), lysozyme is still mostly active and has antibacterial effects. However, before recommendations can be made regarding safe consumption of breast milk that has been contaminated by an infant, future research needs to examine the specific strains of bacteria that grow in breast milk after an infant has contaminated it in order to evaluate the safety of consuming the contaminated breast milk.

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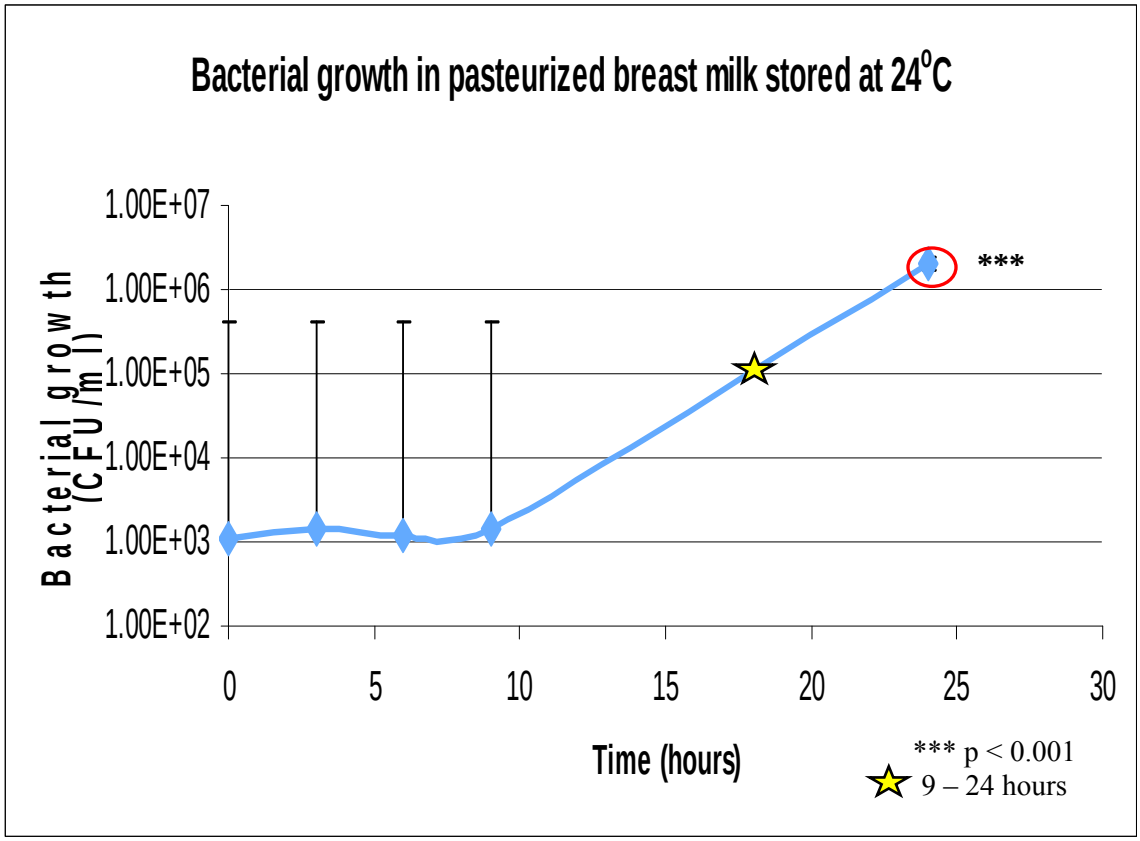

Figure 3.3 Bacterial growth in pasteurized samples stored at 24°C

The red circle indicates the time point at which the change becomes significant.
The yellow star indicates the time at which bacteria counts exceed 10^5 CFU/ml.
Vertical bars show the standard error of the mean.

