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Title: Identifying Differences in the Release of Bioactive Milk Peptides Across the Intestinal Tract Between Term and Preterm Infants

Abstract approved:

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Premature birth interrupts the typical development of the human fetus, leaving the infant born with underdeveloped gastrointestinal and immune systems. Preterm infants have reduced stomach acidity, reduced digestive protease activity, more permeable intestinal membranes, impaired innate and adaptive immune response, and poor microbiome development. Due to these factors, preterm infants are at increased risk for developing a variety of infections and complications, such as necrotizing enterocolitis, sepsis, retinopathy of prematurity, and respiratory distress syndrome. Furthermore, preterm infants are born at an earlier stage of gestation at which they would still be reliant upon the maternal placenta for nutrient delivery and thus are less well equipped to handle the introduction of large quantities of nutrients directly into their gastrointestinal system. The preterm infant's inability to efficiently digest the nutritious components of milk, such as protein, may contribute to their increased risk for developing infectious diseases, as they are less able to release bioactive factors that contribute to their gastrointestinal development. Human milk proteins are cleaved into thousands of peptides as they progress through the gastrointestinal system, many of which have been identified with

bioactivities beneficial to the infant, such as antimicrobial, bifidogenic, immunomodulatory, and antioxidant activities. For these peptides to be active in the infant, they must first be released from their parent protein and then survive additional digestion before reaching their site of activity, often in the intestinal tract or bloodstream. However, if and where along the infant gastrointestinal tract milk bioactive peptides are released is currently unknown. Furthermore, it is unknown how preterm infants may differ from term infants in their ability to release these peptides. The central hypothesis of this research is that preterm infants, due to their impaired gastrointestinal function, will have reduced bioactive peptide release throughout the gastrointestinal system compared with term infants. To test this hypothesis, I performed a series of peptidomic experiments identifying and categorizing the human milk peptides at various stages of infant digestion.

The results of the first experiment identifying human milk and bovine milk fortifier peptides in the human milk and the preterm infant over a period of three hours are presented in Chapter 2. Milk peptide release increased significantly from milk to the stomach and in the stomach over time. The rate of bioactive and potentially bioactive peptide release increased in a similar manner as total peptides. These results contribute to the understanding of how milk proteins are digested and peptides are released in the infant stomach. They also establish the initial release of bioactive peptides as milk is fed to the infant.

The results of the second experiment identifying human milk peptides in preterm and term infant stool are presented in Chapter 3. Whereas the gastric contents represent the beginning of digestion and release of peptides, the stool represents the terminus and

everything that has survived digestion. Over one hundred milk peptides were present in the stool, and thousands more that could derive from proteins known to be in human milk. One bioactive milk peptide was present, and thus may have had the opportunity to be active in the intestinal tract. The stool peptide profiles of preterm and term infants were distinct from each other, with several peptides significantly higher in abundance in one group over the other. This study was the first to determine the capacity for bioactive human milk peptides to survive the entirety of gastrointestinal digestion.

Human milk peptide release was compared from milk, to the stomach, to stool and between term and preterm infants at each site in Chapter 4. Though peptide release was equivalent between infant groups in the milk and stool, peptide abundance was significantly higher in the preterm infants than the term infants. However, term infants had higher abundance of specific milk peptides with antimicrobial activity in the stomach. There was a significant increase in peptide count abundance from milk to stomach, then a decrease from stomach to stool for all infants. These results are the first to compare peptide release after gastrointestinal digestion between preterm and term infants. They suggest that term infants are able to cleave off specific bioactive peptides from the C-terminus of β-casein at higher rates than preterm infants.

Finally, the release of peptides in the infant intestinal tract and the identification of novel antimicrobial peptides are presented in Chapter 5. The bulk peptide extracts of the duodenal/jejunal fluid of preterm and term infants were incubated with *Staphylococcus aureus* and *Escherichia coli* to identify antimicrobial peptides, and *Bifidobacterium infantis* to identify bifidogenic peptides. Several infants had bulk peptide extracts that were highly antimicrobial or bifidogenic. From comparing these extracts

with those that had no activity, seven novel human milk antimicrobial peptides were identified. These results are the first to profile milk peptide release in the intestinal tract and confirm that bioactive peptides are present and potentially active therein.

