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IMPACT OF HOLDER PASTEURIZATION ON THE NUTRITIONAL AND FUNCTIONAL INTEGRITY OF HUMAN MILK PROTEINS AND THE QUALITY OF INFANT FORMULA

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Abstract

Breastfeeding is widely recognized as the optimal method of infant feeding, providing essential nutrients and bioactive compounds that support growth, development, and immune function. Human breast milk is rich in proteins, lipids, carbohydrates, and minerals, which vary throughout lactation to meet the infant's changing needs. Breastfeeding is widely recommended, but when it's not feasible or sufficient, donor human milk (DHM) offers a viable alternative with similar benefits. Collected and pasteurized by milk banks for safety, DHM retains many nutritional properties, though pasteurization may affect its protein content and bioactivity. Therefore, this thesis investigates several literatures about the effects of Holder pasteurization on the composition and bioactivity of human milk, with a particular focus on its protein content. The primary aim of this study is to analyse how Holder pasteurization affects the proteome of human milk, comparing the activity of bioactive proteins before and after pasteurization. The research highlights the significant role of proteins, particularly whey and casein, in infant health, as they contribute to immune defence, tissue repair, and metabolic regulation. The thesis also evaluates the nutritional composition of bovine infant formula, identifying differences and similarities in protein composition and activity between pasteurized human milk and infant formula. The findings indicate that while pasteurization effectively reduces microbial contamination, it can also lead to the denaturation of proteins, compromising their nutritional and functional properties. This degradation may diminish the immune-protective functions of key proteins such as immunoglobulins and lactoferrin, which are critical for infant health. The thesis is structured into five chapters: an introduction to the significance of human milk, an exploration of its composition, an evaluation of the impact of Holder pasteurization, a comparison of human milk and infant formula, and a conclusion that outlines future research directions. This work aims to contribute to the optimization of feeding practices for infants relying on DHM or formula milk, emphasizing the need for improved processing methods that preserve the nutritional integrity of human milk while ensuring safety. Ultimately, this thesis underscores the importance of understanding the effects of pasteurization on human milk to enhance infant nutrition and health outcomes. The insights gained from this study can inform best practices in donor milk processing and the formulation of infant formulas, ensuring that infants receive the highest quality nutrition during their critical early developmental stage.

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Chapter 1
Introduction

1.1 Background studies

Breastfeeding has long been acknowledged as the optimal mode of infant feeding, providing numerous health benefits to infants. Breastfeeding for the first six months of life is universally recognized as the widely accepted standard of infant feeding (WHO, 2003). Human milk (HM) contains a variety of nutrients, including proteins (8–11 g/L), lipids (35–48 g/L), carbohydrates (70–85 g/L), and mineral elements (0.9–1.2 g/L), which play critical roles in infant growth, development, and immune function (Pereira, 2014; Victora et al., 2016). HM also stands as an unparalleled source of nutrition, providing a dynamic range of bioactive compounds, immunological factors, and essential nutrients crucial for optimal growth and development. These macronutrients continuously change in composition and concentration throughout lactation to meet the infant's demanding growth and developmental needs (Zhu & Dingess, 2019).

The nutraceutical and functional properties of proteins, a key component of milk, have been proven to improve human health. Proteins play a central role in supporting various physiological functions, including immune defense, tissue repair, nutrient sequestration, modulation of intestinal microflora and metabolic regulation (Haschke et al., 2016; Newburg & Walker, 2007). These proteins can be broadly categorized into whey and casein fractions, with whey proteins being more abundant in human milk accounting for 60–80% of total protein (Liao et al., 2011). The caseins (CN) of HM consist of αS1-casein (αS1-CN), β-casein (β-CN), and κ-casein (κ-CN), while the whey proteins are made up of hundreds of different proteins covering many different biological functions. The most abundant whey proteins in HM are α-lactalbumin, lactotransferrin, osteopontin, and a range of immune-related proteins like secretory immunoglobulin A (sIgA) (Donovan, 2019; Liao et al., 2017; L. Zhang et al., 2016). Other milk proteins, including lactoperoxidase and lysozyme have significant antibacterial and antiviral characteristics and participate in host immunity (Zhu & Dingess, 2019). Beyond providing essential amino acids for growth, these proteins also contain bioactive peptides that exert non-nutritional functions, further enhancing their significance in infant health (Lönnerdal, 2010). A multitude of factors, including maternal diet, lactation stage, and post-collection processing methods such as pasteurization can influence the composition and bioactivity of these proteins.

Lipids, another essential component of HM, are vital for infant growth and development. While long-chain fatty acids (FAs) originate from either the maternal diet or adipose tissue metabolism, short- and medium-chain FAs are synthesized de novo within the mammary gland (Hachey et al., 1989). In infants, the consumption of lipids from human milk and infant formulas provides approximately 45–55% of the total energy intake. Triacylglycerols (TAGs) comprise 98 wt% of the total lipid fraction of milk, and the digestion of long-chain TAGs and subsequent absorption of long-chain polyunsaturated

fatty acids (LC-PUFAs) are essential for the development of the brain and central nervous system in infants (Bläckberg et al., 1987; Hernell & Bläckberg, 1994). Furthermore, digested lipids can self-assemble into colloidal structures (Salentinig et al., 2011) and assist in the delivery of lipophilic nutrients to the systemic circulation.

Carbohydrates are another vital component of human milk. The two main carbohydrates in human milk are the disaccharide lactose and human milk oligosaccharides (HMOs). Lactose, the most abundant sugar in human milk, serves as a primary source of energy for infants and facilitates the absorption of calcium and other minerals in the infant's intestine. HMOs, on the other hand, are complex carbohydrates formed by the elongation of lactose with monosaccharides such as galactose, N-acetylglucosamine, fucose, and sialic acid (Binte Abu Bakar et al., 2021). Due to their structural similarity to the carbohydrate portions of glycoproteins and glycolipids on epithelial cell membranes, HMOs can inhibit the binding of pathogenic microorganisms to the epithelial cell surfaces in infants, thereby playing a crucial role in infection prevention (Bode, 2006). Additionally, HMOs act as prebiotics, selectively promoting the growth of beneficial gut bacteria such as *Bifidobacteria* and *Lactobacilli*, and have immunomodulatory properties that help protect infants against infections such as necrotizing enterocolitis. Significantly, despite the structural and functional differences between lactose and HMOs, pasteurization does not affect the content of lactose or the bioactivity and composition of HMOs, ensuring that pasteurized donor human milk retains its critical carbohydrate-related benefits (Bertino et al., 2008; Li et al., 2017).

While breastfeeding is widely advocated as the best feeding practice for infants, there are instances where it may not be feasible or sufficient to meet the infant's nutritional needs. In such cases, donor human milk (DHM) serves as a viable alternative, offering many of the same benefits as maternal breast milk. DHM, collected and processed by milk banks, undergoes pasteurization to ensure microbial safety while retaining its nutritional and functional properties to the extent possible (Fugate et al., 2015). However, pasteurization, the most common method used to ensure the safety of DHM, may have implications for the protein content and bioactivity of human milk. Thermal processing, such as Holder pasteurization, involves heating DHM to a specific temperature for a set duration to eliminate harmful bacteria. While effective in reducing microbial contamination, pasteurization can also lead to denaturation or degradation of proteins, potentially compromising their nutritional and functional properties (Moro et al., 2019). Also infant formula is used as another substitute for human milk. Bovine milk has been extensively utilized as a substrate for infant formula despite its distinctive nutritional composition compared to human milk. Notably, the protein content ranges from 32 to 34 g/L, lipids from 33 to 47 g/L, carbohydrates from 34 to 54 g/L, and mineral elements from 4.0 to 4.6 g/L (Pereira, 2014).

In recent years, there has been a growing interest in understanding the impact of pasteurization on the nutritional and immunological properties of human breast milk. While pasteurization effectively reduces the risk of microbial contamination, questions persist regarding its potential effects on protein content, structure, and bioavailability. Such concerns are of particular significance given the critical role of proteins in supporting infant health and development during the early stages of life. Understanding the impact of pasteurization on the protein content and bioactivity of human breast milk is essential for evaluating the efficacy of DHM as a substitute for maternal milk. Comparative analysis between pasteurized and non-pasteurized human breast milk can provide valuable insights into the extent of protein degradation or alteration caused by thermal processing. Such analysis can also inform strategies to optimize the processing methods used for DHM, to preserve protein integrity and bioactivity while ensuring microbial safety.

1.2 Aim and objective

The aim of this thesis is to review the effects of Holder pasteurization on the proteome of human milk, comparing the activity of bioactive proteins before and after pasteurization. It also seeks to review the analysis of proteome in infant formula derived from bovine milk, and to highlight differences and similarities in protein composition and activity between pasteurized human milk and infant formula. By examining these aspects, this review study aims to provide insights into how pasteurization affects the nutritional and functional properties of human milk proteins and how these properties compare to those in infant formula. Ultimately, this review work will contribute to the optimization of feeding practices for infants who rely on donor milk or formula. To achieve this aim, the objectives of this thesis are:

i. To explore the composition of human milk;
ii. To review the impact of Holder pasteurization on human milk proteins;
iii. To compare the proteome of human milk with that of infant formula.

1.3 Thesis structure

The thesis is organized into five chapters, including this introduction chapter. Chapter 2 provides an exploration of the composition of human milk by compiling existing literature, focusing on the variety of proteins, carbohydrates, lipids, and other bioactive compounds. Chapter 3 evaluates the impact of Holder pasteurization on human milk proteins. It describes the Holder pasteurization process, including the temperatures and durations used, and explains the mechanisms by which Holder pasteurization affects the components of human milk, particularly proteins. Furthermore, it analyzes studies that investigate the changes in protein composition and structure resulting from Holder pasteurization and reviews the evidence on how pasteurization affects the bioactivity of key proteins, such as immunoglobulins, lactoferrin, and enzymes. Chapter 4 compares the proteome of human milk with that of infant formula. It reviews studies on the protein composition of infant formula derived from bovine milk, comparing and contrasting the proteomic profiles and bioactive properties of infant

formula and human milk. Additionally, it discusses how these changes may affect recommendations for donor milk processing and the formulation of infant formula. Chapter 5 concludes the thesis and suggests directions for future research to address existing gaps and improve the understanding of Holder pasteurization's impact on human milk.

Chapter 2
Exploring the composition of human milk

2.1 Nutritional composition of human milk

The primary macronutrients in human milk (HM) are carbohydrates, proteins, and fats, excluding water, which comprises about 87-88% (Ballard & Morrow, 2013; Kim & Yi, 2020). These macronutrients provide essential nutritional support for infant growth and development, supplying approximately 65-70 kcal of energy per 100 mL. As shown in Table 2.1, the composition of HM varies significantly from that of bovine milk, particularly in terms of protein content. The components of HM change dynamically according to lactation periods and nursing sessions within a single feeding, adapting to the varying needs of the infant (Martin et al., 2016). Colostrum, the first form of milk produced by the mammary glands is low in fat but high in protein (10%) and is relatively rich in immune-protective components, such as immunoglobulin A (IgA) and lactoferrin, which help prevent neonatal infections (Kim & Yi, 2020). During each nursing session, the milk that is expressed first (foremilk) is thinner with a higher content of lactose, which satisfies a baby's thirst, and following the foremilk, hindmilk is creamier with a much higher content of fat for the baby's needs. Variations are also present with the stage of nursing (age of infant), maternal diet, maternal health, and environmental exposure (Martin et al., 2016). In addition, variation also depends on the processing conditions, such as storage, pasteurization, and containers (Y.-C. Chang et al., 2012; M. H. Kim et al., 2019).

Table 2-1. Composition of human and bovine milk (%) (Guo, 2020)

	Human milk	**Bovine milk**
Protein	1.00	3.40
Casein: Whey protein	30:70	80:20
Fat	3.80	3.50
Lactose	7.00	5.00
Total solids	12.40	12.50
Ash	0.20	0.70

2.2 Carbohydrates

Carbohydrates, which comprise about 7% (60-70 g/L) of HM, account for 40% of the total calorie reserve. Carbohydrates in human milk include disaccharides, oligosaccharides and more complex saccharides such as glycoproteins (Eriksen et al., 2018; Mosca & Giannì, 2017). Lactose, the main carbohydrate in HM, is formed by the combination of galactose and glucose, as illustrated in Figure 2.1. This disaccharide is decomposed and absorbed in the form of monosaccharides by the enzyme lactase. Lactose is present in higher concentrations in HM than in the milk of other species, reflecting the high energy demands of the human brain and showing positive associations with infant weight gain

(Andreas et al., 2015; Eriksen et al., 2018; Mosca & Giannì, 2017). Insufficient lactase may cause lactose malabsorption, but it is relatively rare in exclusively breastfed infants (Martin et al., 2016). It is also the major contributor to the osmolality of HM, and its concentration remains constant in mature milk, helping to maintain a consistent osmotic pressure (Hester et al., 2012; Martin et al., 2016). Additionally, lactose aids in the absorption and attachment of bioactive components such as oligosaccharides, minerals, and calcium (Martin et al., 2016). The concentration of lactose in human milk remains relatively stable, ensuring a consistent supply of this vital nutrient regardless of maternal nutrition, including in cases of malnutrition or energy supplementation (Mosca & Giannì, 2017)

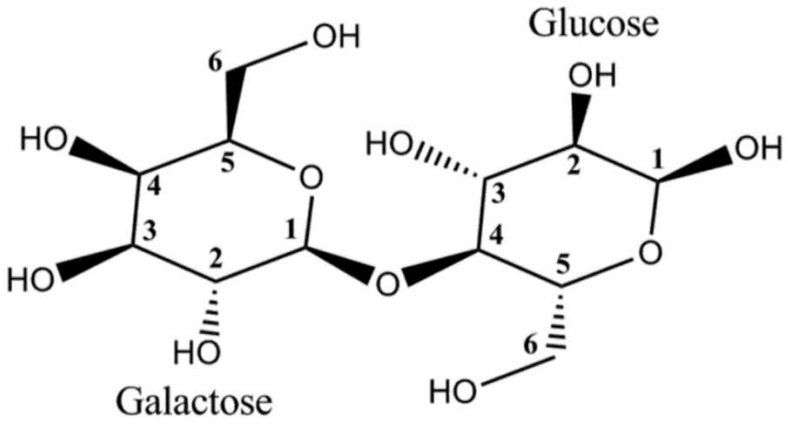

Figure 2-1. Lactose (Guo, 2020)

2.2.1 Human milk oligosaccharides

Human milk oligosaccharides (HMOs) are a crucial component of human milk carbohydrates and represent the third most significant component in breast milk, averaging 12.9 g/L in mature milk and 20.9 g/L at four days postpartum (Coppa et al., 1993). HMOs consist of between 3 to 22 saccharide units per molecule, made up of five different sugars: l-fucose, d-glucose, d-galactose, N-acetylglucosamine, and N-acetylneuraminic acid. Over 200 different types of oligosaccharides have been identified in human milk, all featuring lactose at the reducing end (German et al., 2008).

HMOs function primarily as prebiotics, promoting the growth of beneficial bacteria, such as *Bifidobacterium infantis*, in the infant gastrointestinal tract and protecting the infant from colonization by pathogenic bacteria (Ward et al., 2006). They play a significant role in preventing neonatal diarrheal and respiratory tract infections (Morrow, Ruiz-Palacios, Altaye, Jiang, Guerrero, et al., 2004; Newburg & Walker, 2007). The production of HMOs is genetically determined, with specific profiles resulting from the expression of transferase enzymes in lactocytes.