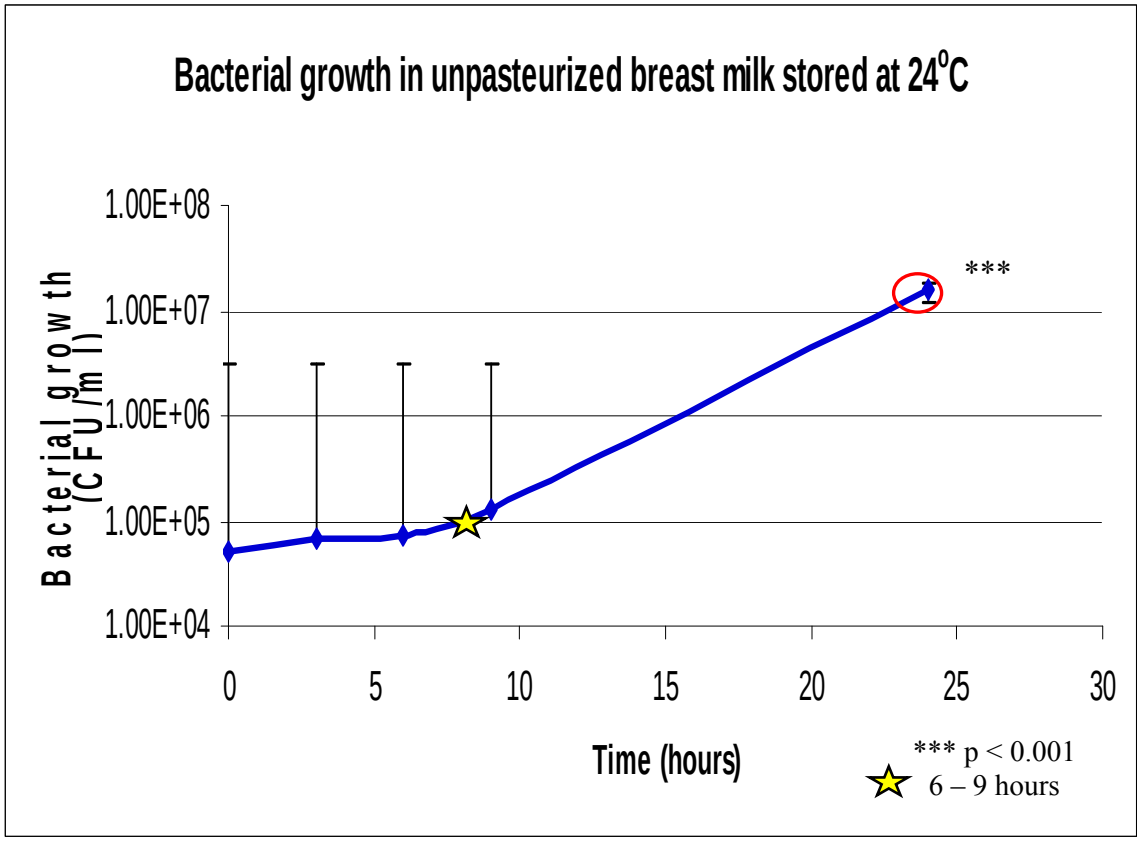

Figure 3.4 Bacterial growth in unpasteurized samples stored at 24°C

The yellow star indicates the time at which bacteria counts exceed 10^5 CFU/ml. Vertical bars show the standard error of the mean.