The summation of these studies is the most detailed map of human milk peptide release across the infant gastrointestinal system to date. Until this point, peptides had only been found in milk and the infant stomach from preterm infants or after *in vitro* digestions of milk. The relevance of the bioactive peptides identified in the literature so far to infant health has not been entirely understood, in part because it was unknown whether the infant was able to release those specific peptides. Peptides in the milk and stomach likely undergo further proteolysis before they reach the intestinal tract where they can act locally or be absorbed in the bloodstream, and peptides from *in vitro* digests may not accurately represent those in in the infant gastrointestinal system, particularly for preterm infants whose systems are less developed and understood. The central hypothesis that preterm infants release fewer bioactive peptides throughout the gastrointestinal system than term infants could only be partly answered, as intestinal samples from only two term infants were able to be collected. In the stomach, it was shown that term infants release one known bioactive peptide at higher abundances than preterm infants and several more potentially bioactive peptides. In the stool, the only identified bioactive peptide was present at similar abundance for both preterm and term infants, though they differed in their total peptide profiles. These studies combine to show that a simple answer for whether term infants release more bioactive peptides than preterm infants may not exist, as peptide release is complicated by stage of digestion, day of life of the infant, starting milk protein/peptide profile, and potentially many other factors.

Identifying Differences in the Release of Bioactive Milk Peptides Across the Intestinal Tract Between Term and Preterm Infants

by
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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Robert L. Beverly, Author

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CONTRIBUTION OF AUTHORS

RLB wrote Chapter 1.

RLB and DCD designed the research in Chapter 2. RLB and MAW conducted the research. RLB analyzed the data and performed statistical analysis. RLB, MAW, and DCD drafted the manuscript. RLB, MAW, and DCD read and approved the final manuscript.

RLB and DCD designed the research in Chapter 3. RLB, RKH, AMM, EAM, and RLM conducted the research. RLB analyzed the data and performed statistical analysis. RLB, RKH, AMM, EAM, RLM, and DCD drafted the manuscript. RLB, RKH, AMM, EAM, RLM and DCD read and approved the final manuscript.

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RLB wrote Chapter 6.

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Chapter 1 – General introduction

Premature birth: rates and burden

Premature birth is defined as any birth before 37 weeks gestational age (GA). The global premature birth rate is about 11% (1) while the United States' rate is slightly less at 10.02%, corresponding to more than 380,000 premature infants born in the United States each year (2). Premature birth is the second highest cause of neonatal mortality and is associated with several physiological and neurological disabilities throughout life (3, 4). Premature birth is classified by varying degrees based upon how many weeks early an infant is born (extremely preterm, <28 weeks GA; very preterm, 28–32 weeks GA; late preterm, 32–37 weeks GA), and the impact of prematurity on health grows more severe with decreasing GA at birth as the infant spends less time developing intrauterinely (3, 5, 6). Treating premature infants has been estimated to have incurred an annual economic burden of 26.2 billion dollars in 2005 (34.87 billion dollars in 2020 after adjusting for inflation) (7), and the total expenditure per infant ranged from $25,000 for infants 35–36 weeks GA to $600,000 for 24 weeks GA (8). Furthermore, premature infants have much longer hospital stays than their term counterparts, from an average of 17 days for late preterm infants to over 60 days for very preterm infants (9, 10), reducing the supply of NICU beds, increasing risk of infection to the infant, and increasing the stress of the parents.

Survival rates for all premature infants have increased tremendously over the past generation with the introduction of therapeutic improvements such as continuous positive airway pressure and antenatal steroids (11). However, extremely premature infants, due to their underdeveloped gastrointestinal and immune systems, remain at increased risk for

developing infectious diseases such as sepsis or necrotizing enterocolitis (NEC), which can have lifelong health impacts (12); and little improvement has been made in reducing the incidence rate of these conditions since 2000 (13).

Physiology of the infant gastrointestinal system

Due to their reduced intrauterine time, premature infants are born with underdeveloped organ systems that perform sub-optimally compared with term infants. Their nervous (14), respiratory (15), cardiovascular (16), and endocrine (17) systems are all impacted and require extensive support to keep them functioning in the NICU, but more critical to understanding the present work is the effect of prematurity on the gastrointestinal (GI) system and microbiome.

