CHAPTER 2

The Composition and Biospecificity of Human Milk

Effective breastfeeding management requires a general understanding of the structure and function of human milk. Many of the recommendations for successful breastfeeding and optimal infant health outcomes are based on using what the clinician knows about the components of human milk, what they do, and how they work. This chapter and Appendix 2-1 provide an overview of the components of breastmilk and of breastfeeding management based on milk composition and function.

Human milk is an intricate and unique fluid that is strikingly different from the milks of other mammals, including the cow. Aggressive marketing of infant formula has blurred the public's perception of the differences between human milk and infant formula. SummerStyles (formerly called HealthStyles) is a private, proprietary national marketing survey that annually collects health-related opinions of adults over 18 years of age. Results from the 2018 survey found that of over 4,000 adults, only 24% agreed that feeding a baby infant formula would increase the risk of illness in the infant and just 25% agreed that lactation reduces the risk of maternal breast cancer (CDC, 2019). YouGov is an international research data and analytics group that researches opinion data. Their 2019 survey of 1,231 American adults found that only 52% agreed that it was much better for an infant to be fed breastmilk than infant formula (YouGov, 2019). Many respondents in these surveys were unaware of the health benefits of breastfeeding and the risks of not breastfeeding. This confusion and lack of clarity regarding the difference between formula and breastmilk can be caused by clever marketing of infant formula, by contradictory and incorrect or misleading internet resources, and by social media postings of opinions that lack evidence and leave mothers vulnerable to formula marketing claims and peer opinions. Many of these interwoven resources are typically not evidence-based and prey on vulnerabilities of new mothers, resulting in families that may be less likely to initiate or sustain breastfeeding.

Hundreds of human milk components interact synergistically to fulfill the dual function of breastmilk, nourishing and protecting infants and young children who are breastfed or who receive human milk. The addition of multiple ingredients into infant formula derived from nonhuman sources cannot

duplicate the health, cognitive, and developmental outcomes seen in infants fed human milk, no matter what formula advertising might claim.

Lactation and Human Milk Composition

Lactation is a highly complex and ancient process involving thousands of mammary genes (Oftedal, 2020). Mammary glands and lactation were inherited from a pre-mammalian ancestor over 200 million years ago in the Jurassic and/or Cretaceous periods (Oftedal, 2012). Lactation is thought to predate placental gestation and mammals themselves. It appears to have evolved in incremental steps as part of the innate immune system, and over time acquired its nutritional function. The mammary gland most likely evolved from apocrine-like glands or mucous skin glands in association with hair follicles that provided hydration and secreted antimicrobial substances to protect the surface of the egg and skin of the newborn (**Figure 2-1**). These glands evolved from the role of providing primarily moisture and antimicrobials to parchment-shelled eggs to the role of supplying nutrients (Oftedal, 2002), replacing the yolk as the primary nutrient source around 170 million years ago. Much later, due to gene sharing and gene duplication events, two antimicrobial enzymes (lysozyme and xanthine oxidoreductase) evolved new functions within the mammary epithelium, which allowed the secretion of fat, whey protein, sugar, and water, resulting in the unique and complex fluid we call milk (Vorbach et al., 2006).

All primary milk constituents evolved before the appearance of mammals. Milk composition and the length of lactation have been modified and adapted to meet the needs of each particular species. For example, the protein content of milk varies among species. In many species, including human, low-solute milk with comparatively low concentrations of protein may be related to a pattern of frequent feeding. Researchers often refer to species that manifest or practice this

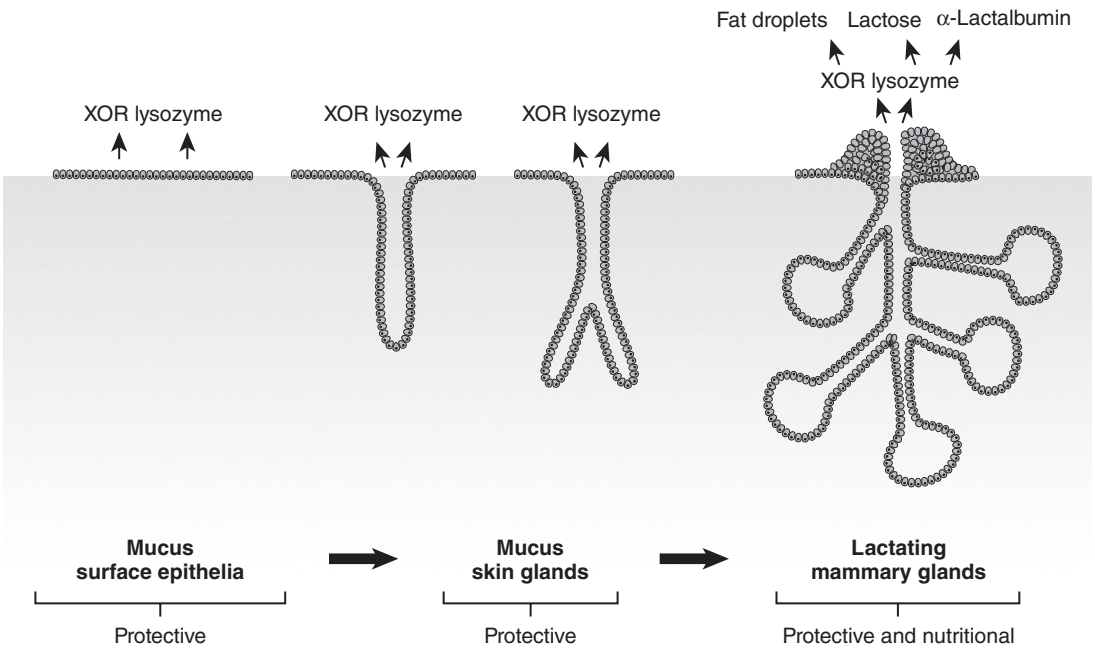

Figure 2-1 Proposed evolution of the mammary gland from a mucous-secreting epithelial gland

Reproduced from Vorbach, C., Capecchi, M. R., & Penninger, J. M. (2006). Evolution of the mammary gland from the innate immune system? *BioEssays, 28*, 606-616. BioEssays by International Council of Scientific Unions; Company of Biologists. Reproduced with permission of John Wiley & Sons Ltd.

concept as "continuous contact" species. Milk varies tremendously in fat content among mammals from less than 1% in the rhinoceros to more than 60% in some seals. Each species has features (e.g., an organ, a behavior, a body system) that serve as major focal points for determining the type, variety, and interactions of the milk components fed to the young. In humans, these focal points include the brain, the immune system, and the acquisition of affiliative behavior.

Human milk composition is not static or uniform like infant formula. Breastmilk is a living dynamic fluid (**Figure 2-2**) that represents an elegant interplay between the needs and vulnerabilities of the infant and the rapid adaptability of the mother's body to provide milk components to meet those needs and support those vulnerabilities:

- Colostrum (1–5 days) evolves through transitional milk (6–14 days) after which human milk is considered fully mature.
- During early lactation, a few hours can show significant changes in milk composition. Lactoferrin for example, decreases significantly over the first three days of lactation.
- Milk composition changes during each feeding as the breast drains and fat content rises.
- Milk composition changes over the course of each 24 hours and over the entire course of lactation.
- Many milk components show a circadian rhythm.
- Milk of preterm mothers differs from that of mothers delivering at term.
- Hundreds of components have been identified in human milk, with some still having unknown roles.
- Hundreds of thousands of immune cells in breastmilk are ingested by the breastfed infant each day.
- Human milk contains stem cells that are involved in the regulation of mammary gland development and tumorigenesis (Thomas et al., 2011).

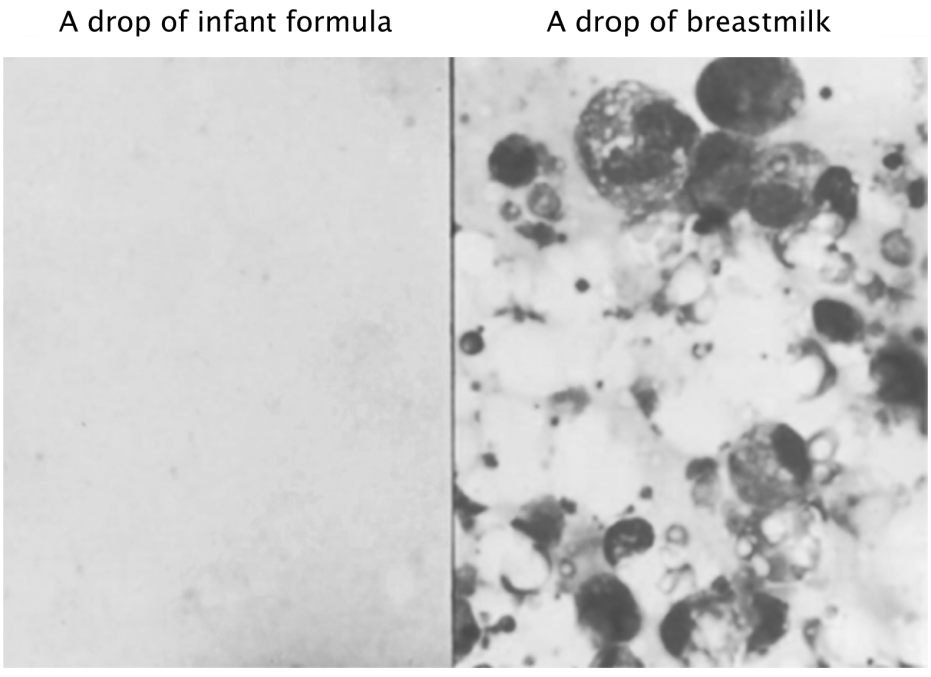

Figure 2-2 Formula and breastmilk magnified and stained. White blood cell infection fighters and fat globules for energy, immunity, and neurodevelopment are seen in magnified and stained breastmilk compared with a breastmilk substitute.

Image from Rebecca Behre, RNC, IBCLC, Gritman Medical Center, Moscow, Idaho.

These stem cells can migrate to different organs to provide active immunity and boost infant development in early life (Hassiotou & Hartmann, 2014).
- Infant formula is an inert nutritional medium with no growth factors, hormones, or live cells like those found in breastmilk.
- Human milk is a biological mediator, carrying a rich variety of bioactive substances intended to grow a brain, construct an immune system, and facilitate affiliative behavior.

Colostrum

Colostrum is the first milk and is present in the breasts from about 12–16 weeks of pregnancy onward. The thick fluid's yellowish color comes from beta-carotene. Colostrum is present in relatively low quantities in the first few days postpartum. Colostrum differs from mature milk in both the nature of its components and in their relative proportions (**Table 2-1**). It is rich in immunologic components such as secretory IgA, lactoferrin, leukocytes, as well as developmental factors such as epidermal growth factor. Colostrum also contains relatively low concentrations of lactose, indicating its primary functions to be immunologic and trophic rather than nutritional (Ballard & Morrow, 2013). Levels of sodium, chloride, and magnesium are higher and levels of potassium and calcium are lower in colostrum than mature milk. When tight junction closure occurs in the mammary epithelium, the sodium to potassium ratio declines and lactose concentration increases, indicating secretory activation, the onset of copious milk production, and the change to transitional milk.

Pancreatic Secretory Trypsin Inhibitor

Pancreatic secretory trypsin inhibitor (PSTI) is a 56-amino acid peptide responsible for protecting the pancreas from autodigestion. Found in highest levels in colostrum at seven times the concentration of mature milk, PSTI protects and repairs damaged intestinal cells as it establishes and maintains infant gut

Table 2-1 Selected Components of Colostrum

Component	Description
Calories	53.6/100 mL (3.38 oz) or 15.85/oz compared with 65/100 mL or 19.23/oz average in mature milk
Volume	0.06–8.11 oz/day over the first three days (based on range of infant intake)
Sodium, fat soluble vitamins, carotenoids, secretory IgA	Higher than in mature milk
Protein	Twice as high as mature milk
Carbohydrates, lipids, lactose	Lower than in mature milk
Pancreatic secretory trypsin inhibitor	Highest concentrations in colostrum
Antioxidants	Higher than in transitional and mature milk
sCD14	Higher than in transitional or mature milk (part of a complex that activates the immune system)
Human milk oligosaccharides	Highest in colostrum and decreasing over time in mature milk

integrity (Marchbank et al., 2009). This high concentration of PSTI in colostrum not only protects and repairs the cells lining the newborn's delicate intestine, but also prepares the gut for handling the "food" to follow. PSTI is not found in commercial infant formulas.

Xanthine Oxidase

Xanthine oxidase is an essential enzyme found in highest concentrations in maternal colostrum. It is located on the outer surfaces of fat globules, attracting pathogens to bind to it and diverting bacteria away from their target, including the digestive tract. A study showed that the combination of breastmilk with saliva containing hypoxanthine and xanthine, generates hydrogen peroxide, which activates the lactoperoxidase system and results in microbial growth inhibition (Sweeney et al., 2018). Endogenous breastmilk xanthine oxidase has been shown to generate the antimicrobial radical nitric oxide, and its influence on the growth of *Escherichia coli* and *Salmonella enteritides* has been examined. Breastmilk, but not infant formula, generated nitric oxide. Xanthine oxidase activity substantially inhibited the growth of both bacteria. The authors concluded that an important natural antibiotic system is missing in formula feeds (Stevens et al., 2000).

Prematurity

The colostrum of mothers delivering preterm is more highly enriched with potent disease protectors than the colostrum of mothers delivering at term. This may occur because the causes of preterm labor, such as inflammation, release signals into the systemic maternal circulation that affect the mammary glands (Trend et al., 2016). Enrichment of specific immune factors varies with the degree of prematurity. Colostrum from mothers delivering before 30 weeks of gestation contains lower concentrations of numerous immune components compared with the colostrum of mothers delivering between 30–37 weeks and 38–41 weeks. For example, while the IgA concentration was higher in colostrum from mothers delivering between 30–37 weeks gestation, the IgA concentration from mothers delivering at less than 30 weeks was significantly lower (Castellote et al., 2011). Perhaps when delivery occurs before 30 weeks, the ability of the lactation process to adapt is blunted or not as efficient as in later preterm and term deliveries (Castellote et al., 2011). Even though some immune factors may be reduced in very preterm colostrum, infant formula contains none of these formidable fighters of infection, leaving infants who are not fed colostrum or human milk much more vulnerable to infections, diseases, and conditions prevented or reduced by breastfeeding or the provision of expressed colostrum and breastmilk. Of interest is that the role of transforming growth factor-β2 (TGF- β2) in the mammary gland may extend beyond its role of facilitating the immune response in the neonate. It has been known for many years that TGFβ suppresses milk secretion (Monks, 2007). In mouse models, expression of TGF-β2 is up-regulated in the mammary gland during pregnancy, but expression rapidly decreases during lactation, and it is implicated in the hormonal regulation of initiation of lactation in association with progesterone. Therefore, persistent higher levels of TGF-β2 in milk from preterm mothers could be associated with, or be a cause of, delayed onset of secretory activation in preterm mothers (Monks, 2007).

Diabetes

Colostrum of diabetic mothers is subject to biochemical and immunological alterations that affect the levels of some of its components. The protein expression involved in immunity and nutrition differs between colostrum of mothers

with gestational diabetes and that of mothers without gestational diabetes (Grapov et al., 2015). Hyperglycemia altered IgG transfer across the placenta and decreases immunoglobulin levels in maternal blood and colostrum (Franca et al., 2012). The colostrum of diabetic mothers is higher in glucose, higher in lipase, lower in secretory IgA and secretory IgG, lower in C3 protein, and lower in amylase (Morceli et al., 2011). It is important that diabetes in mothers be tightly controlled to minimize alterations in colostrum's immune properties.

Smoking

Colostrum of mothers who smoke during lactation has a significantly lower antioxidant capacity than the colostrum of mothers who do not smoke (Napierala et al., 2019). This impairs colostrum's ability to protect the infant from free radicals that contribute to conditions related to oxidative stress to which preterm infants are so susceptible, such as necrotizing enterocolitis (NEC) and retinopathy of prematurity. Certain cytokine (IL-1β and IL-8) levels were found to be significantly lower in the colostrum of mothers who smoke, increasing the newborn's vulnerability to infection (Piskin et al., 2012). Smoking also increases oxidative stress in the mother's plasma and mature milk (Napierala et al., 2019).

Irisin, Adropin, and Copeptin

Colostrum is enriched with irisin, adropin, and copeptin, peptides that are implicated in postnatal adaptation with respect to thermoregulation, vascular adaptation, glucose metabolism, lung function, and fluid homeostasis (Briana et al., 2017). The prime functions of adropin include regulating carbohydrate, lipid, and protein metabolisms by moderating glucose-mediated insulin release. Irisin is an anti-obesitic and antidiabetic hormone regulating adipose tissue metabolism and glucose homeostasis by converting white to brown adipose tissue. Copeptin is a regulator of fluid homeostasis. Early colostrum feeding, particularly of infants born by cesarean section, who are prone to hypothermia, breathing disorders, and dehydration, takes on more urgency in light of the importance of colostrum in body system adaptation and regulation.

Cesarean Delivery

Cesarean delivery can alter the composition of colostrum. Colostrum microbiota composition is strongly influenced by the mode of delivery, as significant differences in terms of biodiversity, bacterial abundance, and microbial interactions have been observed when comparing cesarean section colostrum microbiota with that of vaginal delivery colostrum microbiota (Toscano et al., 2017). Cesarean section colostrum showed a higher abundance of microorganisms of environmental origin compared to vaginal delivery colostrum. Mothers experiencing a cesarean delivery were more exposed to environmental bacteria, which could play a defining role in modulating the microbiota of colostrum (Toscano et al., 2017). A study looking at melatonin levels in colostrum found that melatonin levels were highest in the colostrum of the vaginally delivered group of mothers, lower in the elective cesarean section group, and the lowest in the emergency cesarean group (Namli et al., 2018). Melatonin has not only sleep modulation properties but because of its antioxidant properties it also functions to reduce oxidative stress in neonates with sepsis, asphyxia, respiratory distress, or surgical stress (Sanchez-Barcelo et al., 2011). Avoiding unnecessary cesareans would maximize the melatonin content of colostrum, while the provision of colostrum to infants at risk for oxidative stress would remain an important intervention. The colostrum of mothers delivering by cesarean

is lower in antioxidant status than in mothers who deliver vaginally (Simsek et al., 2015), potentially impeding the ability of colostrum to protect the infant from cellular damage caused by oxidative stress.

Genetics and Environment

Genetic and environmental factors may contribute to the compositional diversity seen in the colostrum of mothers worldwide. Musumeci and Musumeci (2013) reported the compositional differences between colostrum of mothers living in Sicily and those living in Burkina Faso, one of the poorest countries of the African sub-Saharan area. The colostrum of the African mothers was richer in growth factors (IGF-I) that favor intestinal maturation; endorphins and S100B, which protect the brain from the consequences of asphyxia under difficult childbirth conditions; and chitotriosidase (an enzyme produced by activated macrophages), which is protective against gut pathogens, nematodes, and *Candida albicans*. It is thought that these compounds are present at higher levels in African mothers' colostrum due to the precarious conditions of life in this particular country, which exert a selective pressure to preserve the newborn. This protection was also demonstrated by analyzing the oligosaccharides in the African colostrum which showed a characteristic secretion of 2-fucosyl lactose earlier than that found in Sicilian colostrums.

Given the potential stressors on the composition of colostrum, it would seem prudent to assure maximum intake of colostrum for infants who are born by cesarean section, who experienced a difficult or precarious delivery, whose mothers smoke, whose mothers are diabetic, or who were born preterm.

Microbiome of Breastmilk

The human body is host to trillions of microorganisms that inhabit various niches or environments throughout the body. The totality of microorganisms (such as bacteria, fungi, and viruses) that inhabit a particular body environment, their genetic elements, and their interactions with the environment is called the microbiome.

Long thought to be sterile, breastmilk has been shown to contain over 700 species of bacteria (Cabrera-Rubio et al., 2012) and 100–10,000 viable bacteria per mL (Fernandez et al., 2013). Historically, any bacteria that appeared in breastmilk was thought to be pathologic, and mothers were advised to go to great lengths to wash their hands and disinfect their nipples before putting their baby to breast. Around 2003, studies began emerging that described the presence of commensal, physiologic, or normal bacterial populations in human milk (Heikkila & Saris, 2003; Martin et al., 2003). There are several potential origins and mechanisms thought to be responsible for bacterial presence in breastmilk:

- Skin bacteria from the nipple and areola could enter the breast through the nipple pores. It has been shown that during the first 30 days of life, infants who are primarily breastfed receive 27.7% of the bacteria from breastmilk and 10.4% from areolar skin (Pannaraj et al., 2017).
- Bacteria could enter milk through a break in the nipple epithelium when a crack or other damage has occurred.
- A retrograde pathway for bacterial entry into breastmilk could occur as bacteria from the infant's oral cavity is drawn into the breast during milk backflow. Milk has been shown on ultrasound to reverse course and flow back into milk ducts following maximum duct dilation during the milk

ejection reflex. (Ramsay et al., 2006). Similarity between infant oral microbiota and breastmilk microbiota (Bisanz et al., 2015) also supports the retrograde pathway.

