ABSTRACT

ULUS, HANDE ZEYNEP. Processing Human Milk to Increase Nutrient Density for Preterm Infants. (Under the direction of Dr. Jonathan C. Allen).

Breast milk is the optimal food for newborns. Preterm birth might result in problems with the initiation of lactation and limitation of suckling reflexes. Choices to feed preterm infants in neonatal intensive care units are mother's milk, donor milk, or formula. Concerns regarding the selection of feed are catch-up growth and health effects. Even though formula acquires faster catch-up growth, it is not as tolerable as human milk and increases the risk of necrotizing enterocolitis. Preterm infants have better tolerance for human milk, but the lower caloric density of term mothers' milk or donor milk might not meet preterm infant growth needs. Preterm infants have higher protein and energy requirements with a limited stomach capacity. Therefore, the best practice is using breast milk but with a need for increased nutrient density. Aims of this study were to concentrate donor breast milk to have a higher caloric density and protein, but at the same time avoiding side effects of high lactose concentration by precipitating lactose at low temperature. Donor breast milk was obtained from WakeMed Mothers' Milk Bank. Half of the samples were homogenized. Preliminary data found that low-temperature removal of lactose from unprocessed human milk was minimal. Therefore, condensation was applied before lactose removal. Volume reductions were 80%, 60%, 50%, 40%, 30% and 0% respectively. Subsequently, samples were held at 0°C overnight, followed by refrigerated centrifugation for lactose removal at 0°C. Supernatants were separated. Lactose, nitrogen, osmolality, and viscosity were measured. A significant reduction in lactose was achieved. A 30-40% volume reduction is under the American Academy of Pediatrics recommended osmolality for infant feeding. There was no significant reduction in protein. Thus, concentrating human milk in a milk bank setting for

the use of preterm infants might be a simple and low-cost process to achieve a product with higher nutrient density and no non-human components for feeding to preterm infants.

Processing Human Milk to Increase Nutrient Density for Preterm Infants

by
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DEDICATION

To my parents, Hayriye and Rahmi, for everything you did for me through my life and academic career. To my sister, Özge, for making me laugh under any condition. To my roommate, Merve, for all her support. To my teachers, for constantly improving me. To my friends, for making me feel at home away from home.

BIOGRAPHY

Hande was born and raised in Samsun,Turkey. She studied Nutrition and Dietetics in Hacettepe University and she finished her dietetic internship in Ankara, Turkey. Meanwhile, she got the Fulbright Scholarship and was accepted to North Carolina University. During her education, she became interested in human milk research and she had the opportunity to study on the subject. She is planning to stay at NCSU to pursue a PhD degree.

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CHAPTER 1:

Introduction

Breastmilk is the optimal nutrition for newborns and is the recommended way to feed every newborn[1,2]. Preterm infants should receive breast milk, and it should be mother's own milk if it's possible[1]. Human milk banks provide breastmilk to preterm and sick infants when mother's milk is not available. Human milk needs to be fortified to meet the high nutritional demand of a preterm infant[1]. Available fortifiers are cow's milk based powder and liquid, and human milk-based fortifier.

Exclusive human milk diet (human milk + human milk-based human milk fortifier) gives better outcomes for preterm infants for necrotizing enterocolitis[3]. However, commercial human milk based fortifiers are very costly. Therefore, there is a need in human milk banks to increase the nutrient density of human milk in their settings.

Preterm infants have a higher requirement for protein. The focus in the preterm infant nutrition has been shifting from energy alone to characterizing the protein to energy ratio[4]. High energy intake is associated with high fat deposition which might increase non-communicable disease risk in the long term[4]. On the other hand, high protein intake does not have such a side effect. Furthermore, increasing protein intake promotes growth, lean body mass, and long-term developmental outcomes[5].

Mothers, who donate their milk to the milk bank, are often term mothers with surplus milk supply. The composition of term mother's milk is different than preterm mother's milk[6]. Preterm mother's milk is more compatible with preterm infant's requirement. Preterm mother's milk is higher in protein and lower or similar in lactose compared to the term mother's milk, which is closer to the preterm infant's nutritional requirement[6]. Therefore, there is a need to increase energy and protein content in human milk while avoiding excessive levels of lactose.

Lactose is an osmotically active disaccharide. It can be crystallized when lactose concentration exceeds the solubility product constant for a given temperature[7]. The extreme concentration of lactose increases osmolality[8]. Hyperosmolar feeding is one of the proposed risk factors for necrotizing enterocolitis[9]. Therefore, lactose can be removed from milk by crystallizing and precipitating, which would also result in osmolality reduction. Increasing the energy and protein content of the milk can be achievable by concentrating the milk by removing the water from it. Evaporation, freeze-drying, reverse osmosis, and nanofiltration are possible methods to concentrate the milk. In this research, we used evaporation.

The goal of this study was to remove lactose from the human breastmilk efficiently to avoid hyperosmolality while increasing protein content by concentrating it. We hypothesized that there would be no significant difference in lactose, protein and osmolality levels between before (control) and after (supernatant) the chilled centrifugation lactose removal process. To observe concentration effects on the lactose removal we hypothesized there is no significant difference in lactose reduction between different concentration levels.

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CHAPTER 2:

Literature Review

2.1 Preterm birth

The gestational period, under normal circumstances, is expected to last for 40 weeks and any birth that occurs before 37 weeks is a preterm birth. Preterm births can be subdivided into three categories based on the completed weeks of gestation: extremely preterm (<28 weeks), very preterm (< 28-32 weeks) and moderate/late preterm (<32-37 weeks)[1]. The completed weeks of gestation (or the gestational age) have an impact on the chance of survival at the birth. The gestational age is associated with the chance of survival, which means while the amount of time for intrauterine development gets lower, the chance of survival for the newborn decreases[2]. Any birth that happens before the 24th week has approximately 50% chance of survival when managed in neonatal intensive care units that can be found in high-income countries. In most of the low and middle-income countries, outcomes are much worse than high-income countries; births that happen up to the 34th week of gestation have the 50% chance of survival[2].

The high incidence of preterm births directly affects public health outcomes. In 2010, nearly 15 million babies were born prematurely, and 1.1 in every ten births resulted in preterm birth[3]. Health consequences related to preterm birth are linked to 8 Millennium Development Goals (MDG). MDG's are an anti-poverty movement that works on improving the population disparities such as gender equality, primary education, environmental

sustainability, fighting against extreme poverty and hunger and infectious diseases, and also works on reducing child mortality and improving maternal health[1]. Due to actions triggered by Millennium Development Goals, the number of children who died under five years of age decreased from 9.6 million in 2000 to 5.9 million in 2015 with reductions in the primary causes of death such as pneumonia, preterm birth complications, diarrhea, intrapartum-related complications and malaria[4]. Although it is a significant improvement, there is still a lot of work needed, especially to increase healthy outcomes for preterm infants.

Another concern for the newborn baby is birth weight. Children who were born under 2500 g are classified as low birth weight (LBW), under 1500 g are classified as very low birth weight (VLBW) and under 1000 g are classified as extremely low birth weight (ELBW). Neonatologists consider the growth of the baby during gestation and the birth weight. If the infant is very preterm, then it is more likely to be extremely low birth weight (ELBW). Being very preterm, ELBW or intrauterine growth restricted (IUGR) adds more challenge to the newborn's life in the first days, weeks, years, or for even longer time[5].

In the short term, preterm birth and ELBW are the challenges of survival for newborns. Preterm birth is the second largest cause of child death after pneumonia in children under five years of age and directly the biggest reason of neonatal death. When complications are included, it is estimated to cause 35% of neonatal mortality[3]. Preterm birth is the leading cause of child death under five years of age in middle and high-income countries where infections are more likely to be prevented[2].

Besides directly causing death, preterm birth increases the risk for diseases and organ malfunctions due to underdevelopment. The systems that can be affected by prematurity are

lungs and respiratory system, skin, cardiovascular system, immune system, hematologic system, auditory system, ophthalmic system and central nervous system. And the most frequent complications are respiratory distress syndrome, necrotizing enterocolitis (NEC), retinopathy of prematurity (ROP), and neurodevelopmental diseases[6].

2.2 Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is the inflammatory disease of the infant gastrointestinal tract. It is more common in preterm and small for gestational age infants than term and appropriate for gestational age infants[7]. Also, it is the most common GI tract disease in preterm infants and affects 10% of the VLBW infants[8]. It causes destructive changes and may require surgery to remove a portion of preterm infant's gut. Surgery increases the risk of death for the preterm infant. The mechanism for NEC is not well understood, but risk factors are prematurity, enteral feeding, ischemia, and abnormal bacterial colonization in the intestinal tract[8]. Most possible contributors to the risk of NEC are enteral feeding and prematurity[9]. The risk for NEC has been shown to be lower with human milk feeding versus formula feeding[10,11]. Risk factors for NEC are given in Table 1[12].

Table 1: Risk Factors for Necrotizing Enterocolitis

-Prematurity
-Intrauterine growth restriction
-Abruptio placentae
-Premature rupture of membranes
-Perinatal asphyxia
-Low Apgar score
-Umbilical catheterisation
-Hypoxia and shock
-Hypothermia
-Patent ductus arteriosus
-Non-human milk
-Hypertonic feeds
-Rapid introduction of enteral feeds
-Fluid overload
-Pathogenic bacteria
-Polycythaemia
-Thrombocytosis
-Anaemia
-Exchange transfusion
-Cyanotic congenital heart disease

2.3 Importance of nutritional decisions

Nutritional decisions that a pediatrician makes are crucial for every infant. It is important for a preterm infant to catch up to the gestational size of full term infants (termed "catch up growth") and to maintain a stable metabolic state[13]. Preterm infants miss the last weeks of gestation when they could have grown rapidly. It is a challenge to catch up with intrauterine growth rates in a neonatal intensive care unit (NICU). Preterm infants usually fail to grow adequately in the first weeks of life due to intolerance to enteral feeding, unstable

metabolism and weight loss after the birth[14,15]. The goal of nutrition for preterm infants is optimizing growth by avoiding the adverse outcomes such as neurodevelopmental problems, NEC, infections, and the long-term obesity.

One goal for nutritional decisions is to maintain intrauterine growth rate after the birth[16]. Achieving this is challenging because of the reasons mentioned above. In spite of the attempts to catch up with intrauterine growth rate, standard feeding methods provide higher glucose and lipids and significantly lower protein compared to the nutrient accretion of a fetus of the same gestational age[17]. Long-term nutritional interventions for catch-up growth that provide a higher intake of protein were found to be related to reduced neurodevelopmental deficits. On the other hand, instead of long-term aggressive nutritional interventions, maintaining a beneficial undernutrition is related to lower risk for adverse health outcomes such as hypertension, obesity, cardiovascular disease and type 2 diabetes later in life. Due to the change in the body composition, catch-up growth strategy might be beneficial for the short term, but for the long term, aggressive nutritional interventions can be harmful. Even though there are minimal scientific data to create guidelines and strategies for preterm nutrition, decisions should be made individually by monitoring the growth rate[17].

2.4 Nutrient Requirements

As mentioned, there are minimal data to create guidelines for preterm infant nutrition. Guidelines are usually prepared based on LBW infants because data collection is possible for that group[18]. Nutritional decisions in a NICU are often made by monitoring the growth and adverse effects or maintaining the level of nutrient concentration in blood and tissues [18,19].

With the limited number of randomized control trials, American Academy of Pediatrics (AAP) and The European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) stated lower and upper limits and recommendations for macro and micro nutrients. Tables 2. and 3. give the recommended level of intake for macro- and micro-nutrients.

For fluid intake, ESPGHAN stated that recommended intake would be between 135 – 200 mL/kg/day. Considerations when setting the level is that higher intake might increase the risk of morbidity due to complications such as pulmonary dysplasia, and lower intake would increase osmolality and increase renal solute load which would harm the kidney and causes dehydration. Another consideration would be the stomach capacity[18].