HMOs also modulate intestinal epithelial cell responses and act as immune modulators. They alter the intestinal environment by reducing cell growth and inducing differentiation and apoptosis

(Kuntz et al., 2009), as well as modulating immune responses, potentially shifting T-cell responses to a balanced Th1/Th2-cytokine production (Eiwegger et al., 2010).

One mechanism by which HMOs protect against gastrointestinal infection is by acting as receptor decoys. Pathogens bind to HMOs due to their structural similarity to cell surface carbohydrates, preventing pathogens from adhering to intestinal epithelial cells. Instead, pathogens bind to HMOs and are expelled harmlessly from the gastrointestinal tract (Andreas et al., 2015). Studies have shown a significant association between specific 2-linked fucosylated oligosaccharides in human milk and reduced rates of *Campylobacter* diarrhea in breastfed infants. Additionally, low concentrations of lacto-N-difucohexaose in milk have been linked to an increased incidence of *calicivirus* diarrhea (Morrow, Ruiz-Palacios, Altaye, Jiang, Lourdes Guerrero, et al., 2004). HMOs also prevent the adherence of pathogens like *S. pneumonia and Escherichia coli*, providing protection against various bacterial and viral infections (B. Andersson et al., 1986; Cravioto et al., 1991). The structural similarity between specific HMO components and pathogen receptors allows for cross-reactivity, enhancing the protective effects of HMOs (Kobata, 2010; Pritchard et al., 1992). Different pathogen receptors have varying affinities for specific carbohydrate structures. This genetic diversity in HMO production influences the types and timing of microbiota colonization in infants, contributing to the establishment and maintenance of a healthy gut microbiome (Lewis et al., 2015).

2.2.2 Functions of human milk carbohydrate

Carbohydrates in human milk serve multiple essential functions. Lactose provides a significant source of energy for breastfed infants and helps maintain the osmotic pressure in milk alongside mineral constituents (Eriksen et al., 2018; Mosca & Giannì, 2017). It plays a crucial role in enhancing the absorption of minerals, particularly calcium, by being converted to lactic acid by intestinal flora, thereby lowering the pH and increasing the solubility of calcium salts. This effect is bolstered by human milk's low buffering capacity and low protein and phosphorus content. Oligosaccharides support the growth of beneficial intestinal flora such as *bifidobacteria* and *lactobacilli*, which ferment lactose to lactic acid and create a favorable low-pH environment. These oligosaccharides also provide a critical energy source for these bacteria, promoting gut health and potentially supporting immune functions (Jenness, 1979; Kim & Yi, 2020). Lactulose, another disaccharide present in human milk, acts as a growth-promoting factor and energy source for beneficial bacteria like *lactobacillus bifidus* and *lactobacillus acidophilus* (Eriksen et al., 2018).

2.3 Proteins

Proteins are large molecules composed of long chains of amino acids linked together by peptide bonds. During digestion, most proteins are broken down into their component amino acids or small peptide fragments, which are then absorbed into the body. The absorbed amino acids not used for energy production (oxidation) serve as the raw materials for synthesizing new proteins within the body's cells.

A comprehensive review of 43 relevant studies confirms that breastmilk protein content consistently declines as lactation progresses, but also highlights the significant variability that exists, especially in the first few months (Lönnerdal et al., 2017). Human milk proteins account for about 1% (8-10 g/L) of HM, with higher content at birth that decreases to 8-10 g/L at 3-4 months and to 7-8 g/L after six months (Kim & Yi, 2020). These changes in the composition of breastmilk protein correlate well with the evolving nutritional needs of the growing infant (Z. Zhang et al., 2013). Despite the gradual decrease in the total amount of protein in breastmilk throughout lactation, the nutritional quality of the protein, as measured by the ratio of essential amino acids to total amino acids, remains consistent over time. HM proteins are composed of a mixture of whey, caseins, which are the main component and other various peptides, providing crucial amino acids indispensable for infant growth and development, as well as bioactive proteins and peptides essential for many functions (Kim & Yi, 2020). The composition of these proteins is detailed in Table 2.2.

Table 2-2. Protein component of human and bovine milk (%) adapted from (Guo, 2020)

	Human milk	**Bovine milk**
Total caseins	0.3 g/100g	2.6 g/100g
αS1-casein	Trace	40
αS2-casein	Trace	8
β-casein	85	38
k-casein	15	12
Micelle size (nm)	50	150
Whey proteins	0.7 g/100g	0.8 g/100g
α-Lactalbumin	26	17
β-lactoglobulin	_	43
Lactoferrin	26	Trace
Serum albumin	10	5
Lysozyme	10	Trace
Immunoglobulins	16 (IgA)	10 (IgG)
Others	12	24

Caseins, present as micelles, forms clots or curds in the stomach, while whey remains in liquid form and is easier to digest. Caseins are vital for providing amino acids as they digests slowly. They have a unique ability to form stable aggregates with calcium and phosphorus, which increases the concentration of these minerals in milk beyond what can be achieved by solubility alone (De Luca et al., 2016; Mosca & Giannì, 2017). Depending on the stage of lactation, whey can constitute 80% to 50% of the protein content in breast milk (Guo, 2020). The primary whey proteins in human milk

include α-lactalbumin, lactoferrin, and secretory IgA (SIgA) (Donovan, 2019; Jenness, 1979). α-lactalbumin is crucial for lactose synthesis and may facilitate the absorption of calcium and zinc, although only a small fraction of calcium in milk is bound to it. Lactoferrin, another major protein, tightly binds iron, thus limiting its availability to potentially pathogenic microorganisms (Donovan, 2016). SIgA plays a crucial role in preventing infections by binding specific antigens in the infant's gastrointestinal tract. Lysozyme, another milk protein, helps protect the infant by lysing bacterial cell walls (Boniglia et al., 2003).

The amino acid profiles of casein and whey proteins differ, causing the whey/casein ratio in human milk to fluctuate between 70/30 and 80/20 in early lactation and decreases to 50/50 in late lactation (Lönnerdal, 2003). This proportion is significantly greater compared to the milk of other mammals, such as bovine's milk, where whey proteins represent only 18% of milk protein. Traditionally, infant formulas have been high in casein, making them harder to digest compared to human breast milk.

Other proteins include folate-binding protein, bifidus factor, lipase, amylase, alpha1-antitrypsin, antichymotrypsin, and haptocorrin (Guo, 2020). After ingestion, these proteins are broken down into free amino acids for absorption and utilization. Glutamine, the most abundant free amino acid in human milk, is nearly 20 times higher in mature milk than in colostrum (Z. Zhang et al., 2013). Glutamine is vital for providing ketoglutaric acid for the citric acid cycle, possibly acting as a neurotransmitter in the brain and serving as a primary energy substrate for intestinal cells (Agostoni, 2000).

The unique protein profile of breastmilk appears to be optimized to support the infant's growth, long-term development, and protection from disease during this critical stage of life. Some of the proteins in breastmilk are absorbed and others have important biological functions beyond just providing amino acids for growth (Haschke et al., 2016). Both readily absorbed nutritive proteins, and non-absorbed bioactive proteins likely contribute to the many health benefits associated with breastfeeding.

The protein intake of breastfed infants born at term has been used as a reference point to estimate the protein needs of infants during their first year of life (Fomon, 1991; Heinig et al., 1993). Researchers have been able to directly measure the true protein content of breastmilk to quantify these levels. However, the true protein intake from breastmilk does not necessarily equate to the number of amino acids that are available for the infant to utilize for building new body proteins. This is because some of the proteins in breastmilk, particularly the bioactive ones, may pass through the infant's digestive system intact and be excreted in the stool without being broken down and absorbed. So, while breastmilk protein levels decline over time, the proportion of bioactive proteins may increase, which could impact the infant's overall protein utilization (Donovan, 1989)

2.3.1 Bioactive Proteins

In addition to the nutritional proteins, breastmilk contains bioactive proteins and peptides that are not absorbed into the infant's bloodstream. Studying the specific functions of these bioactive components can provide insights into why breastfed infants tend to have lower rates of illness, shorter durations of infections (Dewey et al., 1995), and different gut microbiome compositions compared to formula-fed infants (Isolauri, 2012).

2.3.1.1 Caseins

Caseins in human milk contribute to its amino acid composition. A key functional property of caseins is their ability to form stable aggregates that include calcium and phosphorus, allowing these minerals to be present in higher concentrations than what is achievable through mineral solubility alone. In human milk, caseins form micelles that range from 20 to 55 nm in size, which are much smaller than the micelles in bovine milk, which range from 100 to 150 nm in diameter. The concentration of total casein increases throughout lactation, accounting for about 10-20% in the early stages and rising to 40-50% as lactation progresses (Kunz & Lönnerdal, 1992). Initially, upon the onset of lactation, the concentration of whey proteins is very high, while casein is almost undetectable. As lactation progresses, the synthesis of casein in the mammary gland increases, along with the volume of milk produced. Simultaneously, the concentration of whey proteins decreases, partly due to the increased milk volume (Lönnerdal, 2003).

The primary casein in human milk is β-casein, a highly phosphorylated protein constitute up to 25% of the casein content, or approximately 2.7 g/L in mature breast milk (Cuillière et al., 1999). During digestion, β-casein forms phosphopeptides that can enhance calcium absorption by increasing its solubility, contributing to the high bioavailability of calcium in breast milk (Ferraretto et al., 2001). Additionally, clusters of phosphorylated threonine and serine residues near the N-terminal end of β-casein can lead to the formation of complex calcium ions (Lönnerdal, 2003). These casein phosphopeptides may also aid in the absorption of zinc and other divalent cations.

Another key casein in human milk, κ-casein, is highly glycosylated and plays a role in defense against infections. Its concentration averages around 1.25 g/L in colostrum and transition milk, decreasing to about 1 g/L in mature milk (Cuillière et al., 1999). It has been shown to inhibit the adherence of *Helicobacter pylori* to human gastric mucosa, as well as *Streptococcus pneumoniae* and *Haemophilus influenzae* to human respiratory tract epithelial cells (Hamosh, 2001; Strömqvist et al., 1995). Furthermore, κ-casein promotes the growth of *Bifidobacterium bifidum*. This acid-producing anaerobe suppresses the growth of pathogenic microorganisms in the intestines of breastfed infants, due to the presence of its C-terminus proteolysis product (Guo, 2020).

2.3.1.2 Alpha-lactalbumin

α-Lactalbumin is the primary protein in breast milk, comprises 123 amino acids and four disulfide bridges, making up 20-25% of the total protein content, and it plays several vital roles during the neonatal period (Lönnerdal, 2003; Permyakov & Berliner, 2000). In the mammary gland, α-lactalbumin assists in lactose synthesis, which creates an osmotic pull that aids in milk production and secretion. Additionally, α-lactalbumin binds to divalent cations like calcium and zinc, potentially enhancing the absorption of essential minerals. It also provides a well-balanced supply of essential amino acids crucial for infant growth (Lönnerdal, 2003; Wada & Lönnerdal, 2014). Figure 2.2 shows the structure of Ca^{2+}-bound human α-lactalbumin, highlighting its functional aspects. During digestion, α-lactalbumin forms peptides that temporarily exhibit antibacterial and immunostimulatory properties, potentially offering protection against infections (Wada & Lönnerdal, 2014). A discovered variant of multimeric α-lactalbumin, known as the "molten globule state," has shown anti-infective properties and promotes apoptosis, which may influence mucosal cell turnover and proliferation (Lönnerdal, 2003).

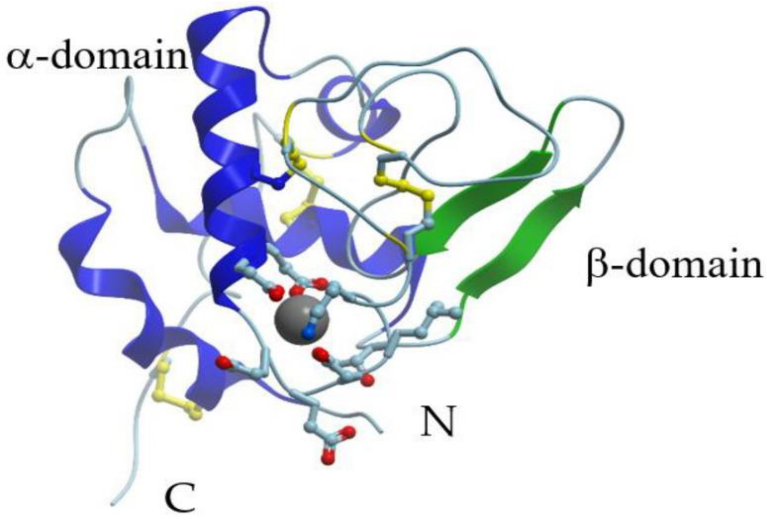

Figure 2-2. X-ray structure of Ca^{2+}-bound human α-lactalbumin. Gray sphere represents bound calcium ion (Permyakov, 2020)

Although Bovine's milk contains α-lactalbumin, it is present in smaller amounts than human milk (2-5% of total protein). α-Lactalbumin undergoes partial digestion in the upper small intestine, forming various peptides that exhibit bioactivity for a time before being fully broken down into amino acids. This slow digestion process allows these transient peptides to perform specific functions within the small intestine. It serves as a rich source of essential amino acids and yields polypeptides with antimicrobial activity, primarily against gram-positive bacteria (Pellegrini, 1999; Wada & Lönnerdal, 2014). Additionally, a folding variant of α-lactalbumin is bactericidal against antibiotic-resistant strains of *Streptococcus pneumoniae* (Håkansson et al., 2000). Due to its antimicrobial properties and other benefits, efforts have been made to enrich breast milk substitutes like infant formula with α-lactalbumin (Lien, 2003).

2.3.1.3 Lactoferrin

Lactoferrin is an 80-kDa iron-binding glycoprotein primarily found in milk and, to a lesser extent, in other exocrine fluids such as bile and tears (Albenzio et al., 2016). Its concentration is highest in colostrum and decreases as lactation progresses. Composed of a single polypeptide chain with two globular lobes represented in figure 2.3, lactoferrin is notably resistant to proteolysis (Baker & Baker, 2009; Lönnerdal & Iyer, 1995). Due to its iron-binding capabilities, it is believed to play a role in iron absorption in the intestinal mucosa, where it is partially broken down, and it also acts as a bacteriostatic agent by sequestering iron from bacteria that require it (Buescher, 2001; Farnaud, 2005). Lactoferrin in neutrophils and its release during inflammation indicate its involvement in immune responses and phagocytic activity (Thai & Gregory, 2020). Additionally, lactoferrin in HM has been shown to enhance the production and release of cytokines such as IL-1, IL-8, tumor necrosis factor α, nitric oxide, and granulocyte-macrophage colony-stimulating factor, which may further influence the immune system (Hernell & Lönnerdal, 2002). It also helps regulate immune responses by inhibiting pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and IL-8, while also suppressing free radical activity (Griffiths et al., 2019; Palmeira & Carneiro-Sampaio, 2016). Beyond its iron-binding properties, lactoferrin may function as a growth factor and bactericidal agent. It has been shown to inhibit microbial adhesion to host cells and exert direct cytotoxic effects against bacteria, viruses and fungi, mainly through the formation of lactoferricin, a potent cationic peptide with bactericidal activity generated during lactoferrin digestion (Griffiths et al., 2019).