- Bacterial translocation from the maternal gut to the breast can occur during pregnancy and lactation. Bacteria-carrying dendritic cells migrate out of the mesenteric lymph nodes in the maternal intestines and into the breasts (Urbaniak et al., 2012). This is referred to as the entero-mammary pathway. It has been demonstrated that colostrum collected even before the first time the infant goes to breast already contains a community of microbes (Damaceno et al., 2017) further supporting the operation of an entero-mammary pathway.
- Breastmilk microbiota can be influenced by many features and determinants such as local pathologies of the breast, mode of delivery, antibiotic receipt, maternal health, and gestational age (Bode et al., 2014). Indirect breastfeeding (i.e., pumped breastmilk) has been shown to contain a depleted complement of a *Bifidobacterial* species (Moossavi et al., 2019), which is normally found in high levels in breastmilk and constitutes the majority of shared taxa between mother's milk and infant stool (Biagi et al., 2017). Both the act of pumping and the milk's lack of contact with the infant's oral cavity may have an impact on shaping and altering the microbiome of breastmilk (Moossavi et al., 2019) as well as the microbiome of the infant's gut (Fehr et al., 2020). Moossavi et al. (2019) also found that *Enterobacteriaceae* and other potential pathogens were enriched in pumped breastmilk.

In the Human Microbiome Project it was found that breastfeeding during infancy was a major life-history characteristic that affected adult bacterial composition (Ding & Schloss, 2014). Infancy is said to be a critical window for bacterial imprinting of breastmilk bacteria (Koenig et al., 2011; Perez et al., 2007) as breastmilk's bacteria that first seed the infant gut influence and select for the bacteria that follow, leaving a footprint that can be detected into adulthood (Ding & Schloss, 2014).

Breastmilk contains a rich diversity of bacteria such as *Staphylococcus, Streptococcus, Corynebacterium, Propionibacterium*, lactic acid bacteria, and *Bifidobacterium* that vary among populations of women depending on maternal characteristics such as body mass index and mode of delivery (Ruiz et al., 2019). Some bacterial strains belonging to the species *Lactobacillus salivarius, Lactobacillus fermentum, Lactobacillus gasseri, Bifidobacterium breve, Bifidobacterium adolescentis*, and *Bifidobacterium longum* subspecies *infantis* have been shown to promote maternal and infant health, including the prevention or treatment of lactational mastitis, the promotion of a normal gut bacterial colonization in preterm neonates, or the amelioration of diarrhea in IBS patients (Ruiz et al., 2019). *Lactobacillus* species and bifodobacteria typically dominate the breastfed infant's gut. Bifidobacteria are nourished by the human milk oligosaccharides (HMO) in breastmilk. HMOs are complex, highly abundant sugars that function as substrates or food for specific microbes, including certain species of *Bifidobacterium*. The microbiomes of newborns and young infants are enriched in genes required for the degradation of those HMOs. This coevolution between bifidobacteria and the infant gut, mediated by HMOs, contributes to the road map for colonization of the gut in early life, which has significant importance to the immune system. Reduction of specific microbes, including *Bifidobacterium* (as seen in the formula-fed infant gut), in early life has been associated with increased risk of allergy and asthma development in childhood, and is suggested to compromise immune function and lead to increased susceptibility to infectious disease. Relatively small amounts of hospital formula supplementation of breastfed

infants during the first days of life results in shifts in microbiota composition, which has been associated with the risk of overweight (Forbes et al., 2018).

Allergy

Allergic diseases are currently among the most common chronic diseases in the world. Atopic dermatitis (AD), food allergy (FA), allergic rhinitis, and asthma are largely determined during the first 1,000 days of life (Anandan et al., 2010). The microbiota is key in the development of allergy and asthma in early childhood, as has been suggested in multiple studies of gut, airway, and skin microbiota (Salameh et al., 2020). Moreover, alterations of the microbiota can precede development of allergies emphasizing a pathogenic role of gut dysbiosis. For example, studies of gut microbiome samples obtained from infants has demonstrated that a low gut microbial diversity during the first month of life precedes the development of AD at two years and asthma at seven years of age (Abrahamsson et al., 2014). Breastmilk shapes the gut microbiota and metabolism favoring the growth of bifidobacteria, enriching the infant gut with IgA, and creating an anti-inflammatory environment that promotes the activity of components and pathways that work to prevent allergy (van den Elsen et al., 2019). Breastfeeding and the provision of breastmilk to neonates is especially important in families with a history of allergic diseases.

Allergic diseases threaten body barriers such as the gut mucosa as in food allergies. A problem, defect, or deficiency in barrier function contributes to the expression of an allergy. At birth, many of the mechanisms that protect the infant's gut barrier physiology may only be marginally functioning:

- *Secretory IgA.* SIgA promotes the clearance of antigens and pathogenic microorganisms from the intestinal lumen by blocking their access to epithelial receptors, entrapping them in mucus, and facilitating their removal by peristaltic activities. IgA down-regulates pro-inflammatory responses normally associated with the uptake of highly pathogenic bacteria and potentially allergenic antigens, and promotes the retro-transport of antigens across the intestinal epithelium (Mantis et al., 2011). IgA secretion in a neonate is very low.
- *Mucus.* A layer of mucus for pathogen and antigen entrapment and elimination produced by goblet cells. Goblet cells in the infant at birth are very low.
- *Tight junctions.* Gut epithelial cells are typically sealed by tight junctions that block pathogens and allergens from entering systemic circulation. However, gut permeability at birth is high.
- *Inflammatory response.* The neonatal gut has a tendency to respond to stimuli with a high inflammatory response. Inflammation plays a critical role in allergy development favoring allergic sensitization to dietary antigens in early life.

Breastmilk components compensate for these vulnerabilities by being rich in IgA, by containing human milk oligosaccharides that attenuate inflammation, through vitamin A and growth factors that help decrease gut permeability, and by promoting the colonization of the gut with microbiota that induce tolerance to antigens. Reduced levels in breastmilk of these myriad factors or the absence of breastfeeding could lead to low-grade inflammation and its resulting prevention of tolerance to dietary antigens. Modifications to breastmilk have been studied, with mixed and uncertain results, to see if increasing the allergen content of the milk would provide allergy protection to eggs and cow's milk,

by maternal vitamin A and fish oil supplementation, and by maternal probiotic administration (Munblit & Verhasselt, 2016).

Nutritional Components of Breastmilk

Breastmilk is a complex, dynamic matrix with a general composition of 87% water, 3.8% fat, 1.0% protein, and 7% lactose. All components of breastmilk are dissolved, dispersed, or suspended in the water portion of the milk. Milk component levels vary depending on the length of time post-birth, maternal diet, maternal health, environmental exposures, mode of delivery, time of day, infant health, infant gender, and if breastmilk is exclusively expressed. The dynamic nature of breastmilk is specifically tailored by each mother to reflect the requirements of her infant.

Water

Infants receiving an adequate amount of breastmilk will automatically consume their entire water requirement. Even in hot or arid climates, human milk provides 100% of water needs (Ashraf et al., 1993). Supplementation with sterile water or glucose water is not appropriate or recommended because it does not provide sufficient nutrition, could lead to weight loss, does not reduce serum bilirubin levels, and might cause hyponatremia (Kellams et al., 2017). With immature kidneys and a strong hunger drive, too much water can cause the loss of sodium, which can affect brain activity, provoke seizures, and lead to a rare condition called water intoxication. Water intoxication may result from inappropriate feeding practices such as giving water bottles to lengthen the time between breast or formula feedings, to relieve respiratory symptoms, to hydrate infants during hot weather, to stretch formula bottles, or for financial reasons. There has been a fad in the health food industry for the use of plant-based milk products, which are low in protein, calories, and fat, and high in water content. Most commonly, soy, rice, almond, and sweet chestnut milk are used as an alternative to dairy products. Parents may give infants these products for financial reasons, to consume a vegan diet, or even to treat a presumed milk allergy, and the marketing behind these products has misled parents to believe that these plant-based beverages are safe alternatives to breastmilk and infant formula. Reports have detailed adverse outcomes in infants due to water intoxication from consuming plant-based milks (Houck et al., 2019). The nutritional quality of plant-based beverages is lower than that of breastmilk, cow's milk, and infant formula, and are not a safe nutritional alternative, especially with the high water content and low nutrient values.

Glucose water provision for neonatal hypoglycemia has been mostly eliminated from hypoglycemia treatment protocols (Abramowski & Hamdan, 2020) to avoid rebound hypoglycemia and in favor of more effective treatments with fewer side effects. Water supplements may discourage feeding at the breast, depress the infant's appetite, contribute to a reduced milk supply, and lead to early formula supplementation. One study reported that infants who were given water or teas in the first seven days of life were three times more likely than other infants to receive non-breastmilk fluids by four weeks of age (Wojcicki et al., 2011). Ready to feed 2-oz bottles of sterile water, 5% glucose water, and 10% glucose water are easily available on websites and are described for use as an initial feeding or for supplemental feedings.

Lipids (Fat)

Lipids are the largest source of energy in breastmilk, providing 40–55% of the total energy of breastmilk and ranging in content from 3.5– 4.5% during lactation. These lipids are present as an emulsion. Triacylglycerides represent the majority of lipids, contributing 98% of the lipid fraction. The rest of the lipids consist of diacylglycerides, monoacylglycerides, free fatty acids, phospholipids, and cholesterol. These are all contained within milk fat lipid globules, with phospholipids, cholesterol, glycolipids, proteins, and glycoproteins forming most of the outer globule membrane and the triacylglycerols housed in the core. Breastmilk contains over 200 fatty acids that are either saturated or unsaturated and either short-, medium-, or long-chained. Lactocytes (milk-making cells) secrete the fat globule including the short chain (SCFA) and medium chain fatty acids (MCFA). The long chain (LCFA) and long chain polyunsaturated fatty acids (LCPUFA) are obtained and imported from the maternal blood stream. LCPUFAs (molecules with a chain length of more than 20 carbon atoms plus 2 or more double bonds) include the omega-3 fatty acid docosahexaenoic acid (DHA) and the omega-6 fatty acid arachidonic acid (AA or ARA). LCPUFAs constitute about 2% of the total fatty acids in breastmilk. A shorthand notation is commonly used when referring to fatty acids. The chemical formula is abbreviated by stating the number of carbons to the left of the colon and the number of double bonds to the right of the colon, for example:

- 16:0 palmitic acid
- 18:2 linoleic acid
- 20:4 arachidonic acid
- 22:6 docosahexaenoic acid

Joining three fatty acids to a glycerol backbone forms the triacylglycerol. The positions occupied by fatty acids along the glycerol backbone commonly appear in very specific positions, which are given stereospecific numbers (sn). Thus, oleic acid usually occupies the sn-1 position on the glycerol backbone, palmitic acid the sn-2 position, and linoleic acid the sn-3 position. The positions of fatty acids along the glycerol backbone influences their availability and how well they are absorbed by the body. This becomes important as these positional preferences of fatty acids are not replicated in infant formula (Sun et al., 2018). Various combinations of vegetable oils are used in infant formula and can adversely affect how the infant absorbs fat and calcium. Calcium/Fatty acid complexes may be formed, known as calcium soaps, which are insoluble, indigestible, and related to the stool hardness seen in formula-fed infants. The formation of calcium soaps may partly explain the substantial differences in the absorption of nutrients (fat and calcium) and bowel habits between breast- and formula-fed infants (Leite et al., 2013).

DHA and ARA are found in high levels in the structural lipids of cell membranes, particularly those of the retina and central nervous system. Their accretion primarily occurs during the last trimester of pregnancy and the first year of life. Infants must make their own DHA and ARA if not provided in their nutritional source. LCPUFAs are synthesized from the essential fatty acids alpha-linolenic acid (ALA) and linoleic (LA) through the elongase and desaturase systems. However, the enzyme systems that are responsible for this are somewhat inefficient during the early months of life. This is why infants fed formula without LCPUFA have a significantly lower plasma or red blood cell levels of DHA and ARA compared with those who were breastfed or fed formula supplemented with LCPUFA, and why DHA and ARA have been added to infant formulas. Fish and

algal oils are the main sources of DHA and ARA added to infant formulas-DHA from fermented microalgae (*Crypthecodinium cohnii*) and ARA from soil fungus (*Mortierella alpina*). DHA in breastmilk is preferentially esterified in the sn-2 position, but algal and fish oils do not have a strong positional specificity and are found in similar proportions at the sn-1, sn-2, and sn-3 positions. The fatty acid blends in formulas that contain palm oil, and palm olein oil can reduce the retention of calcium and fat absorption (Souza et al., 2017). DHA and ARA are considered essential for maturation of the developing brain, retina, and other organs. While formula manufacturers have marketed DHA-supplemented formula as a product to enhance cognitive development, full-term babies fed formula milk supplemented with LCPUFA did not have better outcomes than were reported for full-term babies fed formula milk without LCPUFA (Jasani et al., 2017). Breastfeeding is consistently associated with enhanced cognitive outcomes and higher IQs compared with formula-fed infants (Jasani et al., 2017; Lenehan et al., 2020). The DHA in infant formula may have the same chemical formula as the DHA in breastmilk, but it is structurally different than the DHA in breastmilk and is unlikely to function identically to breastmilk's DHA. A review of the scientific literature, published by the international research network Cochrane, found no clear evidence that the DHA-supplemented formula benefits babies' brain development (Jasani et al., 2017).

Recently, milk fat globule membrane (MFGM) supplements have been added to some infant formulas. The MFGM factions in infant formula are typically extremely low because the fat fraction containing the MFGM is discarded during the formula production process. The MFGM composition confers many of the benefits of breastmilk on immune function and neurodevelopment. However, the MFGM added to infant formula is bovine in origin. The biological functions of MFGM fractions vary significantly in milk from different species and from various ingredient sources. MFGM composition of bovine colostrum and mature milk shows numerous differences from human colostrum and mature milk (Cao et al., 2019). After birth, newborns must adapt to the rapid changes of their environment; thus, a higher diversity of *N*-glycoproteins in colostrum may play important roles in building up the immune system to protect against pathogen infections. Cao et al (2019) identified 164, 134, 74, and 39 *N*-glycoproteins in the human colostrum, human milk, bovine colostrum, and bovine milk, respectively, related to immune system processes. The lack of some *N*-glycoproteins in bovine colostrum and milk compared to human milk as well as differing functions between the two species leave questions remaining as to the efficacy of the addition of MFGMs to infant formula. One study showed that infants fed formula supplemented with MFGM had better health outcomes than those fed a standard formula, but could not match the health outcomes of the breastfed control group (Li et al., 2019).

Human milk fat content is relatively unaffected by maternal diet, but the fatty acid content is. However, studies linking diet and breastmilk fatty acid composition have not shown consistent results. Some studies have shown that women who consume fish and other foods containing high levels of PUFA have relatively higher breastmilk n-3 fatty acids and DHA concentrations compared to milk from women who consume diets that are low in these nutrients (Kim et al., 2017). Pregnant women may be influenced by manufacturers of prenatal supplements that claim it is difficult to consume enough food sources that are high in omega-3 fatty acids. While claims that these supplements will result in breastmilk that will improve infant brain development, they may change the balance of fatty acids in breastmilk and result in potentially unwanted side effects in the recipient infant. A study that looked at supplementing pregnant women with fish

oil capsules showed an increase in the percent composition of eicosapentaenoic acid (EPA) in breastmilk. This was associated with decreased defensive inflammatory mediators in breastmilk and a gut microbiome that had reduced immune priming capability and less colonization resistance (Quin et al., 2020). Because fish oil appeared to alter infant gut microbial composition through changes in breastmilk, these authors recommended further epidemiological studies to clarify whether early fish oil exposures altered infant infectious disease susceptibility. Higher levels of medium chain fatty acids are found in the milk of mothers consuming a low-fat high-carbohydrate diet. Factors that have been reported to influence breastmilk lipid content include maternal age, geographic location, gestational age of the infant, parity, maternal diet during pregnancy and lactation, body mass index (BMI), stage of lactation, time of day, beginning or end of a feeding, smoking, and the number and duration of breastfeedings per day (**Table 2-2**).

Lipids perform other functions besides acting as the largest energy source in breastmilk. Short-chain and medium-chain fatty acids are involved in the maturation of the gastrointestinal tract. Long-chain fatty acids are not only involved in infant visual and brain development but also have antiviral and anti-protozoal effects. Milk phospholipids contribute to the lipid composition of breastmilk. Among the several classes of sphingo- and glycolipids are gangliosides, which contribute to the host defense by binding bacterial toxins. Sphingomyelins in the milk fat globule membrane are involved in the myelination of the central nervous system. The sterol content of human milk ranges from 10–20 mg/dL, rising over the course of lactation, with cholesterol as the major

Table 2-2 Selected Factors Influencing Human Milk Fat Content and Composition

Factor	Influence
Maternal age	Fat content in colostrum is higher in mothers older than 35 years of age (Lubetzky et al., 2015) and is positively correlated with maternal age (Dritsakou et al., 2017).
Gestational diabetes	Fat and energy content of human milk is lower (Shapira et al., 2019).
Within and post-feeding	Fat content rises during a feeding as the breast empties and is highest 30 minutes post-feeding (Hassiotou et al., 2013).
Mastitis	Reduces breastmilk fat and energy content (Say et al., 2016).
Maternal BMI	Increased BMI alters fatty acid concentrations in breastmilk, generally increasing SFA and n6 PUFAs (pro-inflammatory) and decreasing FAs from the n3 series (DHA-anti-inflammatory; de la Garza Puentes et al., 2019).
Length of gestation	Initially higher in preterm milk during the first three weeks or so of lactation (Fischer Fumeaux et al., 2019).
Smoking/Vaping	Contains ~23% lower lipid content (Bachour et al., 2012; Baheiraei et al., 2014) as well as lower amounts of omega-3 fatty acids.
Maternal diet	Lower DHA in milk of mothers with reduced intake of foods that are natural sources of DHA (Barrera et al., 2018).
Method of milk removal	Higher fat content in manually expressed milk (Mangel et al., 2015).
Maternal allergic status	Women with a combination of eczema and respiratory allergy had lower breastmilk levels of several PUFAs including DHA (Johansson et al., 2011).
Storage conditions	Decreases over time when frozen (Orbach et al., 2019). Rises to the top of refrigerated milk forming a high-calorie cream layer.

component. The total cholesterol content of 90–150 mg/L in human milk is in contrast to only 0–4 mg/L in infant formula. Cholesterol is an essential part of all membranes, and considerable amounts are incorporated into myelin in the nervous system during the period of rapid brain growth. It serves as the substrate for the synthesis of bile acids, lipoproteins, vitamin D, hormones, and oxysterols that modulate cholesterol, lipid, and glucose homeostasis, and is required for normal growth and functioning. Breastfed infants' serum cholesterol levels are higher than those of formula-fed infants. This early exposure to the high-cholesterol content of breastmilk may program fat metabolism, improving the body's ability to metabolize fat in later life and positively modifying cardiovascular risk factors. Adolescents who were exclusively breastfed were shown to have lower low-density lipoprotein (LDL—bad cholesterol), lower total cholesterol levels, and lower levels of triglycerides (fats) compared to those who were exclusively formula fed or mixed fed (Hui et al., 2019).

Protein

Protein levels in breastmilk are relatively low compared to some other mammals, approximately 1% on average. Compared with 9 g/L in term breastmilk (6–12 g/L, week 10/12; Gidrewicz & Fenton, 2014), protein content ranges from 12–19 g/L in infant formulas and from 16–27 g/L in follow-up formulas. The high protein content of infant formula has been implicated in the increased risk for obesity in formula-fed infants (Luque et al., 2015). Protein concentration is highest in the early days, declines to relatively stable levels in mature milk, and is higher in preterm milk than term milk. Breastmilk contains over 400 different proteins, which engage in multiple functions—they provide nutrition, possess antimicrobial and immunomodulatory properties, and stimulate the absorption of nutrients (Andreas et al., 2015). There are three major groups of protein, casein, whey, and mucin. The whey/casein ratio in human milk has been shown to fluctuate between 89:11 in colostrum and 65:35 in milk collected 6 to 15 days after delivery. From days 16 through 360, the ratio stabilized to approximately 60:40, ranging from 59:41 to 61:39 (Lönnerdal et al., 2017).

Casein proteins account for 13% of the total protein, are present in casein micelles suspended in solution, and are the lowest in concentration of any species. Three types of caseins are present in human milk, α, β, and κ. The high levels of casein in bovine milk give it the characteristic white color, while the lower concentrations in human milk render a pale or blue appearance. The casein concentration in cow's milk is more than 10 times greater than in human milk, which is why whey protein is added to infant formula to offset the formation of hard curds in the infant stomach. The low casein content of breastmilk is attributed to the slower growth rate of human infants compared to other infant mammals. The casein micelle in breastmilk is the main source of calcium and phosphorous and is necessary for infant bone mineralization. The enzyme protease breaks down caseins into smaller peptides that are not only antimicrobial and immunomodulatory but also antithrombotic, antihypertensive, and have opioid effects. Caseomorphins have structures similar to opioid peptides and may thus affect infant sleep–wake patterns and psychomotor development (Kost et al., 2009). β-casein exhibits antimicrobial activity toward *Haemophilus influenza* and *streptococci*. *H. pylori* is less common in breastfed than in formula-fed infants (Lönnerdal, 2013), as κ-casein inhibits bacterial adhesion, including the adhesion of *Heliobacter pylori.* Caseins may exhibit immunomodulatory activity by regulating chemotaxis (cell movement or migration) and ameliorating inflammation (Chatterton et al., 2013).

Whey proteins represent a major portion of the protein in human milk—90% of the total protein in colostrum and 60% of the total protein in mature milk. Whey proteins comprise a large number of different proteins that include major immunological proteins such as lactoferrin, lysozyme, and secretory IgA, as well as α-lactalbumin and bile salt-stimulated lipase, which have nutritional roles.