The GI system is responsible for the mechanical, chemical, and enzymatic digestion of nutrients so that they can be absorbed and utilized by the infant. The GI system begins development at week 3 of gestation with the initiation of gastrulation and continues over the entirety of gestation and for the first few postnatal months (18). Exposure to growth factors (insulin-like growth factors, epidermal growth factors, transforming growth factors, etc.) in amniotic fluid (19) and human milk (HM) (20) facilitates the development of the GI tract by stimulating cellular proliferation, differentiation, and maturation. Enteral delivery of these growth factors is necessary for intestinal maturation even *in utero* (21, 22), as a swallowing reflex is identifiable in fetuses by week 15 and becomes consistent at weeks 22–24 (23), at which point the fetus is regularly consuming amniotic proteins and amino acids. In the last 15 weeks of development, the intestinal tract length doubles in length, villus length increases, crypt depth increases (18), and tight junctions close (24) under the stimulation provided by

these factors. When an infant is born premature, this critical stage of intestinal development is interrupted; and moreover, the infant no longer receives the majority of its nutrients transplacentally and must thereon rely on its own underdeveloped GI tract to digest and absorb nutrients.

Premature birth affects the digestion, utilization, and regulation of all macronutrients: carbohydrates, lipids, and proteins. Macronutrients are present in food as polymers that must be broken down to their individual monomers or very short chains before they can be absorbed by the body. The GI system accomplishes this through the secretion of various enzymes–glycosidases, lipases, and proteases–at different organs along the GI tract (**Table 1.1**). Each enzyme has specific bonds within the macronutrient structure that it most efficiently cleaves; thus, by the cumulative activity of their entire suite of digestive enzymes, humans are able to break down most foods into their bioavailable components. When one or more enzymes are absent, reduced, or inactive, such as due to the consequences of prematurity, full nutrition is impeded. In terms of protein digestion, premature infants secrete less pepsin (25-27) and have reduced gastric acid secretion and higher gastric pH (25, 26, 28), which impacts the ability of pepsinogen to be cleaved to pepsin, the combined effect of which is diminished protein digestion in the stomach (29, 30). The effect of prematurity on intestinal protein digestion is less certain, as some results from human and animal studies have indicated premature infants secrete lower pancreatic enzymes (31-33), while others found no significant differences in duodenal trypsin activity (34). Though not the focus of the present research, preterm infants do also have reduced digestive lipase (35) activity as well, which can also

indirectly impact protein digestion by inhibiting access to the milk fat globule membrane proteins (36).

Table 1.1. List of digestive enzymes, where they are secreted, and their nutrient activities or site specificities.

Location	Enzyme	Type	Activity/Specificity
Mouth	Salivary amylase	Glycosidase	Starch α-1,4-glycosidic bonds
	Lingual lipase	Lipase	Triglyceride sn-3
Stomach	Pepsin A/C	Protease	Broad specificity
	Gastric lipase	Lipase	Triglyceride sn-3
Intestinal Lumen	Pancreatic amylase	Glycosidase	Starch α-1,4-glycosidic bonds
	Trypsin 1/2/Mesotrypsin	Protease	After Arg, Lys
	Chymotrypsin B/C	Protease	After Phe, Tyr, Trp
	Elastase 2A/2B/3A/3B	Protease	After Ala, Ser, Gly, Val
	Carboxypeptidase A1/2/3	Protease	C-terminal aromatic AAs
	Carboxypeptidase B1/2	Protease	C-terminal Arg, Lys
	Pancreatic triglyceride lipase	Lipase	Triglyceride sn-1 and sn-3
	Pancreatic lipase-related protein 1	Lipase	Unknown
	Pancreatic lipase-related protein 2	Lipase	Triglyceride sn-1 and sn-3, galactolipids
	Carboxyl ester lipase	Lipase	Broad specificity
	Phospholipase A2	Lipase	Phospholipid sn-2
Intestinal Brush Border	Maltase-glucoamylase	Glycosidase	Maltose
	Sucrase-isomaltase	Glycosidase	Sucrose, maltose, and isomaltose
	Lactase	Glycosidase	Lactose
	Trehalase	Glycosidase	Trehalose
	Aminopeptidase A/N/P	Protease	N-terminal AAs oligopeptides
	Dipeptidyl peptidase-4	Protease	N-terminal dipeptides
	Carboxypeptidase P	Protease	C-terminal AAs
	Angiotensin-converting enzyme	Protease	C-terminal dipeptides
	Glutamate carboxypeptidase II	Protease	C-terminal AAs from oligopeptides
	Dipeptidase 1	Protease	Dipeptides
	Meprin A subunit β	Protease	Between acidic AAs
	Neprilysin	Protease	Broad specificity
	Phospholipase B1	Lipase	Phospholipid and diglyceride sn-2
	Alkaline sphingomyelinase	Lipase	Sphingomyelin
	Neutral ceramidase	Lipase	Ceramides