Breastfed infants have been reported to absorb iron more efficiently than those fed with bovine's milk-based formula, a benefit likely attributed to the higher levels of lactoferrin in human milk compared to bovine milk (approximately 1 mg/mL versus 10 µg/mL, respectively). Most of the iron in human milk is bound to lactoferrin, and a receptor specific for lactoferrin, with a higher affinity for human lactoferrin than for bovine lactoferrin, has been identified, further enhancing iron absorption (Albenzio et al., 2016; Lönnerdal, 2013).

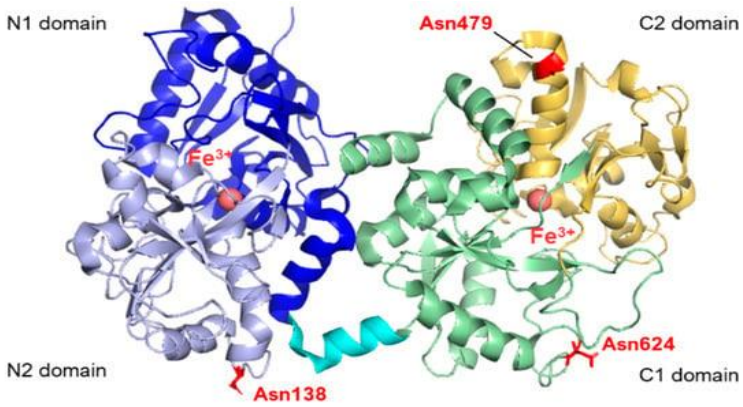

Figure 2-3. The diagram illustrates the structure of human lactoferrin, highlighting its distinct lobes and features. The N-terminal lobes (N1 and N2) are depicted in blue and lavender, while the C-terminal

lobes (C1 and C2) are shown in green and yellow. The connecting α-helix between these domains is colored cyan (Piacentini et al., 2024).

2.3.1.4 Secretory IgA

Mothers' milk is rich in secretory immunoglobulin A (sIgA), particularly during the early stages of lactation. Colostrum, the initial milk produced, varies significantly between mothers but typically contains around 2.0 g/L of sIgA, which decreases to approximately 0.5 g/L in mature milk (Goldman et al., 1982). These antibodies are transmitted in a combined and protected form, remaining inactive until absorbed, which allows them to avoid disrupting beneficial gut bacteria (Lovelady et al., 2003). This process aids in establishing a healthy bacterial flora that suppresses the growth of harmful organisms, thereby enhancing the infant's resistance to infections (Newman, 1995). The absorption of this protein is similar to that of lactoferrin, as it is resistant to proteolytic enzymes in the infant's gut, allowing it to bind effectively to bacterial and viral antigens and thereby inhibiting their attachment to the mucosal lining. This makes it partially excreted intact and partially broken into bioactive peptides (Lönnerdal, 2013). sIgA is abundantly present in the intestinal mucosa of humans and other mammals, playing a crucial role in protecting the epithelium from harmful substances. Acting as a first line of defense, sIgA helps prevent infections by blocking toxins from adhering to the intestinal lining. Also, sIgA antibodies protect the infant without triggering inflammation, a common immune response that can inadvertently damage the infant's delicate digestive system (Goldman & Armond, 1993). In mouse models exposed to *Vibrio cholerae* toxin, sIgA demonstrated a protective effect (Lycke et al., 1999). Also, sIgA can block pathogens by directly recognizing receptor-binding domains, such as in the case of reovirus type 1 Lang. Studies on IgA knockout mice challenged with reovirus showed that oral administration of IgA was as effective as in wild-type mice in clearing the infection (Silvey et al., 2001). Furthermore, sIgA may directly influence bacterial virulence. For example, murine monoclonal IgA targeting *Shigella flexneri* inhibited the bacterial type 3 secretion system, which is essential for the bacteria to invade intestinal epithelial cells (Forbes et al., 2011). The process known as immune exclusion refers to sIgA's ability to prevent pathogens from accessing the intestinal epithelium through mechanisms such as agglutination, entrapment in mucus, and clearance via peristaltic movements (Stokes et al., 1975). In many cultures, especially in the Middle East, South America, and Northern Africa, mothers traditionally apply breast milk to their infants' eyes to treat infections. While this practice has not been scientifically tested, theoretical evidence suggests it could be effective, as it has persisted over time (Newman, 1995). Other immunoglobulins like IgM, and IgG are also present in breast milk but in lower concentrations. Unlike sIgA, these immunoglobulins are more susceptible to digestion and do not persist in the small intestine in the same protective manner as sIgA (Lönnerdal, 2013).

2.3.1.5 Lysozyme

Lysozyme a component of the whey protein fraction in breast milk is an anti-infective enzyme present in human milk, consisting of 130 amino acids. It functions by hydrolyzing the 1–4 linkage between N-acetyl glucosamine and N-acetylmuramic acid in bacterial cell walls, thereby aiding in the

destruction of bacteria (Haschke et al., 2016; Newburg & Walker, 2007). Similar to lactoferrin, lysozyme is also found in other exocrine secretions. It primarily targets gram-positive bacteria, but can also affect certain gram-negative bacteria, which tend to be more susceptible to its action. Unlike other protective proteins in human milk, such as antibodies and lactoferrin, the concentration of lysozyme increases progressively with extended lactation (Aoki et al., 1992). The levels of lysozyme in human milk are significantly higher than in serum and are about 1000 times greater in human milk compared to bovine milk (Hamosh, 2001).

2.3.1.6 Folate binding proteins

Folate-binding protein (FBP) is another significant protein found in human milk, available in both particulate and soluble forms. The soluble form is glycosylated to about 22%, which may enhance its ability to withstand proteolytic digestion (Guo, 2020). Studies have shown that the solids of pooled human and bovine milk contain approximately 2,000 nmol/kg of FBP, while goat milk has been found to contain twice as much. Additionally, the process of freeze-drying or spray-drying milk into powder retains nearly all the FBP (Wigertz et al., 1997). FBP can tolerate low gastric pH and resist breakdown in newborn goats, suggesting that it might be similarly stable in human infants. Research using rat intestinal cells has demonstrated that folate uptake is higher when folate is complexed with FBP compared to when it is in a free form, indicating that FBP might aid in folate absorption (Guo, 2020). It has also been proposed that FBP could slow the release and uptake of folate in the small intestine, promoting gradual absorption and better tissue utilization (Lönnerdal, 2003).

Bifidus Factor: Bifidus factor, also known as B12 binding protein, is one of human milk's earliest identified disease-resistance factors. It supports the growth of beneficial *bifidobacteria*, which are commonly added to yogurt and probiotic supplements. The B12 binding protein binds with vitamin B12 in the intestines, preventing harmful microorganisms from using this essential nutrient (Guo, 2020; Hendricks & Guo, 2014).

2.3.1.7 Haptocorrin

Haptocorrin is a vitamin B12-binding protein present in various body fluids, including breast milk, with concentrations ranging from approximately 5 µg/mL in colostrum to 3 µg/mL in mature milk (Aoki et al., 1992). It is a binding protein in HM believed to be the primary means for allowing vitamin B12 absorption in early infanthood (Burger & Allen, 1974). Haptocorrin binds with vitamin B12 to form the complex holohaptocorrin. This complex can bind to human intestinal brush border membranes, where the haptocorrin-associated vitamin B12 is taken up by intestinal cells (Guo, 2020). Later in life, vitamin B12 absorption is facilitated by intrinsic factor (a glycoprotein) secreted by the gastric mucosa (Seetharam & Alpers, 1982). Although intrinsic factor is present in the stool of young breastfed infants, it is believed that the amount of intrinsic factor may not be sufficient at this age to allow B12 absorption using the corresponding receptor (Adkins & Lönnerdal, 2003). Therefore, infants are

dependent on haptocorrin for absorption of vitamin B12 (Adkins & Lönnerdal, 2002). Research into the possible antimicrobial benefits of haptocorrin suggests it may also inhibit bacterial growth. Structurally, haptocorrin remains unchanged mainly after exposure to digestive enzymes and has been shown to inhibit the growth of *E. coli* in vitro (Adkins & Lönnerdal, 2003). However, a comprehensive study of haptocorrin's effects on 34 commensal and pathogenic bacteria revealed that it only suppressed *Bifidobacterium breve*. This suggests that haptocorrin's antimicrobial activity may be limited to specific strains, indicating that its general antimicrobial properties may not apply universally and warrant further investigation (Jensen et al., 2011).

2.3.1.8 Lipases

Lipase is an enzyme in human milk that supports the digestion and absorption of lipids (Lönnerdal, 2003). The primary source of energy for breastfed infants comes from triacylglycerols, the predominant form of lipid in breast milk. Newborns, especially preterm infants, may have reduced lipase activity, leading to less effective lipid utilization. While infants produce some of this enzyme in their exocrine pancreas, the primary source is the Bile salt-stimulated lipase (BSSL) present in maternal milk. BSSL helps alleviate this issue by hydrolyzing di- and tri-acylglycerols, cholesterol esters, diacylphosphatidylglycerols, as well as micellar and water-soluble substrates, thereby facilitating lipid digestion. In the early 1950s, Freudenberg first demonstrated that human milk contains an inactive lipase, which becomes activated when the chyme reaches the duodenum and encounters bile salts (Freudenberg, 1953). BSSL was purified and characterized in the early 1980s and is known for its broad substrate specificity (Blackberg & Hernell, 1981; Lindquist & Hernell, 2010). However, BSSL is inactivated by the pasteurization of breast milk (Fredrikzon et al., 1978), leading to significantly lower lipid digestion and absorption in preterm infants fed pasteurized donor milk (Y. Andersson et al., 2007).

A randomized phase 3 study recently investigated the addition of recombinant human BSSL to infant formula to determine its effect on growth velocity, presumably by enhancing lipid digestion and absorption. Interestingly, the study found that the benefits on growth were not observed in appropriate-for-gestational-age preterm infants but were present in small-for-gestational-age preterm infants (Casper et al., 2016).

2.3.1.9 Amylases

Amylases are enzymes found in human milk that support the digestion and absorption of sugar molecules (Lönnerdal, 2003). In the absence of pancreatic amylase, human milk (HM) amylase can hydrolyze starch, glycogen, and other related saccharides by cleaving α-1,4 linkages, producing maltose, dextrins, and glucose. The activity of amylase in HM ranges from 1,000 to 5,000 units per liter (Heitlinger et al., 1983), with colostrum containing higher activity levels than transitional or mature milk (Lindberg & Skude, 1982). Amylase activity decreases by approximately 35% after the first trimester of breastfeeding and higher parity may further reduce amylase activity by half. Notably, preterm milk contains amylase activity levels similar to term milk. Beyond its role in aiding digestion,

amylase may also exhibit antibacterial properties by targeting the polysaccharides in bacterial cell walls (Lindberg & Skude, 1982).

Despite the significant amount of α-amylase in human milk, it lacks a corresponding substrate, suggesting its role may be to compensate for the low amylase activity in newborns' saliva and pancreas (Guo, 2020; Hendricks & Guo, 2014).

2.3.1.10 α1-Antitrypsin and Antichymotrypsin

α1-Antitrypsin (A1AT) and antichymotrypsin are protease inhibitors present in human milk that work together to inhibit pancreatic enzymes. In vitro studies have shown that these inhibitors play a crucial role in preventing proteolytic degradation of lactoferrin. However, research suggests that the effect of a1-antitrypsin and anti- chymotrypsin may be only to delay, rather than prevent, the breakdown of proteins, as the total nitrogen balance of breastfed infants is not substantially affected (Lönnerdal, 2003).

The physiological role of protease inhibitors like α1-antitrypsin (A1AT) in breast milk is not fully understood. However, as observed in other mammals, these protease inhibitors may assist in the digestion and/or absorption of bioactive proteins, which are present in relatively high concentrations in colostrum. A1AT is most abundant in colostrum (1.4-5.2 g/L) compared to its levels during the first 26 weeks of lactation (0.07 g/L) and between 26-52 weeks (0.05 g/L) (Mcgilligan et al., 1987). A1AT is also resistant to digestion in the gastrointestinal tract and is found intact in significant amounts in the feces of infants (Davidson & Lönnerdal, 1990).

2.3.1.11 Cytokines

Cytokines play a crucial role in regulating inflammatory processes often associated with infection. They are believed to promote thymocyte proliferation, inhibit IL-2 production from T-cells, and suppress IgE production. Various cytokines in HM have been well-documented over the years. These cytokines include but are not limited to, interleukins (IL) -1β, IL-6, IL-8, IL-10, TNF-α, interferon-γ, transforming growth factor-β, and colony-stimulating factor (Grosvenor et al., 1993). Typically present at deficient concentrations (in picograms), they are present in free form and likely originate from epithelial cells of the mammary glands, activated macrophages, and other cells in HM (Lönnerdal, 2004). Their biological function in infants is thought to complement the infants' cytokine production, which is limited due to the immaturity of their immune systems. Cytokines in HM may help balance Th1 and Th2 responses, thereby providing immunity-related benefits (Lönnerdal, 2003).

2.3.1.12 Growth factors

Several proteins in human milk are associated with the development and function of the infant gut, including growth factors, lactoferrin, and casein-derived peptides. Research indicates that insulin-like growth factors IGF-I and IGF-II stimulate DNA synthesis and promote cell growth in culture,

suggesting they may contribute to the development of the infant gastrointestinal tract (Donovan & Odle, 1994; Playford et al., 2000).

Infants fed formula supplemented with bovine lactoferrin have shown greater weight gain compared to those given unsupplemented formula. Lactoferrin supplementation has been observed to enhance cell proliferation in the small intestine of experimental animals and impact crypt cell development. This suggests that the rapid development of intestinal mucosa in suckling newborns may be partly attributed to the mitogenic effects of lactoferrin. Additionally, breastfed premature infants have been found to excrete intact lactoferrin in their urine, indicating that functionally intact lactoferrin is absorbed by the infant gut (Goldman, 2000). Moreover milk fat globule has been associated with promoting bone growth. This effect is thought to be related to increased calcium intake from milk fat. These findings suggest that milk fat may have a modest, but measurable, impact on bone health (Weinsier & Krumdieck, 2000).

2.3.2 Non-protein nitrogen

Non-protein nitrogen (NPN), which consists of molecules such as urea, creatinine, nucleotides, free amino acids, and peptides, accounts for approximately 25% of the total nitrogen present in human breast milk (Jenness, 1979). This fraction of breast milk, though understudied, contains many bioactive molecules. For instance, nucleotides are considered conditionally essential nutrients during early life, performing critical roles in various cellular processes such as altering enzymatic activities and acting as metabolic mediators (Uauy et al., 1994). Additionally, nucleotides are known to be beneficial for the development, maturation, and repair of the gastrointestinal tract (Uauy et al., 1994), the development of the microbiota, and immune function (Gutiérrez-Castrellón et al., 2007; Singhal et al., 2008). These components contribute significantly to the overall health and development of infants, further highlighting the complexity and importance of human breast milk as the optimal source of nutrition during early life.