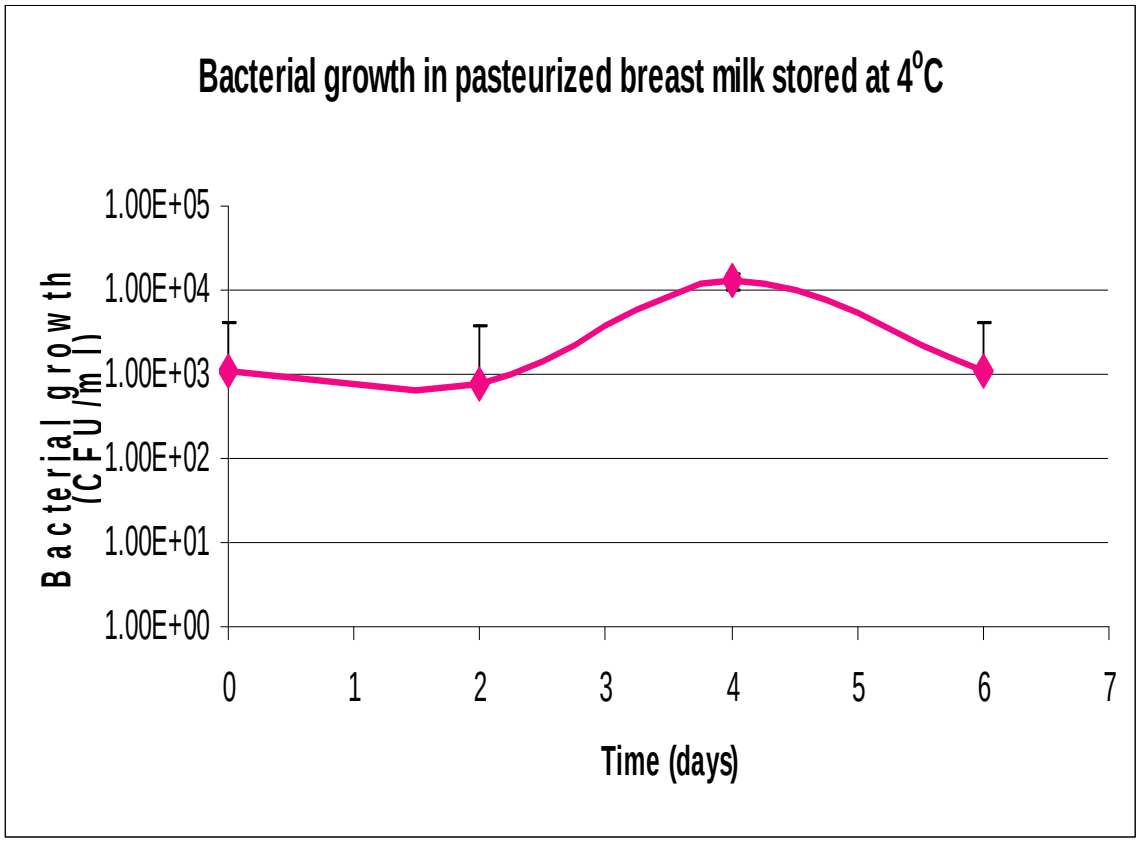

Figure 3.5 Bacterial growth in pasteurized samples stored at 4°C

The yellow star indicates the time at which bacteria counts exceed 10^5 CFU/ml. Vertical bars show the standard error of the mean.