Energy requirement may vary between infants due to activity, thermal balance, growth, absorption, and receive enteral or parenteral nutrition. Also, being SGA, IUGR, and appropriate for gestational age (AGA) makes a difference. SGA preterm infants might need more energy compared to AGA preterm infants, but the ratio between fat-free mass and fat mass should be monitored. Even though it is expected for a preterm infant to deposit more body fat to maintain thermoregulation, body composition should not change dramatically compared to the same gestational age fetus[18,19]. ESPGHAN states a range of 110-135 kcal/kg/day[18]. APA reports a range of 105 – 130 kcal/kg/day, but it can increase if the growth rate is low[19].

Protein intake is essential for optimal growth and neurodevelopmental outcomes. A deficit might cause growth restriction and lower cognitive scores[18,20]. Amino acid composition of the protein is important when calculating requirements but specific needs for

each amino acid is not clear. Therefore, quality of protein may interfere with the need. Protein requirement can be calculated by the need of the fetus at the same gestational age added to excretion of an infant[18]. Randomized control trials are made to define outcomes of the intake levels. ESPGHAN states that intakes in the range of 3 – 4.5 g/kg/day would give acceptable plasma protein results. And weight gain is positively correlated with protein intake up to 4.5 g/kg/day. For ELBW's this number can go up to the upper level of this range due to a higher need for tissue build up. A lower level of 3.5 – 4.0 g/kg/day can meet the requirement of LBW infants[18]. AAP states the requirement of 3.8 – 4.4 g/kg/day for ELBW and 3.4 – 4.2 for VLBW infants. Also, AAP does not recommend the soy-based formulas for preterm infants because it interferes with bone growth[21,22]. Guidelines imply that protein deficit can impair the growth, but a slight excess does not do harm.

Carbohydrate in infant nutrition consists of human milk oligosaccharides (HMO's), which cannot be obtained from cow's milk based infant formula, and lactose. Lactose is the primary carbohydrate in milk. It is a disaccharide and immediate energy source for newborn but excessive amounts of lactose increase osmolality. Lactose enhances calcium absorption[23]. Even though lactase activity is low in premature infants, lactose intolerance symptoms are rare. HMO's are prebiotic carbohydrates that promote probiotic bacteria in the gut[24]. HMO's do not increase osmotic load as much as lactose, and they are abundant in human milk. Cow's milk and currently marketed infant formula do not contain HMO's. They may contain bovine milk oligosaccharide, but it is less abundant and consists of different carbohydrates than HMO's. There are over 200 types of HMO's in human milk[25].

The lower limit of the carbohydrate requirement is calculated through the energy expenditure of glucose-dependent organs to avoid ketosis. The upper limit is calculated by the difference between the energy expenditure and minimum protein and fat intake. ESPGHAN recommends a range of 11.6 – 13.2 g/kg/day carbohydrate intake for preterm infants[18]. AAP states that the recommended range for carbohydrate consumption for ELBW infants is 9-20 g/kg/day and for VLBW infants is 7 – 17 g/kg/day[19].

Table 2. Recommended Maximum - Minimum intakes of macro and micro-nutrients by ESPGHAN

Nutrient	**Per $kg^{-1}.day^{-1}$**	**Per 100 kcal**
Fluid, mL	135-200	
Energy, kcal	110-135	
Protein, g<1 kg body weight	4.0-4.5	3.6-4.1
Protein, g 1-1.8 kg body weight	3.5-4.0	3.2-3.6
Lipids, g (of which MCT<40%)	385-1540	4.4-6.0
Linoleic acid,mg	>55 (0.9% of f.a.)	350-1400
α-linolenic acid, mg	12-30	>50
DHA, mg	18-42	11-27
AA, mg		16-39
Carbohydrate, g	11.6-13.2	10.5-12
Sodium, mg	69-115	63-105
Potassium, mg	66-132	60-120
Chloride, mg	105-177	95-161
Calcium salt, mg	120-140	110-130
Phosphate, mg	60-90	55-80
Magnesium, mg	8-15	7.5-13.6

Table 2 continued

Iron, mg	2-3	1.8-2.7
Zinc, mg	1.1-2.0	1.0-1.8
Copper, µg	100-132	90-120
Selenium, µg	5-10	4.5-9
Manganese, µg	≤27.5	6.3-25
Fluoride, µg	1.5-60	1.4-55
Iodine, µg	11-55	10-50
Chromium, ng	30-1230	27-1120
Molybdenum, µg	0.3-5	0.27-4.5
Thiamin, µg	140-300	125-275
Riboflavin, µg	200-400	180-365
Niacin, µg	380-5500	345-5000
Pantothenic acid, mg	0.33-2.1	0.3-1.9
Pyridoxine, µg	45-300	41-273
Cobalamin, µg	0.1-0.77	0.08-0.7
Folic acid, µg	35-100	32-90
L-ascorbic acid, mg	11-46	10-42
Biotin, µg	1.7-16.5	1.5-15
Vitamin A, µg RE, 1 µg ~ 3.33 IU	400-1000	360-740
Vitamin D, IU/day	800-1000	
Vitamin E, mg (α-tocopherol equivalents)	2.2-11	2-10
Vitamin K, µg	4.4-28	4-25
Nucleotides, mg		≤5
Choline, mg	8-55	7-50
Inositol, mg	4.4-53	4-48

Table 3. Recommended enteral intake recommendation and comparison between ELBW and VLBW infants by AAP

	Consensus Recommendations			
	Weight < 1000 g		**Weight 1000-1500 g**	
	g/kg/day	**g/100 kcal**	**g/kg/day**	**g/100 kcal**
Energy, kcal	130-150	100	110-130	100
Protein, g	3.8-4.4	2.5-3.4	3.4-4.2	2.6-3.8
Carbohydrate, g	9-20	6.0-15.4	7-17	5.4-15.5
Fat, g	6.2-8.4	4.1-6.5	5.3-7.2	4.1-6.5
Linoleic acid, mg	700-1680	467-1292	600-1440	462-1309
Linoleate:Linolenate = C18:2/C18:3	5-15	5-15	5-15	5-15
Docosahexaenoic acid, mg	≥21	≥16	≥18	≥16
Arachidonic acid, mg	≥28	≥22	≥24	≥22
Vitamin A, IU	700-1500	467-1154	700-1500	538-1364
Vitamin D, IU	150-400	100-308	150-400	115-364
Vitamin E, IU	6-12	4.0-9.2	6-12	4.6-10.9
Vitamin K, µg	8-10	5.3-7.7	8-10	6.2-9.1
Ascorbate, mg	18-24	12.0-18.5	18-24	13.8-21.8
Thiamine, µg	180-240	120-185	180-240	138-218
Riboflavin, µg	250-360	167-277	250-360	192-327
Pyridoxine, µg	150-210	100-162	150-210	115-191

Table 3 continued

Niacin, mg	3.6-4.8	2.4-3.7	3.6-4.8	2.8-4.4
Pantothenate, mg	1.2-1.7	0.8-1.3	1.2-1.7	0.9-1.5
Biotin, µg	3.6-6	2.4-4.6	3.6-6	2.8-5.5
Folate, µg	25-50	17-38	25-50	19-45
Vitamin B12, µg	0.3	0.2-0.23	0.3	0.23-0.27
Sodium, mg	69-115	46-88	69-115	53-105
Potassium, mg	78-117	52-90	78-117	60-106
Chloride, mg	107-249	71-192	107-249	82-226
Calcium, mg	100-220	67-169	100-220	77-200
Phosphorus, mg	60-140	40-108	60-140	46-127
Magnesium, mg	7.9-15	5.3-11.5	7.9-15	6.1-13.6

2.5 Options for preterm infant feeding

The following sections describes options to feed preterm infants which are parenteral nutrition and enteral nutrition. For enteral feeding, options are mother's own milk, donor milk, and formula, or a combination. Also fortification also should be applied as necessary.

2.5.1 Parenteral feeding

Infants that are ELBW, VLBW, and IUGR might not be able to establish enteral feeding due to gastrointestinal immaturity. Therefore, it might be necessary to start parenteral

nutrition in the first hours of life to avoid catabolism for a few weeks[19,26]. It is important to start early nutritional support for preterm infants because the protein and energy intake in the first week might affect developmental outcomes measured at 18 months[27]. Early aggressive nutrition, both parenteral and enteral, improves the outcomes in the short term[27]. Early aggressive nutrition can be defined as using a parenteral nutrition method to avoid the interruption of nutrient flow from birth until the establishment of full feeding by providing relatively high amino acids for a short term[28].

Parenteral nutrition helps to meet the nutritional needs when enteral intake is minimal, keeps the infant metabolism anabolic, and increases the chance of survival, but prolonged parenteral nutrition might have detrimental effects such as enteral feeding intolerance, central venous catheter-related sepsis, and parenteral nutrition associated cholestasis[26–28].

A fetus swallows large amounts of amniotic fluid in utero, which enhances the growth and maturity of the intestine[29]. Interrupting enteral intake with long-term exclusive parenteral nutrition causes villus atrophy. Therefore it should be avoided. Trophic feeding is a relatively recent concept that aims to prevent the adverse effects of enteral starvation[26]. As an initiation, a small volume of enteral feeding (10-20 mL/kg/day) is provided for at least five days. Trophic feeding starts gut mobilization and hormone secretion while avoiding a full load of enteral feeding. It enhances the adaptation of the gastrointestinal tract to enteral feeding and prevents feeding intolerance later[27].

The decisions on parenteral feeding and trophic feeding should be made individually. During this period, the newborn should be monitored carefully. Even though catch-up growth

is a controversial concept, failure to thrive should be avoided for optimal long-term growth and developmental outcomes.

2.5.2 Enteral feeding

2.5.2.1 Breastfeeding

Breastmilk is the optimal recommended nutrition for newborns. There are many benefits of breastfeeding for both infant and the mother. For mothers, breastfeeding empowers women, expedites postpartum recovery, decreases the risk for osteoporosis, reduces the risk for cardiovascular diseases and diabetes, protects against ovarian cancer[30]. For infants, breastfeeding reduces the risk of gastrointestinal infection, acute otitis media, respiratory infections, obesity, type 1 diabetes, type 2 diabetes, sudden infant death syndrome, and leukemia[31,32]. Breastfeeding has a population-wide effect on reduction of the risk of blood pressure, hypertension, coronary heart disease and stroke later in life and might be advantageous for cognitive development[32]. Breastfeeding promotes optimal growth.

Human milk meets the requirements of newborns for six months. It is well tolerated by nearly all infants. Human milk protein provides well-balanced amino acids for infants which promotes tissue build up and growth with its high whey-to-casein ratio[33]. Human milk proteins also have the bioactive components that improve immunity, defend against pathogenic bacteria, viruses, and yeast, and promote gut development and function, and enhance absorption[33].

Breastfeeding is recommended feeding norm by the health authorities. ESPGHAN recommends six months of exclusive breastfeeding and continuation of breastfeeding as long

as both mother and child wish. AAP also recommends six months of exclusive breastfeeding followed by complementary feeding for at least one year or longer. WHO recommends breastfeeding to continue for at least for two years or longer[30–32].

Another advantage of breastfeeding is that the contraindications of breastmilk are very rare. Contraindications can be seen due to infectious diseases, medical conditions in mother or infant, restrictive diet or malnutrition, exposure to environmental contaminants, medications and drug use. While some of these factors might interrupt breastfeeding temporarily, such as medication use due to bacterial infections, for most problems it is not necessary to discontinue breastfeeding. The only metabolic condition to contraindicate breastfeeding is classic galactosemia in an infant, which terminates breastfeeding. Breastfeeding is also contraindicated with smallpox and yellow fever vaccinations, HIV, and human T-cell lymphotropic virus I and II infections in the mother. In other conditions continuation of breastfeeding is still possible with some interventions[34].