In healthy adults, proteins can only be absorbed by the GI tract after they have been digested into peptides (di- or tri-) or amino acids. Peptides and amino acids are absorbed by a variety of transporters expressed on the luminal surface of the enterocyte, such as PEPT1 for peptides or SNAT5 for glutamine. Animal studies have shown that low levels of peptide and amino acid absorption begins in the fetus (22, 37). Upon birth, amino acid transport capacity temporarily decreases for the first few days as the intestinal tract rapidly expands in length and surface area, and colonic amino acid transportation is

present potentially as a compensatory response (38). There are few data on differences between preterm and term infants in the expression of amino acid transporters or rates of amino acid absorption. A recent study on jejunal/ileal biopsies showed that PEPT1 expression is present in both preterm and term intestines and increases with postnatal age but that there were no differences in PEPT1 expression by GA at birth (39). Furthermore, neonates (particularly premature infants) have available to them a means of intact protein and peptide absorption through paracellular diffusion due to their looser tight junctions between enterocytes (40-42). Paracellular diffusion is the natural route of IgA absorption (43, 44), but additional permeability can induce and be induced by inflammation and infection ("leaky gut syndrome"), initiating a positive feedback loop that may contribute to the development of NEC (45).

The infant microbiome is a critical feature of the GI system and plays roles in digesting nutrients, communicating with and maturing the immune system, and depending on its composition, conferring either protection from pathogenic infection or increasing susceptibility to it. Initial microbiome development is dependent upon the mode of birth, GA at birth, type of feed, antibiotic administration, and geographic location (46). Bacteria from the mother's urinogenital tract, skin contact, and the hospital environment initiate colonization of the intestinal tract. Due to the importance of vaginal canal bacteria in seeding the infant microbiome, infants born by caesarian section have perturbed microbiomes in early life (47). A healthy infant microbiome starts out with a low diversity of species that consume colonic oxygen, proliferates into one dominated by a few anaerobic commensal species, and reaches a diverse, mature enterotype as breastfeeding ends (48-51). For a healthy infant, the ideal microbiome in the first few

months of life is composed primarily of bifidobacteria and lactobacilli (52). These species selectively receive a rich source of nourishment from indigestible HM oligosaccharides and outcompete pathogenic or proinflammatory species, creating a healthy colonic environment. Such a microbiome is easily achieved in term infants, but preterm infants often have more diversity in their microbiome and lower counts of commensal species compared to term infants (53). An unstable microbiome with a lower abundance of commensal species is implicated in the increased risk that preterm infants have for the development of NEC, sepsis, and other infections (54).

Associated diseases and complications of premature birth

Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is a common and devastating infection of the intestinal tract that affects primarily premature infants (~90% of cases). NEC has an overall estimated incidence of 7% in preterm infants (55), and incidence increases with decreasing GA at birth (56, 57). Mortality also increases with decreasing birth weight and GA to as high as 30% in extremely low-birthweight infants (58). NEC is diagnosed in infants by feeding intolerance and abdominal distension in the early stages, and gaseous cysts, intestinal perforation, and tissue necrosis as it progresses (59). The pathogenesis of NEC is not fully understood and is likely a confluence of several factors caused by an underdeveloped GI tract (discussed above), imbalance in the expression of immune proteins (e.g., an increase in gut toll-like receptor 4 and nuclear factor κB1) (60), systemic ischemia (61), and abnormal early microbiome colonization (also discussed above). One or more triggers are hypothesized to be necessary for NEC to initiate. Hypoxia, limited digestion/gastric motility, dysbiosis, etc. may cause an exaggerated