- Lactoferrin has multiple functions. The highest lactoferrin levels were noted between days 1 and 3 (5.05 mg/ml), decreasing to 3.30 mg/ml in milk expressed at 6 to 15 days and to 1.44 mg/ml in milk collected at 91 to 360 days (Lönnerdal et al., 2017). It binds to the majority of iron in breastmilk and facilitates the uptake of iron into cells. The bacteriostatic effects of lactoferrin are seen in its activity to withhold iron from bacteria that require it for growth. To maintain this bacteriostatic capacity, lactoferrin needs to be in an environment with a low iron concentration. If exogenous iron is added to breastmilk, the benefits of lactoferrin might be impaired, which in turn might increase the risk of infection in newborns (Chan et al., 2007). One study showed adding human milk fortifier that contained iron to term colostrum reduced the bacteriostic action of breastmilk against *E. coli* (Campos et al., 2013). Iron supplements, for a full-term infant who is not iron-deficient, could overwhelm the lactoferrin, causing an overgrowth of intestinal bacteria, resulting in diarrhea and possible microscopic bleeding. Lactoferrin also exhibits antibacterial, antivirus, antifungal, and antiprotozoan activities with its ability to kill *Streptococcus mutans*, *Streptococcus pneumoniae*, *Escherichia coli*, *Vibrio cholera*, *Pseudomonas aeruginosa*, and *Candida albicans.* Lactoferrin modulates the innate and adaptive immune responses and can act as an anti-inflammatory agent. Lactoferricin is a by-product of lactoferrin digestion, which inhibits *Escherichia coli* attachment to intestinal cells (Haschke et al., 2016). Lactoferrin is subject to degradation by high temperatures as seen in the pasteurization of breastmilk. Lactoferrin is severely reduced by flash-heating (74%) and least severely by high hydrostatic pressure (25%). Holder pasteurization, commonly used by milk banks, has been shown to reduce lactoferrin by about 48% (Pitino et al., 2019).
- Lysozyme is another major component of the whey fraction in breastmilk. Lysozyme concentrations vary by duration of lactation, but rather than slowly declining as lactation progresses like many other bioactive proteins, concentrations of lysozyme appear lowest in colostrum and increase through the early months of lactation. This may represent a safeguard adaptation to offset the decline in other protective factors so that the infant receives continued protection from pathogens during the time that solid foods are introduced. Lysozyme lyses the cell walls of most gram-positive bacteria such as *S. aureus.* While lysozyme alone is bacteriostatic, an *in vitro* study showed that in presence of lactoferrin it is also bactericidal and can kill several gram-negative bacteria. The mechanism of action is not fully understood, but it suggests that lactoferrin alters the gram-negative outer cell membrane, enabling lysozyme to break down the inner membrane of the bacteria (Ellison & Giehl, 1991). It also shows inhibition of amoebae and has anti-HIV activity. Human milk lysozyme supports the growth of resident commensal bifidobacteria in breastmilk while inhibiting the growth of adult-like strains of bifidobacteria (Minami et al., 2016). Lysozyme is subject to degradation by high heat treatment in pasteurization methods. Pasteurization of donor human milk by irradiation with ultraviolet-C wavelength showed a higher retention rate of lysozyme than with high heat Holder pasteurization (Christen et al., 2013).

- Secretory immunoglobulin A (IgA) is one of a group of immunoglobulin proteins produced by plasma cells that include IgG, IgA, IgM, IgE, and IgD. IgA shields mucosal surfaces from invasion by preventing adherence of pathogens to the intestinal epithelial surface and neutralizing toxins and viruses. IgA is the major immunoglobulin in human milk, is highest in colostrum, and gradually decreases in milk over time. Median sIgA concentrations decreased from 5.45 mg/ml at days 0 to 5 to 1.50 mg/ml 6 to 15 days after delivery. Concentrations of sIgA remained at or below this level throughout the first year of lactation (Lönnerdal et al., 2017). Maternal immunity against several general pathogens can be transferred through the breastmilk via sIgA, mediated by the enteromammary immune pathway. This process augments infant immunity through the acquired immunity of the mother. sIgA antibodies against numerous bacterial pathogens (e.g., *E. coli*, *V. cholera*, *H. influenza*, *S. pneumoniaie*, *Clostridium difficile*, and *Salmonella*), viruses (rotavirus, cytomegalovirus, HIV, influenza, respiratory syncytial virus), and yeasts (*C. albicans*) have been found in breastmilk, illustrating the breadth of this line of immune defense (Goldman, 1993). To neutralize pathogens in the infant gut, maternal milk antibodies must survive the digestive process through the gastrointestinal tract to reach their site of action. The concentration of IgA is somewhat reduced during the digestive process and from Holder pasteurization but is higher than in pasteurized donor milk (Demers-Mathieu et al., 2019).
- α-lactalbumin is a digestible whey protein that makes up 25–35% of the true protein in breastmilk. It is involved in lactose synthesis and binds zinc, calcium, and iron. α-lactalbumin plays an important role during milk production. It is produced in the epithelial cells of the mammary gland and combines with the enzyme β-1,4-galactosyltransferase to form lactose synthase, which converts glucose and galactose into lactose. Synthesis of lactose is thought to be essential for milk production, creating an osmotic force to draw water into the mammary gland and driving the total volume of milk produced (Layman et al., 2018). During its digestion, peptides appear to be transiently formed that have antibacterial and immunostimulatory properties, adding more protection against infection. In its normal or folded state, this protein has no effect on tumor cells or bacteria. However, when α-lactalbumin unfolds and combines with oleic acid, an omega-9 fatty acid commonly found in human milk, it forms a tumor-fighting complex called human alpha-lactalbumin made lethal to tumor cells or HAMLET. HAMLET forms in the infant stomach when α-lactalbumin comes in contact with digestive acids and combines with breastmilk's oleic acid (Hakansson et al., 1995). HAMLET targets not only cancerous cells but also specific bacteria that target the respiratory tract, including *Streptococcus pneumoniae* and *Haemophilus influenzae* (Hakansson et al., 2011). When HAMLET is used in conjunction with antibiotics, resistant strains of bacteria become sensitive to antibiotics. In conjunction with antibiotics, low levels of HAMLET appear to increase the binding and uptake of the antibiotics by the bacteria and simultaneously inhibit bacterial reproduction (Marks et al., 2012, 2013). HAMLET has broad anti-tumor activity against more than 40 different lymphomas and carcinomas and may contribute to the protective effect of breastfeeding against childhood tumors (Svanborg et al., 2003).
- Mucins are glycoproteins of the milk fat globule membrane. Their diverse functions include regulating cell signaling and transcription and modulating the binding of bacteria to the intestinal mucosa epithelium. There are several present in breastmilk but are not well studied. Mucin 1 appears to

inhibit the ability of a pathogen to bind to its infant host cell surface, and mucin 1 and mucin 4 have been shown to inhibit intestinal cell invasion by *Salmonella* (Liu et al., 2012). Mucins have also been shown to be effective against the binding and infectivity of viruses such as rotavirus (Yolken et al., 1992) and poxvirus (Habte et al., 2007).

Amino Acids

Amino acids are the building blocks of protein. Breastmilk contains essential and nonessential amino acids. Essential amino acids cannot be made by the body and must come from the diet. The nine essential amino acids are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. The nonessential amino acids are alanine, arginine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine, and tyrosine. Amino acids are involved in numerous bodily functions, for example:

- Glutamine, which can be synthesized from glutamate but is conditionally essential, increases by about 20-fold from colostrum to three months of lactation. It has been speculated that glutamate and glutamine supply functional substrates to nervous tissue, protect intestinal growth and integrity, and are essential for immune development.
- Taurine is the second most abundant free amino acid at all stages of lactation. Because humans have a relatively low capacity to synthesize taurine, it is considered essential to normal perinatal development. Taurine is involved in bile acid conjugation, structure and function of retinal photoreceptors, and neurodevelopment. Compared with formula-fed infants, the presence of the more acidic taurine bile acid conjugates in the intestine may favor colonization by *Lactobacillus* and *Bifidobacteria*.
- Tryptophan is required for protein synthesis function, is the primary precursor for the sleep inducers serotonin and melatonin, and is needed for the regulation of immune responses, behavior, mood, appetite, hemodynamics, and growth.
- Phenylalanine is important in the production of molecules such as tyrosine, epinephrine, norepinephrine, and dopamine. However, a rare inherited disorder called phenylketonuria (PKU) causes phenylalanine to build up in the body, and if levels are high enough can cause brain damage in the infant. PKU is caused by a defect in the gene that helps create the enzyme needed to break down phenylalanine. Breastfeeding can continue in the presence of PKU when levels are closely monitored and infants are provided with a phenylalanine-free amino acid–based formula (Kose et al., 2018).

Reproduced from Kose, E., Aksoy, B., Kuyum, P., Tuncer, N., Arslan, N., Ozturk, Y. (2018). The effects of breastfeeding in infants with phenylketonuria. *Journal of Pediatric Nursing*, 38, 27-32.

Enzymes

Enzymes are proteins that act as catalysts to increase the rate of chemical reactions within cells. At the beginning of the 21st century it was already known that human colostrum and milk contained over 70 different enzymes with various roles including immune protection, digestion enhancement, breast function, and the production of breastmilk. Some of them, such as xanthine oxidase, lactoperoxidase, and lysozyme, have active antimicrobial roles. Other enzymes help in the digestion of various substances in the immature digestive tract of newborns such as bile salt-stimulated lipase, pancreatic lipase, and amylase.

Bile Salt-Stimulated Lipase (BSSL)

Bile salt-stimulated lipase (BSSL) represents about 1–2% of total human milk protein. BSSL is activated by bile salts in the intestine allowing it to hydrolyze or break down fats, separating them into free fatty acids and glycerol to aid in fat digestion and absorption. BSSL is not present in cow's milk. Large accumulated amounts of free fatty acids from lipase hydrolysis, especially short and intermediate fatty acids, can cause a rancid flavor in stored breastmilk. Rancid flavor compounds of the free fatty acids caproic acid and lauric acid were found to increase with frozen storage time, with highest levels at 30 days compared with 7 days, gradually increasing over storage time (Hung et al., 2018). BSSL is preserved during refrigeration and freezing but can be inactivated by the heat of pasteurization, which could reduce fat absorption in preterm infants (Andersson et al., 2007). Scalded milk (180°F), boiled milk (212°F), and Holder pasteurization (145°F for 30 minutes) all inactivate BSSL. BSSL has been associated with protection against Norwalk virus and HIV transmission and with efficient use of cholesterol and fat-soluble vitamins. Breastmilk also contains another lipase, lipoprotein lipase, which provides long-chain fatty acids for milk fat synthesis.

Amylase

Amylase is necessary for the infant to digest starch and compensates for immature pancreatic function. Amylase activity in the duodenum of the newborn is only 0.2–0.5% of the adult level. The amylase secreted from salivary glands and the pancreas does not reach adequate levels until two years after birth. At about six months when solid foods are typically started, the infant is still deficient in endogenously produced amylase. Breastmilk helps infants better digest solid foods as amylase activity in human milk is more than 20–40 times higher than in cow's milk. This enzyme is capable of breaking down polysaccharide bacteria capsules, giving it antimicrobial attributes. Amylase is stable when refrigerated. Other infants and toddlers who might benefit from breastmilk amylase are those with pancreatic insufficiency caused by diseases such as cystic fibrosis or malnutrition.

Plasma Platelet-Activating Factor Acetylhydrolase (PAF-AH)

Plasma platelet-activating factor acetylhydrolase (PAF-AH) is an enzyme bound with lipoproteins that degrades not only platelet-activating factor PAF but also PAF-like oxidized phospholipids. Platelet-activating factor (PAF) is one of the most proinflammatory agents thus far described. NEC can be induced within hours after administration of PAF in experimental animals. PAF-AH degrades (PAF) in the blood of neonates during the time that their immune system is not fully developed. The secreted form of PAH-AH is present in breastmilk, but is absent in infant formula. Prematurity, formula feeding, intestinal ischemia, and bacterial colonization may contribute to the pathogenesis of NEC, as these stresses initiate proinflammatory signaling and intestinal injury. Preterm neonates have endogenous PAF-AH deficiency, leading infants with NEC to have increased luminal and systemic accumulations of PAF. PAF-AH catalyzes the hydrolysis of the acetyl group esterifying the sn-2 position of PAF, which eliminates its biological activity (Stafforini, 2009) and endows preterm infants fed with human milk a level of protection from the development of NEC. It is interesting to note that among the species studied, the only one devoid of PAH-AH was bovine milk, leaving cow's milk products unable to substitute for human milk (Park et al., 1983).

Cytokines

Cytokines are a large group of 80 proteins, peptides, or glycoproteins that are secreted by specific cells of the immune system. Cytokines are a category of signaling molecules that mediate and regulate immunity, inflammation, and hematopoiesis. They have a specific effect on the interactions and communications between cells. Cytokine is a general name; other names include lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), and interleukin (cytokines made by one leukocyte and acting on other leukocytes). Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or on distant cells (endocrine action) (Zhang & An, 2007). Cytokines in breastmilk include transforming growth factor- β (TGF-β), interleukin (IL) IL-10, IL-6, IL-1β, IL-4, IL-5, IL-12, IL-13, tumor necrosis factor-alpha (TNF-α), interferon gamma (IFN-γ), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage colony stimulating factor (M-CSF). Milk-derived cytokines affect infant intestinal epithelial proliferation and repair. These activities are essential for maturation and healing of the gastrointestinal tract as well as inducing oral tolerance and allergy reduction (Dawod & Marshall, 2019). Cytokines have been seen to play a role in the growth and differentiation of the mammary gland (Goldman et al., 1996) and can be proinflammatory or anti-inflammatory. Several factors can influence the concentration of cytokines in breastmilk. For example, in a study on subclinical mastitis and the inflammatory status of the breastmilk, subclinical mastitis (a local inflammation in the mammary gland) induced considerable changes in milk proinflammatory cytokines that might affect the infant's immune system and gut microbiota (Tuaillon et al., 2017). This cytokine imbalance is disturbing in part because of the 44 healthy mothers with no signs or symptoms of mastitis, 23% of the women were shown to have subclinical mastitis as evidenced by high levels of a large panel of inflammatory markers in their breastmilk. Infants receiving milk that is high in proinflammatory cytokines may also ingest breastmilk where mineral and trace element composition has been altered (Li et al., 2018).

Nonprotein Nitrogen

Nonprotein nitrogen is an operational term for the remainder of nitrogen in milk once the protein fraction has been removed. Nonprotein nitrogen represents around 20–30% of human milk nitrogen and comprises diverse types of compounds such as nucleotides, urea, aminosugar, and oligosaccharides; free amino acids taurine, arginine, and glutamine, polyamines; and amino alcohols of phospholipids (e.g., choline). These compounds have many different functions. For example, polyamines are involved in cell proliferation and differentiation in many tissues, especially in the intestinal tract tissues. Putrescine, spermidine, and spermine are polyamines present in breastmilk at much higher levels than in infant formula. Spermine is involved in intestinal maturation and gut closure with a protective effect against allergies. Insufficient polyamine intake could play a role in the induction of sensitization to dietary allergens in infancy and childhood (Dandrifosse et al., 2000). Nucleotides play a main role in various metabolic processes, such as energy transfer, nucleic acid synthesis (DNA and RNA), and carbohydrates, lipids, and proteins synthesis. Nucleotides represent 20% of the nonprotein nitrogen in human milk. The intestinal microflora modulation attributed to nucleotides may be due to nucleotides

serving as an energy source of intestinal microflora. Because probiotic bacteria are characterized by a higher growth rate than pathogenic bacteria, they limit the growth of pathogens. Nucleotides received increased attention when they were added to infant formulas in the United States in the 1980s and showed improved outcomes of selected infant health parameters compared to unsupplemented formula. Nucleotides added to infant formula are sourced from yeast RNA hydrolysates, not human derived, and may exhibit a different profile, activity, or function than the combination of nucleotides found in human milk.

Growth Factors

Human milk contains many growth factors that have a wide range of effects on the intestinal tract, vasculature, the nervous system, and the endocrine system.

- Epithelial (epidermal) growth factor (EGF) is higher in colostrum and preterm milk than mature breastmilk and stimulates intestinal maturation and repair. It is also involved in regulation of the mammary, liver, pancreatic and lung development. Heparin-binding growth factor (HB-EGF) is a member of the EGF family, and the primary growth factor responsible for damage resolution following hypoxia, ischemia-reperfusion injury, hemorrhagic shock/resuscitation injury, and necrotizing enterocolitis (Radulescu et al., 2011). This factor is especially important to preterm infants.
- Transforming growth factor beta-1 (TGF-β1) in human milk may play a role in infants' growth and development (Alsharnoubi et al., 2019). There are three isoforms of TGF-β—TGF-β 1, 2 and 3, with TGF-β2 being the most abundant, accounting for up to 95% of content in human milk. The concentration of TGF-β changes during lactation with the highest level in the colostrum. TGF-β regulates IgA production and induces oral tolerance. Cesarean section was associated with increased levels of TGF-β2 in HC (Kociszewska-Najman et al., 2020). TGF-β2 can inhibit the inflammatory response in immature human intestinal epithelial cells and suppress the expression of macrophage cytokines, thereby reducing the risk of NEC in preterm neonates (Brenmoehl et al., 2018). Gut microbial composition in early life varies by levels of TGF-β1 and TGF-β2 during the critical period when microbial succession occurs in parallel with immune system education and metabolic programming. It has been found that TGF-β2-associated bacteria were involved in a wide range of metabolic functions, including steroid and flavonoid biosynthesis, which may contribute to differing metabolic profiles between breastfed compared with formula-fed infants (Hellmuth et al., 2016). A protective effect of TGF-β1 and TGF-β2 has been found against atopic disorders (Oddy & Rosales, 2010; Joseph et al., 2014) with neonatal gut microbiota partially mediating this association. Breastmilk TGF-β concentration explains a portion of variability in gut bacterial microbiota composition among breastfed neonates (Sitarik et al., 2017).
- The enteric or enteral nervous system is the largest component of the autonomic nervous system and is the intrinsic nervous system of the gastrointestinal tract. It orchestrates gastrointestinal function independently of central nervous system input. The enteral nervous system requires brain-derived neurotrophic factor (BDNF) and glial cell-line derived neurotrophic factor (GDNF) for its development (Rodrigues et al., 2011). BDNF enhances peristalsis, which can frequently be impaired in the preterm gut (Boesmans et al., 2008). BDNF, GDNF, and a related protein, ciliary neurotrophic factor (CNTF), can be detected in human milk for up to 90 days following

birth (Fichter et al., 2011). In human cells, breastmilk-derived GDNF increases neuron survival and outgrowth (Fichter et al., 2011).

- Insulin-like growth factor (IGF-1, IGF-II, IGF binding proteins, and IGF-specific proteases) are involved with growth parameters in both term and preterm infants. Breastmilk with high levels of IGF-1 correlated with a high serum level of IGF-1 in premature babies, acting to diminish general growth abnormalities, metabolic disorders, lung and retinal immaturity, and brain developmental abnormalities, which could result in abnormalities in cognitive function (Alzaree et al., 2019).
- Angiogenesis is the process through which new blood vessels are formed and is primarily regulated by vascular endothelial growth factor (VEGF). High levels in colostrum and preterm milk may help regulate vascularization of the retina and contribute to the reduction of retinopathy of prematurity (DiBiasie, 2006).

Hormones

Breastmilk contains many hormones with varying functions that permanently shape infant physiological processes. (**Table 2-3**).

Carbohydrates

The carbohydrate fraction of human milk is composed of lactose, monosaccharides, neutral and acid oligosaccharides, peptide-bound and protein-bound carbohydrates, glucose, galactose, and other complex carbohydrates.

Lactose

Lactose (milk sugar) is the primary carbohydrate in human milk, increasing from 56 ± 6g/L on day 4 to 68.9 ± 8g/L on day 120 (Coppa et al., 1993).

Table 2-3 Selected Hormones in Breastmilk and Their Functions in the Recipient Infant

Hormone	Function
Thyroid hormones: Thyroxine (T4), Triiodothyronine (T3), Thyroid-stimulating hormone (TSH)	Gut development and maturation Brain development
Leptin, ghrelin, insulin growth factor 1 (IGF-1), adiponectin, and insulin	Regulation of appetite, energy balance, growth, body composition, and adiposity
Prolactin	Regulates behavior, the immune system, metabolism, reproductive systems, and many different bodily fluids.
Melatonin	Sleep regulation, increases the phagocytic activity of colostral cells against bacteria.
Glucocorticoids, cortisol	Shape behavioral tendencies and development
Erythropoietin	Increases red blood cells, trophic factor, tightens intestinal junctions
Calcitonin, somatostatin	Growth-regulating hormones
Beta-endorphins	Pain modulators, help newborns deal with the stress of birth and adjust to life outside of the womb
Relaxin	Development of reproductive tissues
Prostaglandins	Protects gastric mucosa from inflammation and necrosis, GI motility, physiologic effects on gastrointestinal tract

It is a disaccharide composed of galactose and glucose and broken down by the enzyme lactase, which is present in the infant by 24 weeks of fetal life. Lactose is specific for newborn growth, it enhances calcium absorption, and is a readily available source of galactose, which is essential to the production of galactolipids such as cerebroside. Galactolipids are essential to central nervous system development and brain myelinization. Infant formula that has had the lactose removed such as soy-based formula or lactose-free formulas lack this important brain development factor. An interesting correlation made decades ago suggested that the amount of lactose in the milk of a species was related to the size of the brain (Kretchmer, 1972). Lactose levels are higher in human milk than in other mammalian species and is not found in other animal or plant sources.