2.5.2.2 Use of breast milk for preterm infant

The use of breastmilk is usually promoted in NICUs. There are many significant long and short term benefits of breast milk for preterm infants. Human milk enhances immature defense systems and neurodevelopmental outcomes of preterm infants. It reduces re-hospitalization rates after NICU discharge. Also, it is associated with lower rates of metabolic syndrome and improved leptin and insulin responses later in life[31]. An exclusively human milk-based diet with appropriate fortification (human milk + human milk-based

human milk fortifier) for VLBW infants meets the growth targets for weight gain, head circumference, and length and prevents EUGR[35].

For breast milk, there are two options in some NICUs; mother's own milk and donor milk. Mother's own milk is the first choice for the infant feeding. The composition of preterm mother's milk and term mother's milk are different [30,36]. Differences are shown in Table 4.

Table 4. Difference in composition of milk of mothers who deliver preterm

Level increased in preterm milk	**Level unchanged in preterm milk**
Total nitrogen	Volume
Protein nitrogen	Calories
Long-chain fatty acids	Lactose (decrease)
Medium-chain fatty acids	Fat
Short-chain fatty acids	Linolenic acid
Sodium	Potassium
Chloride	Calcium
Magnesium	Phosphorus
Iron	Copper
	Zinc
	Osmolality
	Vitamin B_{1-12}

AAP recommends that every preterm infant receive human milk[31]. Mother's own milk fresh or pasteurized is recommended to be the primary diet with appropriate fortification. In the short term, use of mother's own milk in NICU is associated with lower

risk for NEC and late on sepsis[37,38]. Breast milk is protective against retinopathy of prematurity. A positive correlation was observed between the amount of mother's own milk consumed and IQ scores. Breastmilk is well tolerated, so it shortens the time to achieve full enteral feeding. Therefore, it usually reduces the length of the hospital stay[39].

2.5.2.3. Lactation support in NICU

The use of mother's milk in NICU should be encouraged and promoted. Interdisciplinary promotion of breastfeeding increases the rate of human milk feeding in NICU[40]. To increase the number of mothers that supply milk to their infants and the amount of the milk there are actions that need to be taken by healthcare professionals affecting the social environment of the mother. According to Schanler (1999) factors for effective lactation support are "initiating milk expression, maintaining milk volume, psychological support, and lactation, skin-to-skin contact, stress and lactation"[41]. For the first factor, to start milk expression, health care professionals should inform women about the importance of breast milk, expressing breast milk, using the breast pump and significance of regular expression. Table 5. provides the guidelines from Lawrence and Lawrence for initiating milk supply without infant suckling[30].

Table 5. Guidelines for Initiating Milk Supply Without Infant Suckling

1. Begin as soon after delivery as maternal condition permits.
2. Initiate use of electric pump while in hospital.
3. Begin slowly, increasing time over first week.
4. Pump on more regular basis as soon as engorgement is evident.
5. Pump at least five times in 24 hours.
6. Allow a rest period for interrupted sleep of at least 6 hours.
7. Pump a total of at least 100 min/day.
8. Use "double" pump to pump both breasts simultaneously, which can cut total time proportionately.
9. Prepare breasts with warm soaks, gentle stroking, and light massage to maximize production of milk.
10. Encourage skin-to-skin care (kangaroo care).

For the second factor, to maintain milk volume, it is important to monitor the amount of the total milk produced daily and pay attention to abnormal volumes when required. The third factor is giving the psychological support by building a maternal and paternal bond with infant or breastfeeding if it is possible. Fourth factor, Kangaroo care or skin-to-skin contact, is beneficial for both child and parent. It promotes milk production and improves thermoregulation. The fifth factor, relieving maternal stress, enhances milk production performance[41].

2.5.2.4. Donor milk

It is important for every infant to receive mother's own milk but it might not be available despite the efforts to breastfeed or pump due to the mother's health condition. If

mother's own milk is not available, then the options for infant feeding are donor milk and formula. Both AAP and ESPGHAN recommend donor milk as the preferred choice for preterm infant nutrition[31,42].

The main beneficial effect of using donor milk for preterm infants is its preventive effect against NEC[43,44]. A Cochrane review stated that formula increases the risk of feeding intolerance and NEC when compared to donor milk[45]. The same review stated that formula - fed infants have higher weight, length and head circumference at discharge but long-term growth and neurodevelopmental outcomes were not different. Also, donor milk has many observed promising beneficial health effects on sepsis, bronchopulmonary dysplasia, and long-term cardiovascular risk factors but more randomized controlled trials are needed[42,44].

Donor milk should be obtained through donor milk banks, according to public health guidelines[46]. Especially for preterm infants in NICU, a milk bank is the best option to ensure the quality and safety of the milk. In North America, HMBANA sets the standards for human milk banking.

"The Human Milk Banking Association of North America (HMBANA) is a professional association for supporters of non-profit donor human milk banking, and it was founded in 1985 to:

- Develop guidelines for donor human milk banking practices in North America,
- Provide a forum for information sharing among experts in the field on issues related to donor milk banking,
- Provide information to the medical community regarding use of donor milk,

- Encourage research into the unique properties of human milk for therapeutic and nutritional purposes,
- Act as a liaison between member banks and governmental agencies,
- Facilitate communication among member banks to assure adequate distribution of donor milk,
- Facilitate the establishment of new donor milk banks in North America using HMBANA standards."[47]

HMBANA has 26 member milk banks and serves 50 states and 264 cities in the US and three provinces and seven cities in Canada as of 2017[47]. HMBANA milk banks screen the mothers who are volunteering to donate their milk. Mothers who get approved, after phone, medical record and blood screening, ship their expressed, frozen milk to closest milk bank[48]. The milk bank which receives the frozen donated milk, tests in the lab, pools, pasteurizes and dispenses the milk[49]. Holder pasteurization is used in HMBANA milk banks to avoid microbiological risks while maintaining the milk components with the least loss[50]. Table 6 shows the effect of pasteurization on milk components[51].

Table 6. Components of Milk and Effect of the Pasteurization

Component of breastmilk	**Effect of pasteurization**
Amylase	15% reduction
B-cells, T-cells	Abolished
Bile salt-dependent lipase	Abolished
CD14	88% reduction
Fats Linoleic, Linolenic acids Free fatty acids	No effect Reduced 80% increase
Calcium	No effect
Copper	9% reduction – No effect
E. coli inhibition	26% reduction
Epidermal growth factor	No effect
Erythropoietin	Significantly reduced
Immunoglobulins: IgA, sIGA IgG IgM	 0%-48% reductions 34% reduction Abolished
IGF-1, IGF-2, IGF-BP2,3	7%-39% reduction
IL-10	Significantly reduced
Iron	No effect - 15% reduction
Lactate	7% reduction
Lactoferrin	57 – 80% reduction
Lactose	No effect
Lipoprotein lipase	Abolished
Lysine	Significantly reduced
Lysozyme activity	No effect- 24% - 60% reduction
Lymphocytes	Abolished

Table 6 continued

Magnesium	No effect
Mannose-binding lectin	No effect
Oligosaccharides	No effect
Phosphorus	No effect
Potassium	No effect
Protein	No effect – Reduced
Sodium	No effect
TGF-a, TGF-B	No effect
Vitamin A	No effect
Zinc	No effect – 3% reduction

The human milk in milk banks is often milk from mothers of full-term infants. As mentioned before, preterm mother's milk has some compositional differences compared to the term mothers' milk. Both term and preterm mother's milk might need to be fortified depending on the requirement and intake of the infant. Human milk fortifiers are cow's milk based powder, cow's milk-based liquid fortifiers, and human milk-based human milk fortifiers with one nutrient that is high in requirement or multi-nutrient fortification[19,52]. Exclusive human milk-based diet has better outcomes for NEC, morbidity, and mortality in preterm infant[10,53]. There is a need in human milk banks to process and use the donated milk in their settings for preterm infants.

2.6. Osmolality

Osmolality is defined as “the concentration of a solution in terms of osmoles of solute per kilogram of solvent.” Compared to osmolarity, which can be defined as “the concentration of a solution in terms of osmoles of solute per liter of solution.” It is simple and more common to use osmolality (mOsm/kg) for infant feeding[54].

Osmotically active substances, which are vitamins, minerals, mono- and di-saccharides, amino acids and fatty acids in infant feed, add up to the total osmolality of the feed. Osmotic load of carbohydrates is inversely associated with the length of polysaccharide[55]. Therefore, excessive amounts of lactose might cause hyperosmolality and water retention in the gut which may result in dehydration[56]. Also, hyperosmolality is one of the many possible risk factors for NEC[12,54,57]. Willis et al. (1977) observed that extreme hyperosmolar feeds (>1700 mOsm/kg) increased the incidence of NEC[58]. Hyperosmolality was caused by calcium fortification, and the risk was reduced by dilution of the feed to 405 mOsm/kg. The elemental preterm formula which is high in osmolality (650 mOsm/kg), was found to be associated with increased risk for NEC compared to non-elemental preterm formula (359 mOsm/kg)[59].

In 1976, AAP recommended 400 mOsm/L as the upper level for osmolarity[60] which is approximately 450 mOsm/kg[54]. The osmolality of expressed human milk is 296 ± 14[61], and it is isosmotic with blood and interstitial fluid[30]. There is not a more recent recommendation, and this level is still commonly used. However, fortification with fortifier, protein, and multivitamins cause the osmolality of the feed to go beyond this level[62].

Osmolality in basal and fortified human milk was measured by Rosas et al. (2016)

who found 3 of the 4 fortified milk tested exceeded 450 mOsm/kg and osmolality increased within 24 hours after preparation[61]. The osmolality of the fortified human milk increases during storage at 4ºC due to enzymatic activity[63]. As mentioned before, fortification can be done with one nutrient or multi-nutrient, and multi-nutrient fortification might increase osmolality and density above tolerable levels and might cause gastrointestinal tract problems[64]. Therefore, multi-nutrient fortification should be monitored carefully to avoid hyperosmolality.

The osmolality of fortified breastmilk at clinically used levels for many fortifiers exceeds 400 mOsm/kg. The range of osmolality for breast milk (5 mL) fortified with various additives was 242 – 951 mOsm/kg[65]. Also, ESPGHAN stated that with some clinically used fortification applications osmolality levels increase up to 886 mOsm/kg[62]. Even though they did not interpret their results in osmolality; they claimed that combination of human milk, human milk fortifier, additional protein, and multivitamins cause hyperosmolarity. Therefore they should not be applied in combinations[62]. For safe fortification, clear cut-off levels for osmolality is needed[66].

2.7. Lactose

Lactose is a disaccharide that is synthesized in the epithelial mammary gland cells as a molecule of glucose linked to a molecule of galactose with β 1-4 glycosidic bond[67]. Lactose serves as a ready energy source to newborns as mentioned before. Usual lactose concentration in mature human milk range from 6.9% to 7.2%[68]. It is a reducing sugar with optically active, asymmetric carbon atom in glucose part which can exist either as an open-

chain or a hemiacetal ring[67,69]. Therefore, there are two anomers of lactose: α and β lactose[70]. Figure 1. shows the structure of α and β lactose. Some physical properties are different, such as solubility and specific rotation[67].