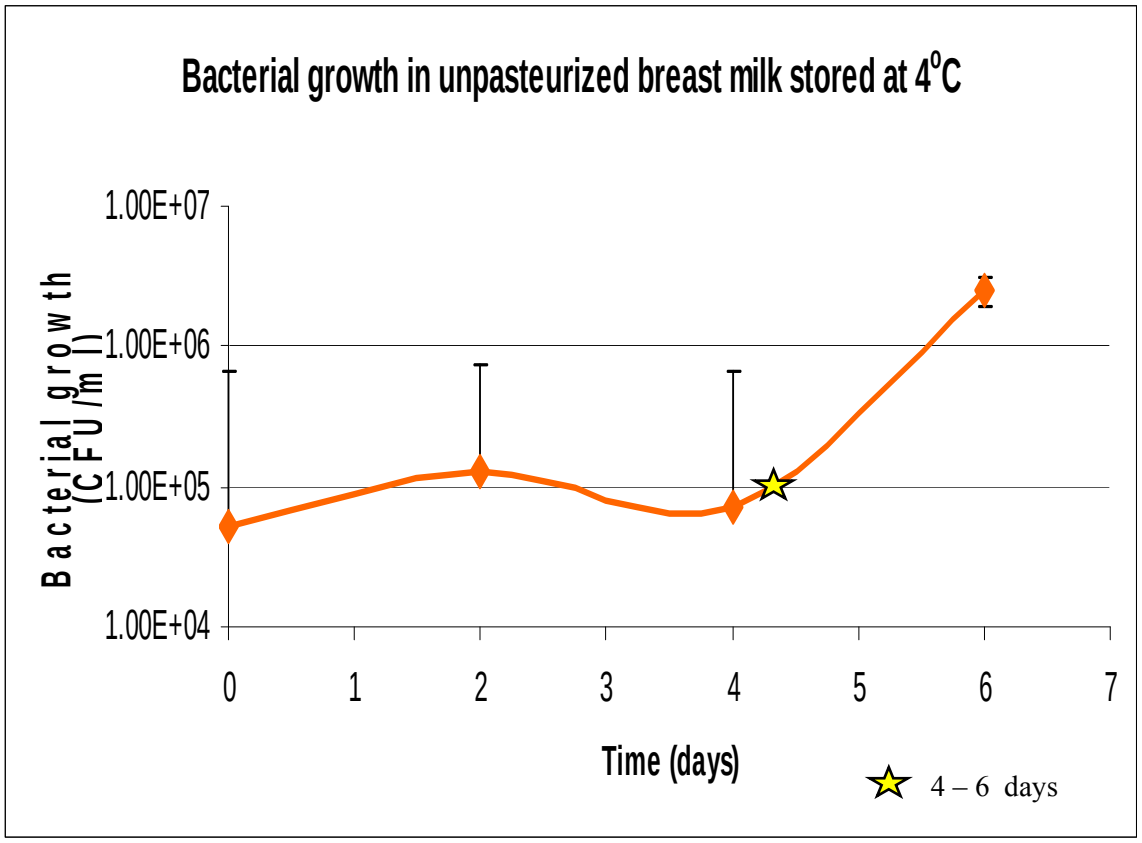

Figure 3.6 Bacterial growth in unpasteurized samples stored at 4°C

The yellow star indicates the time at which bacteria counts exceed 10^5 CFU/ml. Vertical bars show the standard error of the mean.

CHAPTER 4

4.1 Conclusion

Human breast milk provides many nutritional and immunological benefits to the infant during the time of life as it is probably nature's most perfect food. The issue of breast milk storage is one of high importance as breast milk is often stored at home, in the child care center, at the mother's workplace, in donor milk banks, and in hospitals, particularly the NICU. Guidelines for the proper storage of milk need to be established based on evidence. Currently, there is very little evidence on which to base the guidelines. Many studies that have examined the effect of storage temperature and time on protein, fat, free amino ends, fatty acids, lysozyme, and SIgA have not stored breast milk at the times and temperatures used in the present study. Rather, storage time has only been examined in a matter of days, yet storage guidelines are based upon these studies.

Two objectives were investigated in this study. First, the effects of storage temperature and time on components in freshly expressed, human breast milk were determined. Second, the difference in bacterial growth in breast milk contaminated by an infant in mother's own milk and donor milk were determined to investigate if it is safe for infants to drink breast milk from a bottle, store it for a period of time, and then drink from it again.

Participants volunteered for the study with informed consent. The protocol and consent form was submitted to the North Carolina State University Institutional Review

Board and approved. Twelve breast milk samples were collected from 12 women within the community. The samples from women within the community were collected with breast pumps and the entire content of one breast was collected and transferred into sterile containers. The entire content of one breast was collected because milk composition changes from the beginning of feeding to the end of the feeding. Milk collected from women in the community was collected at any time that was most convenient for them during day one of the study.

The samples of milk were placed immediately on ice for transport to the laboratory. Upon arrival at the laboratory, samples of milk were aliquotted for storage in a -20°C freezer, in a refrigerator (4-6°C), and at room-temperature 24°C in stoppered test tubes. Samples were removed at 24 hours, 48 hours, one week, two weeks, and three weeks for analyses and quantitation of proteolysis, lipolysis, and bacterial growth.

Analytical techniques were performed for total protein, free amino groups, total fat, NEFA, lysozyme, IgA, and bacterial growth. The independent variables in this study are stage of lactation, storage temperature, and the length of storage. The dependent variables are the extent of proteolysis, lipolysis, bacterial growth, SIgA activity, and lysozyme activity.

The second part of the study examined the effect of an infant suckling from a bottle of pasteurized breast milk and a bottle of unpasteurized breast milk on subsequent quality of milk remaining in the bottle. Partially consumed bottles were stored at room and refrigerator temperatures and assayed for changes in bacterial load over the course of hours and days.