Oligosaccharides

Oligosaccharides are biologically active carbohydrates that comprise the third largest solid component in breastmilk after lactose and triglyceride. More than 200 neutral and acidic HMOs have been identified that differ in composition from those of any other mammal. HMOs are essentially indigestible by the infant's gut mucosa and are not utilized as a macronutrient. Instead, they are delivered to the gut intact, where they nourish the infant's gut microbiota acting as the infant's first prebiotic. The HMO content in breastmilk is variable, influenced by maternal, environmental, and infant feeding practices, is highest in colostrum, decreases in mature milk, and is genetically predetermined. Concentrations of HMOs measured per mL of milk varies between mothers and decreases between 2 and 12 weeks of lactation, except for 3FL and LNFP III, which increase in concentration as lactation progresses (Borewicz et al., 2020). Human mature milk and colostrum contain 12–13 g/L and 22–24 g/L of milk oligosaccharides respectively, while bovine colostrum collected immediately postpartum contains only around 1 g/L oligosaccharides, and this concentration rapidly decreases after 48 hours. Mature bovine milk contains only trace amounts of oligosaccharides. Oligosaccharides have water-soluble cell surface analogs that can inhibit pathogen binding to host cell receptors on the surface of the intestinal cell. Oligosaccharides can inhibit the binding to their intestinal cell receptors of such bacteria as *Streptococcus pneumonia*, *Haemophilus influenza*, *E. coli*, and *Campylobacter jejuni*. They essentially act as decoys through their ability to mimic intestinal cell receptors, preventing bacterial, viral, or protozoan parasite pathogens from attaching to their respective receptors in host cells. When these pathogens attach to the HMOs rather than the intestinal epithelial cells, they are escorted out of the body in the feces rather than proliferating and causing the infant to become ill. HMOs are antimicrobials that act as bacteriostatic or bacteriocidal agents (Bode, 2015). HMOs are potent disease fighters. HMOs and their metabolic products, such as sialic acid, have a role in brain development, neuronal transmission, and synaptogenesis. HMOs are a source of sialic acid, which is an essential nutrient for optimal brain development and cognition.

Lactating mothers differ genetically in their ability to produce protective oligosaccharides, thus influencing their infant's susceptibility to the acquisition of diseases. Each woman has a unique composition and concentration of HMOs in her milk. This wide-ranging variety is highly dependent on the activity of the Secretor (*Se*) and Lewis (*Le*) genes in the mammary glands. *Se* genes encode the enzyme α1-2-fucosyltransferase (FUT2) and *Le* genes encode the enzyme α1-3/4-fucosyltransferase (FUT3), both of which are involved in the biosynthesis of fucosylated HMOs (Kumazaki & Yoshida, 1984; Johnson & Watkins, 1992). Mutations on the *Se* gene can inactivate FUT2, designating

those mothers as nonsecretors, with milk from nonsecretor (Se−) women containing no or only traces of α1-2 fucosylated HMOs. Mothers with a functional FUT2 gene are called secretors. Mutations on the *Le* gene inactivate FUT3, and consequently, milk from Lewis-negative (Le−) women contain no or only traces of α1-4 fucosylated HMOs (Kobata, 2010). Lack of the FUT3 enzyme can have negative consequences. The nonsecretor mothers secrete lower HMOs than secretor mothers. Infants of women who do not have the FUT3 enzyme show delayed colonization of *Bifidobacteria* and show more differences in the metabolic activity of their microflora, especially *Streptococcus* (Wiciński et al., 2020). Based on the activity of the FUT2 (*Se*) and FUT 3 (*Le*) enzymes in lactating woman, HMO composition can be classified into four different phenotypes (**Box 2-1**) (Tonon et al., 2019).

Besides the influence of the *Se* and *Le* genes on the composition of HMOs, other factors may contribute to the variability of HMOs, even within the same phenotype, such as gestational age, lactation stage, mode of delivery, parity, BMI, as well as environmental factors such as geographic location and exercise. Harris et al. (2020) reported that the oligosaccharide 3'-sialyllactose (3'-SL) is related to and can improve infant metabolic health and cardiac function and is increased in milk of human mothers who exercise. Feeding exercise-induced higher levels of 3'-SL to infant mice negated the detrimental effects of a high-fat diet on body composition and metabolism. Higher levels of 3'-SL may contribute to the lower occurrence of obesity, type 2 diabetes, and cardiovascular disease seen in those who have been breastfed. Some of the HMOs' effects on infant health have been related to specific structures and usually in a dose-dependent manner. For example, higher concentrations of α1-2 fucosylated HMOs have been related to a lower risk of allergy at two and five years of age in infants with high hereditary allergy risk (Sprenger et al., 2017) and lower amounts of disialyllacto-N-tetraose (DSLNT) in human milk is associated with the development of NEC in preterm infants (Autran et al., 2018). *Bifidobacteria* are established earlier and more often in infants breastfed by secretor mothers than in infants fed by nonsecretor mothers (Lewis et al., 2015). *Bifidobacteria* are protective against enteric infections, reduce inflammation and gut permeability, and a thriving *Bifidobacteria* environment in the gut keeps pathogens in check. Infants breastfed by nonsecretor mothers had 10 times fewer *Bifidobacteria* and were delayed in establishing a *Bifidobacteria*-laden microbiota, possible due to

Box 2-1 Four Phenotypes of HMOs

1. Se+Le+, the most common, containing α1-2 and α1-4 fucosylated HMOs, such as 2'-fucosyllactose (2'-FL) and lacto-N-difuco-hexaose I (LNDFH I)
2. Se−Le+, which contain α1-4 fucosylated HMOs, such as lacto-N-difuco-hexaose II (LNDFH II), but does not contain α1-2 fucosylated HMOs, such as 2'-FL, lacto-N-fucopentaose I (LNFP I), difucosyllacto-N-hexaose c (DFLNH c) and LNDFH I
3. Se+Le−, which contain α1-2 fucosylated HMOs, such as 2'-FL and LNFP I, but does not contain α1-4 fucosylated HMOs, such as DFLNH c, LNDFH I and II
4. Se−Le−, the least common phenotype, containing neither α1-2 nor α1-4, but only α1-3 fucosylated HMOs, such as 3'-fucosyllactose (3'-FL) and difucosyl-para-lacto-N-neohexaose (DFpLNnH). This phenotype may have lost some of its defensive capabilities, rendering it less protective for the infant.

difficulties in the infant acquiring a species of *Bifidobacterium* that is able to consume the specific milk oligosaccharides provided by the mother (Lewis et al., 2015).

Some infant formulas are currently supplemented with oligosaccharides. While biotechnological means exist for the production of commercially available oligosaccharides, the composition and abundance of HMOs found in human milk have not been artificially reproduced. 2'FL has been added to some infant formulas, but its origin is not human. One fermentation process to commercially produce 2'FL uses genetically modified *E. coli* bacteria (LSRO Solutions, 2017). Other technologies use enzymatic treatment and genetic engineering of fungi, yeasts, or bacteria. Mixtures of galactooligosaccharides (GOSs) and fructooligosaccharides (FOSs) or inulin have been added to infant formulas. HMOs are complex glycans composed of five different monosaccharides, while FOSs and GOSs are much simpler structures. GOSs and FOSs are different structurally from HMOs found in human milk (Wiciński et al., 2020). While some GOS- and FOS-enriched formulas may mimic human milk in composition, they do not match it in performance (Baumann-Dudenhoeffer et al., 2018). Researchers reviewed 41 randomized controlled trials of prebiotic-supplemented formula and concluded that while the products seemed safe, they didn't lead to tangible health benefits (Skorka et al., 2018). In a formula company-funded trial of a formula containing manufactured 2'-fucosyllactose and lacto-N-neotetraose, babies receiving these additives had a lower rate of bronchitis than babies receiving unsupplemented formula (10% vs. 28%), as well as lower rates of lower respiratory tract infections (19% vs. 35%) and antibiotic use (42% vs. 61%) in the first year of life (Puccio et al., 2017). These results were a comparison between the test formula and a control formula, not a comparison with breastfeeding outcomes. Adding higher doses and/or different combinations of oligosaccharides to infant formula may result in unwanted or health-challenging outcomes. It has been shown that higher levels of 2'-fucosyllactose, lacto-N-tetraose and a third oligosaccharide in breastmilk of mothers in India were associated with a greater incidence of symptomatic rotavirus infections (Ramani et al., 2018) in their babies, and that in cell culture experiments, the oligosaccharides increased the infectivity of a virus strain that causes severe gastrointestinal infections in infants. Specific oligosaccharides or mixtures of them in breastmilk have been correlated with excessive weight gain (Larsson et al., 2019) and risk of allergies (Miliku et al., 2018) in breastfeeding infants. Randomized controlled trials of adding oligosaccharides into infant formula are usually restricted to structurally simple compounds such as 2'fucosyllactose and lacto-N-neotetraose (Chouraqui, 2020). More research is needed on which oligosaccharides are selected to be put into infant formula, the doses of each, the specific combination of oligosaccharides, and their respective ratios. Marketing claims regarding formula additives are often company-funded, may have poor quality of evidence, often provide no evidence available to the public, and when the results of randomized trials are made public, they may be limited by small sample sizes, poor follow-up, and provide unpersuasive results (Hughes et al., 2017).

Because oligosaccharides present in human milk can modulate the microbiota of breastfed infants, it might be possible that HMOs could also modulate the bacterial communities in the breast itself, as the breast has its own microbiome. While the breastmilk of secretor women is rich in 2'FL and other α-1,2-fucosylated HMOs, nonsecretor women lack the functional FTU2 enzyme, resulting in milk that does not contain α-1,2-fucosylated HMOs. Certain HMOs in breastmilk (2'FL and lacto-N-fucopentaose) have been shown

to enrich the growth of *Streptococcus mitis* and *Streptococcus oralis*, two species abundant in human milk that possess fucosidase, a gene than enables digestion of fucose moieties. However 2'FL does not confer a growth advantage to *S. epidermidis* nor *S. aureus*, the former a causative agent for subclinical mastitis and the latter of which is a common causative agent of clinical mastitis. HMO-driven proliferation of *Streptococcus* commensals may serve to maintain bacterial diversity in the milk microbiome, allowing commensals to outcompete potential pathogens such as *S. aureus*. In this way, HMOs may serve to help prevent the bacterial dysbiosis that leads to the development of mastitis (Meyer et al., 2019). In essence, some mothers may be protected from mastitis by their own HMOs.

Vitamins

Human milk provides a full complement of water-soluble and fat-soluble vitamins. The fat-soluble vitamins are A, D, E, and K. The water-soluble vitamins are ascorbic acid (vitamin C), thiamin (vitamin B_1), riboflavin (vitamin B_2), pyridoxine (vitamin B_6) folate, pantothenate, biotin, and vitamin B_{12}. The concentration of water-soluble vitamins in human milk shows variations reflecting stage of lactation, maternal dietary intake, and delivery before term. The breast cannot synthesize water-soluble vitamins, so their origins lie in the maternal plasma, derived from the maternal diet. In mothers who are adequately nourished, vitamin supplementation in higher than physiological doses either has no effect or is transient.

Ascorbic acid (Vitamin C)

Vitamin C is a key antioxidant in breastmilk. The average mature milk vitamin C concentration ranges from 50–90 mg/L in mothers consuming an adequate amount of vitamin C in their diet. Milk concentrations are not markedly increased with routine daily multivitamin supplementation. Vitamin C levels are generally higher in colostrum by 10–20 mg/L compared with mature milk. Levels are relatively stable until after 12 months postpartum when they begin to decrease slightly and reach 30% of previous levels by 18–24 months postpartum. Donor milk undergoing Holder pasteurization (62.5°C for 30 minutes) has lower vitamin C levels than that reported in fresh milk and lower than unpasteurized milk. Pooled and pasteurized donor milk from milk banks in Ontario and British Colombia, Canada, had average vitamin C levels of only 17.7 mg/L (range 1.9–43.2 mg/L) and 21.7 mg/L (range 0–68 mg/L), respectively (Castro et al., 2019). Preterm infants consuming donor human milk that has been Holder pasteurized may need vitamin C supplementation. The flash-heat method of treating breastmilk to reduce HIV transmission (placing a container of expressed milk in a water bath and brining the water to a rolling boil) does not change the milk vitamin C content (Israel-Ballard et al., 2008). Freezing (–20°C) freshly expressed mature milk does not change milk vitamin C levels for at least 3 months of freezer storage. After 6 to 12 months of freezing (–20°C), vitamin C levels can decrease by 15–30%. Storage at –80°C preserves vitamin C levels for up to 8 months, with 15% loss by 12 months. Mothers who smoke have lower milk vitamin C levels than those who do not (Dror & Allen, 2018a). Mothers whose diets are deficient in vitamin C or who smoke may benefit from enriching their diet with vitamin C–containing foods or consuming a supplement to correct a known deficiency (Drugs and Lactation Database, 2019).

Handling of expressed breastmilk can alter the vitamin C content of the milk. Francis et al. (2008) found that various bottle-feeding systems showed measurable decreases in the mean concentration of ascorbic acid over a 20-minute sampling period (the approximate time of a feeding). Those bottles with the largest milk-to-air surface had the greatest decreases in mean concentration of ascorbic acid over time. The air moving through the milk and the formation of bubbles on the surface of the milk could be factors in the observed decreases of ascorbic acid concentration, which ranged from 1% decrease to a 76% decrease depending on the bottle system and the size of the milk surface that was exposed to air. Ascorbic acid is also subject to photodegradation as minimization of exposure to light provides protection for nutrients that are susceptible to oxidation. A study looked at the decreases in ascorbic acid relative to the color of the container that the breastmilk was placed in (Francis & Dickton, 2015). It was found that ascorbic acid in breastmilk samples that were placed in the control and amber colored containers maintained ascorbic acid levels above 90% of the original ascorbic acid concentration. In the remaining clear or green and blue containers, the concentrations of ascorbic acid decreased to less than 65% of the baseline level. The concentrations of ascorbic acid consistently decreased significantly over a relatively short period of time when stored in translucent containers. This suggests that to retain as much ascorbic acid as possible during expressing, storing, and feeding, breastmilk should be placed in amber-colored bottles or the bottle should be covered with an opaque material to retain as much ascorbic acid as possible. Caregivers may wish to avoid shaking bottles of expressed breastmilk, which can increase the milk-to-air surface, may wish to use bottle systems with lower milk-to-air surface interfaces, and may wish to consider using amber-tinted bottles or covering the bottle with an opaque material for expressing, storing, and feeding purposes.

Thiamin (B_1)

Thiamin is essential for the use of carbohydrates in pyruvate metabolism and in fat synthesis. Deficiency in the breastfeeding mother has been associated with beriberi in infants. Breastmilk concentrations and infant status are strongly dependent on maternal intake and status.

Riboflavin (B_2)

Riboflavin is involved in oxidative intracellular systems. Levels are 36 mg/dL in human milk and much higher in cow's milk. Maternal riboflavin status is dependent on intake of animal source food and green vegetable consumption. Maternal riboflavin deficiency rapidly results in low milk concentrations of the vitamin.

Pyridoxine (B_6)

Pyridoxine is important to DNA synthesis needed to form the cerebrosides in the myelination of the central nervous system. Human milk contains 12–15 mg/dL of B_6 and is higher in cow's milk. Exceptionally high levels of pyridoxine (way above normal supplementation levels) have been shown to reduce prolactin levels in women with hyperprolactinemia (Witwit, 2019). An older study reported that women who took oral contraceptives with high estrogen concentrations for long periods of time prior to pregnancy had reduced levels of B_6 in their breastmilk (Roepke & Kirksey, 1979).

Vitamin B_{12}

Vitamin B_{12} is critical to the infant's developing nervous system. The vitamin occurs almost exclusively in animal tissue, is bound to protein, and is minimal to absent in vegetable protein. Infants born to vitamin B_{12}–deficient mothers have compromised liver stores at birth and consume milk with a lower vitamin B_{12} concentration, placing them at increased risk for vitamin B_{12} deficiency and its neurologic and developmental consequences (Dror & Allen, 2018b). B_{12} levels are highest in colostrum and reach a nadir at three to four months postpartum. Lactating mothers acquire vitamin B_{12} primarily through the intake of animal source, fermented, and vitamin B_{12}–fortified foods. A lactating mother consuming a diet without meat or dairy products may have breastmilk deficient in vitamin B_{12} unless other B_{12} food or supplement sources are provided. A cluster of symptoms indicating a B_{12} deficiency in an exclusively breastfed infant is most commonly diagnosed between 4 and 10 months of age and includes anemia, irritability, infections, hypotonia, microcephaly, refusal to suck, failure to thrive, apathy, anorexia, movement disorders, and gross developmental delay or regression. Mothers of infants experiencing these symptoms should be asked if they are consuming a diet where meat and dairy products are absent. Mothers, their milk, and infants can be tested for B_{12} status and treatment instituted, if necessary, with oral or intramuscular vitamin B_{12}. Breastfeeding need not be abandoned.

Vitamin A

Dietary vitamin A is obtained in mainly two forms, retinol (preformed vitamin A) or provitamin A carotenoid (precursor of vitamin A). It is available in foods either as preformed vitamin A; as retinyl ester, abundant in some animal-derived sources such as liver, eggs, dairy products, and fatty fish; or as provitamin A carotenoids, mainly β-carotene, abundant in dark colored fruits and vegetables such as green leaves, carrots, ripe mangos, and other orange-yellow vegetables. This group of carotenoids offers other health benefits, including antioxidant, anti-inflammatory, and immunomodulatory properties. Vitamin A is necessary to maintain epithelial tissues, vision, and immune function. The concentration of vitamin A in human milk decreases over the course of lactation; it is maximal in the colostrum and reaches a plateau in mature milk. In healthy mothers, the vitamin A concentration varies from 5–7 μM in colostrum, 3–5 μM in transitional milk, and 1.4–2.6 μM in mature milk. In some developing countries where vitamin A food sources may lack vitamin A or its precursors, breastmilk may be deficient in vitamin A. During lactation, if breastmilk does not provide the neonate with appropriate vitamin A levels, the immune system might be affected, raising the risk for infectious diseases, night blindness, depressed immunity, squamous metaplasia of mucous epithelium in several organs, hyperkeratosis, and disturbances in cell differentiation, organ development, and growth and reproduction. Vitamin A is essential for mammary gland formation during the embryonic period and for mammary gland development during puberty, pregnancy, and lactation as well as for lactation sufficiency (Cabezuelo et al., 2020).

Vitamin D

The major forms of vitamin D present in breastmilk are cholecalciferol (vitamin D_3), ergocalciferol (vitamin D_2), and their respective 25-hydroxylates (25-OH) also known as calcidiols. Vitamin D is a prohormone that is synthesized

in humans following skin exposure to ultraviolet B radiation in the range of 280–320 nm. In comparison to sunlight, diet provides less than 10% of the body's vitamin D requirement in unsupplemented individuals. Vitamin D is necessary to maintain calcium homeostasis and bone health. Vitamin D deficiency or low intake is detrimental to the health of mothers and children because of the increased risk of osteomalacia in adults and rickets and delayed growth in infants and children. Deficiencies are also associated with increased risk of autoimmune diseases in adults and children and lower respiratory tract infections in children. While breastmilk contains all the nutrients an infant requires, vitamin D may be lacking, as the natural method for acquiring sufficient vitamin D relies on-skin exposure to sunshine. Women in northern latitudes, cold climates, who use sunscreen, have dark skin, or wear clothing that covers large portions of the skin may be deficient in vitamin D themselves. If a breastfeeding mother is vitamin D deficient, her breastmilk may not contain the recommended levels of vitamin D for infants, as the vitamin D content of human milk is dependent on the mother's vitamin D status. Infants solely breastfed by women with vitamin D intakes of 400 IU/day (the recommended amount) typically attain a circulating 25(OH)D concentration in the marginally sufficient to severely deficient (< 12.5 nmol/L) range (Ziegler et al., 2006). Due to concern about skin cancer, professional organizations recommend that infants avoid sun exposure, wear sunscreen, and exclusively or partially breastfed infants receive a 400 IU supplement of vitamin D daily (Wagner et al., 2008). Although the recommendation to supplement breastfed infants with vitamin D drops has been published for decades, it seems that this recommendation is frequently not followed (Ahrens et al., 2016). When data for infants 0 to 11 months in the 2009–2016 NHANES set was analyzed, it was found that only 27% of U.S. infants met the AAP recommended guidelines for vitamin D supplementation (Simon & Ahrens, 2020). Parents complain that the drops are difficult to administer and inconvenient, that breastmilk has all the vitamins that are needed, that the baby spits them out or does not like them, that the infant demonstrates gastrointestinal distress from the drops, or that their healthcare provider did not mention the necessity of vitamin D supplementation (Umaretiya et al., 2017). Many mothers prefer to supplement themselves rather than their infants (Umaretiya et al., 2017). An alternative strategy to improve infants' vitamin D intake to recommended levels is to supplement the breastfeeding mother with high doses of vitamin D (Wagner et al., 2006). High-dose (6,400 IU/day) vitamin D_3 supplementation of breastfeeding mothers was shown to safely and significantly increase maternal circulating 25(OH)D and vitamin D from baseline compared to control mothers who received 300 IU vitamin D_3/day. Infant levels achieved exclusively through maternal supplementation were equivalent to levels in infants who received oral vitamin D supplementation. Thus, a maternal intake of 6,400 IU/day of vitamin D elevated circulating 25(OH)D in both mother and nursing infant to optimum levels (Wagner et al., 2006). Some may think that the low concentrations of vitamin D renders human milk incomplete or defective and necessitated the recommendation of vitamin D supplementation for breastfeeding infants to remedy this defect. Hollis et al. (2015) demonstrated in a randomized controlled trial that maternal vitamin D supplementation alone with 6,400 IU/day safely supplies breastmilk with adequate vitamin D to satisfy the requirement of the nursing infant. The problem may not be in the blueprint of human milk but in the dietary vitamin D recommendation relative to the lactating mother. The current recommendation of 400 IU vitamin D per day for lactating women seems unable to elevate blood concentrations high enough to transfer optimum

levels into breastmilk. The Hollis study again demonstrated that with appropriate vitamin D intake, the lactating mother can fully transfer from her blood to her milk the vitamin D required to sustain optimal vitamin D nutrition in the nursing infant with no additional supplementation required for the baby. While this intervention may not be suitable for every situation, it offers a potentially more convenient method of assuring optimal vitamin D levels in both mother and infant and may reduce the struggles of getting sufficient vitamin D drops into an infant without under-dosing or overdosing possibilities (Ketha et al., 2015).