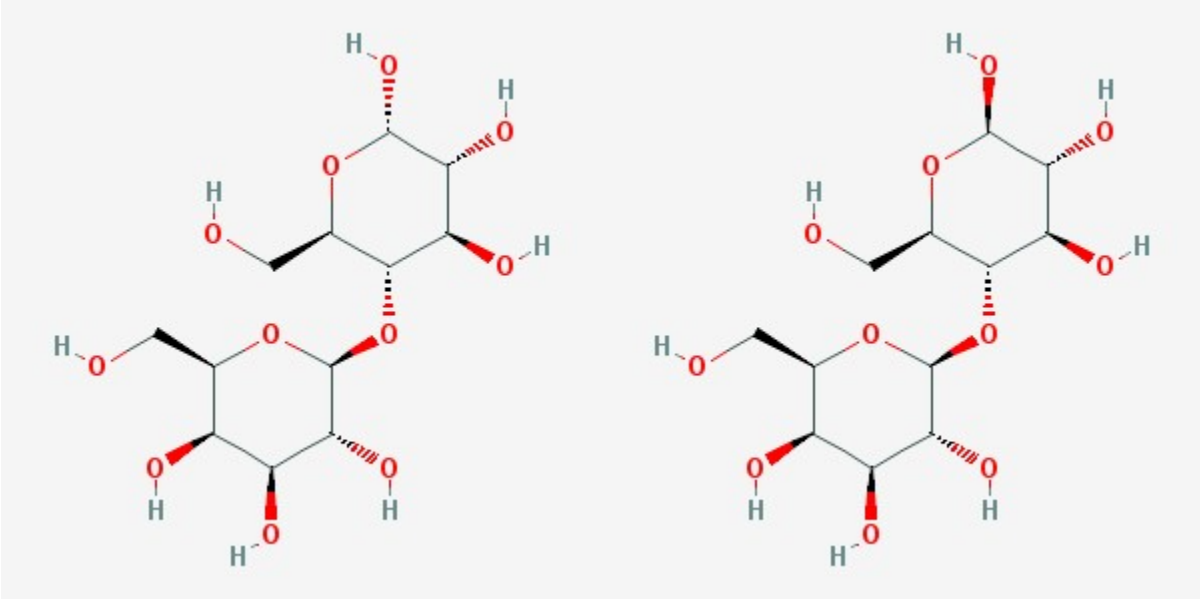

Figure 1. 2 D chemical structure of α lactose (on the right) and β lactose (on the left)

Lactose molecules can switch between α and β forms (mutarotation), to establish equilibrium, depending on the temperature and concentration[69]. At body temperature, two parts of α-lactose and three parts of β-lactose are in equilibrium[71]. Under certain circumstances, lactose crystallizes in the solution. Saturation of solution, temperature, pH of the solution and other components of milk affects the crystallization. In order to initiate the crystallization, solution should be saturated[69]. Figure 2, displays a solubility curve of lactose in water.

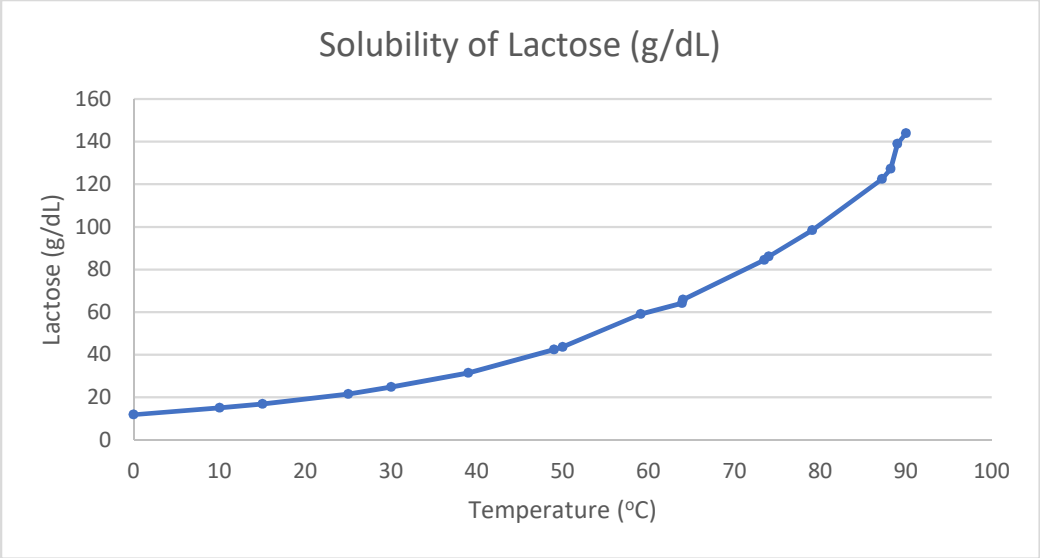

Figure 2. Final solubility of lactose in water

Final solubility for both α and β lactose is shown in Figure 2[72]. In order to initiate lactose crystallization concentration of lactose should be above the solubility curve. The solubility of lactose is proportionally associated with temperature. Thus, an attempt to crystallize lactose should be made at low temperature with saturated solutions.

At temperatures under 95 ºC, α-lactose is the isomer that crystalizes[69]. Therefore, the following reaction occurs:

B-lactose $\longleftrightarrow$ α-lactose $\longleftrightarrow$ α-lactose hydrate crystals[72]

Once lactose crystalizes, it is possible to precipitate the crystals by centrifugation.

2.8. Viscosity

Rheological characteristics of liquids differ depending on Newtonian behavior or non-Newtonian behavior. For a Newtonian fluid, the rate of strain is exactly proportional to the applied stress[73]. A single value can describe Newtonian viscosity. Newtonian viscosity is temperature dependent, but it is constant for the shear rate, whereas non-Newtonian fluids are dependent on shear rate[67]. These fluids can be shear thinning or shear thickening as shown in Figure 3.

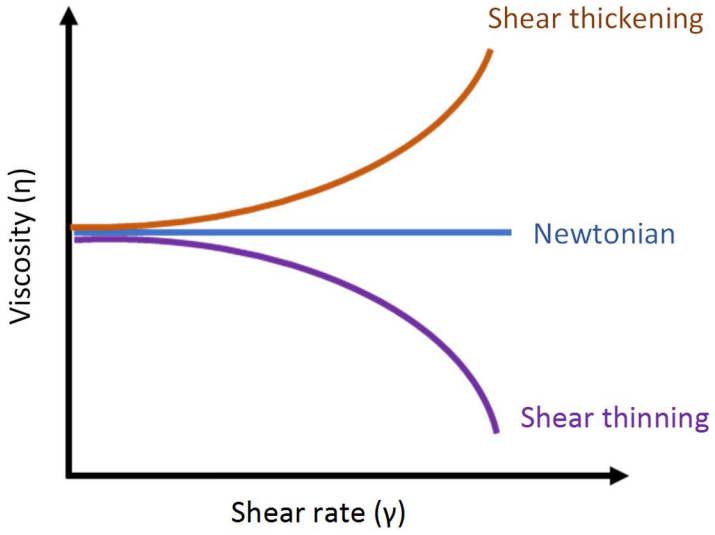

Figure 3. The viscosity of Newtonian and Non-Newtonian fluids.

Fresh skim milk and whole milk usually show Newtonian behavior[67]. However, condensed milk might exhibit shear-thinning properties, which are a non-Newtonian fluid

behavior, depending on the amount of total solid[67]. The viscosity of Non-Newtonian fluids can be described as a function of shear rate. Cream and other dairy products derived from milk with minimal processing are non-Newtonian fluids[67].

2.9. Evaporated milk

There are different methods and technologies for increasing the milk concentration. Methods that are commonly used are evaporation of the milk under vacuum, freeze drying and membrane filtering[74]. Vacuum evaporation for evaporated milk production in industrial setting is illustrated in Table 7[74].

Table 7: Manufacturing stages of evaporated milk

Receiving and Selection of Milk
Preliminary Treatment Clarification, Fat separation / standardization)
Pre-heating (115 – 128ºC, 1 – 6 min)
Vacuum Evaporation (45 – 70ºC)
Homogenization (P1=15 – 25 mPa; P2=5 – 10 mPa)
Packaging
Sterilization (100 – 120ºC, 15 – 20 min or 140ºC, 3 s)
Storage (20ºC)

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CHAPTER 3:

Material and Methods

Donated human breast milk was obtained from the WakeMed Mother's Milk Bank (Cary, NC). Milk banks experience milk shortages, therefore categorizing the milk by the factors that affect milk composition such as being expressed at the beginning or end of the feeding, time of the day, infant's age, maternal diet, gestational age at birth[1–3], is not always applicable. Therefore, the milk was not categorized according to these factors in this research.

The milk was frozen at -20 ºC when received and kept frozen until use. It was thawed in a shallow shaking water bath (Thermo Scientific Precision) at 27 ºC for 30 minutes. Thirty-six samples of 200 mL each were prepared from separate milk donations collected in 60-180 mL containers. Samples from different mothers were pooled to achieve the required 200 mL volume. Homogenization, evaporation, and centrifugation were applied in order to achieve a lactose-reduced human milk concentrate. Eighteen of these samples were homogenized, while the remaining eighteen were not. A benchtop homogenization machine (Niro Soavi, Panda2K, Italy) was used to do two-stage homogenization. After homogenization, the samples were condensed with a rotary evaporator (Buchi R-300 Rotavapor™). The water bath of the rotavapor was set to 40 ºC to avoid protein denaturation. The rotation rate of the rotavapor was set to 30 rpm, and inner pressure was set to 33 mbar, which efficiently removed water from the milk. Six different levels of volume reductions

were applied to the samples. There were three replicates for each combination of condensation and homogenization levels. Volume reductions were specified as 80%, 60%, 50%, 40%, 30%, 0%, respectively. These reduction levels were selected depending on the results of our preliminary experiments. In the preliminary experiments, we observed that there is limited lactose precipitation in the samples with zero condensation, which shows an increase in lactose concentration was necessary to initiate crystallization. Also, the samples, which are over-condensed, may be too saturated to build lactose crystals; therefore, we decided to observe a variety of condensation levels. Different condensation levels were achieved by changing time in the rotavapor. For the 30% volume reduction, the samples were evaporated for 18 minutes. For the 40%, 50%, 60%, 80% volume reductions, average time spent at the rotavapor was 30, 45, 60 and 75 minutes, respectively. As mentioned before, initial volume was measured at 200 mL for each sample. After the volume reductions, final volumes measured were respectively 40 mL, 80 mL, 100 mL, 120 mL, 140 mL and 200 mL for decreasing levels of condensation (from 80% to 0%). These samples were divided into two tubes: one tube underwent chilled centrifugation as a lactose removal process, and the other one was kept as control. The volumes that underwent centrifugation were 20 mL for the 80% volume reduction and 35 mL for the other levels. Centrifuge tubes were kept at -20 ºC overnight before centrifugation. Then, the centrifugation was applied at 0 ± 2 ºC, 3500 rpm for 60 minutes. During the centrifugation process, the samples thawed slowly, and lactose crystals precipitated at the bottom.