inflammatory response, which cannot be well regulated by the infant's immune system, thus leading to further intestinal damage and opening the way for pathogens or opportunistic species to establish an infection (62). Treatment for NEC involves intravenous feeding and broad-spectrum antibiotics, with surgery as a last resort due to poor outcomes. The optimal prevention for NEC is early feedings of mother's own milk with HM-based fortifier (63), and probiotics and prebiotics have recently been investigated as preventative therapies (64).

Neonatal sepsis

Neonatal sepsis is a systemic infection that occurs in infants less than 28 days old. Neonatal sepsis can be further categorized as early-onset (occurring within 72 hr from birth) or late-onset (occurring over 72 hr from birth). A recent meta-analysis calculated the global incidence of neonatal sepsis at 2.2% of livebirths (65), with increased cases in middle-income countries compared with high-income (though the true rate is likely larger, as the authors concluded that there wasn't enough data to assess low-income countries). Overall mortality is 18% for early-onset and 11% for late-onset sepsis (23). Neonatal sepsis incidence, like NEC, increases with decreasing GA at birth, but the rate of increase is much larger for late-onset than early-onset (66). Pathogens responsible for sepsis are predominantly Gram-positive, including *Staphylococcus aureus*, coagulase negative staphylococci, and group B streptococci (66, 67), but incidence of Gram-negative cases from *Escherichia coli* has also risen in recent years (68). Additional causal pathogens are *Listeria monocytogenes*, *Klebsiella pneumoniae*, and some *Candida* species (67, 69). The infant may acquire the pathogens responsible for early-onset sepsis *in utero* or from the mother's vaginal canal during birth, while late-onset sepsis

pathogens are more usually acquired from the hospital or community setting (70). As with NEC, HM feeding has a dose-response relationship with reducing incidence of neonatal sepsis in preterm infants (71). Isolated lactoferrin (an antimicrobial milk protein in both human and cow's milk) (72) and probiotics (73) have also been successfully applied to reduce sepsis incidence in several randomized controlled trials.

Retinopathy of prematurity

Retinopathy of prematurity is a condition where the retina has detached from the eye of the premature infant. Retinopathy is one of the leading causes of infant blindness, with the proportion varying by country due to differences in development of neonatal care and screening (74). The condition arises as the infant transitions from a relatively low-oxygen environment in the womb to a high-oxygen environment outside. Hyperoxia, oxidative damage, physiological immaturity, and sudden cessation of amniotic growth factors cause the retinal vasculature to underdevelop in phase 1 retinopathy (75). Phase 2 occurs as the now hypoxic environment of the eye leads to over-proliferation of poor, leaky vasculature, and the development of scar tissue (75). Premature infants that receive a diet of exclusively or mainly HM are less likely to develop retinopathy over those who are fed primarily formula (76, 77). HM contains antioxidative (78) and immunological (79) factors (mostly proteins and vitamins) that may protect the infant retina from oxidative damage and prevent or inhibit development of retinopathy.

Respiratory distress syndrome and bronchopulmonary dysplasia

Respiratory distress syndrome (RDS) and bronchopulmonary dysplasia (BPD) are both diseases of the lung and airway that primarily affect preterm infants. RDS is an acute condition mainly due to the preterm infant's inability to produce surfactant, and is

diagnosed by tachypnea, chest retractions, and noisy breathing, all indicating the infant's struggle to sufficiently oxygenate (80). There are many underlying causes of RDS of which multiple can present at the same time, including interruption of lung or trachea development by preterm birth, congenital malformations, cardiovascular disease, sepsis, pneumonia, central nervous system injury, electrolyte imbalance, etc. (81). BPD is a chronic condition diagnosed by treatment with >21% oxygen for at least 28 days. BPD often occurs as a result of RDS but can also be caused by other factors such as mechanical trauma, hyperoxia, or infection (82). BPD is caused by interruption in the development of alveolar and vascular tissues, leading to reduced surface area and inefficient gas exchange (82). Sepsis is an important predictor for BPD. Systemic bacterial infiltration and the resulting inflammation can damage the fragile tissues within the lung to initiate the disease or can aggravate just enough to require oxygen therapy and mechanical ventilation, which can cause barotrauma and further inflammation inside the lungs in a self-perpetuating cycle (83). Unsurprisingly, given what has previously been discussed in the present paper, a few recent meta-analyses of cohort and randomized controlled trial studies have shown HM feeding is protective for BPD (84, 85), though none have yet been performed for RSD.