2.3.3 Glycoproteins

A glycoprotein is defined as a protein that has one or more glycans covalently bonded to its polypeptide backbone, typically through N- or O-linkages (Joshi et al., 2018). Protein glycosylation is one of the most significant post-translational modifications (PTMs) found in human milk proteins (Froehlich et al., 2010). It is estimated that as much as 70% of the proteins in human milk are glycosylated. The levels of expression and glycosylation of glycoproteins in human milk can fluctuate during lactation and in response to various biological factors (Orczyk-Pawiłowicz et al., 2014). The glycosylation of proteins in milk is particularly noteworthy due to its implications for proteolytic susceptibility and its role as a competitive inhibitor of pathogen binding, as well as an immunomodulator in the gut. Consequently, glycosylated proteins in human milk play a crucial role in developing the infant's gut and immune system (Georgi et al., 2013).

2.3.4 Other Enzymes

Human milk contains a complex mix of proteolytic enzymes, zymogens, protease activators, and inhibitors, with the overall proteolytic activity being determined by the interactions among these components. These enzymes each serve distinct functions, and the primary proteases in human milk include plasmin, trypsin-2, cathepsin-D, neutrophil elastase, thrombin, kallikrein, and various amino- and carboxypeptidases (Dallas, Murray, et al., 2015). These proteases are secreted in inactive forms and later activated by protease activators like tissue-type plasminogen activator (t-PA) and urokinase-type activator (u-PA) (Dallas, Murray, et al., 2015). Notably, human milk contains higher levels of enzymes than bovine milk (Shahani et al., 1980), and the enzymes in human milk are structurally distinct from those in other bodily fluids. This difference, attributed to the more organized tertiary structure of human milk enzymes, leads to greater hydrophobicity, making them more resistant to proteolysis and denaturation in the infant's gastrointestinal tract (Dallas, Murray, et al., 2015; Nielsen et al., 2017; Shahani et al., 1980).

Research has shown that the neutral pH of human milk provides a buffering effect in the infant's stomach, raising the pH and limiting proteolysis by pepsin (Dallas, 2012). This increase in pH is believed to play a crucial role in promoting bacterial colonization in the gut, with milk proteases like cathepsin-D and plasmin remaining active in the infant's stomach to aid in digestion (Dallas, 2012). The presence of proteases in human milk may be an evolutionary adaptation designed to enhance digestion in infants (Zhu & Dingess, 2019). The effective digestion of the human milk proteome in the infant's gastrointestinal tract is vital, as inadequate digestion can lead to incomplete amino acid hydrolysis, which is essential for growth and could increase the infant's protein requirements. Additionally, undigested proteins reaching the colon may contribute to the overgrowth of protein-fermenting bacteria like *Clostridium perfringens* and various Bacteroides species. This could negatively impact the population of beneficial carbohydrate-consuming bacteria, such as *Bifidobacteria*, in the infant's gut (Dallas, Murray, et al., 2015).

2.4 Lipids

Lipids, secreted by mammary epithelial cells, account for almost 50% of the nutritional supply in human milk (HM) and are the second most prevalent macromolecule, playing a crucial role in infant growth and the development of the central nervous system (Kim & Yi, 2020). Long-chain polyunsaturated fatty acids (LCPUFAs) delivered through HM significantly influence the development of the retina and brain cortex in infants (Mosca & Giannì, 2017). Sphingomyelins, which are vital for the myelination of the central nervous system, contribute to neurobehavioral development (Delplanque et al., 2015). In general, HM contains 3.5-4.5% fat, primarily in the form of triglycerides, with the fat content being influenced by dietary habits, maternal diet, and other factors, and it increases significantly from colostrum to mature milk. The fatty acid profile of HM is detailed in Table 2.3, which compares the composition of fatty acids in human and bovine milk. While the fatty acid profile of HM remains

stable during a single lactation, the total fat content can increase 2–3 times from foremilk to hindmilk (Saarela et al., 2005). Most fatty acids in HM fall within the C10-C18 range, with a small proportion of unsaturated long-chain fatty acids. The amounts of LCPUFAs and their precursors are primarily determined by maternal diet, which can affect infant adiposity and body fat percentage (Eriksen et al., 2018). Furthermore, short-chain fatty acids in HM serve as important sources of calories and play a crucial role in gastrointestinal maturation (Mosca & Giannì, 2017).

Table 2-3. Fatty acid profile in human and bovine milk (%, w/w).

	Human	**Bovine**
Saturated		
Butyric (4:0)	_	3.5
Caproic (6:0)	_	1.9
Caprylic (8:0)	_	1.3
Capric (10:0)	1.4	2.5
Lauric (12:0)	6.2	2.8
Myristic (14:0)	7.8	10.7
Palmitic (16:0)	22.1	27.8
Stearic (18:0)	6.7	12.6
Total	48.2	65.6
Monounsaturated		
Palmitoleic (16:1)	3.1	2.5
Oleic (18:1)	35.5	26.5
Gadoleic (20:1)	0.96	Trace
Cetoleic (22:1)	Trace	Trace
Total	39.8	30.3
Polyunsaturated		
Lineoleic (18:2)	8.9	2.9
Linolenic (18:3)	1.2	1.6
Parinaric (18:4)	_	Trace
Arachidonic (20:4)	0.72	Trace
Eicosapentenoic (20:5)	Trace	Trace
Total	10.82	4.5

2.4.1 Milk fat globule

The lipid droplets, known as fat globules, are natural oil-in-water emulsion and enveloped by a biological membrane rich in bioactive substances, which serves as the interface with the intestinal tract. The secretion of Milk Fat Globules (MFG) by the mammary epithelium includes a diverse collection of proteins and lipids bound to the membrane in milk (Lopez et al., 2019). There is broad scientific

consensus recognizing the importance of human milk fat globules in infant nutrition. The physical structure of these fat droplets can influence digestion, postprandial metabolism, and potentially prevent fat accumulation in adults (Baars et al., 2016; Baumgartner et al., 2017).

The milk fat globule membrane (MFGM) is the membrane that encases lipid droplets as they are secreted into the alveolar lumen of the lactating mammary gland (Cavaletto et al., 2008). As illustrated in Figure 2.4, this membrane surrounding the MFG is not just a passive barrier but an active player in infant health, carrying complex lipids, including phospholipids and sphingolipids, that contribute to brain development and immune function. The intricate structure of the MFGM, with its proteins and glycoproteins, supports intestinal health, enhances nutrient absorption, and provides defense against pathogens. As research continues to unveil the multifaceted roles of MFGM, these insights drive the evolution of infant formulas to better replicate the functional benefits of human milk, ultimately supporting optimal growth and development in infants (Brink & Lönnerdal, 2020).

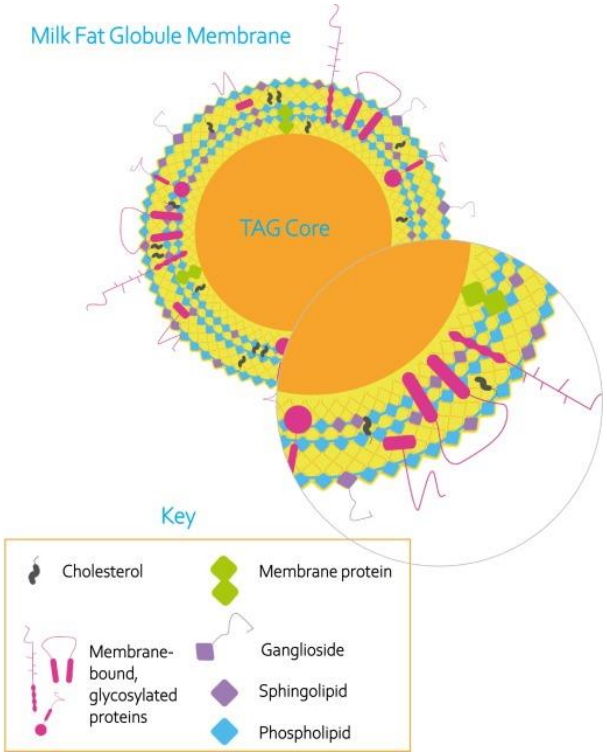

Figure 2-4. Milk fat Globule Membrane (Brink & Lönnerdal, 2020)

2.4.2 MFGM Lipids

The Milk Fat Globule Membrane (MFGM) contains a variety of complex lipids, including glycerolipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine) and sphingolipids (sphingomyelin and gangliosides), which, while not abundant, have significant structural and functional roles (Lopez et al., 2019). Glycerolipids and sphingolipids are amphipathic in nature and are vital for membrane integrity and function (Brink & Lönnerdal, 2020).

The phospholipids in MFGM are a crucial source of choline, an essential nutrient involved in metabolism and the construction of membranes in the brain and nervous tissue. Newborns need substantial amounts of choline for rapid organ growth and cell membrane biosynthesis, with the European Food Safety Authority (EFSA) recommending an adequate intake of 130 mg per day for the first six months of life (EFSA, 2013; Meyers, 2006). Sphingolipids, which make up about half of the complex lipids in the MFGM, play important roles in cell signaling. The digestion of sphingomyelin, the main sphingolipid in breast milk, produces ceramide, sphingosine, and sphingosine-1-phosphate. These metabolites are involved in the regulation of cell growth, differentiation, apoptosis, and the migration of immune cells (Nilsson, 2016).

Gangliosides are another significant group of lipids in MFGM, consisting of a hydrophobic ceramide and a hydrophilic oligosaccharide chain that includes sialic acid residues. Initially isolated from the brain, gangliosides are abundant in neural tissues but are also present in various vertebrate tissues and fluids. Breast milk is rich in gangliosides, which increase their concentrations in the intestinal mucosa, plasma, and brain, playing a crucial role in tissue development, particularly the small intestine (Park et al., 2005; Suh et al., 2004). The concentration and types of gangliosides in breast milk change during lactation, with disialogangliosides abundant in colostrum and monosialogangliosides making up 85% of total gangliosides in mature milk, indicating different functionalities at various stages of infant development (Rueda et al., 1998).

Gangliosides are also important for immunity and protection against infections. They contribute to the proliferation, activation, and differentiation of immune cells and have immunomodulatory effects, particularly in early lactation when disialoganglioside levels are highest (Brønnum et al., 2005). Gangliosides have a growth-promoting known impact on beneficial bacteria like *bifidobacteria* and *lactobacilli*, and they protect against pathogens such as *Giardia muris* and *Giardia lamblia* (Suh et al., 2004; Zúñiga et al., 2018). Gangliosides also improve the absorption of polyunsaturated fatty acids ω3 and ω6 compared to saturated fatty acids, enhancing the nutritional quality of breast milk (Park et al., 2006).

2.4.3 MFGM Proteins

More than 100 different proteins have been identified in the MFGM, all secreted through a unique process by mammary epithelial cells (Lee et al., 2018). These proteins vary in their attachment to the membrane: some, like lactoadherin, are weakly adhered; others, like mucin, have a peripheral distribution; while butyrophylin is more integrated within the fat globule. The distribution of these proteins can change based on the size of the fat globule and the timing of secretion, particularly in premature births (Peterson et al., 1996). Butyrophylin, for instance, becomes more abundant as the fat globule increases in size.

Many MFGM proteins though make up just 1–4% of the total protein content in milk (Cavaletto et al., 2008), are highly glycosylated, which helps them resist the acidic environment and pepsin activity in the infant's stomach, preserving their functionality in the intestine. Proteins such as mucin and lactoadherin support intestinal development by promoting the health of the intestinal epithelium and providing antiviral and antibacterial activities. Butyrophylins are linked to immune response regulation, while bile salt-activated lipase is crucial for lipid digestion. These proteins collectively play essential roles in supporting infant health and development through their structural, functional, and bioactive properties(Zhu & Dingess, 2019).

2.4.4 Functions of human milk lipid

Lipids in human milk serve multiple critical functions essential for infant nutrition and development. They act as a primary energy source and reserve, crucial for supporting the rapid growth and weight gain of infants. Lipids also facilitate the absorption and transport of fat-soluble vitamins and other compounds, contributing to the effective delivery of these essential nutrients (Guo, 2020). Human milk contains essential fatty acids, such as linoleic acid (18:2 ω-6) and α-linolenic acid (18:3 ω-3), which are vital for physiological processes including the development of the nervous system and visual acuity. The presence of long-chain polyunsaturated fatty acids (PUFAs) like docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) supports the development of brain and retinal tissues (Lönnerdal, 1986; Brink & Lönnerdal, 2020; Lee et al., 2018). Cholesterol, another lipid component in human milk, plays a role in cell membrane formation and hormone synthesis. Phospholipids function as emulsifying agents and stabilizers for the milk fat globule membrane, aiding in digestion and absorption, while also binding essential minerals and interacting with digestive enzymes (Guo, 2020).

2.5 Vitamins

Human milk contains all the essential water-soluble and fat-soluble vitamins. Compared to bovine milk, human milk has higher levels of vitamins A, E, C, nicotinic acid, and inositol but lower levels of vitamins B1, B2, B6, B12, K, biotin, pantothenic acid, and choline as shown in Table 2.4. Generally, human milk provides sufficient amounts of most vitamins necessary for average infant growth, except for vitamin D and possibly vitamin K. Additionally, exclusively breastfed infants of mothers following a strict vegetarian diet may need vitamin B12 supplementation to prevent deficiency, which can lead to severe and permanent neurological damage (Guo & Hendricks, 2008).

Table 2-4. Vitamin content of human and bovine milk (mg/L) (Guo, 2020)

Vitamin	**Human**	**Bovine**
Vitamin A	0.53	0.37
Carotene	0.24	0.21
Cholecalciferol (1.))	0.001	0.0008
Tocopherol (E)	5.4	1.1

Vitamin K	0.015	0.03
Thiamin (B1)	0.15	0.42
Riboflavin (B2)	0.37	1.72
Pyridoxine (B6)	0.10	0.48
Cobalamin (B12)	0.0003	0.0045
Niacin	1.7	0.92
Folic acid	0.043	0.053
Ascorbic acid (C)	47	18
Biotin	0.007	0.036
Pantothenic acid	2.1	3.6
Inositol	300	160

2.5.1 Fat-soluble vitamins

Fat-soluble vitamins are essential for infant health, and maternal nutrition and supplementation influence their levels in human milk. Vitamin A, which includes retinyl esters, retinol, and β-carotene, is crucial for various physiological processes. Adequate maternal nutrition generally ensures sufficient vitamin A in human milk, but supplementation around childbirth can enhance vitamin A levels in milk, especially when maternal intake is inadequate (Guo, 2014). Vitamin D is vital for bone metabolism and may impact immune function and mental health. However, human milk often contains insufficient vitamin D, potentially leading to deficiencies in exclusively breastfed infants, particularly in areas with limited sunlight exposure. Maternal supplementation with 400-2000 IU of vitamin D daily is recommended, with higher doses being more effective in achieving satisfactory vitamin D levels in infants (Guo, 2014; Parker et al., 2017). Vitamin E, known for its antioxidant properties, is usually sufficient in the milk of term infants but may be inadequate for preterm infants, who might require vitamin E-enriched formula to prevent deficiencies (Guo, 2014). Finally, vitamin K, essential for blood clotting and bone health, is in low amounts in human milk. Newborns who are born with low vitamin K levels, increasing their risk of hemorrhagic disease. Supplementation after birth is necessary to prevent this condition, and while maternal intake can influence milk vitamin K levels, the results are mixed (Guo, 2014; Guo & Hendricks, 2008). While human milk provides essential vitamins, appropriate supplementation can address potential deficiencies and support optimal infant health.