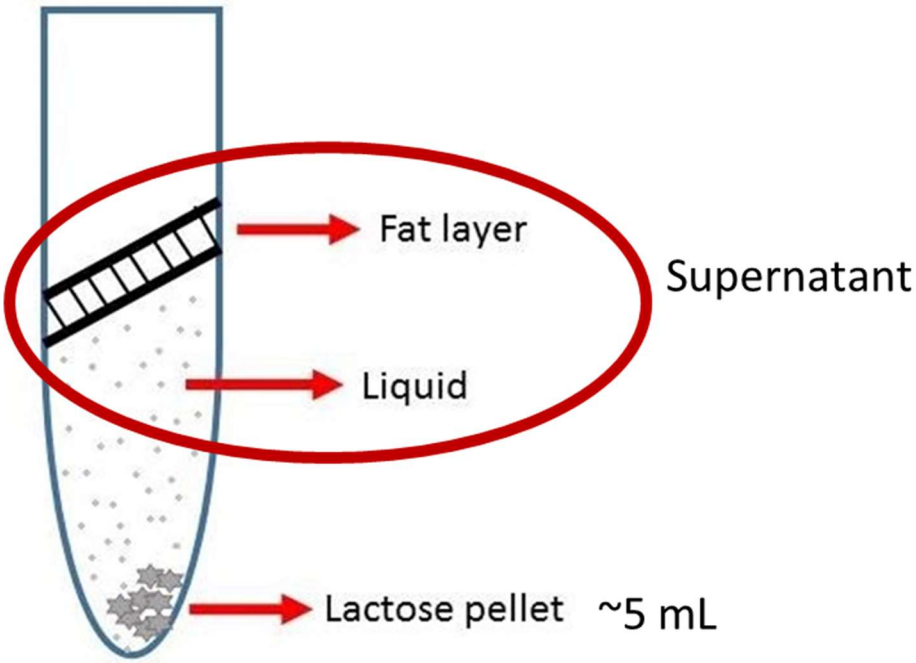

Figure 4: Separation in a non-homogenized sample after the centrifugation

Figure 4 shows the separation in a centrifuge tube after the centrifugation was applied. The tube consists of a fat layer (only for non-homogenized milk) at the top, liquid, and a lactose pellet at the bottom. The part that does not include the lactose pellet is called "supernatant". The supernatant was removed carefully to another tube for measurements. Approximately 5 mL of fluid was left at the bottom in order not to disturb the lactose pellet and to prevent lactose from dissolving back to supernatant while removing the supernatant. Then, the lactose pellet was discarded. The measurements of supernatants were compared to the controls.

Lactose, protein, osmolality, and viscosity of each sample were measured. Lactose measurement was performed with the R-biopharm Lactose/D-galactose enzymatic kit. First, the samples were prepared for kit application by denaturation, precipitation, and filtration of proteins. For this procedure, 1 mL of each sample was mixed with 20 mL of water, and 1 mL of trichloroacetic acid (3M) then was incubated for 10 minutes. Then, the solution was neutralized with 1M sodium hydroxide and was brought up to 100 mL. Subsequently, the solutions were filtered with 0.45 µm nylon filters (EMD Millipore Corp. Germany) in order to prepare for the kit application. The lactose in solution was oxidized and coupled to NADH formation by the following enzymatic reactions.

$$\text{Lactose} + H_2O \xrightarrow{\beta-galactosidase} \text{D-glucose} + \text{D-galactose}$$

$$\text{D-galactose} + NAD^+ \xrightarrow{\beta-galactos\ \ dehydrogena} \text{D-galactonic acid} + NADH + H^+$$

NADH formation stoichiometrically corresponds to the amount of lactose in the initial solution. The amount of lactose was calculated by measuring the difference in light absorption depending on the increase in NADH level. Measurements were taken at 340 nm wavelength with a spectrophotometer (Thermo Multiskan MCC Fisher Sci USA) in a 96-well plate with blanks and standard curve.

Total nitrogen analysis was performed to measure protein (Vario Macro cube CHN and Rapid N Exceed, Elementar Analysensysteme Germany). In breast milk, non-protein

nitrogen consists of 20-25 % of the total nitrogen whereas in bovine milk it is only 3-5%[4]. Therefore, the conversion factor for bovine milk, which is 6.38, overestimates the protein in human milk. A correction is necessary for the conversion factor for application in human milk. With this correction, 5.18 was used as the conversion factor[5]. The aim for the protein measurement is to see if there is a significant nutrient loss into the lactose pellet. Protein requirement increases for the preterm infants, and a goal of the project was to increase protein concentration; hence, the protein was measured to observe the effect of the lactose removal process.

Osmolality (mOsm/kg) was measured with a freezing point depression osmometer, Advanced Micro Osmometer Model 3300 (Advanced Instrument Inc., Norwood, MA, USA) which requires 20 μL of each milk sample for the measurement. The instrument was calibrated with standard solutions of 50 mOsm and 850 mOsm. The measurement was repeatable.

Viscosity measurement was performed with an Anton Paar modular compact rheometer MCR 302 at 25ºC (Anton Paar GmbH, Austria). Parallel plate and double-gap concentric cylinder geometry were used. Most of our samples were non-Newtonian fluids, so we report flow curves instead of one value.

In this study, we investigated the effects of volume reduction and lactose removal on the lactose and osmolality levels of human milk. We represent the volume reduction as condensation, which is a fixed treatment factor and has six categories (80%, 60%, 50%, 40%, 30%, and 0%). Other fixed treatment factors are centrifugation, and it has two categories (control and supernatant) and homogenization which has two categories (homogenized and

non-homogenized). The dependent variables are the lactose, protein, osmolality and viscosity measurements of the human milk.

In our study, data sets included 72 observations each for the lactose content, protein contents, and the osmolality levels. Thirty-six subjects' milk donations were randomly assigned to six categories of condensation. Then, the lactose, protein, and osmolality measurements were taken from each subject before and after applying lactose removal. There are two repeated measurements on each subject. We use the GLM procedure in SAS Version 9.4 (SAS Inc. Cary, USA) to perform Wilcoxon and paired t-test for lactose, protein, and osmolality measurements.

3.1 References

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CHAPTER 4:

Results and Discussion

4.1 Lactose

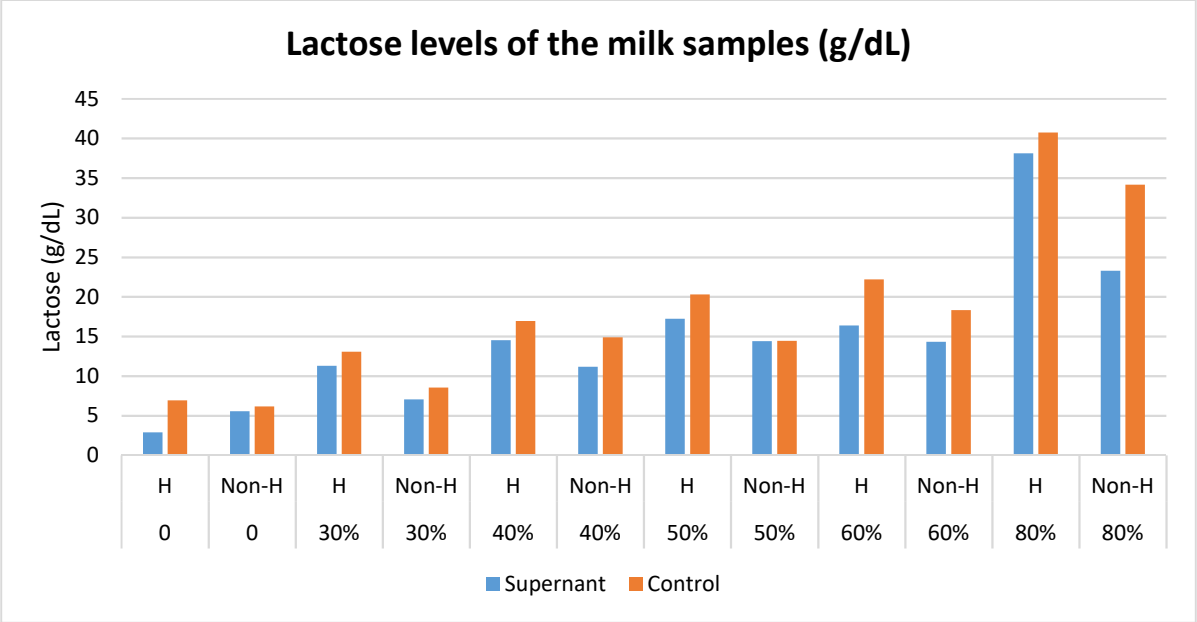

Figure 5. Comparison of average lactose levels for homogeneity, condensation and lactose removal

Figure 5 displays the variability in the lactose content of the milk samples before and after the lactose removal process, homogenization, and condensation were applied. The horizontal axis shows the combinations of categories for the homogeneity and condensation variables. The vertical axis indicates the amount of lactose content (g/dL) for each combination. Orange columns represent the lactose amounts for milk samples that were not centrifuged (control), whereas blue columns represent the lactose levels for milk samples that

were centrifuged (supernatant). The results show that the lactose amount for the supernatant group is usually smaller than those for the control group except for the NonH-50% category. However, the lactose amount in the control and supernatant groups appears to vary for the homogenized and non-homogenized milk. In order to figure out if homogenization had an influence on the lactose precipitation, we compared the lactose content between the control-H and control-NonH groups as well as the supernatant-H and supernatant-NonH groups. Because the lactose measurements do not come from a normal distribution, we applied the nonparametric Wilcoxon signed rank test. We specified the significance level as $\alpha=0.05$. The Wilcoxon test statistic for the control groups (W=370, *P*-value=0.2482) cannot reject the null hypothesis that the average lactose amounts in control-H and control-NonH groups are not significantly different from each other. Similarly, the Wilcoxon test statistic (W=360, *P*-value=0.4064) for the supernatant groups cannot reject the null hypothesis of equality of the average lactose content for the homogenized and non-homogenized milk. Thus, we can conclude that homogenization does not have an impact on the lactose content.

As can be seen in Figure 5 and 6, the categories of condensation are not equally spaced, so the dramatic increase in the lactose content from 60% to 80% category might seem misleading at first glance. Our preliminary results showed that handling over-condensed samples was problematic. Therefore we preferred to apply no more than 80% volume reduction by condensation. Figure 6 illustrates the comparison of lactose trendlines between the control and supernatant groups. As expected, the amounts of lactose for control and supernatant increased proportionally as the volume reduction increased. The rate of increase for control is larger than the rate of increase for supernatant (Figure 6). We also

investigated whether there was a difference in the lactose content before and after applying the lactose removal process. In accord with the previous results, there was no statistical difference between the H and NonH categories, so we pooled the data together. Then, we used a one-sided paired t-test for testing the null hypothesis that the difference between lactose measurements for control and supernatant is zero versus the alternative hypothesis that the difference is greater than zero. The paired t-test statistic is 5.7614 and the *P*-value < 0.0001, which shows that the null hypothesis can be rejected at the significance level of 0.05. We can conclude that lactose removal had a significant effect on the lactose content and the supernatant group had relatively lower lactose amounts than those for the control group.

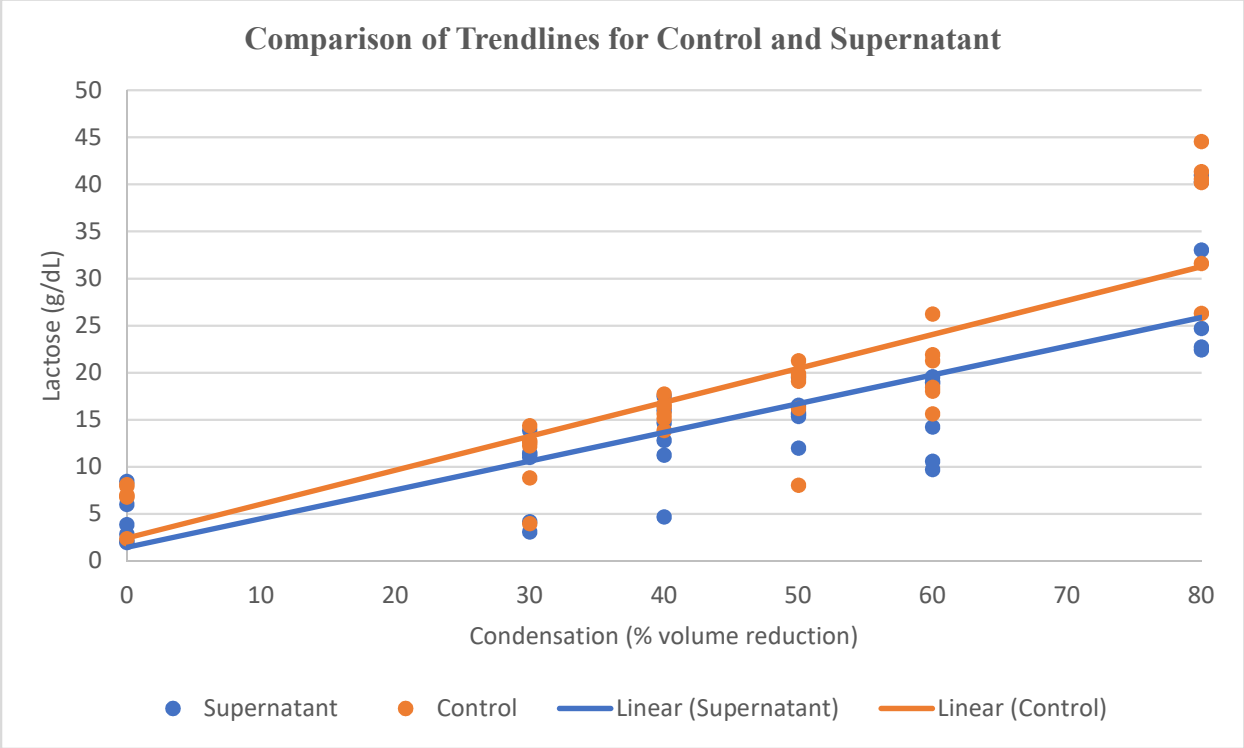

Figure 6. Comparison of lactose trends for the control and supernatant groups

4.1.1 Statistical Analysis

In this section, we use the GLM procedure in SAS Version 9.4 (SAS Inc. Cary, USA) to perform two-way repeated measures ANOVA results for lactose measurement. Data input and ANOVA table can be found in Appendix.