More data are needed on the ability of stored milk to reduce its bacterial load when it is newly contaminated by an infant.

Pasteurized breast milk was compared to unpasteurized breast milk from individual mothers in order to study the differences in the bacterial growth in these two groups of milk once they have been contaminated by an infant. It is imperative to study this question in *pasteurized breast milk* because many infants drink banked breast milk, which is pasteurized. This is especially true in neonatal intensive care units. Many infants who consume banked breast milk are premature and/or sick, and this is a population of concern. However, findings may also be applied to individuals' own *unpasteurized milk* because the infants will also consume one or two ounces from a bottle containing their own mother's unpasteurized milk.

This study was aimed at benefiting premature and sick infants consuming banked breast milk. The findings may be applicable in neonatal intensive care units, as well as childcare facilities, milk banks, and in the home. Because banked milk is expensive and the number of mothers donating is low, it would be beneficial to infants in neonatal intensive care units if the banked milk could be safely stored for a longer period of time after the infant has already drank from the bottle. This would prevent a great deal of banked breast milk from being thrown away, when the infants could still safely consume the milk and benefit from it. The study is not focusing on consumer practices.

Based on the data in this study, it appears that breast milk may be stored longer than the current recommendations. Results of this study provide evidence that breast milk may be stored in the freezer at -20°C for longer than three weeks, in the refrigerator at 4°C for up to

2 weeks, and at room temperature (24°C) for up to 48 hours. However, the impacts of increased NEFA are controversial and must be taken into consideration when storing breast milk. Also, specific strains of bacteria should be examined.

Based on the data, after a baby has drunk from and contaminated a bottle of breast milk, it appears that unpasteurized breast milk can be stored up to 6 – 9 hours and pasteurized breast milk between 9 – 24 hours at 24°C. Unpasteurized breast milk can be stored between 4 – 6 days and pasteurized breast milk can be stored longer than 6 days at 4°C. Also, the data show that bacteria originating from the mother are greater than bacteria originating from contamination by an infant. However, more research is needed with a larger number of participants and focusing on the growth of specific strains of pathogenic bacteria in breast milk contaminated by an infant and stored for a certain amount of time and at various temperatures.

Although the breast milk samples in the present study can be stored for the indicated amount of time without detrimental changes to the components in breast milk that were examined, more research needs to be done in order to make definitive guidelines. Safe limits for bacterial concentration in human milk used to feed premature infants have never been established (Jones and others 2000; Law and others 1989) but they need to be established in regards to breast milk. In addition to establishing safe limits for bacterial concentration in human milk, specific strains of bacteria need to be identified in breast milk that has already been contaminated by an infant, in both pasteurized and unpasteurized breast milk.

The affects of NEFA on infants in regards to fat, fatty acid, and calcium absorption, as well as breast milk jaundice needs to be determined as there are many conflicting results. It has been shown that NEFA may increase bioavailability of fat to the infant when duodenal bile salts are low (Hernell and Blackberg 1982) but that NEFA may bind calcium (Patton and Carey 1979) and form fatty acid soaps (Lavine and Clark 1987). NEFA have been implicated in the development of breast milk jaundice in the neonate (Hargreaves 1973; Lavine and Clark 1987; Poland, Schultz, Garg 1980) but the association could not be found in other studies (Constantopoulos, Messaritakis, Matsaniotis 1980; Jalili and others 1985). The antiviral effects of NEFA must also be considered as a positive consequence of increased NEFA (Ogundele 2000; Thormar and others 1987).

This study provides scientific evidence for time limits for safe breast milk storage at several temperatures. The data suggest that existing guidelines are conservative. Comparing bacterial counts in the two experiments in this study indicates that an infant suckling from a bottle of breast milk contributes no more bacteria than does pumping, and refeeding from the same bottle at a later time should be acceptable. Additional research with a larger sample size should be conducted to confirm these experiments.