2.5.2 Water-Soluble Vitamins

Water-soluble vitamins are not effectively stored in the body, so maternal dietary intake significantly influences their levels in human milk. Vitamin B_1 (thiamine) content in human milk averages 0.15 mg/L, and supplementation in adequately nourished women does not substantially increase these levels, indicating a transfer limit. However, maternal thiamin deficiency can result in low thiamin levels in milk. Vitamin B_2 (riboflavin) levels in milk are affected by maternal intake; supplementation with 2 mg/day can increase milk riboflavin levels, and 2.5 mg/day is sufficient to

maintain adequate levels during lactation. Vitamin B_7 levels in milk range from 5–12 mg/L and are not significantly affected by the supplementation of other B vitamins. Still, they do increase with direct B_7 supplementation if initial levels are low. Vitamin B_6 concentrations in milk vary with maternal intake; levels are about 210 µg/L with an intake around the RDA (2.5 mg/day) and 120 µg/L with lower intake, but excessive supplementation can suppress lactation. Vitamin B_9 levels in milk rise with lactation time, and supplementation can help increase levels in women with low socioeconomic status and intake. Vitamin B_{12} levels in milk are also influenced by maternal intake, with deficiencies more common in vegetarian mothers or those with low socioeconomic status. While well-nourished women do not see increased milk B_{12} levels with supplementation, women with low B_{12} status can benefit from it (Guo & Hendricks, 2008). Vitamin C levels in human milk are typically 50 mg/L and are not significantly increased by high supplementation, indicating an upper transfer limit. Vitamin B_3 and B_5 levels in milk correlate with maternal intake, and choline, which is critical for infant growth and development, reflects maternal dietary intake, especially from animal sources (Guo, 2020). Human milk contains mostly water-soluble forms of choline (84%), with lipid-soluble forms comprising up the rest (Fischer et al., 2010). Maternal choline intake influences milk choline levels, which correlate with infant choline status and may aid brain development (Ilcol et al., 2005). Taurine, essential for neurodevelopment, is abundant in human milk, and deficiency in preterm infants can lead to significant health issues, so formula supplementation is standard (Chawla, 2018; Heird, 2004). Creatine, vital for brain development, is provided by human milk or synthesized de novo by the infant (Edison et al., 2013). Carnitine, necessary for fat metabolism, is present in human milk at 60-70 nmol/mL, and soy-based formulas are often supplemented to match these levels due to the importance of carnitine for neonatal development (Guo, 2020).

2.6 Minerals

Minerals in human milk exist in various chemical forms, including inorganic ions, salts, and as constituents of proteins, fats, and nucleic acids. These minerals are vital for numerous physiological functions, such as structural components of body tissues and essential parts of enzymes and biologically important molecules (Tan et al., 2020). The primary minerals in human milk include sodium, potassium, chloride, calcium, magnesium, phosphorus, and sulfur (Taravati Javad et al., 2018). Citrate, while not a mineral, is a water-soluble component of human milk that can bind minerals. The concentration of minerals in human milk changes throughout lactation; sodium and chloride levels decrease, while potassium, calcium, magnesium, and free phosphate levels increase. These concentrations are also influenced by the mother's nutritional status, environmental factors, and other variables(Tan et al., 2020).

2.6.1 Macrominerals in Human Milk

Macrominerals in human milk, such as sodium, potassium, chloride, calcium, magnesium, and phosphorus, play critical roles in various physiological functions (Aumeistere et al., 2017). As shown

in Table 2.5, sodium, the principal extracellular cation, regulates osmolarity, acid-base balance, active transport across cells, and membrane potential. Potassium, the primary intracellular cation, is vital for nerve impulse transmission, blood pressure maintenance, and skeletal muscle contraction. Chloride, the principal extracellular anion, is essential for fluid and electrolyte balance. The concentrations of sodium, potassium, and chloride decrease throughout of lactation from 480, 740, and 850 mg/L in colostrum to 160, 530, and 400 mg/L, respectively, with no significant correlation to maternal intake (Picciano, 2000).

Calcium, comprising 1.5%-2% of an adult's body weight, primarily exists in bones and teeth as calcium phosphate. At the same time, the rest is involved in regulatory functions such as heart rhythm, hormone secretion, blood coagulation, nerve conduction, muscle contraction, enzyme activation, and membrane integrity. Human milk supplies approximately 200 mg of calcium daily, generally adequate for term infants but may not suffice for preterm infants. Despite supplementation, maternal calcium intake does not significantly affect milk calcium content. The calcium level in human milk rises from 250 mg/L on day 1 to about 300 mg/L by day 5 and remains steady until day 36, but can decrease by 30% between the first and ninth months of lactation (Kılıç Altun et al., 2018; Sánchez et al., 2020)

Magnesium is essential for neuromuscular transmission, muscle contraction, protein and nucleic acid metabolism, and enzyme function. It supports skeletal growth along with calcium and phosphate. Human milk contains approximately 30-35 mg/L of magnesium, about 30% higher in colostrum than in mature milk. Dietary magnesium intake does not significantly influence its concentration in human milk (Aumeistere et al., 2017; Guo, 2014; Taravati Javad et al., 2018).

Table 2-5. Mineral composition of mature human and bovine milk (Picciano, 2000)

Mineral	Mature human milk	Bovine milk
Sodium (mg/L)	207 ± 94	580
Potassium (mg/L)	543 ± 78	1400
Chloride (mg/L)	453 ± 53	1040
Calcium (mg/L)	259 ± 59	1180
Magnesium (mg/L)	31.4 ± 5.9	120
Phosphorus (mg/L)	142 ± 25	930
Iron (µg/mL)	0.4–0.76	0.2–0.6
Zinc (µg/mL)	1–3	4
Copper (µg/mL)	0.2–0.4	0.05–0.2
Manganese (ng/mL)	3–6	21
Iodine (ng/mL)	12–178	70–219
Fluoride (ng/mL)	4–15	19
Selenium (ng/mL)	15 –20	10
Aluminum (ng/mL)	4–14	27

Chromium (ng/mL)	0.2–0.4	5–15
Molybdenum (ng/mL)	1–2	22
Cobalt (μg/L)	— 0.1	

Phosphorus is critical for various biological functions, including forming structural components like calcium phosphate in bones and teeth and being part of lipids, proteins, carbohydrates, and nucleic acids. The phosphorus content in human milk increases from 100 mg/L on day 1 to 170 mg/L by day 8, then decreases to 130 mg/L by day 36 (Aumeistere et al., 2017).

2.6.2 Trace Elements in Human Milk

Trace elements, or microminerals, constitute less than 0.01% of body mass but are essential for various physiological functions. These elements in human milk include iron, zinc, copper, manganese, selenium, iodine, fluorine, molybdenum, aluminum, cobalt, chromium, and nickel(Taravati Javad et al., 2018).

Iron is crucial for oxygen transport, storage, and utilization, being a key component of hemoglobin, myoglobin, and cytochromes. Iron deficiency anemia affects about 30% of the global population. The mean iron concentration in human milk is 0.3 mg/L, decreasing from 1 mg/L in colostrum to 0.3-0.6 mg/L in mature milk. Dietary iron intake does not correlate with milk iron concentration, supplementation up to 30 mg/day does not affect its levels. Iron in human milk is primarily bound to lactoferrin, a protein with a high affinity for ferric ions (Guo, 2020; Guo & Hendricks, 2008).

Zinc is essential for growth, development, immune function, and numerous metabolic processes, acting as a cofactor for various enzymes. Zinc deficiency, first reported in the Middle East, can lead to dwarfism, impaired sexual development, and anemia. The mean zinc concentration in mature human milk is about 2 mg/L, though it varies widely from 0.65-5.3 mg/L. Dietary zinc intake does not significantly affect its concentration in human milk, and supplementation does not alter zinc levels in zinc-adequate diets (Flynn, 1992; Picciano, 2000).

Copper is vital for iron utilization and acts as a cofactor for enzymes in glucose metabolism, hemoglobin synthesis, and connective tissue formation. Mature human milk contains about 0.3 mg/L of copper, decreasing from 0.6 mg/L in early lactation to 0.21-0.25 mg/L by week 20. Maternal dietary intake does not significantly influence copper concentration in human milk (Taravati Javad et al., 2018).

Manganese is a cofactor for glycosyl transferases and other enzymes, including mitochondrial superoxide dismutase and pyruvate carboxylase. It is abundant in foods, and dietary deficiency is rare. In mature human milk, manganese concentration averages around 10 μg/L, decreasing with lactation

progression. Manganese is mainly bound to lactoferrin in human milk, but its low concentration means it occupies a minimal fraction of lactoferrin's binding capacity (Guo & Hendricks, 2008).

Selenium is a crucial part of the enzyme glutathione peroxidase, which works with other antioxidants to protect cells from oxidative damage. Human milk selenium concentration is about 16 μg/L, higher in colostrum at 41 μg/L. The selenium content of human milk correlates with maternal plasma selenium levels and glutathione peroxidase activity, indicating maternal selenium status influences key milk selenium levels (Guo, 2014).

Iodine is necessary for thyroid hormone synthesis and impacts basal metabolism and reproduction. Iodine deficiency can cause goiter, while excess iodine reduces thyroid iodine uptake. Human milk in the United States has a mean iodine concentration of 142 μg/L, with intake correlated to dietary iodine, such as iodized salt (Picciano, 2000).

Molybdenum is a crucial component of several enzymes, including aldehyde oxidase, xanthine oxidase, and sulfite oxidase. The molybdenum content in human milk decreases from 15 μg/L on day 1 to about 2 μg/L by one month. Dietary molybdenum deficiency is rare, observed only in patients on long-term parenteral nutrition (Picciano, 2000).

Chromium is essential for glucose tolerance, and deficiency is noted only in long-term parenteral nutrition patients. Mature human milk has a mean chromium content of 0.27 μg/L. Cobalt, part of vitamin B12, is found in human milk at about 0.1 μg/L, with dietary supplementation affecting milk levels only in cobalt-deficient diets (Picciano, 2000; Taravati Javad et al., 2018).

Fluoride, while not essential, protects against dental caries and is found in human milk at about 16 μg/L. Excessive fluoride can cause fluorosis. Infants on breast milk or formula with nonfluoridated water may require fluoride supplements (Flynn, 1992; Picciano, 2000).

Chapter 3
Impact Of Holder Pasteurization On Human Milk Proteins

3.1 Pasteurization

Human milk is widely regarded as the gold standard for infant nutrition and recommended as the benchmark against which all other infant feeding practices should be measured (Meek & Noble, 2022). It is uniquely tailored to deliver the essential nutrients, bioactive components, and immune-enhancing factors vital for a newborn's growth and development. In cases where a mother's milk is unavailable, donor human milk (DHM) is considered the next best option, particularly for premature or low-birth-weight infants weighing less than 1500 grams (De Oliveira et al., 2016).

Human milk banks play a crucial role by providing donor milk, a preferable alternative to infant formula. Several guidelines have been established to implement and regulate human milk banks (HMB) to ensure the provision of high-quality donor human milk. These regulations address the handling, processing, and storing of breast milk, aiming to maintain microbiological safety and preserve its nutritional quality (Gharbi et al., 2023).

While various sterilization methods were initially employed, pasteurization is the most recognized and effective technique for sanitizing milk. Milk received at the human milk bank undergo pasteurization. The ideal pasteurization process includes a rapid heating phase, followed by a phase where the temperature is held constant, and concludes with a rapid cooling phase (Moro et al., 2024). Holder Pasteurization (HoP) is the most commonly used method, involving low temperature and extended time (+62.5°C for 30 minutes). Other methods include thermal processes such as High-Temperature Short-Time (HTST) pasteurization, as well as non-thermal techniques like High-Pressure Processing (HPP), Microwave Irradiation and Ultraviolet C radiation (Moro et al., 2019, 2024; Wesolowska et al., 2019).

3.2 Holders pasteurization

Thermal pasteurization is mandated for donor human milk in many countries, including neonatal intensive care units and human milk banking programs, to ensure microbial safety. The safety of banked milk is ensured through Holder pasteurization (HoP), also known as the LTLT method (low temperature, long time; 62.5°C for 30 minutes) (Guerra et al., 2018). This pasteurization process is necessary to inactivate pathogenic bacteria and viruses, as well as to minimize spoilage, making the milk safe for feeding to infants. Two freeze-thaw cycles are used in the process: the first occurs between milk expression and pasteurization, and the second occurs between pasteurization and administration (De Oliveira et al., 2016). However, it is essential to note that heat treatment, while it ensures safety, reduces some beneficial components like immunoglobulins, lactoferrin, lysozyme, lactoperoxidase, lipase and certain vitamins. Although the overall nutritional value of the proteins remains mostly intact, their bioactive functions and antimicrobial properties are affected (Lima et al., 2017; Picaud & Buffin, 2017)

3.3 Changes in protein concentration

There is a significant reduction in key proteins such as sIgA and lactoferrin in pasteurized milk with a minimal impact on lysozyme (J.-C. Chang et al., 2013). The sIgA content gets significantly reduced, decreasing to approximately 48% to 60% of its original concentration (Akinbi et al., 2010; Contador et al., 2013). Pasteurization also reduce lactoferrin concentration by 44% and the concentration of lactoperoxidase by 82% compared to fresh milk. On the other hand, proteins like α-lactalbumin and serum albumin are more stable and do not exhibit significant changes in concentration after pasteurization (Akinbi et al., 2010; Peila, Coscia, et al., 2016). Figure 3.1 shows the concentrations of bioactive proteins in different forms of human milk, highlighting these variations. It is important to note that the measured protein concentration may remain unchanged even if there is a reduction in biological activity due to partial denaturation (Lima et al., 2017).

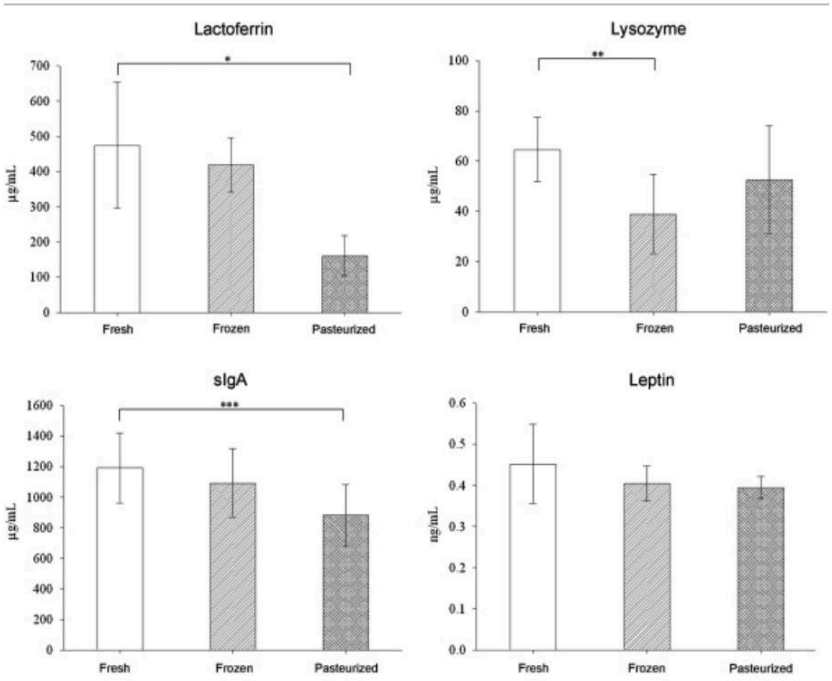

Figure 3-1. Concentrations of bioactive proteins in different forms of human milk (J.-C. Chang et al., 2013)

3.4 Protein Content

The composition of the human milk (HM) protein fraction varies among mothers and changes throughout lactation. The protein content in term milk is estimated to be approximately 0.9 to 1.2 g/dL, which is typically higher in preterm milk (Ballard & Morrow, 2013). However, the accurate protein content of HM is often overestimated due to its high proportion of non-protein nitrogen (Lönnerdal, 2003). In a study by Vieirà et al. (Vieira et al., 2011), the average protein content in donor milk (DM), assessed using an infrared analyzer, was significantly reduced by Holder pasteurization (HoP). This finding was also observed in colostrum (Koenig et al., 2005). Conversely, other studies did not observe any significant change in protein content (Hamprecht et al., 2004; Ley et al., 2011; Silvestre et al.,

2006), even when total nitrogen content was measured indirectly (García-Lara et al., 2013). Most of the studies reviewed suggest that HoP does not significantly affect the protein content of DM. A statistically significant reduction was reported in only two studies, and even then, one study involving mature milk (Vieira et al., 2011) noted a very slight reduction (-3.9%), similar to the findings of studies that claimed no effect on total protein content.