4.1.2 ANOVA Results for Lactose

The correlation coefficient between control and supernatant is 0.42 with a *P*-value of 0.02, which means that correlation between the repeated measurements is statistically significant at the significance level of 0.05. This justifies that the repeated measures ANOVA is an appropriate analysis method for these data.

Table 8: Repeated Measures ANOVA Results for Lactose.

VARIABLE	F VALUE	P-VALUE
CONDENSATION	38.75	<0.0001 *
LACTOSE REMOVAL	16.52	0.0003 *
CONDENSATION*LACTOSE REMOVAL	1.02	0.4208

*** Significant at <.05 level

The results show that there was a significant condensation effect (F=38.75, *P*-value<0.0001), indicating that the average lactose content was different for at least one

category of condensation. Additionally, there was a significant centrifugation effect (F=16.52, *P*-value=0.0003), indicating that the average lactose content changed after the lactose removal procedure was applied. The mean lactose content for control and supernatant are 18.05 g/dL and 14.68 g/dL, respectively. This means that we observed a significant decrease in lactose amounts when centrifugation was applied. On the other hand, the interaction effect between centrifugation and condensation does not seem to be statistically significant (F=1.02, *P*-value=0.4208). This means that the effect of the chilled centrifugation procedure on the lactose content is not different for the categories of condensation.

4.2 Protein

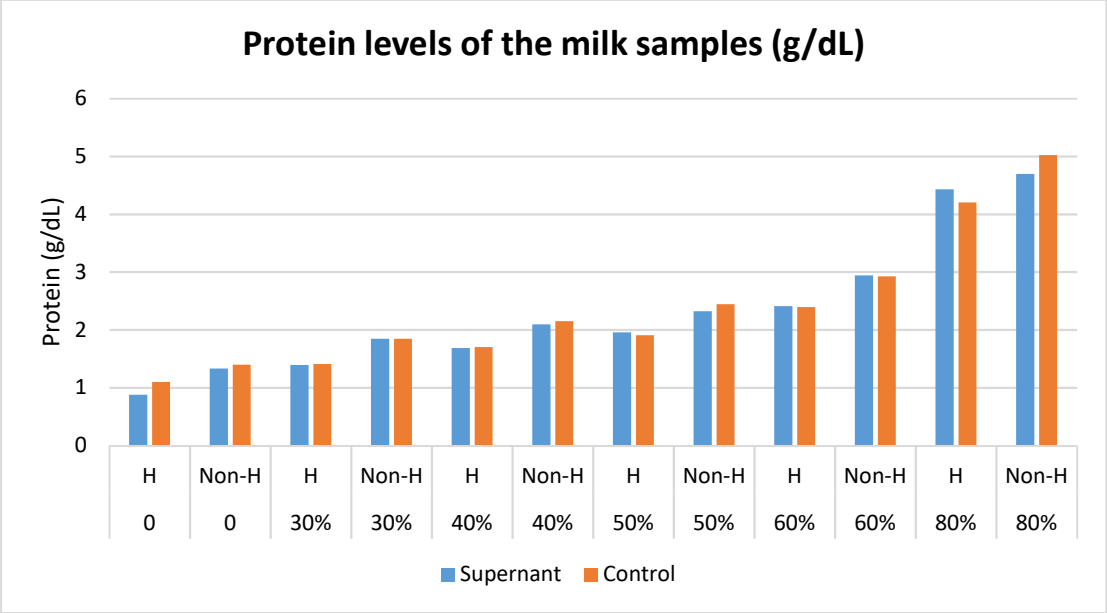

Figure 7. Comparison of average protein levels for homogeneity, condensation and lactose removal

Figure 7 illustrates the variability in the protein content of the milk samples before and after lactose removal process, homogenization and condensation are applied. According to the results, the protein amounts for the supernatant and control groups are very similar to each other for almost all combinations of homogeneity and condensation. In general, the protein amount for the homogenized milk seems to be lower than those for the non-homogenized milk. This observation could be due to greater loss of fat by adherence to processing equipment without homogenization. Due to the violation of the normality assumption, we again applied the Wilcoxon test to the control and supernatant groups to see whether the protein content differed between the NonH and H categories. The Wilcoxon test statistics for the control groups (W=281.5, *P*-value=0.1054) and the supernatant groups (W=285, *P*-value=0.1341) cannot reject the null hypothesis of equality of the average protein content of the homogenized and non-homogenized milk. As we expected, homogenization did not cause a noticeable change in the protein amount. Furthermore, we observed that the protein amount increased proportionally as the volume reduction increased. As in the previous case, we used a two-sided paired t-test to figure out whether there is a difference in the protein content before and after applying the lactose removal process. The null hypothesis indicates that the difference between protein measurements for control and supernatant is zero versus the alternative hypothesis that the difference is not equal to zero. The paired t-test statistic is 0.66, and the *P*-value is 0.5151, which shows that the null hypothesis cannot be rejected at the significance level of 0.05. Thus, we can conclude from these results that lactose removal did not seem to have an impact on the protein content of the milk, which implies that lactose removal did not cause protein loss.

4.3 Osmolality

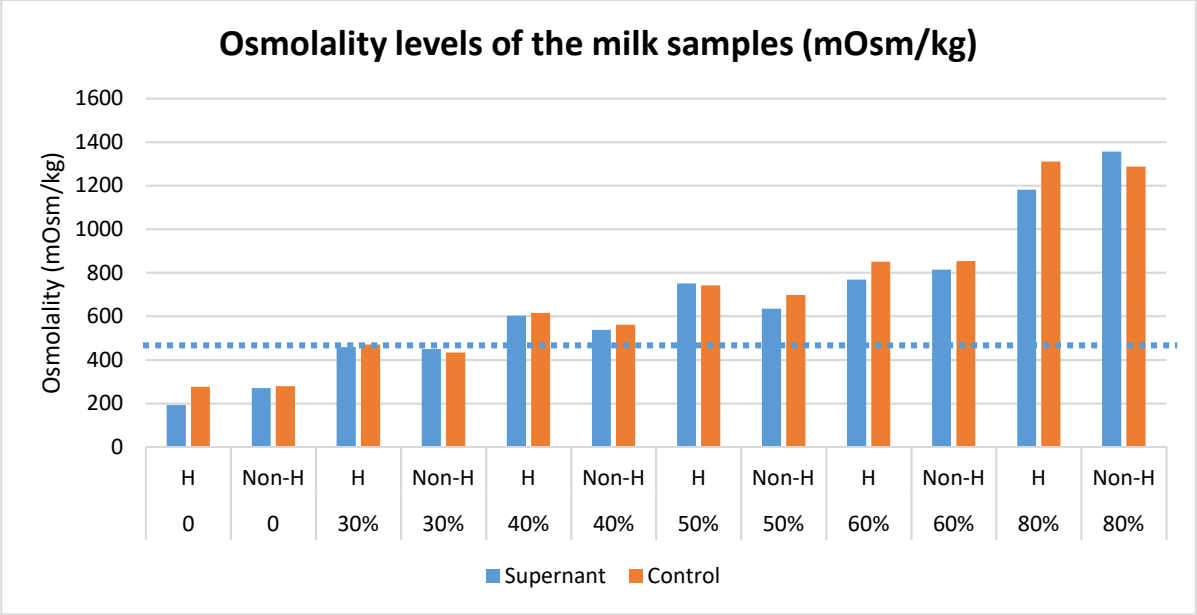

Figure 8. Comparison of average osmolality levels for homogeneity, condensation and lactose removal. The dotted line indicates the AAP recommendation for osmolality in infant feeding

Figure 8 shows the variability in the osmolality of the milk samples before and after the lactose removal process, homogenization, and condensation were applied. The results do not indicate a particular pattern in osmolality levels for the supernatant and control groups. We observed that osmolality levels for the homogenized and non-homogenized milk seem similar to each other. We statistically investigated whether homogenization influences osmolality for the control and supernatant groups. The Wilcoxon signed rank tests statistic for comparing the control-H, and control-NonH groups are W=346.5 with the *P*-value=0.6792. This shows that the homogenization process did not have an effect on osmolality in the control groups. In the same way, the Wilcoxon test result for the

supernatant groups (W=340.5 and p-value=0.8210) confirms that homogenization did not significantly change the osmolality levels in the supernatant groups. Moreover, we observed that osmolality increased gradually as the volume reduction increases. We applied a one-sided paired t-test for testing the null hypothesis that there was no difference in the osmolality levels before and after applying the lactose removal process versus the alternative hypothesis that the difference is greater than zero. The paired t-test statistic is 3.51 and the *P*-value < 0.0001, which shows that the null hypothesis can be rejected at the significance level of 0.05. We can conclude that lactose removal had a significant effect on the osmolality level and the supernatant group had relatively lower osmolality amounts than those for the control group.

The blue dotted line on the osmolality graph shows the AAP recommendation for upper level of osmolality in feed for newborns[1]. Even though, there is not a specific recommendation for preterm infants this value is widely accepted. However, fortification applications that add powdered nutrients to human milk to meet the higher nutrient requirements of preterm infants might result in higher osmolality in feed than this recommendation. The 30% and 40% volume reduction in our study had osmolality less than the AAP recommendation for maximum osmolality.

4.3.1 Statistical Analysis

In this section, we use the GLM procedure in SAS Version 9.4 (SAS Inc. Cary, USA) to perform two-way repeated measures ANOVA results for osmolality. We applied Tukey HSD as multiple comparison. Data input and ANOVA table can be found in Appendix.

4.3.2 ANOVA Results for Osmolality

The correlation coefficient between control and supernatant is 0.96 with a *P*-value less than 0.0001, which means that correlation between repeated measurements was statistically significant at the significance level of 0.05.

Table 9: Two-way ANOVA results.