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APPENDICES

Appendix A. Informed Consent Form for Part One of the Study

North Carolina State University
Informed Consent Form

Principal Investigators: Jonathan C. Allen, April Fogleman, Mary Rose Tully

Purpose of the study

You are invited to participate in a research study. The purpose of this study is to gain understanding on the keeping quality of human milk for mothers who express milk the baby would not consume, and feed it later, such as in a day-care setting. Samples of your donated breast milk will be subjected to experimental procedures and analyzed for nutritional and bacterial counts. The object of the study is to determine the optimal storage temperature and time for human breast milk and to learn more about bacteria counts in breast milk.

What will happen if you take part in the study?

If you participate in this study, we will ask you to express the entire contents of one breast. The sample will be collected at a time and location that that is mutually agreeable, such as your home or workplace. Our graduate assistant will be present to collect the breast milk samples after you have expressed the milk. Bacterial cultures and experimental procedures will be conducted in our laboratory. You will be asked questions about the age and weight of your baby.

Benefits

Knowledge will be gained to help others in learning the best time and temperature combination to store breast milk. This knowledge may help the participants for breast milk storage later in life.

Risks

There are no known risks associated with participating in this study.

Compensation

You will not receive anything for participating in this study.

Confidentiality

The information in the study records will be kept strictly confidential. Results of our analysis will be secured and is available only to the researchers for this study unless you specify otherwise. No reference (oral or written) will be made that can link the participants to the study.

If you have questions about the study

If you have any questions at any time about the study or the procedures, you may contact the researcher, April Danielle Fogleman, at 216 Schaub Hall, NCSU, Raleigh, North Carolina 27695, or by phone (919) 247-7971. You may also contact Dr. Jonathan Allen, at 218 Schaub Hall, NCSU, Raleigh, North Carolina 27695 or by phone (919) 513-2257.
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), or Joe Rabiega, IRB Coordinator, Box 7514, NCSU Campus (919/515-7515).

Your participation in this study is voluntary; you may decline participation without penalty. If you decide to participate, you may withdraw from the study at anytime without penalty. If you withdraw from this study before data collection is completed your data will be destroyed.

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 withdraw at any time.

*Participant's Signature*_____*Date*_____
*Investigator's Signature*_____*Date*_____

Appendix B. Informative Letter to Participants about the Study

Hi Ladies,

Thank you for your willingness to participate in this project. I would like to give you some more details. As I mentioned in my previous email, I am trying to determine the best time and temperature combination to store breast milk without losing nutritional quality (such as protein, fat, and the immune factors) and without causing an increase in bacterial growth.

The research we are doing on the donated breast milk samples will look at three factors

1. The effect of storage time and temperature of human breast milk on milk protein breakdown. We will measure total protein, free amino acids, and the activity of immunological factors such as secretory IgA and lysozyme. The amount of amino acids available for protein synthesis is important for premature babies, but an increase in protein degradation signals a decline in milk quality.

2. The effect of storage time and temperature of human breast milk on the total fat and free fatty acid content of the milk. The amount of total fat and free fatty acids in human milk are important to infant health because high levels of free fatty acids are not as available for absorption. Free fatty acids are more easily oxidized when they are not in the triglyceride form. Calcium soaps are not absorbable, so calcium availability for absorption is decreased as well. Both the milk itself and bacteria that may grow contain lipase that can elevate free fatty acids during prolonged storage, signaling reduced milk quality.

3. The effect of storage time and temperature of human breast milk on bacterial contamination. Normally, human milk anti-bacterial factors will reduce the bacterial count after its secretion. With a high bacterial load or after prolonged storage, the antibacterial factors could be used up, bacterial counts could again increase, or the milk could be less effective at attacking bacterial pathogens in the infant's gut. More data are needed on the ability of stored milk to reduce its bacterial load when it is newly contaminated. For example, if an infant reuses a bottle or if milk is stored at certain temperatures will bacterial growth increase?

We would appreciate your assistance with the following

On July 21st, we need you to pump the entire contents of one breast as you normally would and immediately put the milk into sterile container(s) that will be provided. You will then place the sterile container(s) containing the milk into the refrigerator. We will send two sterile containers in the mail to your home a few days before July 21st so that you can express the milk anytime during the day of July 21st that is convenient for you.