Vitamin E

Alpha-tocopherol is the major isoform of vitamin E present in the human diet and in breastmilk. Vitamin E functions as an antioxidant and is required for muscle integrity and resistance of erythrocytes to hemolysis. In mothers not taking a vitamin E supplement, average alpha-tocopherol levels in colostrum range from 20–50 micromol/L. At one to two weeks postpartum, milk levels are 7–14 micromol/L, and beyond two weeks they are 3–9 micromol/L (Lima et al., 2014). Hindmilk levels are higher than foremilk. Maternal obesity and smoking are associated with lower milk alpha-tocopherol levels. The higher the pregestational BMI (relative to normal) and the greater the gap between the real and recommended gestational weight gain, the lower the concentration of retinol tends to be (Sámano et al., 2017). Vitamin E concentrations in mature milk were significantly lower in smokers than in nonsmokers. Maternal smoking favors peroxidation events in newborns. If the concentration of antioxidants (vitamin E) in smokers' breastmilk is also lower, it might aggravate the peroxidation problems of their newborns (Ortega et al., 1998).

Vitamin K

Vitamin K is naturally present in human milk in concentrations of 1–9 mcg/L (2.2–20 nmol/L). Similar levels have been reported in colostrum. Levels are approximately 1 mcg/L higher in hindmilk than foremilk. Three forms of vitamin K are known: vitamin K_1 (phylloquinone), vitamin K_2 (menaquinones), and vitamin K_3 (menadione). Vitamin K_1 (phylloquinone) is the major circulating form and is primarily provided by dietary sources, such as green leafy vegetables. Vitamin K_2 (menaquinones) is found in the diet, particularly in egg yolks, chicken, beef, vegetables, and fermented products. Vitamin K is essential for the synthesis of blood clotting factors. Vitamin K deficiency may cause unexpected bleeding (0.25–1.7% incidence) during the first week of life in previously healthy-appearing neonates (early vitamin K deficiency bleeding [VKDB] of the newborn [formerly known as classic hemorrhagic disease of the newborn]). Late VKDB, a syndrome defined as unexpected bleeding attributable to severe vitamin K deficiency in infants 2 to 12 weeks of age, occurs primarily in exclusively breastfed infants who have received no or inadequate neonatal vitamin K prophylaxis. In addition, infants who have intestinal malabsorption defects such as cholestatic jaundice or cystic fibrosis or infants with gallbladder problems that prevent them from absorbing fat-soluble vitamins may also have an increased risk for late VKDB. Babies are born with very limited amounts of vitamin K because the vitamin K levels transferred from the mother to the child across the placenta are quite low and the gut flora of neonates is immature, limiting the amount of vitamin K that can be synthesized from that source. Infant vitamin K levels are lowest at days 2–3 and do not reach adult levels until about six months of age. Breastmilk has very low levels of vitamin K and

almost all babies who develop VKDB are exclusively breastfed (Shearer, 2009). Because parenteral vitamin K has been shown to prevent VKDB of the newborn and young infant and the risks of cancer have been unproven, the American Academy of Pediatrics (AAP) recommends that vitamin K_1 should be given to all newborns as a single, intramuscular dose of 0.5–1 mg within six hours of birth (Committee on Fetus and Newborn, 2003).

Minerals

The essential minerals or trace elements include iron, zinc, copper, iodine, selenium, calcium, phosphorus, magnesium, potassium, and sodium, with many other minerals and trace elements present in breastmilk. Minerals play numerous physiological roles in the body including, iron, zinc, copper, and iodine, which are essential for optimal brain development; calcium, phosphorus, and magnesium are required for adequate bone health; selenium, zinc, and iodine function in a crucial role in thyroid hormone metabolism; and potassium and sodium are important electrolytes involved in water fluxes and enzymatic functions. Zinc, copper, and selenium contents were found to be significantly lower in preterm milk than in term milk when compared at an equivalent infant developmental stage (Sabatier et al., 2019). Zinc and copper contribute to cellular and molecular processes and are essential for growth, the immune response, and cognitive function. The serum zinc concentration in term and preterm infants is high at birth and then progressively decreases, correlating with normal growth. Selenium is important for optimal functioning of antioxidant systems, and its serum concentration increases in healthy term breastfed infants after birth. Therefore, the observed differences of concentrations of these three trace elements in preterm human milk and term human milk at the same postmenstrual age may be physiologically relevant and require further discussion regarding recommendations for fortifying preterm breastmilk (Sabatier et al., 2019).

Sodium

Sodium levels vary in breastmilk, with mean content measured at 42.73 mmol/L (SD = 22.12 mmol/L; range, 14.50–120.00 mmol/L; Galipeau et al., 2012). Sodium levels have also been measured as 7 mEq/L or 16 mg/dL. Several factors impact the sodium levels in breastmilk including stage of lactation, maternal age, length of gestation, insulin use, gestational diabetes, ethnicity, breastfeeding frequency, daily breastmilk intake, mastitis, and weaning. The presence of gestational diabetes increased the risk of an elevated breastmilk sodium, and a higher breastfeeding frequency was associated with a lower level of breastmilk sodium (Galipeau et al., 2012). Sodium levels are highest in colostrum and quickly fall during the early days postpartum because the gland is undergoing the transition between pregnancy, when the junctions are open, and full lactation, when the junctions have closed. The ratio of breastmilk sodium to potassium (Na:K) dramatically declines as lactation progresses through colostral, transitional, and mature milk production stages. Declining milk Na:K is an objective biomarker of mammary gland progress toward copious mature milk production over the first week postpartum. During pregnancy, involution, mastitis, and the first four days postpartum, the junctions between the alveolar cells (milk-making cells) have large gaps that remain open, allowing sodium and chloride to enter the milk space, drawing water along with them. During the first four days postpartum, lactose and potassium are also thought to move

from the milk space to the blood. The net result is that under these conditions, milk has greatly increased concentrations of sodium and chloride and decreased concentrations of lactose and potassium. After four days postpartum under normal circumstances the alveolar cells swell under the influence of prolactin, which closes these gaps. However, during the first few days postpartum, breastmilk sodium levels can remain high if the infant transfers little colostrum and/or there is a delay in lactogenesis II (onset of copious milk production). Breastmilk sodium concentration has been shown to be inversely related to the daily intake of milk during the first three days postpartum (Manganaro et al., 2007). One study observed that 42% of 196 exclusively breastfeeding mothers with a milk supply concern at day 7 had concurrent biochemical evidence of less progress toward mature milk production (Murase et al., 2017). Furthermore, among exclusively breastfeeding mothers with elevated milk Na:K at day 7, the adjusted odds of stopping breastfeeding by day 60 were significantly greater than for women without elevated milk Na:K, irrespective of perceived milk supply concern at day 7. The observed prevalence of elevated Na:K was twofold greater in the mothers with milk supply concerns (42% versus 21%). Essentially, sodium concentration in breastmilk during at least the first week postpartum serves as a biomarker, with high sodium levels indicative of infant feeding inadequacy, insufficient milk removal, a delay in lactogenesis II, and insufficient milk production. Thus, the concern regarding milk sufficiency may not always be maternal misperception.

Calcium

Calcium is an important constituent of bone and bone mineralization and plays a critical role as a messenger in cell-signaling pathways. Calcium concentrations in breastmilk range between 100 and 300 mg/L. To promote normal growth, infant formula must contain double the concentration of calcium as what is found in breastmilk. It is thought that this is due to decreased absorption of the calcium in formula and a factor in breastmilk that aids intestinal absorption. Bone calcium deposition rate is greatest in infancy and is a direct function of intestinal absorption. The calcium to phosphorus ratio (Ca:P) may be an important determinant of calcium absorption and retention because of the regulatory mechanisms, which control calcium and phosphorus homeostasis within the body. In breastmilk, the Ca:P is approximately 2:1, with similar ratios in infant formulas; however, absolute quantities are higher in infant formulas to account for the differing bioavailabilities. Generally, maternal diet does not appear to influence the concentration of calcium in milk unless the mother is significantly malnourished. There is a normal increase in the concentration of calcium in breastmilk during the first week of lactation and a subsequent gradual decline until late lactation. The application of a physicochemical model has demonstrated that the total concentration of calcium is dependent on the concentrations of both casein and citrate, and change in the concentration of citrate is the predominant factor in breastmilk (Kent et al., 2009). Calcium and phosphorus supplements may be given to breastfed or breastmilk–fed preterm infants as preterm infants are born with low skeletal stores of calcium and phosphorus, and breastmilk may not always supply enough of these nutrients for some of these infants. Lactation also affects the maternal calcium movement. Calcium uptake in the maternal duodenum is enhanced during lactation. Pregnancy itself may lead to maternal bone loss, but if followed by lactation, lactation will have a protective effect on bone density while the duration of lactation and parity may modulate its effect (Salari & Abdollahi, 2014).

Iron

Iron is the most abundant trace element in the human body and serves multiple functions. Approximately two-thirds of iron is utilized as functional iron, which is found in hemoglobin (Hb; 60%), myoglobin (5%), and in heme and nonheme enzymes. The key function of iron in hemoglobin is oxygen transportation, which is essential for cell respiration. Iron in myoglobin is required to store oxygen in muscles. As a component of tissue enzymes, iron is important in energy production and immune system functioning. Because of its high presence in multiple brain regions, iron plays an important role in essential neurologic processes such as neurotransmitter synthesis and myelination. At birth, full-term healthy infants have liver iron stores of about 75 mg/kg, a high blood volume, and Hb concentration in proportion to their body weight. Hb iron and iron stored in the liver present at birth are the most important iron sources during the first few months of life for full-term infants, particularly breastfed infants. During the first few months of life, infants experience a physiological decline in their blood volume and Hb concentration as well as liver depletion of iron stores. Breastmilk contains a relatively low amount of iron (mean iron content = 0.35 mg/L) with a bioavailability of 45–100%. Most healthy breastfed infants born at term do not need exogenous iron until around six months of life (Domellöf et al., 2014). Preterm infants whose liver stores of iron were interrupted by an early birth may need additional iron supplements much earlier than a healthy full-term baby. Iron deficiency and iron deficiency anemia can have negative effects on neurodevelopmental outcomes. Despite ferrous sulfate being a well absorbable form of iron, the cow's milk proteins available in infant formula have an inhibitory effect on iron absorption, which is why much higher amounts of iron are added to formulas due to its poor bioavailability. Iron-fortified cereals are the most common source of iron during the complementary feeding period. Ferric pyrophosphate and elemental iron are the two types of iron added to these cereals, but have low bioavailability. This is why pureed meats might be a better source of iron for first solid foods, as the absorption of heme iron from meat is several-fold higher than the non-heme iron from fortified infant cereal (Friel et al., 2018). The AAP recommends that breastfed infants should be supplemented with 1 mg/kg per day of oral iron beginning at four months of age until appropriate iron-containing complementary foods are introduced into the diet (Baker et al., 2010). However, not all experts agree (Raj et al., 2008), as the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) Committee on Nutrition guidelines do not recommend general iron supplementation for breastfed infants (Domellöf et al., 2014). Unless the infant is anemic, preterm, or has other medical issues, iron supplementation is usually not needed during the first six months of life. The high lactose and vitamin C content of breastmilk helps with iron absorption. Delayed umbilical cord clamping at birth has been associated with beneficial effects on infant hemoglobin at different durations of follow-ups and demonstrated a subsequent reduction of anemia without unacceptable side effects (Qian et al., 2019).

Side effects have been noted when infants who are iron replete are supplemented with additional iron (Lönnerdal, 2017). These effects include decreased growth (both linear growth and weight), increased illness (usually diarrhea), interactions with other trace elements such as copper and zinc, altered gut microbiota to more pathogenic bacteria, increased inflammatory markers, and impaired cognitive and motor development. For example, lactoferrin is largely unsaturated with iron but has a high affinity for it. Lactoferrin uses a mechanism

to withhold iron from iron-requiring pathogens in the infant gut, which protects against infection. Saturating lactoferrin with unneeded supplemental iron can eliminate its growth-inhibiting effect against pathogens such as *E. coli*, leaving the infant gut more prone to dysbiosis, inflammation, and bacteremia.

Zinc

Zinc is essential as a component of at least 300 enzymes that have both catalytic and structural roles. It also plays a role in metabolism of proteins, carbohydrates, and lipids. Further, it is essential for cell differentiation and body growth. Many DNA-binding proteins are zinc complexes. Zinc concentration in colostrum ranges from 8–12 mg/mL and in mature milk from 1–3 mg/mL, and in the majority of situations is completely sufficient for normal infant growth, health, and development. However, zinc concentrations in pooled donor human milk may be lower than expected based on time postpartum when the milk was donated (Young et al., 2019). Zinc levels decrease as time postpartum increases. While banked donor human milk is fortified in NICUs, this is not done when banked donor human milk is used in the level I nursery or when parents purchase donor human milk. In these situations, infant zinc levels may need to be monitored more closely if the banked donor milk is used long term or as a large portion of the infant's diet.

Zinc deficiency in breastfed infants due to low levels of zinc in breastmilk can occur (Liew et al., 2017). The infant may present with persistent perioral, neck, and/or groin rashes, poor weight gain, irritability, poor appetite, slowed growth and development, diarrhea, or immune system deficiencies. Milk zinc levels can be low despite normal zinc levels in maternal serum. Transient neonatal zinc deficiency (TNZD) may be the result of mutations in the maternal SLC30A2 gene, which encodes the ZnT2 transporter—the protein vehicle that imports zinc into the milk-secreting cells in the breast (Golan & Assaraf, 2020). Without this transport mechanism, zinc cannot be effectively secreted into the milk. This loss of function in the zinc transporter SLC30A2 may be more common than previously thought, with one estimate of the frequency of TNZD-causing mutations in the general population being at least 1 in 2,334 exclusively breastfed infants at risk of developing TNZD (Golan et al., 2019). Various mutations of ZnT2 have also been shown to affect breastmilk production. Women who have mutations in ZnT2 may have difficulty breastfeeding because zinc is necessary for the growth of the mammary glands and the function of mammary epithelial cells and secretion pathways (Alam et al., 2015). ZnT2 mutations can be one cause of insufficient milk production. If women harboring loss of function mutations in *SLC30A2/ZnT2* also suffer from low milk supply, their infants may have been supplemented with infant formula, which would mask the zinc deficiency in the milk. TNZD-causing mutations may also be genetically inherited (Liew et al., 2017), with a history of insufficient breastmilk in the maternal grandmother supporting the possibility of a genetic contribution to insufficient milk production. Zinc supplementation of the infant will restore normal zinc levels supporting breastfeeding to continue.

Iodide (Iodine)

Adequate iodine intake is required for the synthesis of thyroid hormones. Sufficient breastmilk iodine levels are particularly important for proper neurodevelopment in nursing infants. Because breastfed infants are reliant on maternal dietary iodine intake, recommendations for maternal dietary iodine intake during lactation range from 250–290 μg/d. The median breastmilk iodine levels

in women in the United States range from 35–155 μg/L. In iodine-sufficient areas, breastmilk iodine concentrations are generally adequate to meet infants' iodine nutritional needs. Substances such as perchlorate, thiocyanate, and nitrate can decrease the entry of iodine into the thyroid gland and lactating breast. Maternal exposures to thiocyanate, a metabolite of cyanide that is produced as a by-product of cigarette smoke, and nitrate, which is produced naturally and is present in many prepared foods, are able to decrease breastmilk iodine availability (Laurberg et al., 2004). Smoking during the period of breastfeeding can dose-dependently reduce breast-milk iodine content to about half and, consequently, exposes the infant to increased risk of iodine deficiency (Laurberg et al., 2004). Substances such as perchlorate, thiocyanate, and nitrate may competitively inhibit the sodium-iodide symporter responsible for iodide transport in the lactating mammary gland. Smoking during the period of breastfeeding increases the risk of iodine deficiency–induced brain damage in the child (Pearce et al., 2007). Because exposure to thiocyanate and nitrate is ubiquitous, the additive effects on iodide uptake may be important when assessing iodine availability (Leung et al., 2011). During the early days of life, the infant thyroid very actively accumulates iodide for thyroid hormone synthesis. Thiocyanate from the mother may divert part of the iodine in the infant from thyroid uptake to renal excretion. The American Thyroid Association has recommended that women in North America receive dietary supplements containing 150 μg of iodine daily during pregnancy and lactation and that all prenatal vitamins contain 150 μg of iodine (Becker et al., 2006). Infants of breastfeeding mothers who smoke may need to have their iodine levels monitored and to be given iodine supplementation if necessary while they continue to breastfeed.

Trace Elements

Copper, selenium, chromium, manganese, nickel, fluorine, molybdenum, cobalt, and magnesium all appear in adequate amounts in human milk. Manganese (Mn) functions both as an essential nutrient and a toxic element. Breastmilk Mn content ranges from 2–11 μg/L. Infant formulas can contain excessively high amounts of Mn (Frisbie et al., 2019). Frisbie et al. (2019) analyzed 25 different infant formulas for Mn content and found that all of the 25 products purchased in the United States for this study had a measured Mn concentration that was greater than the 5 μg of Mn/100 kcal that the Food and Drug Administration (FDA) requires as a minimum level for infant formulas. The concentrations of Mn in these 25 products ranged from 26–860 μg of Mn/100 kcal. A Mn concentration of 26 μg/100 kcal is about 32 times greater than that of breastmilk (Koletzko et al., 2005). The product with 860 μg of Mn/100 kcal has about 1,000 times more Mn than breastmilk (Koletzko et al., 2005) and was labeled "toddler powder," which is not regulated by U.S. laws regarding infant formula. Soy is a source of the relatively high maximum concentration of Mn in soy formulas and was measured in this study at 170 μg/100 kcal. An Mn concentration of 170 μg/100 kcal is about 200 times greater than that of breastmilk (Koletzko et al., 2005). If infant formula is reconstituted with tap water, Mn from the tap water may further increase the total Mn content of the formula and elevate the total Mn burden for the infant (Ljung et al., 2011). Excessively high Mn exposure has been associated with adverse effects on infant and child neurodevelopment (Lucchini et al., 2017). A meta-analysis showed significantly higher overall peripheral manganese levels in children diagnosed with attention-deficit/hyperactivity disorder (ADHD) than in controls (Shih et al., 2018).

Fluorine (Fluoride)

Fluoride can occur naturally in water and, in some communities, it is added to water supplies to reach the recommended concentration of 0.7mg/L for the prevention of tooth decay. Breastmilk contains extremely low concentrations of fluoride (0.005–0.01mg/L) due to the limited transfer of fluoride from maternal plasma into breastmilk (Zohoori et al., 2018). Formula-fed infants living in areas containing fluoridated water have an approximate 70-fold higher fluoride intake than exclusively breastfed infants due to formula powder reconstitution with fluoridated water (Zohoori et al., 2018; U.S. Environmental Protection Agency, 2010). A relationship has been found between exposure to fluoridated water and the prevalence of ADHD (Malin & Till, 2015). Two mechanisms for this association have been put forth by these authors; silicofluoride-treated water has the ability to corrode lead-containing plumbing, with lead, a known factor in affecting the risk of developing ADHD (Braun et al., 2006); and exposure to fluoridated water may contribute to ADHD via suppression of the thyroid gland. Fluoride reduces thyroid gland activity (Klein et al., 2001) and hypothyroxemia has been associated with ADHD and is considered a potential cause of the disorder (Vermiglio et al., 2004). In a study of 398 mother-child dyads, it was found that for each 0.5mg/L increase in water fluoride concentration, a decrease of 4.4 full-scale IQ points was noted among preschool children who were formula-fed in the first six months of life, and a 9.3 point decrement was reported in performance IQ (nonverbal intelligence; Till et al., 2020). The authors of this study recommended that during the first six months of life, fluoride exposure should be limited by using non-fluoridated water or water with a low fluoride content to reconstitute infant formula. Some commercially available bottled nursery water contains added fluoride. Parents should be advised that breastfed infants do not require additional water or fluoride and should check with their primary care provider about the safety of such products advertised for young infants.