VARIABLE	F VALUE	P-VALUE
CONDENSATION	21.77	<0.0001 *
LACTOSE REMOVAL	17.4	0.0002 *
CONDENSATION*LACTOSE REMOVAL	3.87	0.0079 *

*** Significant at <.05 level

According to Table 9, there was a significant condensation effect (F=21.77, *P*-value<0.0001), indicating that the average osmolality was different for at least one category of condensation. The F test is significant for centrifuge (F=17.40, *P*-value=0.0002), showing

that the average osmolality changed after lactose removal was applied. The mean lactose content for control and supernatant was 691.04 mOsm/kg and 656.74 mOsm/kg, respectively. This means that we observed a significant decrease in osmolality when centrifugation was applied. Furthermore, the interaction effect between centrifugation and condensation is statistically significant (F=3.87, p-value=0.008). This means that the effect of centrifugation on osmolality is different for the categories of condensation. We also performed one-way ANOVA to investigate which categories of condensation had different amounts of osmolality change. In this case, our treatment factor is condensation with the same categories and the dependent variable is the osmolality difference between control and supernatant.

We obtained the same F test statistic and *P*-value for the interaction effect in Table 8. Tukey's HSD test for multiple comparisons determined that the highest difference in osmolality between control and supernatant was obtained at the 40% condensation level, which is followed by 0% and 60%, respectively. Additionally, the difference in the 40% condensation level is statistically different than those for the 30%, 50%, and 80% condensation levels.

4.4 Viscosity

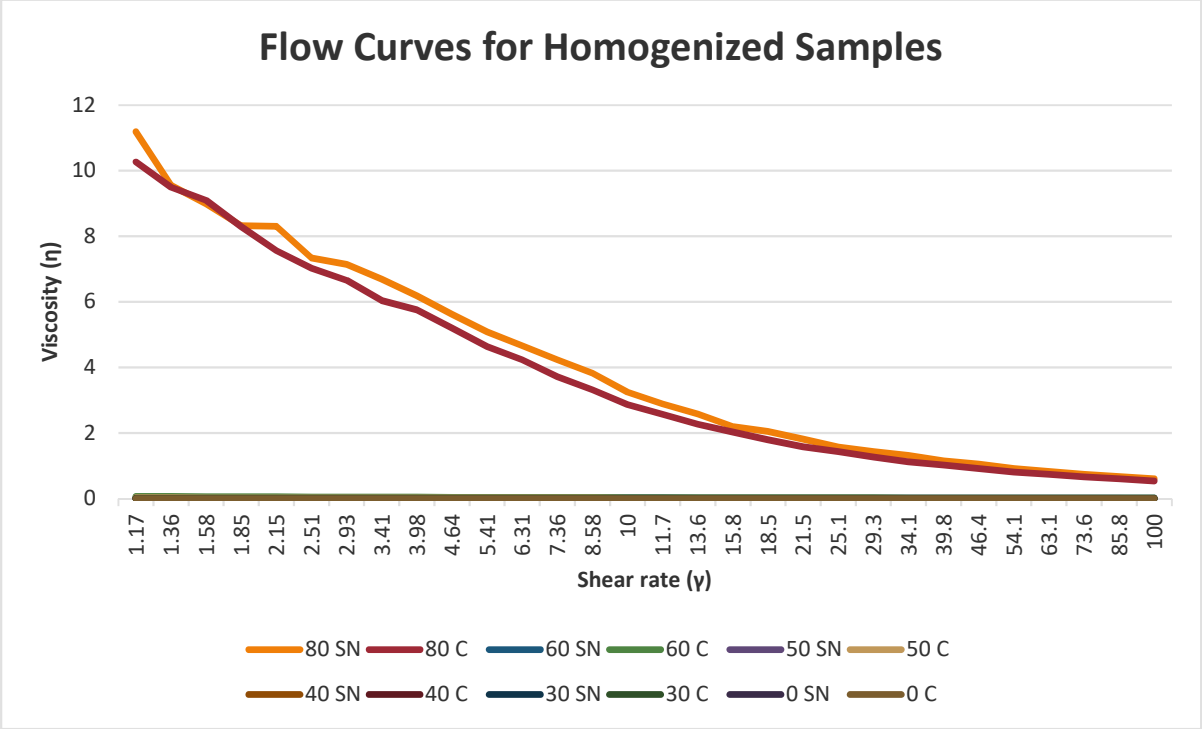

Figure 9. Flow curves for all homogenized samples

In Figure 9 through 12, the vertical axis shows apparent viscosity in units of Pa·s , and horizontal axis shows shear rate is in the 1/s unit for all the viscosity data. As mentioned before, flat flow curves represent Newtonian fluid whereas, a curve with a slope represents non-Newtonian behavior. Concave curves like we observe at homogenized, 80% condensed, samples indicate shear thinning liquids. As shown in Figure 9 the flow curve for the 80% condensation level is dramatically higher than the rest of the condensation levels. To be able to observe flow curves of less condensed samples we excluded 80% and 60% condensation

data and graphed the 50%, 40%, 30% and 0% flow curves separately, which is shown in Figure 10.

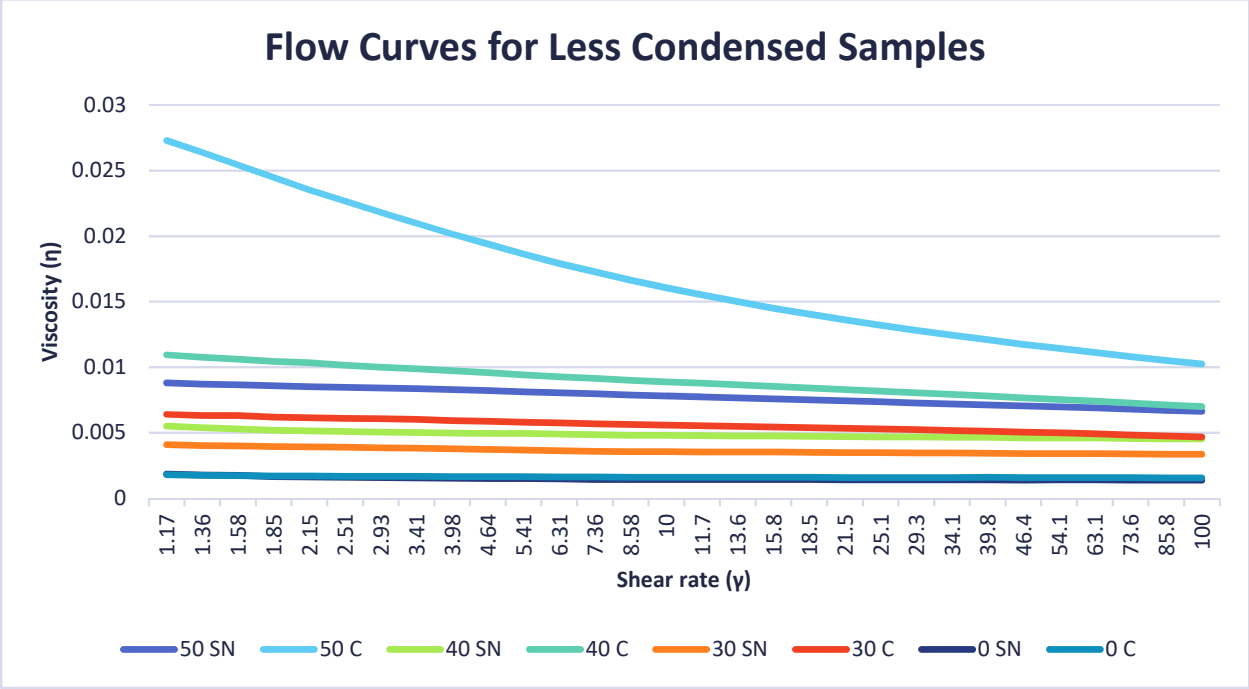

Figure 10. Graphics of flow rate for lower condensation levels of homogen samples

The flow rate for homogenized samples are shown in figures 9 and 10. As shown in Figures 9 and 10, lower condensation levels had lower viscosity. The 80% condensed samples have very high viscosity and non-Newtonian behavior. Viscosity gradually decreased as condensation decreased. Also, apparent viscosity was higher in controls compared to supernatants for each pair, except for no condensation. It is also apparent that non-Newtonian behavior is stronger in more condensed samples.

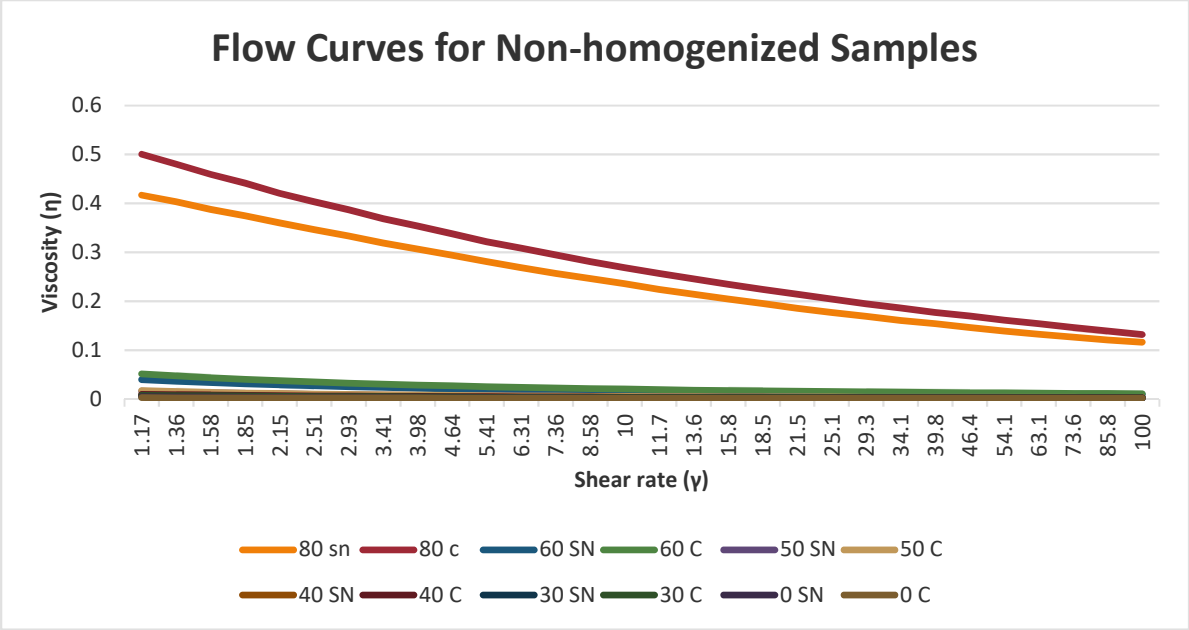

Figure 11. Graphics of flow rate for all non-homogenized samples

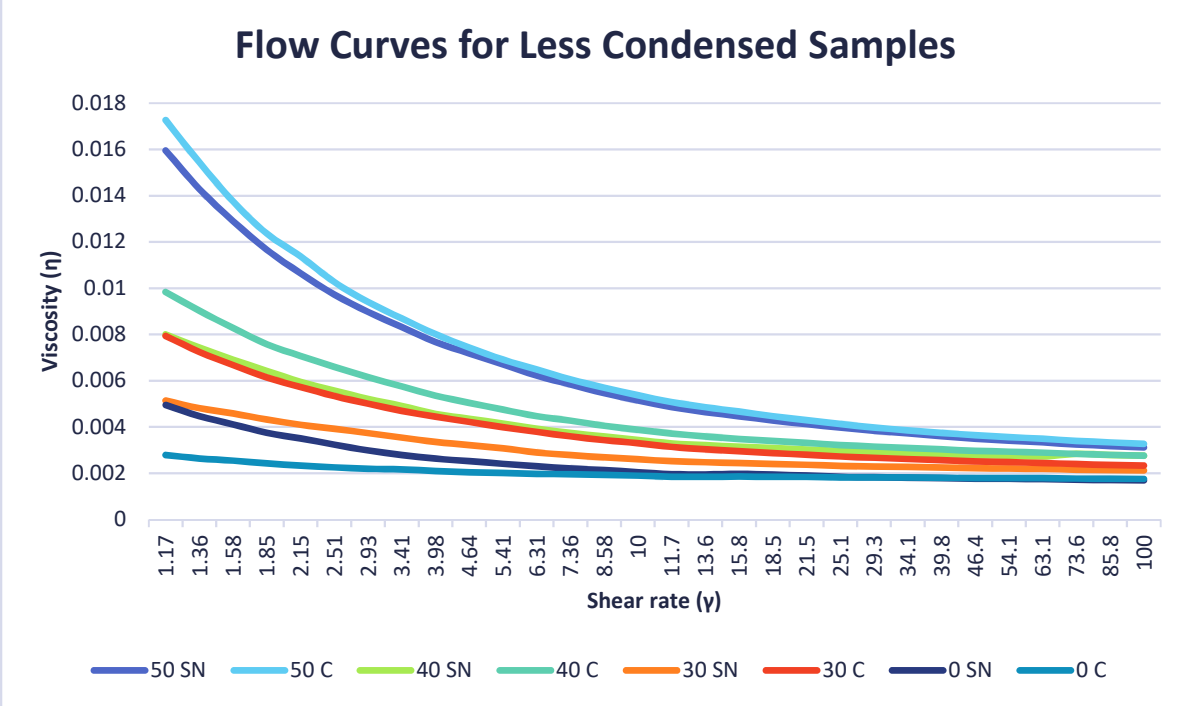

Figure 12. Graphics of flow rate for lower condensation levels of non-homogenized samples