On the container please write your name, the time of milk expression, and the date of your child's birth (so we know your stage of lactation). On the same day, I will drive to your home or work (or wherever is most convenient for you) and pick up the sample of milk at a given time. We will give you a consent form for participating in our research study at that time.

If you are still interested in participating, please send me the following information so I can plan when and where to pick up your sample of milk on July 21st. All information will remain confidential.

- Place (address) and time you would like me to pick up your milk sample

Please let me know if you have any questions. Thank you for your help!

Sincerely,
April Fogleman

Appendix C. Informed Consent Form for Part Two of the Study

North Carolina State University
Informed Consent Form

Principal Investigators: Jonathan C. Allen, April Fogleman, Mary Rose Tully

Purpose of the study

You are invited to participate in a research study. The purpose of this study is to gain understanding on the keeping quality of human milk for mothers who express milk the baby would not consume, and feed it later, such as in a day-care setting. Samples of your donated breast milk will be subjected to experimental procedures and analyzed for bacterial counts. The object of the study is to determine how long breast milk can be stored after a baby has drunk from the bottle as well as to observe the difference in bacterial growth in breast milk contaminated by an infant in mother's own unpasteurized milk and mother's own pasteurized milk.

What will happen if you take part in the study?

If you allow your baby to participate in this study, we will ask that you arrange a time for the research assistant to come to your house and collect a bag of your pumped breast containing 2 - 3 ounces of milk. The research assistant will bring this milk to the laboratory at NC State and pasteurize it according to a specific protocol called the holder pasteurization method, in which the milk will be transferred into a sterile container, held and gently agitated in a water bath at 66°C for 30 minutes. Pasteurization will destroy any pathogenic (disease causing) microorganisms that may have been originally present in the milk. After pasteurization, the milk will be immediately cooled to and held at refrigeration temperature (4°C).

On the day of the experiment, the researcher will bring the pasteurized milk back to you at a convenient location and you will be asked to prepare bottles of both the pasteurized milk and your own, unpasteurized breast milk (approx. 2 – 3 oz). You will then be asked to feed you baby 1 oz from each bottle. The bottles will be taken away with approximately 1 – 2 oz. of fluid remaining and provided back to the researcher. Feeding can continue if necessary using any other milk the mother has available.

If it is alright with you, we would like to perform the initial bacterial analysis in your home. It would require minimal table space and it only takes 5 minutes. However, if this is not okay we can perform the initial bacterial analysis elsewhere.

Benefits

Knowledge will be gained to help others in learning how long a bottle may be stored for reuse after the baby has drunk from it. The study is aimed at benefiting infants consuming unpasteurized breast milk as well as premature and sick infants consuming pasteurized banked breast milk. The pasteurized milk will be representative of pasteurized donor banked milk because it will be pasteurized the same way as in the Wake Med milk bank.

The findings will be applicable in neonatal intensive care units, as well as childcare facilities, milk banks, and in the home. Because banked milk is expensive and the number of mothers donating is low, it would be extremely beneficial to infants in neonatal intensive care units if the banked milk was stored for a longer period of time after the infant has already drank from the bottle. This would prevent a great deal of banked breast milk from being thrown away, when the infants could benefit from it.

Risks

There are no known risks associated with participating in this study.

Compensation

You will not receive anything for participating in this study.

Confidentiality

The information in the study records will be kept strictly confidential. Results of our analysis will be secured and is available only to the researchers for this study unless you specify otherwise. No reference (oral or written) will be made that can link the participants to the study.

If you have questions about the study

If you have any questions at any time about the study or the procedures, you may contact the researcher, Dr. Jonathan Allen, at 218 Schaub Hall, NCSU, Raleigh, North Carolina 27695 or by phone (919) 513-2257.

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), or Joe Rabiega, IRB Coordinator, Box 7514, NCSU Campus (919/515-7515).

Your participation in this study is voluntary; you may decline participation without penalty. If you decide to participate, you may withdraw from the study at anytime without

penalty. If you withdraw from this study before data collection is completed your data will be destroyed.

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 withdraw at any time.

*Participant's Signature*_____*Date*_____
Investigator's Signature_____Date__