3.5 Protein denaturation and aggregation

The extent of protein denaturation during pasteurization is influenced by the proteins' physicochemical properties and the specifics of the thermal process used. Holder pasteurization (HoP) leads to significant denaturation and aggregation of proteins in human milk, particularly affecting whey proteins and caseins (CNs). During HoP, proteins lose their natural structure—secondary, tertiary, or quaternary and unfold (Sergius-Ronot et al., 2022). Figure 3.2 shows the impact of pasteurization on proteins and other component of milk. The unfolding due to pasteurization promotes thiol-catalyzed disulfide-bond interchanges, resulting in the aggregation of proteins into larger complexes. These changes alter the solubility, structural integrity and functionality of the proteins and increase the overall volume of protein particles due to the formation of new aggregates.

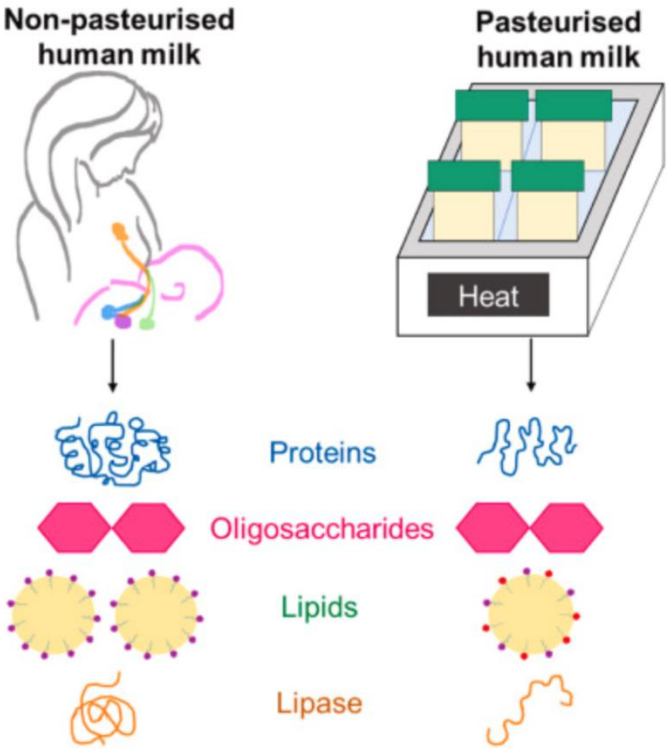

Figure 3-2 Diagram showing the impact of pasteurisation of human milk on the structure of proteins, oligosaccharides, lipids and lipase (Binte Abu Bakar et al., 2021).

One significant effect of HoP is the denaturation of whey and milk fat globule membrane (MFGM) proteins, which can lead to their redistribution into the casein fraction, where they become "trapped" in casein micelles. This entrapment caused by heat-induced denaturation, allows these proteins to interact with and become incorporated into the casein micelles (J. Zhang et al., 2023). HoP has been shown to cause 32 proteins, not initially found in the whey fraction, to become trapped in the casein fraction (Dewan et al., 1974).

The denaturation caused by HoP can also lead to a loss of bioactivity, particularly in heat-sensitive proteins like lactoferrin, immunoglobulins, and enzymes such as bile salt-stimulated lipase (BSSL). For instance, the antimicrobial activities of lactoferrin and immunoglobulins are significantly reduced after HoP, diminishing the milk's protective properties against infections. Aggregation also affects the integrity and functionality of the MFGM, which is essential for delivering fat and associated bioactive molecules to the infant.

3.6 Changes in protein bioactivity

3.6.1 IgA (Immunoglobulin A)

IgA is crucial for the immune protection of infants. HoP leds to a substantial reduction in IgA levels. The impact of HoP on the concentration of different immunoglobulin (Ig) classes in donor milk (DM) has been extensively studied since 1977 (Ford et al., 1977). IgA and secretory IgA (sIgA) are the most thoroughly investigated classes, with nearly all studies reporting a reduction in their levels following HoP. Mayayo et al. demonstrated that the most significant decreases in IgA levels during HoP occurred within the first 5 minutes of treatment, with reductions of 45%. The remaining 25 minutes of treatment resulted in less than 10% further reductions (Escuder-Vieco et al., 2021; Mayayo et al., 2014, 2016). Significant decreases in IgA levels were detected using methods such as Enzyme-Linked Immunosorbent Assays (ELISA) (Contador et al., 2013; Permanyer et al., 2010; Viazis et al., 2007) and Radial Immunodiffusion Assays (RIA) (Goldsmith et al., 1983; Liebhaber et al., 1977). Some older studies also noted reductions in IgA levels (Evans et al., 1978; Ford et al., 1977), though they lacked statistical significance, potentially due to weak experimental designs. Similar detrimental effects on IgAs have been observed in colostrum samples (Espinosa-Martos et al., 2013; Koenig et al., 2005; Sousa et al., 2014).

Other Ig classes have been less frequently studied, with mixed results due to the low concentrations and difficulty detecting these proteins in milk samples. However, most studies noted some degree of reduction. IgM and IgG levels showed a significant decrease or were degraded entirely in mature DM following pasteurization (Contador et al., 2013; Czank et al., 2009; Goldsmith et al., 1983; Liebhaber et al., 1977). Recent studies confirmed IgG and IgM's low resistance to pasteurization, including in colostrum (Espinosa-Martos et al., 2013; Koenig et al., 2005; Sousa et al., 2014). Specifically, IgG subclasses exhibited varying degrees of thermal resistance: IgG1 remained unaffected,

while IgG4 was reduced, and IgG2 and IgG3 were undetectable in both fresh and pasteurized samples (Espinosa-Martos et al., 2013). The findings indicate that HoP consistently reduces all classes of immunoglobulins, likely due to the complex structure of these proteins (Peila, Coscia, et al., 2016).

3.6.2 Lactoferrin

Lactoferrin is an iron-binding protein that inhibits the growth of iron-dependent pathogens by reducing the availability of free iron. Additionally, it can disrupt bacterial cell membranes by binding to the lipid-A portion of lipopolysaccharides on the bacterial cell surface (Ochoa & Cleary, 2009). Lactoferrin is sensitive to thermal denaturation and significantly decreases during HoP within the first 5 minutes of treatment, with reductions of 70%. The remaining 25 minutes of treatment resulted in less than 10% further reductions (Escuder-Vieco et al., 2021; Mayayo et al., 2014, 2016). Multiple studies using various techniques, such as ELISA, RIA, and monospecific antisera(Goldsmith et al., 1983; Sousa et al., 2014), have investigated lactoferrin, consistently reporting reductions in its concentration ranging from 35% to 90%. However, the reduction was only reported as significant by Christen and collegues (Christen et al., 2013). A reduction in the lactoferrin-containing band was also noted using a semi-quantitative protein electrophoresis method (Christen et al., 2013). Since the bactericidal activity of lactoferrin is mediated by bactericidal peptides formed during digestion, some activity may be retained in pasteurized HM despite the reduction in protein levels (Lönnerdal, 2003). A recent survey using non-reducing protein electrophoresis reported that lactoferrin aggregation, rather than degradation, occurs after donor human milk pasteurization with HoP (Mayayo et al., 2014). It remains unclear whether this aggregation decreases lactoferrin's bactericidal activity.

3.6.3 Lysozyme

Lysozyme plays a crucial role in antibacterial defense. HoP also reduced its levels, affecting the milk's bacteriostatic properties. Several studies reported reductions in its concentration after pasteurization, ranging from 20% to 85%. Ford and colleagues (Ford et al., 1977) tested lysozyme's biological activity but found no significant differences post-pasteurization. However, other researchers observed a substantial reduction in lysozyme activity following HoP, even in colostrum (Gibbs et al., 1977; Sousa et al., 2014; Viazis et al., 2007). In these studies, lysozyme activity was consistently assessed using a *Micrococcus lysodeikticus*-based turbidimetric assay, which measures the extent to which bacterial growth is inhibited by lysozyme-containing samples. Since DHM is a complex mixture of several antibacterial enzymes and factors, it is difficult to determine if the reduction in activity is solely due to decreased lysozyme concentration.

3.6.4 Enzymes

Enzymes and their activity are often considered markers for assessing the effectiveness of thermal treatments due to their varying responses to heat. Some enzymes, such as lactoperoxidase and alkaline phosphatase, are technological markers for pasteurization in bovine milk. However, data on

lactoperoxidase in human milk (HM) are limited, as its concentration is typically below the detection threshold of commercial kits (Ford et al., 1977; Sousa et al., 2014). Similar to what has been observed in bovine milk, alkaline phosphatase inactivation after Holder pasteurization also occurs in human milk (Hamprecht et al., 2004).

Given the compensatory role of several HM enzymes in aiding nutrient digestion in newborns (Hamosh, 1994), researchers have focused on the activity of lipase and amylase enzymes in milk. Studies have shown complete degradation in both concentration and enzymatic activity for lipoprotein lipase and bile salt-dependent lipase following pasteurization (Cavallarin, 2011; Hamprecht et al., 2004; Henderson et al., 1998). In contrast, amylase activity is only partially retained after HoP (Henderson et al., 1998). The clinical significance of changes in these enzymes' activity remains to be fully understood. In particular, the potential impact on nutrient absorption, especially lipid digestion, when feeding pasteurized versus raw HM, cannot be entirely dismissed.

Muramidase activity refers to the enzymatic activity of lysozyme, the enzyme that breaks down bacterial cell walls, contributing to the antimicrobial properties of human milk. Peroxidase activity plays a role in the body's immune response by catalyzing reactions that produce antimicrobial substances. The heat treatment during pasteurization causes partial denaturation of these enzymes, leading to a significant decrease in their biological activity. Muramidase activity in pasteurized milk was 50% to 76% lower than in fresh human milk. Similarly, peroxidase activity was diminished by 69% to 88% in pasteurized milk (Akinbi et al., 2010).

3.6.5 Bile salt dependant lipase (BSSL)

Given that BSSL is a heat-sensitive enzyme that begins to inactivate at temperatures as low as 45°C (Wardell et al., 1984), the substantial loss observed after all thermal treatments was anticipated. Wardell et al. demonstrated that even brief exposure to 55°C can inactivate the enzyme. The levels and activity of BSSL were nearly eliminated following HoP, consistent with previous findings (Escuder-Vieco et al., 2021; Peila, Moro, et al., 2016).

3.6.6 Cytokines

Cytokines in human milk (HM) are primarily anti-inflammatory, potentially reducing the impact of infections (Lönnerdal, 2003). The effect of pasteurization on various cytokines has been studied, particularly in colostrum (Espinosa-Martos et al., 2013). Pasteurization had some affect on Interleukin (IL)1β, IL2, IL4, IL5, IL6, IL10, IL12, IL13, IL17, Interferon (IFN)-γ, Tumor Necrosis Factor (TNF)-α, and Monocyte Chemotactic Protein (MCP)-1. Interestingly, IL7 levels increased significantly post-pasteurization, possibly due to its release from cellular or fat compartments into the aqueous fraction. However, Macrophage Inflammatory Protein-1β (MIP-1β) levels were significantly reduced (Espinosa-Martos et al., 2013). In mature donor milk, IL2, IL4, IL5, IL12, and IL13 remained unaffected after pasteurization, while IL10, IL1β, IFN-γ, IL6, and TNF-α were all decreased (Delgado

et al., 2014; Ewaschuk et al., 2011; Untalan et al., 2009). Additionally, a significant increase in IL8 was observed following Holder pasteurization (HoP) in mature DHM (Ewaschuk et al., 2011). Overall, cytokines show varying levels of thermal resistance, and the biological significance of these changes in specific contexts has yet to be fully explored.

3.6.7 Growth Factors

Only a few studies have investigated how pasteurization affects growth factors (GFs) in HM, with each study typically focusing on a specific GF. Transforming Growth Factor (TGF)-β2 was found to be stable in colostrum (Espinosa-Martos et al., 2013), while Epidermal Growth Factor (EGF) and TGF-β1 showed no significant differences pre-and post-pasteurization (Untalan et al., 2009). Heparin-Binding Epidermal-like Growth Factor (HB-EGF) was unaffected by heat, but Hepatocyte Growth Factor (HGF) was significantly reduced (Ewaschuk et al., 2011). Insulin-like Growth Factors (IGF)-1 and 2, along with IGF-binding proteins 2 and 3, were reduced to varying degrees by pasteurization. Granulocyte-macrophage colony-stimulating factor (GM-CSF) concentrations increased significantly post-pasteurization. At the same time, granulocyte-colony stimulating factor (G-CSF) was undetectable in all samples, both before and after HoP, in colostrum and in mature milk (Espinosa-Martos et al., 2013; Ewaschuk et al., 2011). As with cytokines, the limited number of studies and the variability between different growth factors make it difficult to draw generalized conclusions about the effects of HoP.

3.7 Comparison of pasteurized and not pasteurized human milk

The fat concentration in raw human milk slightly decreases after pasteurization. Similarly, the protein content shows a minor reduction from raw milk to pasteurized milk. In contrast, the lactose concentration remains relatively stable as seen in Table 3.1. Pasteurization leads to slight reductions in fat and protein concentrations, while the overall impact on lactose is minimal, which indicates that the primary energy source for infants is largely preserved. However, the observed reductions in protein levels affects the bioactivity of heat-sensitive proteins such as immunoglobulins and lactoferrin, which are crucial for immune function and overall infant health (Vieira et al., 2011).

Table 3-1. Comparison of mean fat, protein and lactose concentrations (mg%) in human milk through the studied processes. (Vieira et al., 2011)

	Raw		**Pasteurized**		**Thawed**		
	Mean ±Sd	**Median**	**Mean±Sd**	**Median**	**Mean±Sd**	**Median**	**P^a**
Fat	2.17±1.46	1.72	2.05±1.46	1.67	2.00±1.45	1.60	<0.001
Protein	1.03±0.39	0.95	0.99±0.42	0.92	0.97±0.41	0.89	<0.001
Lactose	6.36±0.51	6.49	6.28±0.54	6.48	6.34±0.55	6.48	0.427

3.8 Impact on infant health

3.8.1 Antibacterial impact

The significant loss of lactoferrin after HoP could reduce the milk's antibacterial and antioxidant properties and its ability to enhance iron absorption in infants. The overall reduction in the concentration and activity of host defense proteins due to Holder pasteurization might affect the clinical outcomes for infants, potentially making them more susceptible to infection-related complications than those fed fresh mother's milk (Akinbi et al., 2010). However, feeding donor human milk (DHM) to very-low-birth-weight (VLBW) infants instead of formula improves feeding tolerance and offers protection against necrotizing enterocolitis (NEC), although the slower growth often associated with DHM compared to formula feeding is a significant concern (Quigley et al., 2019).