The flow rate for non-homogenized samples are shown in Figures 11 and 12. Similarly with homogenized samples, viscosity of 80% samples were dramatically higher than the rest of the condensation levels. Therefore, in order to be able to observe flow curves for less condensed samples, 60% and 80% levels were excluded in Figure 12. Viscosity increased as volume reduction increased in nonhomogenized samples as well. Also, viscosity of controls appears to be higher than the supernatants, similar to the data from homogenized samples. It is apparent that non-Newtonian behavior is stronger in more condensed samples. Also, Newtonian character is more distinct in homogenized samples.

Viscosity of feed might interfere with use of condensed human milk in a NICU. High viscosity of feed may cause less fluidity in intragastric (IG) tubes[2]. Accuracy of enteral feed pumps depends on the feed's viscosity. Highly viscous feed lowers the flow rate[2]. Feeding pumps set at different flow rates may be inaccurate with non-Newtonian fluids. Delivering fortified feeds with pump should be done by taking viscosity into account[3]. Viscosity of feeds should be evaluated to determine their efficiency in NICU.

4.5 Discussion

Similar studies focused on either one condensation level or different processing methods[4,5]. Grance, et al.[4] applied a lactose removal procedure to non-condensed milk and used freeze-drying subsequently to lactose removal. However, we were not able to reproduce similar lactose removal rates with their method in our preliminary research. Valentini[5], claimed that the level of condensation where lactose removal can be initiated is 75% of

volume reduction. We showed lactose crystallization is achievable in lower levels of condensation. Also, handling 75% condensed milk can be challenging in a milk bank setting.

We used similar methods for lactose removal process with those in the literature. We applied lactose removal on various levels of condensation to evaluate the condensation effect. We expected higher lactose reduction as volume reduction increases. Even though we observed an increase in lactose reduction (Figure 6) it was not statistically significant. This might be due to a limitation on lactose mutorotation in lower temperature or effect of viscosity on crystallization.

We observed a combined effect of condensation and lactose removal on osmolality. Reduction in osmolality due to lactose removal was statistically significant at 40% volume reduction. Our aim in lactose reduction is to reduce osmolality in concentrated milk with increased nutrient density. We achieved statistically significant lactose reduction, but may be able to have a greater reduction with lower osmolality after further research.

4.6 Reference

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CHAPTER 5:

Conclusions

Use of human milk is the first choice for feeding premature infants in NICU[1]. Kangaroo care and breastmilk expression should be encouraged and promoted[2]. When mother's milk is not available despite the effort, milk should be obtained from a milk bank. However, breast milk obtained from a milk bank might not meet the high nutritional demand of preterm infants[3]. Therefore, there is a need to increase the nutrient density of a portion of the breast milk processed in milk banks to meet the need of preterm infants.

The aim of this study was to provide affordable, nutrient dense, exclusive human milk-based feed for preterm infants in a NICU with processing methods that could be performed in a milk bank setting. In order to meet this need, we condensed the milk. Subsequently, we applied a lactose removal process. We used various levels of condensation and measured lactose, osmolality, protein and viscosity levels. We made the comparisons between control samples that were not subjected to lactose removal and the supernatant fraction of samples that were subjected to the lactose removal process.

We observed a significant decrease in lactose and osmolality after lactose removal for all the condensation levels. Furthermore, we showed that protein was conserved during the lactose removal process and it increased proportionally with volume reduction. Even though lactose removal was not significantly different for various condensation levels, osmolality reduction was significantly different for the 40% condensation level followed by 60% and 0%. The 40% volume reduction meets the AAP recommendation for maximal osmolality of

450 mOsm/kg[4]. There was a 63% increase in protein at 40% volume reduction compared to no condensation. Homogenized samples with 40% volume reduction retain Newtonian flow characteristics with minimal increase in viscosity. These findings are promising to establish a process that could be used in a human milk bank to increase nutrient density in feeds.

Future research is needed to define optimal growth in preterm infants. Also, a cut-off level of osmolality for preterm infants and relationship between osmolality and necrotizing enterocolitis should be defined. In order to establish a procedure to increase the nutrient density of human milk in a milk bank setting, future research should have a larger sample size to confirm repeatability, since variability in the composition of human milk is a limitation. Further investigation for viscosity and rheological properties of condensed milk is needed to optimize its use in NICU. Also, more research is needed to investigate the microbiological properties of this nutrient-dense feed.

5.1 Reference

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APPENDIX

Appendix A: Statistical Analysis for Osmolality and Lactose Measurement

Data from Protein, Osmolality and Lactose Measurement

Observation	Homogeneity	Condensation	Centrifuge	Protein	Osmolality	Lactose
1	Non-H	80	Supernatant	5.5426	1320.0	22.4383
2	Non-H	80	Supernatant	4.8951	1391.5	22.7360
3	Non-H	80	Supernatant	3.6519	952.5	24.7205
4	Non-H	60	Supernatant	3.4706	879.5	14.2356
5	Non-H	60	Supernatant	2.8231	827.0	9.7374
6	Non-H	60	Supernatant	2.5382	734.5	18.9985
7	Non-H	50	Supernatant	2.5641	639.0	15.7571
8	Non-H	50	Supernatant	2.3310	673.0	12.0196
9	Non-H	50	Supernatant	2.07200	585.5	15.3602
10	Non-H	40	Supernatant	1.99430	444.5	4.7099
11	Non-H	40	Supernatant	2.20150	577.5	11.2588
12	Non-H	40	Supernatant	2.09790	592.5	17.5101
13	Non-H	30	Supernatant	2.04610	446.5	3.0892
14	Non-H	30	Supernatant	1.65760	411.0	4.1807
15	Non-H	30	Supernatant	1.83890	492.5	13.8718
16	Non-H	0	Supernatant	1.42450	274.0	2.0970
17	Non-H	0	Supernatant	1.26910	250.0	6.0329

Data table continued

18	Non-H	0	Supernatant	1.29500	284.5	8.4805
19	H	80	Supernatant	4.29940	1207.5	40.2974
20	H	80	Supernatant	4.55840	1180.0	40.9801
21	H	80	Supernatant	4.42890	1149.5	33.0526
22	H	60	Supernatant	2.59000	800.0	10.5978
23	H	60	Supernatant	2.36035	753.5	19.5873
24	H	60	Supernatant	2.28827	762.0	18.9425
25	H	50	Supernatant	1.96840	748.0	15.6046
26	H	50	Supernatant	1.96581	759.0	16.5529
27	H	50	Supernatant	1.93473	743.0	19.4735
28	H	40	Supernatant	1.78969	659.5	12.8357
29	H	40	Supernatant	1.62911	569.5	14.6943
30	H	40	Supernatant	1.63688	577.5	16.0218
31	H	30	Supernatant	1.35457	453.0	11.3943
32	H	30	Supernatant	1.38306	447.0	11.0150
33	H	30	Supernatant	1.44004	474.5	11.4702
34	H	0	Supernatant	0.91168	213.5	3.8786
35	H	0	Supernatant	0.88319	171.5	2.8570
36	H	0	Supernatant	0.83398	198.5	1.9717

Data table continued

37	Non-H	80	Control	4.99870	1245.0	26.3081
38	Non-H	80	Control	6.44910	1329.5	44.5657
39	Non-H	80	Control	3.62600	1051.0	31.6002
40	Non-H	60	Control	3.26340	873.5	15.6579
41	Non-H	60	Control	3.03030	867.0	18.0393
42	Non-H	60	Control	2.48640	819.0	21.2807
43	Non-H	50	Control	2.71950	693.0	8.0505
44	Non-H	50	Control	2.38280	696.5	16.2201
45	Non-H	50	Control	2.22740	700.0	19.0977
46	Non-H	40	Control	2.27920	547.0	13.8718
47	Non-H	40	Control	2.17560	592.5	15.1287
48	Non-H	40	Control	1.99430	541.5	15.6579
49	Non-H	30	Control	2.07200	457.5	3.9823
50	Non-H	30	Control	1.70940	419.0	8.8443
51	Non-H	30	Control	1.76120	422.0	12.7472
52	Non-H	0	Control	1.34680	281.0	2.3947
53	Non-H	0	Control	1.39860	281.0	8.1498
54	Non-H	0	Control	1.45040	275.5	7.9513
55	H	80	Control	4.32530	1300.0	41.3594

Data table continued

56	H	80	Control	4.22170	1291.5	40.2215
57	H	80	Control	4.06630	1327.0	40.6008
58	H	60	Control	2.61590	829.0	26.2631
59	H	60	Control	2.27747	846.0	18.4494
60	H	60	Control	2.28956	876.5	21.9390
61	H	50	Control	1.93732	749.0	19.8908
62	H	50	Control	1.92696	739.0	21.2942
63	H	50	Control	1.85962	738.0	19.6632
64	H	40	Control	1.82336	677.0	17.7667
65	H	40	Control	1.62911	574.0	16.6287
66	H	40	Control	1.66278	595.0	16.4012
67	H	30	Control	1.40896	462.5	12.6081
68	H	30	Control	1.37270	466.5	12.2288
69	H	30	Control	1.45040	481.0	14.3908
70	H	0	Control	1.15773	275.5	6.8072
71	H	0	Control	1.06708	277.5	7.0115
72	H	0	Control	1.07485	281.0	6.9094

LACTOSE TWO WAY ANOVA
The SAS System
The GLM Procedure

Repeated measures analysis of variance tests of hypotheses for between subjects effects

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Condensation	5	5773.135768	1154.627154	38.75	<.0001
Error	30	893.920882	29.797363		

Repeated measures analysis of variance univariate tests of hypotheses for within subjects effects

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Centrifuge	1	205.0994717	205.0994717	16.52	0.0003
Centrifuge*condensation	5	63.6043999	12.7208800	1.02	0.4208
Error	30	372.3672258	12.4122409		

OSMOLALITY TWO WAY ANOVA

The SAS System

The GLM Procedure

Repeated measures analysis of variance tests of hypotheses for between subjects effects

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Condensation	5	5477491.028	1095498.206	21.77	<.0001
Error	30	1509487.333	50316.244		

Repeated measures analysis of variance univariate tests of hypotheses for within subjects effects

Source	DF	Type III S S	Mean Square	F Value	Pr > F
Centrifuge	1	21183.68056	21183.68056	17.40	0.0002
Centrifuge*condensation	5	23572.23611	4714.44722	3.87	0.0079
Error	30	36526.33333	1217.54444		