Human milk for the premature infant

HM is the optimal food for both preterm and term infants. HM provides the most appropriate balance of nutrients for term infant health and development, and it even has a limited capacity to compensate for preterm birth with a corresponding increase in protein and energy content (though supplemental protein and vitamins are still needed to match intrauterine growth rates). Term HM contains a mean macronutrient content of

approximately 9–15 mg/mL of protein, 30–50 mg/mL of lipids, and 67–78 mg/mL of carbohydrates (86, 87). Furthermore, HM is a rich source of vitamins A, C, and E, several B vitamins, and most major minerals (88). The nutrient composition of HM is not static however, and changes significantly over the duration of breastfeeding, over the period of a day (from morning to evening), and even over one single feeding. The earliest milk produced in the first 5 days is called colostrum, and is a watery substance high in protein, immunoglobulins, and salt (89, 90). Colostrum proceeds into transitional milk as protein content decreases and lipid content increases and settles into a high-fat mature milk around day 15 (91, 92). Other factors influence the nutrient composition of HM, including mother's age, health, diet, and geographic region (93). For example, higher maternal fat intake correlates with higher breast milk fat content, and consumption of different types of fats (saturated, monounsaturated, or polyunsaturated) increases the percentage of those fats in the milk (94).

As discussed above, HM feeding is protective to the preterm infant for the various diseases and complications that can develop post-partum. In fact, any HM feeding compared with exclusive formula feeding reduces overall mortality rates in preterm infants up to their discharge from the hospital (95). The benefits of HM extend beyond preventing infant morbidity and mortality only in the hospital and affect the infant throughout life. Though formula feeding is capable of achieving similar infant growth rates as HM (96, 97), HM feeding improves protein balance (97), body composition, and obesity rates (98). These benefits have been shown to last into childhood (99-101), and a few studies indicate they may last into adulthood as well (102, 103); though it is my opinion that the evidence for such results is much weaker, and careful studies are

required to further elucidate the effect. HM may also have an effect on allergic disease development, though its effects are contentious as many of the earlier establishing results were inhibited by methodological issues in accurately assessing infant allergy (104). Recent studies have conflicted over whether HM reduces odds of developing specific diseases such as asthma, eczema, and hay fever (104, 105). Such ambiguity has extended to the role of HM in reducing food allergies as well (106). HM has a suite of immunological compounds and proteins that have the potential to provide immune support and contribute to HM's myriad protective benefits.

Human milk proteins

Milk proteins from all mammalian species, including humans, can be divided into two categories: caseins and whey. The caseins make up around 40% of mature HM and are composed of α_{s1}-casein, β-casein, and κ-casein. Together they form spherical casein micelles, an important macrostructure that ensures the casein proteins remain soluble and aids in the delivery of calcium and phosphorus to the infant. The whey proteins are a much more diverse group of proteins numbering in the hundreds, with the largest constituents among them being α-lactalbumin, lactoferrin, osteopontin, immunoglobulins, lysozyme, and serum albumin (107). Whey proteins have good solubility in water and are found dissolved in the aqueous fraction or associated with the milk fat globule membrane. Every protein in HM serves a function, whether it be as an amino acid delivery system or as an intact bioactive protein. Because each protein secreted in HM incurs a cost of energy and resources to the mother, natural selection processes would ensure that they provide at least equal benefit to the infant.