Defense Agents in Human Milk

The immune system of human milk is a complex interplay between milk factors, the matrix of human milk, synergistic activities of defense components, difference in resident gut microflora of the infant, and individual differences in mothers and infants. It is designed to compensate for the physiologic immaturity of the infant immune system. Breastfeeding maintains the immunological link between the mother and infant. For example, during direct breastfeeding, infant saliva reacts with breastmilk to produce reactive oxygen species, while simultaneously providing growth-promoting nucleotide precursors that regulate early oral and gut microbiota. One study demonstrated that fresh breastmilk contained 27.3 ± 12.2 μM of hydrogen peroxide (a well-known antimicrobial agent), but mixing infant saliva with breastmilk generated an additional > 40 μM hydrogen peroxide, sufficient to inhibit growth of the opportunistic pathogens *Staphylococcus aureus* and *Salmonella spp* (Al-Shehri et al., 2015). This immunological link can also be seen in the responsiveness of leukocytes to maternal or infant infections. Within the first one to two weeks postpartum, leukocyte numbers decrease to a low baseline level in mature breastmilk. This baseline level is maintained throughout lactation unless the mother and/or her infant became infected. Hassiotou et al. (2013) showed that when either the mother or the infant experienced an infection, leukocyte numbers significantly increased up to 94% leukocytes out of

total cells. Upon recovery from the infection, baseline values were restored. Mastitis resulted in a significant increase in milk leukocyte levels, whose detection in breastmilk may be useful in the assessment of the health status of the lactating breast. Human milk provides the recipient infant with several tiers of protection, resulting in the reduced incidence of many diseases and adverse conditions long after breastfeeding has ceased (Hanson, 2002). A suite of factors with immunological, hormonal, enzymatic, trophic, and bioactive activity are present in breastmilk and offer passive protection to the infant during a vulnerable and critical period of time (**Table 2-4**). Breastmilk is rich in maternal cells. Macrophages and leukocytes, which are abundant during early lactation, are important cellular components. Breastmilk feeding is a mechanism of transmission of immunocompetence from the mother to her infant (Chirico et al., 2008) and aids in the education of the immune system. Breastmilk is often referred to as an infant's first immunization or vaccine. It has been shown that breastfed infants compared to formula-fed infants may show a better response to several vaccines (polio, diphtheria, tetanus, measles, mumps, rubella, *Haemophilus influenzae* type b [Hib], and pneumococcal serotypes 6B and 14; Dorea, 2009).

The thymus gland is instrumental during infancy and early childhood in the production and maturation of T-lymphocytes or T cells (a type of white

Table 2-4 Anti-infective and immunological components in human milk

Adaptive immunity compounds	Immunoglobulins sIgA (11S), 7S IgA, IgG, IgM, IgE, IgD, free secretory component, antiidiotypes
Innate immunity agents	Complement, chemotactic factors, properdin factors, interferon, α-fetoprotein, antistaphylococci factors, mannose binding lectin, ß-defensin-1, antiadherence substances (oligosaccharides, mucins, lactadherin, glycolipids and glycosaminoglycans, *K*-casein), milk fat globule, hormones and growth factors (prolactin, cortisol, insulin, thyroxin, prostaglandins, vascular-endothelial growth factors, nerve growth factor, TGF, erythropoietin), antiviral factors (fatty acids and monoglycerides), migration inhibition factor, α -lactalbumin
Cytokines, chemokinees, and receptors	IL-1ß, IL-2, IL-4,IL-5,IL-6,IL-8,IL-12, IL-13, IL-16, IL-18, IFNy, TNF α, G-CSF, M-SCF,GM-CSF,GROα, monocyte chemotactic protein-1,TGFß1 and-2, sCD14, Toll-like receptor, sFas, sFasL
Antiinflammatory factors	IL-10, TGF ß2, glucocorticoids , antioxidants (α-tocopherol, ß-carotene, lutein, vitamin E, catalase, glutathione peroxidase,), lactoferrin, IL-1Ra, soluble TNFα receptors I and II, CD59
Prebiotics	Bifidus factor, oligosaccharides
Histocompatibility antigens	
Carrier proteins	Lactoferrin, transferrin, vitamin B-12 binding protein, steroid binding protein
Enzymes	Lysozyme, lipoproteinlipase, leukocyte enzymes, antiproteases, platelet-activating factor-acetyl-hydrolase
Others	Nucleotides, long-chain polyunsaturated fatty acids
Cellular	
Total counts	Colostrum,1-3 $\times 10^9$/L; mature milk,~1×10^8/L
Cell types	Macrophages, ~60%; neutrophils,~25%; lymphocytes,~10%; epithelial cells

Reproduced from Chirico, G., Marzollo, R., Cortinovis, S., Fonte, C., & Gasparoni, A. (2008). Antiinfective properties of human milk. *Journal of Nutrition, 138*, 1801S–1806S.

blood cell). T cell functions include directly killing infected host cells, activating other immune cells, producing cytokines, suppressing unwanted immune responses to self-antigens, and regulating the immune response. The thymus involutes with age and by puberty it begins to shrink and becomes replaced with fatty tissue over time, leading to impaired responses to pathogens and vaccines and to increased susceptibility to infections, autoimmune diseases, and malignancy as people age. It has been shown that the thymus is significantly larger in breastfed infants compared to formula-fed infants (Hasselbalch et al., 1996; Hasselbalch et al., 1999). CD4+ and CD8+ cells (subsets of T cells) show higher percentages in breastfed infants. A positive correlation was found between thymus size and CD8+ cells with an increased number of breastfeedings per day at 8 months correlating with an increase number of CD4+ cells at 10 months of age (Jeppesen et al., 2004).

While milk of secretor women is rich in 2'-fucosyllactose and other α1-2-fucosylated HMOs, nonsecretor women lack a functional FTU2 enzyme resulting in milk that does not contain α1-2-fucosylated HMOs. Interestingly, some strains of *Staphylococcus*, the major cause of mastitis, bind to 2'-fucosyllactose (Lane et al., 2011). Therefore, it is possible that susceptibility to suffer from mastitis is determined not only by the bacterial composition of the human milk, but also by the blood group and corresponding type of HMOs in the milk.

Stem Cells

Cregan et al. (2007) first identified cells with mammary stem/progenitor properties in human milk. Since then it has been shown that pluripotent stem cells (cells that have the capability to develop into any of the 200 cell types in the body) are present and active in the lactating breast (Hassiotou et al., 2012). Animal studies have shown that breastmilk stem cells as well as immune cells survive the neonatal gut, migrate into the blood, and from there travel and integrate into various organs, including the thymus, liver, pancreas, spleen, kidneys and the brain where they appear to turn into specialized cells of each specific organ (Hassiotou et al., 2015). Stem cells whose destiny is the thymus are thought to facilitate both the cellular tolerance between the mother and the infant and contribute to the maturation of the infant's immune system (Molès et al., 2018). Stem cells that migrate to the brain have been shown in animal studies to become specialized brain cells of two types: neuronal and glial, the two main brain cell types (Aydin et al., 2018). It has been speculated that mammary stem cells may support tissue homeostasis, regeneration, and brain development in early life (Twigger et al., 2013). Much remains unknown regarding the function of mammary stem cells. However, in the dairy cow it has been shown that there is a known decline in milk production as lactation progresses, which was attributed to a steady rate of epithelial cell death (Capuco et al., 2001). Developing treatments to maintain and/or boost mammary stem cell proliferation was proposed as a strategy to maintain and/or increase bovine milk production (Capuco et al., 2012). This raises a tantalizing future possibility that an intervention for human breastmilk insufficiency may arise from using mammary stem cell enhancement techniques (Hassiotou & Hartmann, 2014).

MicroRNA

MicroRNAs (miRNA; ribonucleic acid) are a class of noncoding RNAs (do not code for proteins) that play important roles in regulating gene expression. More than 1,400 miRNAs have been identified in breastmilk, with miRNA

hsa-miR-148a-3p consistently being the most abundant miRNA in breastmilk (Benmoussa & Provost, 2019). This particular microRNA has been shown to suppress the activity of genes in tumor cells involved in proliferation, leading to speculation that miRNA-148a may have a protective effect against cancer in infants (Golan-Gerstl et al., 2017). They are crucial modulators of many normal functions, such as cardiac function and other cardiovascular processes, immune protection such as regulation of B and T cell differentiation and development, innate/adaptive immune response, and tissue function. They are involved in all major biological processes including cell differentiation, cell cycle, apoptosis, immunity, development, and disease. MiRNAs can tolerate an infant's gastrointestinal environment and be absorbed into the intestine, where they can influence the infant's developing immune system. The storage and freeze-thawing of breastmilk did not denature the miRNAs, a dietetically important finding for low-birth-weight babies and other hospitalized infants who are usually given freezer-stored breastmilk (Kosaka et al., 2010). The microRNA strands found in the breastmilk of mothers giving birth at full-term differ from those seen in the milk of mothers who give birth to premature infants. Carney et al. (2017) showed that milk from mothers who delivered early is richer in the miRNAs that target mRNA involved in metabolism. This suggests that the miRNA composition of a mother's milk adapts to favor enhanced growth in premature infants. They play significant roles in the development and normal functioning of the lactating mammary gland (Alsaweed et al., 2015). The distinct changes of milk miRNA in response to the status of the mammary gland together with data supporting the mammary origin of milk miRNA highlight their potential diagnostic value as noninvasive and easily accessible biomarkers of mammary gland function and health to facilitate timely management of lactation difficulties such as mastitis and maintenance of breastfeeding for longer periods (Alsaweed et al., 2016). When compared with human milk, miRNAs are found in much lower amounts and types in infant formula (Alsaweed et al., 2016).

Chrononutrition (or Can Breastmilk Tell Time?)

Just as the body experiences recurring daily physiological rhythms, so too does breastmilk. The temporal variation in human milk composition acts like an internal timekeeper that provides time-of-day information to the infant (**Figure 2-3**).

For example, human milk contains higher levels of cortisol during the morning and activity-promoting amino acids cycle higher during the day, which probably function to promote alertness, feeding behavior, and catabolic processes in infants (Pundir et al., 2017; Sánchez et al., 2013). A study of 23 exclusively breastfeeding mothers who expressed milk before and after each breastfeeding session over 24 hours, showed distinct diurnal variation in cortisol. Cortisol levels were an average of 330% higher in morning milk (2.97 ng/ml between 4:00 a.m. and 10:00 a.m.) compared to late afternoon and evening milk (0.69 ng/ml between 4:00 p.m. and 10:00 p.m.; Pundir et al., 2017). However, human milk during the nighttime contains low levels of these activity-promoting components and instead provides high levels of melatonin and tryptophan, which promote sleep, relax digestion, and facilitate cell restoration (Engler et al., 2012; Illnerova et al., 1993). Melatonin is almost undetectable in breastmilk during the day, but rises before nighttime sleep, peaking in the early morning hours (Illnerova et al., 1993). Twenty-one

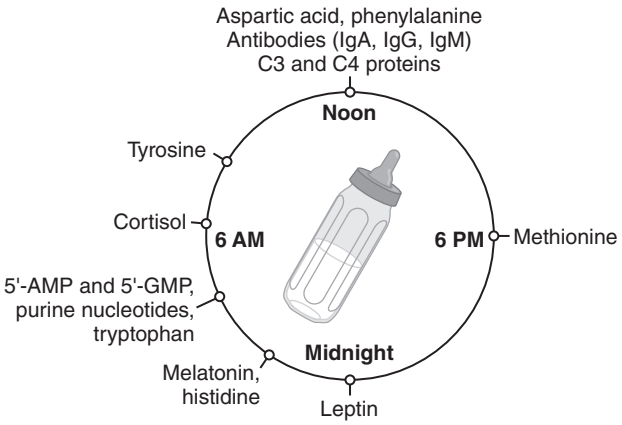

Figure 2-3 Human milk component fluctuations across 24 hours

mothers collected breastmilk samples five times in a 24-hour period between day 5 and 10 postpartum. The median melatonin concentration in daytime milk (10 a.m. to 10 p.m.) was 1.5 mg/L and the median concentration in nighttime milk (10 p.m. to 10 a.m.) was 7.3 ng/L (Katzer et al., 2016) with peak levels occurring round 3 a.m. (Qin et al., 2019). Regular daily variation in milk melatonin emerges within the first days after birth (Sánchez et al., 2013). To ensure maximal levels of melatonin in breastmilk at night, the mother could be advised to sleep in a dark room and breastfeed under low lighting, as maternal exposure to light can cause melatonin suppression in breastmilk (Brainard et al., 2015). This helps assure that the infant receives an adequate amount of melatonin, which can have an impact on the entrainment of the infant's circadian rhythms, as well as providing powerful antioxidant, anti-inflammatory and immune regulatory effects (Anderson et al., 2016). Many immune components exhibit higher activity during the day and follow a diurnal pattern (Franca et al., 2010). It is during infancy that the stage is set for circadian programming, and the body's circadian rhythms are established. The circadian pattern develops to be in harmony with the child's day/night environment. This harmony promotes the proper functioning and synchronization of all the body systems. Nutritional components, hormones, and immune factors all vary in concentration over a 24-hour time frame (**Table 2-5**).

The hormonal composition of breastmilk shows significant changes over the day. For example, leptin regulates energy balance by inhibiting hunger. In a study of 19 mothers who expressed milk samples before and after each feeding for 24 hours, leptin levels were significantly higher in milk collected between 10 p.m. and 4 a.m. than milk collected between 4 a.m. and 10 p.m. (Cannon et al., 2015). Immune components are generally higher in daytime milk than at night.

Circadian rhythms are important in sleep-wake cycles, respiratory rate, body temperature, digestion, metabolism, hormone release, and immune function. Dysregulated circadian rhythms can affect sleep and immune function. It has been reported that infants with colic have blunted cortisol rhythms, as seen by lower morning and higher evening cortisol levels, compared to infants without colic (White et al., 2000). Infants are not born with a completely functioning circadian clock. The variations in milk components over the day may act as environmental cues for the acquisition of infant circadian biology. Feeding directly from the breast matches maternal and environmental rhythms.

Table 2-5 Selected Human Milk Component Variation over 24 Hours

Milk Component	Concentration Variation
Nucleotides	Higher nocturnal levels of 5'AMP and 5'GMP, which facilitate the release of GABA and melatonin, relaxing and sleep-inducing nonprotein nitrogen fractions (Sánchez et al., 2009).
Tyrosine, methionine, phenylalanine, aspartic acid, glycine	These activity-promoting amino acids all show peak levels in daytime milk (Sánchez et al., 2013).
Total fat content	Mature term and preterm milk expressed at night has higher total fat content than daytime expressed milk (Moran-Lev et al., 2015).
Iron Vitamin E	Peaks around noon. Peaks in the evening (Barkova et al., 2005).
Magnesium Zinc Potassium	Highest levels in the morning (Karra & Kirksey, 1988).
Sodium	Highest in early morning hours (Keenan et al., 1982).
Leptin	Peak levels at night and early morning (Cannon et al., 2015).
Cortisol	Levels are highest in the morning (Pundir et al., 2017).
Melatonin	Is almost undetectable in breastmilk during the day, rising before nighttime sleep, peaking in the early morning hours (Illnerova et al., 1993).
sIgA,	Highest around noon.

However, many infants receive expressed breastmilk, which may not be circadian matched at the time it is ingested by the infant. A study in the United States reported that 85% of breastfeeding mothers fed their infants with previously expressed human milk at least some of the time, and 25% of these infants consumed previously expressed breastmilk daily (Fein et al., 2008). The same study also found that approximately 6% of breastfeeding mothers in the United States did not feed their infant directly at the breast, but only fed their infants expressed milk. Rather than feeding milk that is not matched to the time of day, provision of circadian-matched expressed breastmilk to infants may enhance infant health outcomes for both term and preterm infants. In light of what is known regarding daily oscillation in milk components, infants who are at a high risk of infection or who are actively fighting infections might benefit from milk selectively collected during the day, as key immune factors are generally higher in milk expressed during the day compared to the night. Daytime milk expressed around noon is especially enriched with sIgA, providing protection against bacterial and viral infections, neutralizing toxins, and acting to increase free radical levels. Day (compared to night) colostrum and mature milk also contain larger quantities of phagocytes that function to engulf and destroy harmful microorganisms, foreign particles, and cellular debris, in addition to playing an important role in maintaining cell lineages that facilitate long-term defense against specific pathogens (Field, 2005). Provision of

circadian-matched breastmilk would be important in the NICU to promote the acquisition of circadian body rhythms in preterm infants. Mothers and other care providers can be advised to label expressed milk with the time of day that it was expressed, and select the stored milk that best corresponds with the current time that the infant is being fed.

Human Milk Fortification

The immunological factors in breastmilk are important for all infants, but they are essential for preterm or ill infants whose immature immune systems are challenged by an early birth and some of the adverse conditions that may accompany it. Nutrient fortification of preterm mother's milk is seen in NICUs when an infant's nutritional needs exceed the capacity of breastmilk to provide selected nutrients that support a particularly desired growth velocity. Inadequate nutrition or poor postnatal growth of low birth-weight or very low birth-weight infants has been associated with neurocognitive impairments (Chan et al., 2016). Protein is often the limiting factor in human milk, and any shortfall could affect growth. Inadequate intake of protein is particularly responsible for decreased fat-free mass gains, which may lead to poor neurocognitive outcomes (Ramel et al., 2016). Thus, protein supply needs special attention in early life, and meeting the requirements can be challenging. Insufficient intake of other nutrients may provoke specific deficiency states, such as osteopenia (due to insufficient intake of calcium and phosphorus) and to various micronutrient deficiencies, such as zinc deficiency. Fortification of human milk is designed to increase the concentration of nutrients to enable the infant to grow at the same rate as a fetus (Ziegler, 2014).

There are several approaches to human milk fortification. Standard fortification is the most utilized method seen in NICUs. It involves adding a fixed amount of multinutrient fortifier to 100 mL of human milk to reach the recommended nutrient intakes as determined by the manufacturer of the product. However, growth problems have sometimes been found to persist with this method of fortification (Brown et al., 2016). Standard fortification does not account for the variability of human milk macronutrient content, as well as variety of each infant's unique requirements, which can lead to protein undernutrition (Arslanoglu et al., 2019). One reason for possible protein undernutrition with standard fortification is that the protein content of preterm human milk is highest during the first three weeks postpartum but can decrease significantly after that (Maly et al., 2019). Standard fortification products assume that the higher level of protein in the expressed milk remains constant and may not account for the decline in protein concentration after about three weeks. This would make it difficult to reach the recommended protein intakes with many infants (Maly et al., 2019).

The concept of individual fortification encompasses two methods of fortification: adjustable fortification and targeted fortification. Adjustable fortification involves determining protein intake and supplementation based on each infant's metabolic response. Various protocols have been created, usually with standard fortification initiated using a multinutrient fortifier that is guided by blood urea nitrogen (BUN) levels as a surrogate for assessing protein adequacy. If the BUN level is below a predefined threshold value, extra protein is added in the form of a protein supplement. If the BUN level is above a specified value suggesting excessive protein, the level of fortification is reduced.

In targeted fortification, the macronutrient composition of breastmilk is analyzed and is fortified to assure that the infant receives the recommended

amount of each nutrient. This method requires the use of a milk analyzer, which can be an expensive piece of equipment. It can be time-consuming but allows clinicians to conduct real-time analysis of human milk and add modular products as needed. Addition of a protein fortifier to expressed breastmilk for 30 days based on milk analysis showed a greater early gain in fat-free mass compared with infants in a standard fortification group (Parat et al., 2020). In a prospective observational study, very low birth weight preterm infants 32 weeks of gestational age and younger were randomized into two groups according to the method of breastmilk fortification (targeted fortification vs. adjustable fortification). Anthropometric measurements (daily weight gain and weekly head circumference) were performed in both groups weekly for four weeks to compare their growth. Individualized protein fortification using the targeted method had more positive effects on short-term growth compared with the adjustable fortification method (Bulut et al., 2020).

There are a number of commercial fortifiers available that differ by the source of the milk (bovine or human) and by the nutrient composition. Bovine-based multinutrient fortifiers contain differing amounts of protein, energy, minerals, lipid, trace elements, vitamins, and electrolytes. Single-nutrient fortifiers that contain only protein, lipids, or carbohydrates are available and used for individual fortification. Pasteurized human milk–based fortifiers are on the market and are created by concentrating heat-treated human milk and then adding vitamins and minerals. Various caloric densities of this fortifier allow for individual adjustment based on growth or BUN. A novel human milk–derived cream supplement has also been produced whose use showed an earlier discharge and the most benefit to infants with bronchopulmonary dysplasia (Hair et al., 2016). Using a human milk–based fortifier allows for the infant to be fed an exclusive human milk diet. An advantage of an all human milk diet was shown in a study where an exclusively human milk–based diet was associated with a significant reduction in the rates of NEC and surgical NEC compared with dietary exposure to bovine milk–based products. This study found a reduction in NEC of 50% and in surgical NEC of almost 90% in infants fed an exclusive human milk diet compared with a diet containing bovine milk–based products (Sullivan et al., 2010). Lucas et al. (2020) reported that in their study, cow's milk–derived fortifier was associated with a more than fourfold increased relative risk of NEC and an over fivefold increased risk of severe morbidity comprising NEC surgery or death. Use of a cow's milk–based fortifier has the potential to alter the anti-infective properties of breastmilk. One study compared the effects of a powdered bovine-based fortifier and a human milk–based fortifier on the antimicrobial activity of breastmilk toward *Enterobacter sakazakii*, *Escherichia coli, Clostridium difficile*, and *Shigella sonei*. Human milk inhibited the growth of all the test organisms. This antibacterial activity was almost totally inhibited by the addition of a bovine protein-based human milk fortifier, while it remained unaffected by the addition of a human breastmilk–based fortifier (Chan et al., 2007). Schlotterer et al. (2019) demonstrated that the addition of an acidic, bovine-based fortifier added to holder pasteurized donor human milk significantly reduced the lysozyme and IgA levels. Caution should be noted when adding a preparation to human milk, such as a fortifier, that neutralizes the milk's ability to destroy harmful bacteria, especially if the potential for bacterial contamination resides in the fortifier itself. To further minimize bacterial proliferation in fortified breastmilk, hang time for continuous feedings at room temperature should be limited to a maximum of four hours (Steele & Collins, 2018). Over time, the osmolality of fortified human milk can increase (by up to 4%) and the size of milk fat globules may become

altered (possibly impacting fat digestion) if the time lag after fortification exceeds 12 hours (Takahashi et al., 2012).