3.8.2 Digestive impact

The slower growth in infants fed DHM is thought to be due to alterations in the nutrient content and bioactivity of the milk following processing, caused by Holder pasteurization (HoP). These alterations may impact the milk's digestibility and absorption, leading to suboptimal growth outcomes (Pitino et al., 2023). While HoP is effective in inactivating pathogens, it also significantly reduces the activity of bile salt-stimulated lipase, an enzyme crucial for fat digestion.

The reduction in bile salt-stimulated lipase and other lipases significantly impairs fat digestion and absorption in neonates. This is critical because neonates rely heavily on these enzymes for efficient nutrient absorption with their immature digestive systems. The decrease in lipase activity is linked to reduced lipolysis kinetics, which may contribute to slower infant growth (O'Connor et al., 2015; Piemontese et al., 2012).

3.8.3 Immunoprotective Biomolecules

An increase in immunoprotective biomolecules in the MFGM after HoP treatment might be due to protein aggregation from the serum, which could have implications for neonatal immunity.

3.9 Other Pasteurization Methods

3.9.1 High-Temperature Short-Time pasteurization

High-Temperature Short-Time (HTST) was the first alternative to Holder Pasteurization (HoP) tested for enhancing the nutritional and immunological quality of milk, with its introduction in the dairy industry in 1930s (Holsinger et al., 1997). This method typically involves heating thin layers of milk in continuous flow systems at 72°C for 15 seconds. HTST has shown promising results when applied to donor human milk (DHM) treatment (Escuder-Vieco et al., 2018). Research indicates that HTST is at least as effective as HoP in ensuring the microbiological safety of human milk, while also being superior in preserving its antioxidant potential, lactoferrin content and structure, vitamins B and C, and certain

cytokines. It effectively eliminates any vegetative cells present in raw human milk samples, including both Gram-negative and Gram-positive bacteria and yeast, although it does not eliminate spore-forming bacteria like *Bacillus cereus*, similar to HoP (Moro et al., 2024). HTST treatment is superior to HoP in preserving the nutritional quality of donor human milk (DHM) because it minimizes the loss of thermosensitive components, such as phospholipids, polyunsaturated fatty acids (PUFAs), and Bile Salt-Stimulated Lipase (BSSL), while also maintaining higher levels of bioactive factors like immunoglobulins, including secretory IgA (sIgA) and lactoferrin. The process needs instrument implementation to be extended to a larger use in DHM bank (Escuder-Vieco et al., 2018, 2021).

3.9.2 High Pressure Processing

High-Pressure Processing (HPP) is a promising non-thermal technique increasingly used in the food industry. This method applies high hydrostatic pressure, typically ranging from 400 to 800 MPa, for short durations (5-10 minutes), effectively inactivating pathogenic microorganisms while preserving the nutritional integrity and sensory qualities of the product (Considine et al., 2007; Huppertz et al., 2006). HPP can be applied to both solid and liquid foods, making it versatile for various applications, including donor human milk. One of the significant advantages of HPP is its ability to inactivate a wide range of microorganisms. Studies have shown that HPP effectively destroys vegetative cells of bacteria such as *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella spp.* at pressures between 300 and 400 MPa. This level of microbial inactivation is comparable to that achieved through thermal pasteurization methods like Holder Pasteurization (HoP) (Permanyer et al., 2010; Viazis et al., 2008; Windyga et al., 2015). However, while HPP is effective against many pathogens, it may not completely inactivate bacterial spores, which can survive high-pressure treatments unless combined with thermal processes (Demazeau et al., 2018). In terms of nutritional quality, HPP has been found to better preserve bioactive compounds compared to traditional thermal methods. Components such as immunoglobulins (IgA, IgM, IgG), lactoferrin, lysozyme, and various cytokines remain largely intact after HPP treatment (Escuder-Vieco et al., 2018; Sousa et al., 2014). This preservation is crucial as these bioactive factors contribute significantly to the health benefits of human milk. HPP also minimizes changes to thermosensitive components like phospholipids and polyunsaturated fatty acids (PUFAs), which are vital for infant development. It also maintains the sensory qualities of human milk better than HoP. Research indicates that the changes in volatile components and overall taste are less pronounced with HPP compared to thermal methods, making it a more favorable option for maintaining the natural characteristics of milk (Contador et al., 2015; Garrido et al., 2015). However, it is essential to note that while pressures below 600 MPa do not significantly affect the lipid fraction of HM, higher pressures may lead to undesirable changes in fatty acid content and increase the risk of lipid oxidation products (Martysiak-Żurowska et al., 2017; Moltó-Puigmartí et al., 2011) . Despite its advantages, the implementation of HPP in human milk processing faces challenges, primarily related to equipment costs and scaling. The initial investment for HPP technology can be significantly higher than traditional

methods like HoP, with estimates suggesting that pascalized donor milk could cost about 130% more than heat-treated milk (Moro et al., 2019).

3.9.3 Microwave treatment

Microwave heating (MWH) involves using a controlled microwave device that maintains a constant temperature for a short duration. Research by Malinowska-Panczyk and colleagues showed that while baseline levels of lipase and transforming growth factor beta 2 (TGF-β2) decreased similarly to those in Holder pasteurization (HoP), lactoferrin and IgA were better preserved with MWH. Furthermore, MWH effectively inactivated inoculated microbiota at temperatures of 62.5°C or 66°C for 5 or 3 minutes, respectively (Malinowska-Pańczyk et al., 2019; Martysiak-Żurowska et al., 2021). Additional studies revealed that samples treated with MWH at 62.5°C for 5 minutes maintained enzyme activities comparable to those subjected to HoP. The concentrations of macronutrients, fatty acids, lipid peroxides, and α-lactalbumin after MWH were similar to those found in raw milk, with no formation of furosine (Martysiak-Żurowska et al., 2019). After treatment at 2450 MHz with a maximum power of 300 W, at 60°C for 30 seconds, Leite and colleagues observed a reduction in microbial contamination in experimentally inoculated human milk, specifically against *Staphylococcus aureus* and *Salmonella typhimurium* at concentrations of 10^6 CFU/mL, comparable to the effects achieved through HoP. They also reported that immunoglobulins and lactoferrin levels did not significantly decrease after microwave treatment at 60°C for 30 seconds or 65°C for 15 seconds; although, HoP resulted in substantial reductions in IgA, IgG, IgM, and lactoferrin concentrations (Leite et al., 2019, 2022). These findings suggest that microwave heating can be an effective alternative to traditional pasteurization methods while preserving the nutritional and bioactive components of human milk more effectively (Moro et al., 2024).

3.9.4 Ultraviolet C radiation

Short-wavelength ultraviolet C (UV-C) radiation, which operates within a wavelength range of 200–280 nm, is increasingly recognized as a treatment method in the food industry. This technique effectively penetrates microorganisms, damaging their nucleic acids and preventing essential physiological processes, without altering the organoleptic properties of the food being treated. However, UV light has limited penetration depth in food materials, only a few millimeters, which means that cloudy liquids like milk cannot be effectively treated unless exposed in thin layers (Moro et al., 2024). Recently, studies have enhanced understanding of UV-C's effects on DHM. Kontopodi and colleagues demonstrated that UV-C could be a viable alternative to Holder pasteurization (HoP), achieving sufficient microbiological inactivation while better preserving bioactive components. Their research resulted in reduction of *Enterobacter cloacae*, *E. coli*, and *Staphylococcus epidermidis*, with minimal impact on levels of IgA, lactoferrin, lysozyme, and bile salt-stimulated lipase (BSSL) (Kontopodi et al., 2022). UV-C treatment also preserved insulin levels in DHM (Mank et al., 2021). UV-C at 2,259 J/L effectively reduced *S. aureus* growth without affecting the bioactivity of immune proteins or

producing enterotoxins (Almutawif et al., 2019). Despite its potential, the lack of appropriate equipment for evaluating UV-C technology in human milk banks remains a significant challenge. There are currently no published data on UV-C's effectiveness against bacterial spores present in human milk; however, studies indicate its ability to eliminate spores in various liquids like orange juice and distilled water (Assal et al., 2023; Colás-Medà et al., 2021). This suggests potential for future research into UV-C's application for ensuring the safety and quality of donor human milk while preserving its nutritional and bioactive properties (Moro et al., 2024).

Chapter 4
Comparing Proteome of Human Milk and Infant Formula

4.1 Introduction

Although human milk is rich in essential nutrients and diverse bioactive ingredients, making it the first functional food in an infant's life, it is not always a viable or acceptable option. In some situations, breastfeeding may be impractical, insufficient, or unavailable. Therefore, finding a suitable alternative becomes crucial.

In such cases, infant formulas are essential for providing proper nutrition. The industry's primary goal is to replicate the composition of human milk as closely as possible (Floris et al., 2010). Extensive knowledge of human milk composition has significantly contributed to the development of infant formulas. Table 4.1 presents a comparison of the composition of human milk, bovine milk, and infant formula milk, highlighting differences and similarities in their nutritional profiles. However, there are differences in digestibility and bioavailability between breast milk and infant formula nutrients. Also the composition of human milk is not static; it changes throughout the breastfeeding period and is influenced by various factors such as the environment and the mother's diet (Lönnerdal, 2004, 2014). Today, companies and research centers are dedicated to improving the quality of infant formulas. Their focus is on matching the macronutrient and micronutrient levels of human milk and incorporating bioactive compounds to make formulas as similar to human milk as possible (Martin et al., 2016). The ultimate goal of infant formula development is to achieve similar physiological effects to those observed in breastfed infants rather than replicating the exact composition of human milk in every detail (Gómez Gallego et al., 2009)

The latest trend in infant formula production emphasizes the inclusion of functional ingredients that naturally occur in human milk. Ingredients like probiotics, prebiotics (oligosaccharides), proteins such as lactoferrin and α-lactalbumin, nucleotides, and polyunsaturated fatty acids (mainly docosahexaenoic and arachidonic acids) are being added to enhance the functionality of these formulas (Joeckel & Phillips, 2009). Research has shown that infant formulas enriched with these bioactive components are more effective than those without additions (Aly et al., 2016).

Table 4-1. Composition of Human milk, Bovine milk and infant formula milk adapted from (Guo, 2020)

	Human milk	Bovine milk	Infant formula
Protein	1.00	3.40	1.8 - 3.0
Casein:Whey protein	30:70	80:20	40:60 - 50:50
Fat	3.80	3.50	3.5 - 4.5
Lactose	7.00	5.00	7.0 - 8.0
Ash	0.20	0.70	0.5 - 1.0

4.2 Proteomic composition of Infant Formula

4.2.1 Protein source and composition

Milk from various animals especially bovine milk has been explored as substitute for breast milk. Infant formulas utilize proteins from bovine milk, which is modified to adjust the protein composition to better match human milk. However, there is a differing protein composition between human milk and bovine milk. Human milk whey:casein ratio is about 60:40 in mature human milk while bovine milk has a higher casein content, around 20:80 (whey:casein) (Donovan, 2019; Kunz & Lönnerdal, 1992).

4.2.2 Protein content

The protein content in infant formula has historically been significantly higher than in human milk. Although this gap has narrowed in recent decades, the difference persists (Lönnerdal, 2014). The higher protein intake in formula-fed infants results in significantly elevated levels of most plasma amino acids and blood urea nitrogen compared to breastfed infants. This has raised concerns about potential metabolic stress on developing organs like the liver and kidneys. More troubling is the significantly higher serum insulin levels in formula-fed infants, likely due to increased concentrations of branched-chain, insulinogenic amino acids (valine, leucine, isoleucine) (Räihä & Axelsson, 1995).

When the protein content in infant formula is reduced, the amino acid composition becomes crucial to ensure that the serum levels of essential amino acids in formula-fed infants remain comparable to those in breastfed infants. When reducing the protein content in infant formula, tryptophan often becomes the first limiting amino acid (Lönnerdal, 2003). The addition of free amino acids (tryptophan for example) has been the strategy used to address this. However, this free amino acid is likely absorbed more rapidly than those bound within proteins, which require proteolysis before the body can take them up. This rapid absorption may lead to faster delivery to target tissues like the liver and brain, which may have metabolic implications. Still, clinical studies have shown that reducing the overall protein content in infant formula while increasing the proportion of α-lactalbumin can result in plasma tryptophan levels comparable to those found in breastfed infants (Davis et al., 2008; Sandström et al., 2008).

4.2.3 Protein requirement in infant milk

The Directive 2006/141/EC specifies the allowable sources of protein for use in infant formula (IF) which includes bovine milk protein. The directive also outlines the currently permitted minimum and maximum protein content in IF (European Commission, 2007). These regulations are compared with the recommendations provided by the Scientific Committee on Food (SCF) in (Scientific committee on food, 2003a), as shown in Table 4.2.

Table 4-2. Recommended protein content in infant formula

Bovine milk	**Directive 2006/141/EC**		**SCF**	
Unit	**Minimum**	**Maximum**	**Minimum**	Maximum
g/100 kcal	1.80	3.0	1.80	3.0
g/100 kJ	0.45	0.7	0.45	0.7

Currently permitted minimum and maximum amounts of protein in infant formula (European Commission, 2007; Scientific committee on food, 2003a).

4.3 Infant protein requirement

The average requirement (AR) and a population reference intake (PRI) for protein for infants using a factorial approach were established by EFSA (EFSA NDA Panel, 2012, 2013). This approach calculates the requirement as the sum of the protein needed for maintenance and the protein required for growth, adjusted for the efficiency of dietary protein utilization. In Table 4.3, the protein intakes deemed adequate for most of infants are presented, based on nutrient requirements and dietary intakes for infants and young children in the European Union.

Table 4-3. Protein Reference Intake (PRI) for Infants

Age (months)	**PRI (g/kg body weight per day)**	**Body weight (kg)[(a)]**		**PRI (g/day)**	
		Boys	**Girls**	**Boys**	**Girls**
1 to < 2	1.77	4.5	4.2	8	7
2 to < 3	1.50	5.6	5.1	8	8
3 to < 4	1.36	6.4	5.8	9	8
4 to < 5	1.27	7.0	6.4	9	8
5 to < 6	1.21	7.5	6.9	9	8
6 to < 7	1.15	7.9	7.3	9	8
7 to < 8	1.27	8.3	7.6	11	10
8 to < 9	1.23	8.6	7.9	11	10
9 to < 10	1.19	8.9	8.2	11	10
10 to < 11	1.16	9.2	8.5	11	10
11 to < 12	1.14	9.4	8.7	11	10

(a): 50th percentile of WHO Growth Standards (EFSA NDA Panel, 2013)

There is no PRI for the age group from birth to less than one month due to insufficient data for the first month of life. However, it is reasonable to assume that protein requirements during the first month are not significantly different from those in the second month of life.