Human milk can be lyophilized (freeze-dried) and used as an additive to increase the levels of macronutrients and micronutrients available to preterm infants. It has been shown that such freeze-dried breastmilk has osmolality and levels of certain macronutrients and micronutrients compatible with the nutritional needs of very low birth-weight infants (Oliveira et al., 2019). Freeze-dried human milk concentrate used as a fortifier eliminates some of the side effects of using cow's milk–based fortifiers such as gastrointestinal problems and the introduction of foreign proteins. Compared to simple freezing, lyophilization of human milk is reported to better preserve nutritional characteristics and the integrity of several immune components while preventing oxidative deterioration (Cortez & Soria, 2016). The production of a concentrate using freeze-dried human milk was shown to have good nutritional stability and the retention of the original fatty acid profile during six months of frozen storage (Bomfim et al., 2018).

Fortifiers are available in liquid and powder forms. Powdered infant formula as well as powdered fortifiers are not sterile. The CDC recommends the use of liquid products over powder in the NICU setting in an effort to reduce contamination and infection risk (CDC, 2002). A 2002 FDA letter to healthcare professionals warned that premature infants and infants with underlying medical conditions could become infected with *Cronobacter* and recommended that powdered infant formulas be avoided in NICUs unless there was no alternative (FDA, 2002). Sterile liquid fortifiers have also been recommended by the Academy of Nutrition and Dietetics (Steele & Collins, 2018) and the American Society for Parenteral and Enteral Nutrition (Boullata et al., 2017). *Cronobacter sakazakii* infection has been reported in the NICU from the use of a powdered fortifier product (Foodborne Illness Outbreak Database, 2007). *Cronobacter* has been known to cause meningitis, septicemia, and necrotizing enterocolitis in neonates. This is especially concerning since it has been shown that 90% of invasive pediatric *Cronobacter* infections have been in infants who had received powdered infant formula (Jason, 2012). *Cronobacter* can also infect healthy, term (not just hospitalized preterm) young infants (Jason, 2012). Invasive *Cronobacter* infection is extremely unusual in infants not fed powdered infant formula or powdered human milk fortifiers. However, it has occurred in exclusively breastmilk-fed infants and has been associated with expressed breastmilk from pump collection kits that were not appropriately cleaned and sanitized (Bowen et al., 2017; Sundararajan et al., 2018).

One study reported on the use of a mobile milk cart for the preparation and fortification of breastmilk in a hospital that lacked a central space for the management of human milk nutrition for its preterm infants (Barbas, 2013). A best practice concept for the provision of human milk in the NICU is the use of a centralized facility or a human milk management center that allows staff to analyze breastmilk, perform creamatocrits, conduct nutrient analysis, fortify milk under aseptic conditions, make skim milk, and tailor the milk to meet each infant's needs (Spatz et al., 2014).

Breastmilk Treatment

Breastmilk is often treated, handled, and stored, potentially affecting some of its nutrients and bioactive factors. Bacterial growth and contamination are also of concern when handling human milk, especially for use with preterm or ill infants.

Refrigeration

Recommendations for breastmilk storage times and temperatures vary according to different sources. Macronutrient content (protein, carbohydrate, and fat) of expressed breastmilk refrigerated at 4°C (39.2°F) was examined at six different times following birth (Paduraru et al., 2019); 3 ± 1 days (colostrum), 7 ± 1 days (transitional milk), 14 ± 1 days, 21 ± 1 days, 30 ± 1 days (mature milk), and 60 ± 1 days. Fresh milk was analyzed the same day, within two hours after expression. Refrigerated milk was analyzed the next 24, 48, and 72 hours, after rewarming to room temperature. Samples of milk frozen at –20°C (–4°F) were thawed at room temperature 1, 2, 4, 8, and 12 weeks after sampling. After 72 hours of refrigeration, macronutrient and energy levels were preserved with minimal changes. Slutzah et al. (2010) studied milk composition over a 96-hour period and found little change in the composition of fresh milk refrigerated for four days at 4°C/39.2°F. Term colostrum may be kept frozen for three months, whereas milk from day 14 to day 21 could be frozen for one month; mature milk expressed after 30 days of lactation can be kept for two months, as macronutrient changes occurred following each of these times. Refrigeration for up to 72 hours is preferable to freezing for longer than two weeks, as freezing reduced the protein content more than by refrigeration (Paduraru et al., 2019).

Several components of breastmilk contribute to protecting the infant against the harmful effects of oxidative stress such as vitamin C and E and enzymes, including superoxide dismutase, catalase, and glutathione peroxidase. Refrigeration and freezing of breastmilk cause a significant decline in the levels of individual antioxidants, including vitamins C, A, and E. A study that subjected transitional and mature milk to refrigeration and freezing showed that the total antioxidant capacity of milk was reduced by 10–20% and 15–30% following a 48-hour and one-week storage period respectively (Xavier et al., 2011). This emphasizes the importance of using fresh expressed breastmilk for preterm infants or milk that has been stored only for short periods of time to ensure that a robust antioxidant system is retained in the milk, as preterm infants are highly subject to conditions related to oxidative stress.

Bacteriological and immunological characteristics were studied in four groups of expressed milk samples: freshly expressed unfed milk, freshly expressed leftover milk, previously frozen unfed milk, and previously frozen leftover milk. Bacteriological and immunological characteristics of all four categories of milk remained stable during storage at 4°C/39.2°F (refrigerator temperature) for six days. The quality of each group declined when stored at 24°C/75°F (room temperature) for longer than three hours (Fogleman et al., 2018). Since breastmilk contains so many species of bacteria, a study was conducted to explore changes in the major bacterial groups in samples of milk stored for six days at 4°C/39.2°F. Cold storage did not markedly change total viable bacterial load and HMO profiles were stable, illustrating that the process of microbial transfer from the refrigerated milk to the infant gut was not adversely affected (Schwab et al., 2019).

Human cytomegalovirus (HCMV) belongs to the β-herpesvirus family and plays a major role in disease acquisition in immunosuppressed patients such as preterm infants. A breastmilk–acquired HCMV infection usually causes no clinical symptoms in healthy neonates and has a low probability of causing deafness and nervous system sequela. However, the risk of serious sequela from HCMV may increase in pathologically jaundiced infants (Hou et al., 2020). Virus shedding into breastmilk of immunocompetent healthy breastfeeding mothers occurs in nearly every seropositive mother at any time point

during lactation, but usually ends two to three months after birth (Hamprecht et al., 2008; Prendergast et al., 2019). Transmission via breastfeeding can lead to severe symptomatic HCMV infection in preterm infants, with infants whose birth weight is below 1,500 g or who are less than 32 weeks of gestational age at highest risk. Refrigeration and freezing of milk from preterm seropositive mothers may reduce the risk of transferring HCMV to the infant but does not eliminate it (Hamprecht et al., 2008). Freezing milk at –20°C/–4°F has not been shown to produce a reduction in viral titre (Omarsdottir et al., 2015). Only heat inactivation eliminates virus infectivity, and short-term heat inactivation is most preservative of nutrients and bioactive factors in the milk. Although Holder pasteurization (62.5°C/144.5°F for 30 minutes) will inactivate HCMV, it also decreases the immunological components of the milk. Rapid high-temperature treatment of human milk (72°C/161.6°F for 5 to 15 seconds) has been shown to eliminate HCMV infectivity without destroying many of the anti-infective capabilities of the milk (Lawrence, 2006). Short-term heat inactivation for five seconds at 62°C/143.6°F has also been recommended (Hamprecht & Goelz, 2017). High power (750 W) microwaving was shown to completely eliminate HCMV in samples from 31 HCMV-seropositive mothers (Ben-Shoshan et al., 2016). Low-power microwave irradiation (500 W) had a 13% failure rate while three-day freezing and one-day freezing of infected breastmilk samples had failure rates of 7% and 20% respectively. Ehlinger and colleagues (2011) studied alternative methods to reduce HCMV shedding into breastmilk as a method to lower the potential HCMV transmission to preterm infants. Rather than treat the milk, antibody-based maternal vaccines were proposed as a useful approach to protect vulnerable infants against acquisition of symptomatic HCMV infection. Vaccines to prevent HCMV infection are currently under development (Plotkin & Boppana, 2019). Ultraviolet-C (UV-C) irradiation at 254 nm has been investigated as an alternative treatment method and has been shown to better preserve components such as lactoferrin, lysozyme, and secretory IgA compared with Holder pasteurization. Full replication was ablated in one study by various treatment doses. However, evidence of viral immediate early proteins within the cells was never completely eliminated, indicating that some viral gene transcription was still occurring (Lloyd et al., 2016).

Freezing

Long-term storage of human milk by freezing at various temperatures can affect macronutrient content and bioactive factors in breastmilk. Conflicting results in studies have led to different storage recommendations. In a study on 137 samples of term and preterm breastmilk, stored at −20°C/–4°F and −80°C/–112°F for 4, 12, and 24 weeks, it was reported that fat and energy content were consistently higher in the −80°C samples compared with the paired −20°C samples at each time point. Comparison of the differences in macronutrients content over time (4 vs. 24 weeks) revealed a significant loss of fat (0.3 g/100 mL [7.9%], $p = 0.001$) and energy (2.3 kcal/100 mL [3.3%], $p = 0.03$) in the −20°C group. Fat and protein were also significantly decreased over time in the −80°C group. Long-term storage of breastmilk at −80°C was associated with better fat and energy preservation compared with storage at −20°C in this study (Orbach et al., 2019). Ahrabi et al. (2016) examined the integrity (pH, bacterial counts, host defense factors, nutrient contents, and osmolality) of freshly expressed and previously refrigerated human milk subjected to long-term freezer storage. Milk pH, total bacterial colony count, and Gram-positive colony counts decreased significantly with freezer storage

($P < .001$); bacterial counts decreased most rapidly in the refrigerated frozen group. The Gram-negative colony count decreased significantly over time ($P < .001$). Nonesterified fatty acid concentrations increased significantly with time in storage ($P < .001$). This could potentially affect the taste and odor of the milk. Freezing for up to nine months at –20°C did not affect total protein, fat, lactoferrin, secretory IgA, or osmolality in either group. Takci et al. (2012) analyzed the bactericidal activity of human milk on *Escherichia coli* and *Pseudomonas aeruginosa* and determined the changes in bactericidal activity following freezing at –20°C and –80°C for one month and three months. Freezing at –20°C for one month did not cause statistically significant alteration in bactericidal activity, but storage for three months lowered the degree of bactericidal activity significantly against *E. coli*. Bactericidal activity was protected when the samples were stored at –80°C. Freezing at –20°C and –80°C for one month and three months did not cause any significant change in bactericidal activity against *P. aeruginosa*. Storage by freezing at –80°C was concluded to be more appropriate to keep bactericidal capacity of stored human milk greater than one month if affordable and available, especially in intensive care settings.

Human breastmilk lipolysis (fat breakdown) increases with frozen-storage duration, which can contribute to the development of a rancid flavor in breastmilk. Lipolysis of 7-day frozen breastmilk was shown to far exceed the adult threshold for detecting rancid flavor in dairy products, while the lipolysis in the 30-day frozen milk reached the intolerable level (Hung et al., 2018). Many mothers are advised to "use the oldest milk in the refrigerator or freezer first," but this recommendation might be best implemented in some instances only when the breastmilk has been frozen for less than 7 days. Even though infants' flavor sensitivity may differ from adults, extreme lipolysis in breastmilk may increase the probability of infants refusing it. When mothers find that their expressed and stored milk consistently has an off odor or soapy or metallic taste, they are often advised to scald the milk after expressing it to reduce the high-lipase activity. However, scalding the milk at 82°C/180°F, while inactivating lipase, may also degrade other nutrients in the milk. Providing scalded milk as the total nutrition source for an infant may be problematic. Mothers can be advised to mix fresh breastmilk half and half with the stored milk to see if the infant accepts the mixture before being advised to scald the milk. The change in milk odor or taste can happen in a matter of a few hours or in some expressed milk at 24 hours or longer. Mothers can determine when their milk changes odor or taste by tasting expressed milk hourly, which will reveal their personal timeline. This may help in determining how quickly the milk should be fed to the infant. If the infant accepts the milk, then no scalding is necessary and the milk is safe to consume. Parents should also determine if the off odor or taste in the breastmilk is due to maternal medications or something the mother ingested that has flavored the milk such as anchovies, old vegetable oils, or fish oil supplements.

Recommendations from the Academy of Breastfeeding Medicine (Eglash et al., 2017) and the Human Milk Banking Association of North America (Jones, 2019) for breastmilk storage for term infants can be found in **Box 2-2**.

Freeze Drying (Lyophilization)

Refrigeration and freezing are the most common approaches to short- and long-term storage of breastmilk. However, lyophilization is used as a storage technique for complex products such as drugs, biological tissues, and some foods and may be of value for milk banks and others wishing to use stored

Box 2-2 Human Milk Storage Recommendations for Term Infants

The Academy of Breastfeeding Medicine recommends:

- Room temperature (16–29°C/60–85°F), 4 hours or 6–8 hours under very clean conditions.
- Refrigerator (4°C/39.2°F), 4 days or 5–8 days under very clean conditions.

The Human Milk Banking Association of North America recommends:

- Room temperature: < 6 hours.
- Refrigerator: < 5 days for a term infant and < 8 days for an older infant.
- Freezer: 3 months ideal; ≤ 6 months optimal; ≤ 12 months in a deep freezer (–20°C/–4°F); infant may require supplemental vitamins if consuming breastmilk that has been frozen for long periods of time and not receiving any fresh breastmilk.
- Previously frozen milk that has been thawed in the refrigerator but not warmed can be stored at room temperature for < 4 hours and in the refrigerator for < 24 hours, but should not be refrozen.
- Breastmilk from an incomplete feeding can be refrigerated ≤ 4 hours and not refrozen.
- Thawed previously frozen pasteurized donor human milk ≤ 4 hours at room temperature, ≤ 48 hours in a refrigerator, and not to be refrozen.
- Small cooler, Styrofoam cooler, or insulated lunch container with frozen ice packs or frozen gel packs (15°C/59°F): 24 hours. (Hamosh et al., 1996)

human milk. Salcedo et al. (2015) compared the effect of lyophilization with that of human milk storage methods (−20°C, −80°C) in terms of pathogenic bacterial abundance. Microbiological content, bactericidal activity, sialic acid, and ganglioside contents, as well as protein, fat, and lactose concentrations were assessed in 125 human milk samples. Lyophilization and storage at −80°C significantly reduced the content of mesophilic aerobic microorganisms and *Staphylococcus epidermidis* when compared with storage at −20°C. Bactericidal activity was not significantly modified by lyophilization when compared with freezing at either −20°C or −80°C. Bactericidal activity was not correlated with fat, protein, or lactose content, but was significantly correlated to ganglioside content. The bactericidal activity was significantly greater in mature milk and in milk from women with term delivery than in milk from early lactation (days 1–7 postpartum) and milk from women who delivered preterm, respectively. Proteins, glucose, triglycerides, polyphenols, and markers (nitrites, superoxide anion, hydroperoxides, lipoperoxides, and γ-glutamyl transpeptidase) were analyzed after freeze-drying and retained their functionality and integrity (Cortez & Soria, 2016). While freeze-drying as a breastmilk storage approach may help extend the shelf life of expressed milk and help with storage space constraints, it could cause a reduction in beneficial bacterial counts. Freeze-drying has also been shown to reduce vitamin C concentration by 30% (Martysiak-Żurowska et al., 2017). This may be of concern as vitamin C can be further degraded by exposure to light (Francis & Dickton, 2015).

Microwaving

Refrigerated or frozen breastmilk is sometimes microwaved by parents to quickly heat or thaw it, however microwaving human milk can potentially destroy some of its nutritional and bioactive components. The microwaving effect on breastmilk is not well studied. Quan et al. (1992) tested 22 freshly frozen human milk samples for lysozyme activity, total IgA, and specific secretory IgA

to *Escherichia coli* serotypes 01, 04, and 06 when heated by a microwave for 30 seconds at a low- or high-power setting. Microwaving at high temperatures (72°C/161.6°F to 98°C/208.4°F) caused a marked decrease in activity of all the tested anti-infective factors. *E. coli* growth at ≥ 98°C was 18 times that of the control sample of human milk. Microwaving at low temperatures (20°C/68°F to 53°C/127.4°F) had no significant effect on total IgA, specific IgA to *E. coli* serotypes 01 and 04, but did significantly decrease lysozyme and specific IgA to *E. coli* serotype 06. Even at 20°C/68°F to 25°C/77°F, *E. coli* growth was five times that of the control sample of human milk. Triglyceride and carotenoid concentrations in human milk were shown to remain stable after low-temperature microwave heating (Tacken et al., 2009). High-power microwaving (750 W) has been shown to eradicate HCMV in breastmilk (Ben-Shoshan et al., 2016). It has long been known that warming bottles in a microwave has the potential for causing palatal and other burns in the infant's mouth and throat (Hibbard & Blevins, 1988), which is why parents are advised not to microwave bottles of breastmilk. Yet a 2013 study showed that 10% of parents heated their expressed breastmilk in a microwave (Labiner-Wolfe & Fein, 2013). Given the potential for alterations in nutritional and bioactive factors in breastmilk and the possibility of inflicting burns, microwaving of breastmilk to heat a bottle for a feeding should be discouraged. Parents can be advised to place the bottle of expressed breastmilk under warm running water or in a bowl of warm water. Human milk is delivered to the infant at body temperature, leaving little reason to heat breastmilk beyond nature's thermostat. Many full-term babies are perfectly content to drink cold breastmilk right from the refrigerator. If a cream layer has formed at the top of the bottle, parents can gently swirl the milk or gently stir it to distribute the fat layer more evenly throughout the milk.

Bottle Warmers

Many parents use commercially available bottle warmers to heat milk prior to a feeding. One study showed that electrical-based bottle warmers can heat breastmilk to a temperature exceeding 80°C/176° F, a temperature at which some beneficial human milk properties could disappear (Bransburg-Zabary et al., 2015). This study demonstrated that larger milk portions could be overheated (above 40°C/104° F), that it was difficult to determine when the milk reached the desired temperature, and that the bottle warmer created heat zone islets with high temperatures compared with other cooler areas within the bottle. Even warming the milk just to 37°C brings the fat to its melting point This facilitates changes from solid fat, which is present at 4°C refrigerator temperature, to liquid or oil fat. Oil fat appears to adhere to the side of the container at 37°C/98.6°F more than it does at 4°C, therefore lowering the fat content of the milk (Eglash et al., 2017). Overheating during the warming process can cause denaturation and inactivation of some of breastmilk's bioactive proteins and decrease fat content (Eglash et al., 2017). While the breastmilk can be overheated so too can the nipple. Parents should be cautioned that some of the bottle warmers on the market have a high potential for erratic performance, may overheat the bottle such that it needs to be cooled for several minutes before feeding the infant, can burn parents' fingers, and can reach such high temperatures that the plastic bottle partially melts and becomes deformed. Bottle warmers have a history of being recalled for overheating, melting, smoking, and catching on fire (U.S. Consumer Product Safety Commission, 2016). Using a bottle warmer is often thought to be a quicker solution to warming bottles, but by the time the device

is prepared, preheated (if necessary), and the heating time and cooling times are added, this may not warm a bottle any quicker than running it under tap water. The potential for altering human milk properties should be considered, especially if the infant is receiving exclusively pumped breastmilk or is preterm.

Fractionation

Breastmilk can be separated or fractionated to provide skim milk for conditions such as chylothorax or to provide extra calories through use of the high-fat portion of the milk for infants who need to gain weight. Mothers can separate their milk to deliver milk with a higher fat and energy content to help infants gain weight. This process involves pumping the breasts for about two minutes after the milk starts flowing steadily, the pump is turned off and this milk is placed into a separate container. This first milk is generally termed foremilk and is lower in fat. The mother then resumes pumping until the milk flow stops and pumps about two minutes more. This milk is often called hindmilk and its higher calorie count can be used for infants who may need more calories for growth. A creamatocrit can be performed using a creamatocrit analyzer to determine the fat and calorie content of the milk. Mothers can also skim off the cream portion of breastmilk that rises to the top of a container of refrigerated milk.

A refrigerated centrifuge can be used to separate the lipid portion of the milk when preparing fat-free breastmilk for use in infants with chylothorax. The elimination of long-chain fatty acids from the diet of the infant is part of the nutritional treatment for this condition. Alternately, expressed breastmilk can be refrigerated and left undisturbed for 8 to 12 hours, allowing the fat portion to rise to the top of the container. Drewniak & Fenton (2013) analyzed 31 human milk samples that were separated into fatty and low-fat milk layers using three methods: 24-hour refrigerator storage (2°C) followed by using either a spoon or syringe/syphon to extract the low-fat portion of the milk, milk centrifuged at 3,000 rpm for 15 minutes at room temperature and milk spun in the refrigerated-centrifuge at 3,000 rpm for 15 minutes at 2°C. Cold centrifugation milk processing and skimmed milk syringe extraction methods achieved the best rate of fat reduction.