4.4 Health Consequences

4.4.1 Protein Intakes and Growth

Several studies generally indicate that protein concentrations in formulae of 1.8-1.9 g/100 kcal, derived from intact milk protein, support normal growth when these formulae are fed as much as the infant desire without restriction. A study involving infants fed with a low-protein IF containing 1.77 g protein per 100 kcal and a follow-up formula (FOF) providing 2.2 g protein per 100 kcal during the first year of life showed no statistically significant differences in weight-for-length and body mass index (BMI) at 24 months, compared to a breastfed reference group (Koletzko et al., 2009). Similarly, another study found no significant differences in weight gain, length gain, and head circumference at four months of age between infants fed with an IF containing 1.9 g/100 kcal protein and those fed with an IF containing 2.2 g/100 kcal protein (Trabulsi et al., 2011).

4.4.2 High Protein Intakes

In infants, an excessively high protein intake (approximately 20% of total energy intake) can disrupt water balance, especially when no other liquids are consumed or when extrarenal water losses are increased (EFSA NDA Panel, 2012). It has been suggested that a high protein intake may lead to heightened insulin secretion and increased levels of insulin-like growth factor (IGF)-1 and IGF-binding protein (IGFBP)-1 (Axelsson, 2006). Additionally, high protein intake has been linked to accelerated growth and a higher BMI in childhood (Hörnell et al., 2013; Koletzko et al., 2009; Weber et al., 2014).

4.4.3 Protein Quality

The protein quality of infant formula (IF) refers to its ability to meet the body's metabolic needs for amino acids and nitrogen. Digestible indispensable amino acid scores (DIAAS) are used to evaluate the true ileal digestibility (Mathai et al., 2017). Amino acid reference patterns are crucial for evaluating protein quality by comparing the amino acid composition of food with a standard reference. Since breast milk from a healthy, well-nourished mother meets the amino acid needs of infants for the first six months, breast milk's amino acid profile is used as the ideal reference for products that substitute for breast milk.

The levels of essential and conditionally essential amino acids per energy value in infant and follow-on formulas are based closely on the amino acid content of human milk (Darragh & Moughan, 1998; Räihä & Axelsson, 1995; Villalpando et al., 1998). Due to the lower cysteine formation from cystathionine in the transsulfuration pathway, L-cysteine is considered a conditionally essential amino acid for neonates, meaning methionine cannot fully substitute for cysteine. Including both cysteine and methionine in infant and follow-on formulas is essential. The ratio of methionine to cysteine should not exceed two unless safety and suitability are clinically demonstrated (Viña et al., 1995).

Tyrosine is synthesized through the hydroxylation of phenylalanine in the liver. Studies on human neonates show a substantial capacity for phenylalanine hydroxylation (van Toledo-Eppinga et al., 1996;

House et al., 1998), but it is unclear how well neonates can manage high phenylalanine and low tyrosine intake via this process. Infants may need a direct dietary source of tyrosine because low phenylalanine hydroxylase activity in some neonates can lead to hyperphenylalaninemia and tyrosine deficiency (House et al., 1998; Van Toledo-Eppinga et al., 1996). Both tyrosine and phenylalanine in infant and follow-on formulas are required, with the tyrosine to phenylalanine ratio not exceeding two unless clinical evaluation ensures safety and suitability (Scientific committee on food, 2003b).

4.5 Effects of Processing on Nutritional Value of Protein

The nutritional value of protein is affected by its amino acid composition, protein hydrolysis, and heat treatment—especially in the presence of iron, vitamin C, and lactose. Heat processing, such as pasteurization, homogenization, and spray drying, impact the properties of infant formula, primarily due to interactions between proteins and carbohydrates, proteins and lipids, as well as protein-protein interactions. These thermal processes also affect other components and alter the physicochemical, structural, and reconstitution properties of infant formula (Sun et al., 2018). Heat treatment during production promote Maillard reaction and leads to protein aggregation or denaturation, as proteins are susceptible to heat (Nunes et al., 2019). Maillard reaction, a chemical process between the carbonyl group of reducing sugars and free amino acids, can occur during the heat treatment of infant formula, resulting in the formation of Schiff's base, such as lactosyl-lysine (Nunes et al., 2019). Lysine is the most reactive amino acid, although sugars can also react with histidine, arginine, tryptophan, and methionine (Kamdem & Tsopmo, 2019; Mehta & Deeth, 2016). Schiff's bases are chemically unstable and can undergo further isomerization, known as the Amadori rearrangement, leading to the formation of lactulosyl-lysine (the Amadori product). This process can reduce lysine bioavailability, resulting in nutritional loss (Mehta & Deeth, 2016; Zenker et al., 2020). Prolonged heating under acidic or neutral conditions can generate various furfural compounds like 5-methyl-2-furaldehyde, 5-hydroxymethylfurfural, 2-furyl-methyl ketone, and 2-furaldehyde (Chávez-Servín et al., 2015; Shen et al., 2022). Advanced glycation end-products, such as N^{ε}-carboxymethyl-lysine, may have potential pro-oxidant and pro-inflammatory effects on health (Elmhiri et al., 2015). Aldehydes, reductones, furfurals, and other intermediates may react with amines to form high molecular weight dark polymeric compounds called melanoidins (Singh et al., 2021). Melanoidins can chelate metals, which may impair the absorption and metabolism of essential minerals like Zn, Ca, Cu, Mg, and Fe (Rannou et al., 2016; Roncero-Ramos et al., 2016). Newer techniques have been developed to study the generation of these undesirable compounds during thermal processing, including the prediction of advanced glycation end-products (AGE) using the Molecular Transformer model curated with literature data (Yang et al., 2023).

Protein aggregation results in increased viscosity, reduced emulsion stability, and diminished overall performance during processing (Joyce et al., 2017). Protein aggregation often leads to the loss of biological activity, which encourages protein coagulation (Borad et al., 2017; Qian et al., 2017; Souza et al., 2015). Among the proteins, casein is more heat-stable compared to whey (Fox et al., 2015).

However, intense heating of casein can cause its breakdown, leading to dephosphorylation and the loss of its protective function, particularly when glycosylated. Since β-casein plays a crucial role in the bioavailability of zinc and calcium, its functionality loss could lead to potential problems in infants (Lönnerdal et al., 2017).

Whey proteins are more susceptible to heat, which can alter their nutritional and functional properties (Golkar et al., 2019). The solubility of milk proteins decreases when whey proteins interact with casein micelles, leading to their aggregation or dissociation. This, in turn, affects the functionality and hydrophobicity of reconstituted products (Borad et al., 2017; Souza et al., 2015). The most sensitive proteins are immunoglobulins (Igs), which are present in lower concentrations (McCarthy et al., 2022). Upon heating, the bioavailability of zinc and calcium decreases as α-lactalbumin loses its ability to bind these minerals. Although human milk does not contain β-lactoglobulin, bovine milk-derived infant formula, when exposed to heat, can cause this protein to become insoluble or denatured (Rafe & Razavi, 2015) .To prevent β-lactoglobulin aggregation during thermal processing, increasing the concentration of α-lactalbumin may be an alternative (Buggy et al., 2017). Heat-induced protein denaturation can also influence protein allergenicity by exposing or masking epitopes, depending on the intensity of the heat treatment (Golkar et al., 2019).

4.6 Comparative Proteomics of Human and Bovine Milk

Recent comparative proteomic analyses of human and bovine milk have revealed significant differences and similarities between the two, shedding light on the evolutionary adaptations that have tailored these milk to meet the specific needs of human infants and calves (Beck et al., 2015; Reinhardt et al., 2012; Reinhardt & Lippolis, 2008). These studies highlight significant differences in the proteomic profiles of human and bovine milk, reflecting evolutionary adaptations to meet the specific nutritional and immune needs of human infants and calves.

One study identified 268 proteins in human milk and 269 in bovine milk, with 147 shared proteins between the two species (Hettinga et al., 2011). A similar study found 379 quantified proteins, with 93 shared proteins between the two (L. Zhang et al., 2016), indicating some overlap but also significant species-specific differences in protein content and function. Both studies classified the proteins using Gene Ontology (GO), with human milk showing a higher abundance of immune-related proteins, such as lactoferrin (LTF) and immunoglobulin A (IgA), lysozyme (LYZ), essential for mucosal immunity which provides infants with robust defense mechanisms (Hettinga et al., 2011). By contrast, though initially rich in immunoglobulins, bovine saw a rapid decline in these proteins after the first few days of lactation (L. Zhang et al., 2015). However, bovine milk was richer in antibacterial proteins, including lactoperoxidase (LPO) and cathelicidins, reflecting the environmental challenges faced by ruminants (Lemay et al., 2009).

As mentioned, a key difference between human and bovine milk is the variety and concentration of immune-related proteins. Lactoferrin (LTF), complement 3 (C3), and osteopontin (SPP1) were abundant in both species, but their concentration decreased more rapidly in bovine milk compared to human milk, suggesting longer-lasting immune protection from human milk (Liao et al., 2011; Q. Zhang et al., 2013). In another study, LTF was firmly bound to the milk fat globule membrane (MFGM) in human milk, where it likely contributes to protecting the mammary gland and providing immune defense to the infant (Cho et al., 2000; Hettinga et al., 2011).

Proteins like CD14, part of the Toll-like receptor complex that detects bacterial components, were present in both species' colostrum but disappeared from commercial bovine milk after processing. The persistence of CD14 and other immune factors, such as complement proteins, in human milk underscores the specialized role of human milk in providing long-term immune support (Hettinga et al., 2011).

Another distinctive feature is the presence of serine protease inhibitors (SERPINA1 and SERPINA3) significantly higher in human milk, which protect bioactive proteins like lactoferrin and immunoglobulins from premature digestion in the infant's gastrointestinal tract (Hettinga et al., 2011; L. Zhang et al., 2016). These inhibitors are less prominent in bovine milk, which may reflect the different nutritional and developmental needs of calves compared to human infants (Stelwagen et al., 2009).

Bovine milk contained a higher concentration of transport proteins, such as β-lactoglobulin (LGB), especially lipid transporters, which are absent in human milk. This absence contributes to the lower concentration of transport proteins in human milk overall (Hinz et al., 2012; Lönnerdal, 2003). Human milk, however, is enriched in enzymes like bile salt-activated lipase (BSSL) exclusively and lipoprotein lipase (LPL), which are vital for nutrient digestion in infants, who have an underdeveloped digestive system (Piemontese et al., 2012). BSSL plays a crucial role in lipid digestion, compensating for the low pancreatic lipase activity in human infants before weaning (Dallas, Smink, et al., 2015; Lindquist & Hernell, 2010). In contrast, bovine milk is rich in ribonucleases (RNASE1 and RNASE4), which are more suited to the digestion requirements of calves. Calves experience a significant increase in pancreatic lipase within days after birth, reducing their reliance on BSSL for lipid digestion (L. Zhang et al., 2015).

A more significant number of quantified MFGM proteins compared to milk serum proteins in both human and bovine samples have been identified. This is because MFGM originates from the epithelial cells where milk fat is synthesized and secreted (Lu et al., 2014; McManaman & Neville, 2003). For the development of infant formulas, the isolation and inclusion of key bioactive proteins from bovine, can help bridge the gap between bovine milk-based formulas and the nutritional complexity of human milk (L. Zhang et al., 2016).

Bovine milk has been widely used as a base for infant formulas, but the absence of certain key bioactive proteins, such as BSSL and higher concentrations of LTF and IgA, limits its nutritional equivalence to human milk. However, lactoferrin extracted from bovine milk is used as a functional ingredient in infant formula due to its known benefits in neonatal development and immune function (Lu et al., 2018). Another promising bioactive protein, osteopontin (SPP1) (Schack et al., 2009), found in higher concentrations in yak milk than in cow or goat milk, could offer alternative sources of beneficial proteins for infant formula production (L. Zhang et al., 2016).

Chapter 5
Conclusion and Recommendations

5.1. Conclusion

This thesis has examined the impact of Holder pasteurization on the composition and bioactivity of human milk proteins, contributing significantly to our understanding of the nutritional and functional integrity of human milk. The findings underscore the critical importance of human milk as the optimal source of nutrition for infants, particularly in the first months of life, when their growth and development are most rapid. Human milk is uniquely designed to meet infants' nutritional needs, providing essential macronutrients such as proteins, fats, and carbohydrates and a variety of bioactive compounds that support immune function and overall health. The dynamic composition of human milk, which varies throughout lactation and even within a single feeding session, reflects the infant's changing needs. This adaptability is crucial for promoting optimal growth, cognitive development, and protection against infections. While Holder pasteurization effectively eliminates harmful pathogens, it may inadvertently compromise the bioactivity of crucial proteins. For instance, the denaturation of immunoglobulins, lactoferrin, and other bioactive proteins can diminish their protective functions, vital for the infant's immune defense. The comparative analysis with bovine infant formula highlighted the significant differences in protein composition and functionality, reinforcing that human milk provides unique benefits that are not entirely replicated in infant formulas.

Moreover, the thesis emphasizes the necessity for ongoing research into the optimizing pasteurization processes to minimize the loss of bioactive compounds. This is particularly relevant for donor human milk, which is increasingly utilized as a substitute when maternal breastfeeding is not feasible. There should be a balanced approach that prioritizes both microbial safety and the preservation of the nutritional quality of human milk. This thesis not only elucidates the effects of Holder pasteurization on human milk but also serves as a call to action for researchers, healthcare providers, and policymakers to prioritize the integrity of human milk in infant feeding practices. This implications extend beyond academic inquiry; they have real-world significance for the health and well-being of infants who rely on donor milk or formula. As we move forward, it is imperative to continue exploring innovative methods that can enhance the safety and nutritional quality of human milk, ensuring that all infants receive the best possible start in life. By fostering a deeper understanding of the nuances of human milk composition and the effects of processing methods, we can better support the nutritional needs of infants in diverse feeding scenarios.

5.2. Recommendations and Future Research Ideas

i. Future research should focus on exploring alternative pasteurization methods that are already in use on other foods in order to minimize the degradation of bioactive proteins in breastmilk while ensuring microbial safety.

ii. A combined approach to pasteurization methods can also be implemented. This can be achieved by sequentially applying different pasteurization methods where one method is utilized until it reaches its threshold for bioactive loss, then followed by another method that addresses its limitations. This way, nutrient degradation can be minimized while maintaining safety standards.

iii. Studies can be conducted to track the health and developmental outcomes of infants fed pasteurized human milk versus those fed non-pasteurized milk or infant formula. This can provide valuable data on the long-term effects of pasteurization and infant formula on infant health.

iv. Pooled milk could be sourced from mothers who are at the same stage of lactation. Given that milk composition varies significantly throughout the lactation period, this ensures that the pooled milk provides a consistent and appropriate nutrient profile tailored to the specific needs of infants at similar feeding stages. The nutritional content will align better with the developmental requirements of growing infants.

v. How maternal diet, health, and environmental factors influence the composition of human milk and its response to pasteurization needs to be checked. This can provide insights into optimizing milk quality for donor banks.

vi. Future research should also focus on formulating infant formulas that better mimic the nutritional and bioactive profiles of human milk, potentially incorporating elements that compensate for any losses incurred during pasteurization.

vii. Expand research to include a broader range of milk sources from various mammalian species by investigating and comparing the compositions of milk from different mammals, such as cows, goats, sheep, camels, and buffaloes. Researchers can identify unique bioactive components and nutrients that may complement one another. This approach is similar to the practice of combining plant-based foods to create a balanced diet. By sourcing bioactive proteins and essential nutrients from multiple mammalian milks, it is possible to formulate a more nutritionally complete infant formula.

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