Pasteurization

The nutritional and therapeutic properties of human milk are an indispensable part of an infant's start in life, especially for preterm and ill infants. Initiating lactation in preterm mothers and maintaining the mother's milk supply for NICU infants remains challenging. Donor milk has become the preferred way of feeding newborns who cannot receive their mothers' own milk. Microbiological safety is of concern when using pooled donor human milk to feed these fragile infants. Pasteurization of donor human milk is designed to eliminate bacterial pathogens as well as viruses such as cytomegalovirus (CMV), hepatitis B virus (HBV), human T lymphotropic retrovirus (HTLV I and II), as well as HIV 1, 2; rubella virus; and herpes virus (HCV). Holder pasteurization eliminates all bacteria except *B. cereus*. Milk banks belonging to the Human Milk Banking Association of North America use this method to pasteurize breastmilk and screen for and discard any batches that are positive for *B. cereus* postprocessing. There are several types of pasteurization processes (Wesolowska et al., 2019) that affect various nutrients and bioactive factors in the treated milk to a greater or lesser extent:

- Low-temperature long-time (LTLT) pasteurization, also known as Holder pasteurization, is commonly used by human milk banks for breastmilk

pasteurization. Milk is incubated for 30 minutes at 62.5°C/144.5°F in a water bath or other device that ensures effective heating and followed by rapid cooling. There is a significant variability in the data reported by the scientific literature relative to the effects of Holder pasteurization on human milk components. Macronutrients remain relatively intact, but various beneficial components can be compromised or destroyed completely (Peila et al., 2016).

- Energy and protein content are relatively not affected.
- IgA is reduced in both mature milk and colostrum; IgM is almost completely degraded; IgG1 is not affected but IgG 4 is reduced and IgG2 and 3 were undetectable.
- Lactoferrin can be reduced by 35– 90%.
- Lysozyme concentration can be reduced by 20–80%.
- Lipoprotein lipase and bile salt dependent lipase are almost completely degraded while amylase retains partial activity.
- Cytokines are variously affected with some not significantly affected and others such as macrophage inflammatory protein-1β (MIP-1β), IL10, IL1β, IFN-γ, IL6, and TNF-α showing decreased activity.
- Some growth factors are decreased such as hepatocyte GF (HGF), insulin-like GF (IGF)-1 and 2, as well as IGF binding proteins 2 and 3.
- The hormones insulin, adiponectin, and erythropoietin concentrations are significantly decreased.
- There is a higher retention of fat-soluble vitamins A, D, and E, and more heat sensitivity of the water-soluble vitamins, especially vitamin C.
- Lactose and oligosaccharides appear stable after pasteurization.

- High-temperature short-time (HTST) pasteurization or flash heat pasteurization heats breastmilk to 72°C/161.6°F for 5–16 seconds and seems to be more effective than the Holder method in eliminating bacteria and viruses with lipid envelopes (HIV, HTLV), as well as model viruses for HCV and hepatitis B virus that cannot be otherwise deactivated (Orloff et al., 1993). Flash pasteurization is often used in countries with a high risk of HIV infection and limited access to Holder pasteurization equipment. It was adopted as a simple, universally accessible method and does not require any sophisticated equipment. Escuder-Vieco et al. (2018) reported that HTST processing at 72°C for at least 10 seconds efficiently destroyed all vegetative forms of microorganisms present initially in raw donor milk, although sporulated *Bacillus sp.* survived this treatment. Alkaline phosphatase was completely destroyed after HTST processing at 72°C and 75°C, but γ-glutamil transpeptidase showed higher thermoresistance. Furosine concentrations in HTST-treated donor milk were lower than after Holder pasteurization, and lactulose content for HTST-treated donor milk was below the detection limit of analytical method (10 mg/L). The authors concluded that HTST pasteurization could achieve the microbiological safety objectives established in a milk bank while having a lower impact on heat damage to the milk.
- High hydrostatic pressure pasteurization inactivates pathogenic microorganisms by applying hydrostatic high pressure (usually 400–800 MPa) during short-term treatments (< 5–10 minutes). The 200 + 400 MPa variant of high-pressure pasteurization was reported to be the best option of high pressure to preserve several metabolic hormones and immunocomponents of human milk such as leptin, adiponectin, insulin, growth factor, IgG, and lactoferrin to a much greater extent than Holder pasteurization (Wesolowska et al., 2018). High-pressure pasteurization better preserved the activity of bile salt stimulated lipase and free fatty acids compared with

Holder pasteurization. All variants of high-pressure processing slightly changed the β-carotene and lycopene content (it was increased), whereas lutein + zeaxanthin content was decreased by 40.0% (at 600 MPa, 200 + 600 MPa, 450 MPa) up to 60.2% (at 100 + 600 MPa) compared with a 15.8% decrease in the Holder pasteurized samples (Wesolowska et al., 2019). Exposure to pressures below 600 MPa has not been found to influence the content or composition of the lipid fraction of HM. However, increasing pressure above this limit might result in undesirable changes in the content of selected fatty acids in human milk (Moro et al., 2019).

- Ultraviolet radiation (UV) irradiation (200–280 nm wavelength) is classified as a nonthermal disinfection method. UV-C has a high germicidal effect, between 250 and 270 nm, and is capable of destroying bacteria, viruses, protozoa, yeasts, molds, and algae, although it has a low penetration capacity that limits its use to liquid foods and flat surfaces. UV light cannot penetrate milk or other cloudy foods. Therefore, these substances must be presented to the irradiation system as a thin layer, decreasing the practicality of this process when large volumes of donor human milk are being processed by human milk banks (Moro et al., 2019).
- Ultrasonic processing (20–100 kHz) is an emerging technology for the preservation of foods through the induction of inertial cavitation. Inertial cavitation results in the formation of microscopic bubbles, which rapidly collapse and produce shock waves and localized heating that disrupt the cellular membranes of bacteria. One study looked at different ultrasound settings on the elimination of *Escherichia coli* and the retention of BSSL activity. The findings report that the viability of *E. coli* could be reduced by $\log_{10}$ 5 with a minimal loss of activity of BSSL by applying 13·8 kJ of energy in 12 ml of human milk using high ultrasound power over a short exposure time to ensure that the temperature remains below the critical level for protein denaturation (Christen et al., 2012). Improvement in microbial inactivation can be achieved when ultrasound is combined with mild heating, referred to as thermoultrasonic processing. Czank et al. (2010) evaluated the effect of ultrasonic and thermoultrasonic processing on artificially contaminated human milk samples using an ultrasonic cell disruptor that produced acoustic waves of 150 W peak power mounted onto a precision water bath, which was heated at 45°C and 50°C. The thermoultrasonic processing was considerably more effective than ultrasounds alone against inoculated *S. epidermidis* and *E. coli*. Lysozyme retention after the ultrasonic processing was approximately 65%, and lower after thermoultrasonic processing. Lipase activity was sensitive to both treatments, with a 30% activity being retained in the mildest applied conditions.
- Air and water pasteurizers are used less commonly, have little published research, and do not produce the same temperature patterns as seen in Holder pasteurization. Air-based heat pasteurization caused bottles to reach target temperatures at different times compared with water-based heating, which exposed milk bottles to lower heat but still often higher than Holder pasteurization (Buffin et al., 2017).
- Retort processing involves heating low acid foods in hermetically sealed containers to extend their shelf life, a process commonly used in the canning industry. Pooled milk is heated to 121°C/249.8°F for 5 minutes at 15 pounds per square inch above atmospheric pressure resulting in a product that could be shelf stable for several years without the need for refrigeration. Meredith-Dennis et al. (2018) compared the macronutrient content (protein, carbohydrate, fat, energy), immune-protective protein, and HMO

content of human milk between Holder pasteurized breastmilk and retort sterilization. All of the following nutrients and bioactive factors were significantly lower in milk subjected to retort processing compared with Holder pasteurization: total protein concentration, fat and energy, carbohydrate, IgA, IgG, IgM, lactoferrin, α-lactalbumin, α-1-antitrypsin,, casein, lysozyme, and tested HMOs. Using retort-processed human milk could be problematic, especially for preterm or ill infants or if this milk were the only source of nutrition for an infant. Lima et al. (2017) reported that human milk processed via Holder pasteurization retains more sIgA activity and lysozyme activity (87% and 54%, respectively) than retort-processed shelf-stable human milk (11% and 0%, respectively). Retort processing of donor human milk results in a commercially sterile product, including the elimination of *B. cereus*, but there is a significant loss of nutrients and bioactive factors in achieving commercial sterility. **Table 2-6** summarizes the advantages and disadvantages of several breastmilk pasteurization methods.

Table 2-6 Advantages and Disadvantages of Several Breastmilk Pasteurization Methods

Processing Technique	Advantages	Disadvantages
Low-Temperature Long-Time Pasteurization (LTLT), known as Holder Pasteurization (HoP)	■ Best known methodology ■ Recommended in all international guidelines for the constitution of Human Milk Banks ■ Well-established antimicrobial and antiviral activity ■ Retention of many beneficial and protective effects of human milk	■ Reduction/disruption of important nutritional and immunological factors of human milk ■ Ineffective against bacterial spores (*Bacillus cereus*) ■ Need of regular requalification of the pasteuizer
High-Temperature Short-Time Pasteurization (HTST Pasteurization)	■ Utilized dairy industry since 1930s ■ Less thermal stress (processing time in seconds and not in minutes) ■ Better retention of sIgA and lipase activity in comparison to HoP ■ Smaller loss in antioxidant potential than HoP	■ Prototypes have been used for comparative studies ■ No device available on the market today ■ Ineffective against bacterial spores (*Bacillus cereus*)
High Pressure Processing (HPP)	■ No thermal stress (processing at low temperature) ■ Better retention of some important biological components (lipase, lysozyme, lactoferrin, IgA) in comparison to HoP ■ Higher microbial safety	■ Antiviral activity needs a more deep evaluation ■ Investment and operating costs are significantly higher than a conventional pasteurizer ■ Scaling down of the equipment represents a practical problem ■ Dimensions and weight of the apparatus make difficult the placing in human milk banks
Ultraviolet-C irradiation (UV irradiation)	■ Emerging food preservation technique that retains higher quantities of bioactive components ■ Better retention of IgA in comparison to HoP ■ Effective on elimination of *Bacillus cereus* spores	■ Application of UV-C technology is difficult in human milk ■ Only few preliminary reports are available ■ Antiviral activity has to be evaluated ■ Lack of appropriate equipment in a human milk bank setting

Moro, G. E., Billeaud, C., Rachel, B., Calvo, J., Cavallarin, L., Christen, L.,... & Picaud, J-C. (2019). Processing of donor human milk: update and recommendations from the European Milk Bank Association (EMBA). *Frontiers in Pediatrics, 7*, 49. doi: 10.3389/fped.2019.00049

Further research is needed on alternate pasteurization technologies compared with Holder pasteurization. Limited microbiological data is available on nonthermal technologies relative to their efficacy for use with donor milk, especially considering the strict human milk banking requirements of no bacterial growth after pasteurization (Peila et al., 2017).

Storage Containers

If healthy term infants are obtaining most of their milk directly from the breast, the type of storage container used for occasional feedings of expressed breastmilk is probably not clinically significant. However, human milk is a living fluid and remains biologically active during storage. All commonly used storage containers can affect the composition of the milk to a greater or lesser extent. Storage containers take on more significance if the milk will be used with preterm or ill infants, with infants who receive a substantial portion of their feedings as expressed milk, or who receive only expressed breastmilk.

Johnson et al. (2019) analyzed breastmilk protein, fat, and carbohydrate content after being frozen in a standard freezer (–20°F) for 30, 60, and 180 days in milk storage containers of five commonly used materials—low-density polyethylene bags (Pump & Save breastmilk bags, Medela, McHenry, Illinois), polypropylene hard plastic (VoluFeed, Abbott, Lake Forest, Illinois), food-grade stainless steel (Kiki, Pura Stainless, Santa Barbara, California), food-grade silicone (BabyPods, Mastrad Inc., Paris, France), and borosilicate glass (Avent Natural baby bottle, Philips, Sudbury, United Kingdom). Results showed that in frozen breastmilk stored between 0–180 days, silicone retained significantly more protein than low-density polyethylene bags ($p = 0.001$) and more total calories (kcal/oz) than polypropylene containers and low-density polyethylene bags ($p = 0.046$ and 0.013, respectively). The authors calculated that a premature infant would receive on average, 0.2 g per dL less protein if low-density polyethylene bags were utilized for milk storage. That is, a preterm infant with a minimum intake of 150ml/kg/day would receive approximately 0.3g/kg less protein per day (10% less than the recommended protein intake). Such a loss of protein may represent a situation that preterm infants can ill afford. If low-density polyethylene bags are used for storing breastmilk for term or preterm infants, the milk should be used in less than 60 days to avoid high protein losses. Fat loss has been reported to be up to 9% (polyethylene bag with a polyester outer layer) in some containers (Chang et al., 2012). This fat loss was approximately 2.7 kcal/dL, which accounts for 4% of the total calories found in human milk. Fat can be sticky and adhere to more flexible plastic such as polyethylene bags. Parents may complain that it is difficult to get the fat off the sides of the bottle or bag. The bottle or bag can be gently swirled under warm running water or placed in a bowl of warm water and swirled to partially liquify the fat, reducing its adherence. If a significant amount of fat still remains adhering to the bottle or bag, some parents have used a small silicone spatula to scrape the sides of the bottle or turned the storage bag inside out and scraped the sides of the bag to return as much fat as possible to the feeding container.

The bactericidal activity of breastmilk can be affected by the type of storage container. Takci et al. (2013) reported that bactericidal activity of breastmilk against *E. coli* was significantly reduced at 24 and 48 hours of refrigeration in polyethylene bags compared to glass bottles. In an older study, Goldblum et al. (1981) showed that lysozyme and lactoferrin concentrations were decreased in Pyrex glass bottles and polypropylene bags but not in polyethylene bags during 24 hours of refrigeration. Neutrophils, macrophage, and lymphocyte numbers

were decreased in all containers, but cell count increased following 24 hours of refrigeration. The increase in cell count was significantly higher in the glass bottles, indicating that these cells adhered to the walls of the container during the early hours of refrigeration but later released over a longer period of time. Also found was that secretory IgA and its antibodies to *E. coli* antigens were significantly decreased in milk that had been stored in polyethylene bags. Glass bottle storage of expressed breastmilk may better preserve the bactericidal activity of human milk against *E. coli* during short-term refrigerator storage. High loses (60%) of secretory IgA has been reported when breastmilk is stored in polyethylene for 48 hours (Garza & Nichols, 1984).

Breastmilk should not be stored in plastic specimen containers, steel containers, any container that is not specified as food grade, plastic sandwich bags, or any plastic container that contains bisphenol A (BPA). The U.S. FDA has banned the sale of BPA-containing bottles and food packaging for infants (FDA, 2014). Parents should avoid using any plastics with a recycle code of 3 or 7 or with PC imprinted on them. Parents and clinicians should also be aware that many BPA-free plastic products still contain multiple chemicals with estrogenic activity that can leach into their contents. Leaching is accelerated if the product is exposed to common-use stressors such as UV in sunlight, microwave radiation, or moist heat from boiling or dishwashing. Many plastic products have numerous plastic parts. Baby bottles can have 3–10 different plastic parts in various combinations (bottle, nipple, anti-colic item, sealing ring, liner bag, cap, etc., with each part having different and unique blends of 5–30 chemicals. A study that assayed more than 100 component parts from more than 20 different baby bottles, including many advertised as BPA-free, indicated that extracts from at least one bottle component of each baby bottle always demonstrated estrogen activity (Yang et al., 2011). Plastic baby bottles may be BPA-free but they are not necessarily free of estrogenic activity, resins, phthalates, solvents, colorants, inks, and other additives that may disrupt cellular processes or act as endocrine-disrupting compounds.

Some mothers who are mostly or exclusively pumping use the pitcher method of storing pumped breastmilk. To make more room in the refrigerator or to avoid having to use a large number of bottles, all the milk that is pumped by the end of the day is stored in a covered pitcher or large container and then decanted into the next day's feeding bottles. Any freshly expressed milk is cooled first before adding it to the larger container. This method involves multiple exposures of the stored milk to potential contaminants, light exposure every time the refrigerator door is opened, several transfers of milk to various containers, and the possible loss of nutrients or anti-infective properties depending on the type of container used. This method may be acceptable for normal healthy full-term infants but if milk is being collected for preterm or ill infants, milk from each pumping session should remain in the container into which is was expressed and use the sterile containers provided by the NICU.

Glass or hard plastic containers seem preferable for storing human milk (Jones, 2019). Some mothers use mason jars or jelly jars that have been carefully cleaned and have solid tight- fitting lids. Parents can use glass baby bottles that are made with pharmaceutical-grade borosilicate glass that is heat- and thermal shock–resistant. These bottles can go from the refrigerator to a container of warm water and can also be sterilized in boiling water. Many glass baby bottles are compatible with breast pumps. Glass containers should be regularly checked for any cracks or chips and discarded if any are found. These containers can be covered with items such as baby bottle sleeves that cover a good portion of the bottle to reduce photodegradation of light-sensitive milk components.

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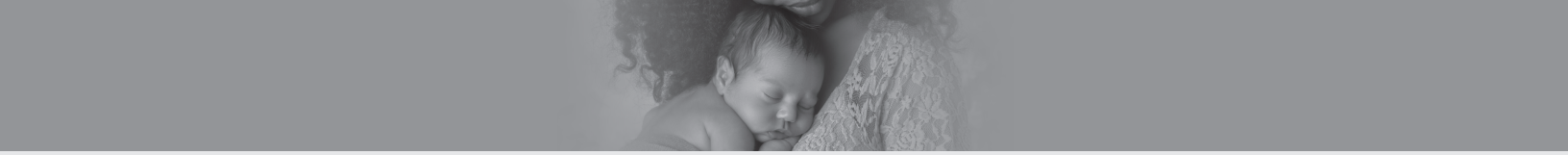

Appendix 2-1

Summary of Interventions Based on the Biospecificity of Human Milk

1. Help parents understand that infant formula and human milk are not equivalent. The addition of multiple ingredients into infant formula derived from non-human sources cannot duplicate the health, cognitive, and developmental outcomes seen in infants fed human milk, no matter what formula advertising might claim. If parents want or need to use infant formula, the decision should be made in conjunction with the infant's primary healthcare provider and the formula used should be one that has been cleared by the U.S. Food and Drug Administration.
2. Mothers should be discouraged from smoking or vaping as smoking depletes colostrum and mature milk of their antioxidant capacity.
3. Given the potential stressors on the composition of colostrum, it would seem prudent to assure maximum intake of colostrum for infants who are born by cesarean section, who experienced a difficult or precarious delivery, whose mothers smoke, whose mothers are diabetic, or who were born preterm.
4. The infant gut microbiome is shaped and educated by the bacteria in breastmilk. Infant formula ingestion should be avoided if possible, especially during the early days and weeks post-birth, as formula supplementation can alter this process and increase the susceptibility to infectious disease and autoimmune diseases and conditions such as obesity, asthma, diabetes, and allergies.
5. Breastfed infants do not require additional water, glucose water, or fluoridated water supplements.
6. Vitamin C in breastmilk can be depleted by refrigeration, freezing, heat treatment, maternal smoking, feeding bottles with large milk-to-air surfaces, and exposure to light. Better preservation of vitamin C can be achieved by minimizing refrigeration and freezing times, avoiding heating breastmilk in a microwave or bottle warmer, not shaking containers of breastmilk, covering containers used to express, store, and feed breastmilk to reduce photodegradation, and helping mothers to avoid smoking. Some infants may need vitamin C supplements if consuming only expressed breastmilk, especially if it has been pasteurized and frozen.

7. A lactating mother consuming a diet without meat or dairy products may have breastmilk deficient in vitamin B_{12} unless other B_{12} food or supplement sources are provided. Mothers, their milk, and infants can be tested for B_{12} status and treatment instituted, if necessary, with oral or intramuscular vitamin B_{12}.
8. Mothers deficient in vitamin D may also produce milk with vitamin D deficiency. Supplementing the mother with 6,400 IU/day of vitamin D can optimally elevate both the mother's and infant's vitamin D levels.
9. A suite of bioactive components is present in breastmilk and are not found in infant formula. Provision of human milk to all infants, especially preterm, ill, or compromised infants is critical for optimal health and developmental outcomes.
10. The temporal variation in human milk composition is important in establishing circadian rhythms in infants. Mothers and other care providers can be advised to label expressed milk with the time of day that it was expressed, and select the stored milk that best corresponds with the current time that the infant is being fed.
11. Stored breastmilk can develop off odors and tastes due to lipolysis (fat breakdown), which increases with frozen-storage duration. Providing scalded milk as the total nutrition source for an infant may be problematic due to loss of some nutrients and bioactive components. Mothers can be advised to mix fresh breastmilk half and half with the stored milk to see if the infant accepts the mixture before being advised to scald the milk.
12. Breastmilk should not be stored in plastic specimen containers, steel containers, any container that is not specified as food grade, plastic sandwich bags, or any plastic container that contains bisphenol A (BPA). Glass or hard plastic containers seem preferable for storing human milk.