The human casein content is on average 55% β-casein, 35% κ-casein, and 10% α_{s1}-casein, though there is a large degree of variation among mothers and over time (108). The caseins are amphipathic, highly-phosphorylated and glycosylated proteins with plastic, open structures (109). For β-casein and α_{s1}-casein, phosphorylation sites are located in concentrated Thr/Ser regions along the protein that allow for chelation of Ca^{2+} ions. Such sites are found in the N-terminus of β-casein within the motif "TIESLSSS" (amino acids 18–25), which exists in six phosphorylation variants (110), in α_{s1}-casein "SESSEPIPLES" (amino acids 31–41), which exists in four variants (111), and in α_{s1}-casein "SSISSSS" (amino acids 85-91), which exists in seven variants (111). Human κ-casein is also phosphorylated but not to the same density as the other casein proteins and so does not have the ability to bind Ca^{2+}. Instead, its main function is to stabilize the HM micelle structure through its hydrophilic, glycosylated C-terminus (also known as "glycomacropeptide") that forms the surface "hairy layer" of the micelle (112). Human micelles are much smaller than other species and form very fine curds when precipitated in the infant stomach. The smaller curd size is believed to regulate the rate of gastric emptying and to increase surface area exposure to proteases for accelerated digestion (113).

The number of unique proteins in HM is estimated to be around 400 (114), of which only three are the caseins. The remainder are all grouped together as whey proteins, despite tremendous differences in abundance and activity. The single most abundant protein in HM is α-lactalbumin (accounting for 22% of total protein), which combines with β-1,4-galactosyltransferase to form lactose synthase in the mammary gland and also provides an enriched pool of tryptophan to the infant (115). Lactoferrin is

the second most abundant protein and has several functions within the infant, including iron-binding for delivery to enterocytes, antimicrobial activity against pathogens, and growth-stimulating activity for select commensal species (116, 117). There is some evidence that supplementation with bovine lactoferrin lowers risk of late-onset sepsis in preterm infants and lower-quality evidence that it reduces risk of NEC (118, 119), but similar studies have not yet been performed with human lactoferrin. HM immunoglobulins (Ig) secreted in milk are predominantly secretory IgA (IgA bound to the secretory component of the transporter protein polymeric Ig receptor), with some secretory IgM, IgA, IgM, and IgG (107). Secretory IgA resists digestion and binds antigens in the GI system or crosses the intestinal barrier to contribute to systemic defense in the naïve infant adaptive immunity (120, 121). Lysozyme is another antimicrobial protein and glycosidase that cleaves bacterial cell wall carbohydrates to facilitate lysis (122). Osteopontin is a multi-functional protein with a diverse range of immune cell and enterocyte stimulating and suppressing effects that can influence intestinal development (123). In addition to these most abundant proteins, whey proteins also include varying amounts of nutrient-degrading enzymes, enzyme inhibitors, and enzyme promoters; growth factors and peptide hormones; mucins; and a variety of metabolic and structural proteins from the maternal mammary gland and blood cells.

Peptidomics: uncovering a new aspect of milk

All HM proteins have some bioactivity in the intact state and are a source of amino acids as they are digested. However, once digestion has initiated and before it has completed, the most common form of most milk proteins is neither the intact protein nor their component amino acids but the thousands of unique peptides that exist as an

intermediary. The study of these peptides is called peptidomics, and it can be used to identify naturally occurring hormone or other bioactive peptides, to understand protein processing and degradation in the cell, to identify biomarkers of disease, and in the case of the present dissertation, to characterize how food proteins are digested, i.e., HM for the infant.

Peptidomics has become an important tool in the field of nutrition, as it enables researchers to investigate deeper into the functional components of the foods we consume. Over the years, peptidomic studies have been performed to characterize the peptidomes of several foods and food products, but milk and milk product (cheese, yogurt, formula, etc.) peptides have been characterized since the first conceptualization of the field. Milk is recognized as the ideal food in which to find naturally-occurring bioactive peptides, as everything in milk has the potential to impart beneficial health effects to the young of that species. The peptidomic studies of milk peptides can be further broken down into two broad categories: those focused on identifying novel bioactive peptides in milk, and those focused on surveying the entirety of peptides released in milk or during processing of milk.

Milk bioactive peptides

The investigation of bioactive milk peptides has been an evolving process first initiated in 1950, when Mellander and Isaksson identified the first known bioactive peptides from bovine milk (124, 125). These peptides were discovered from casein after undergoing pepsin and pancreatin digestion, and were highly phosphorylated; they were later dubbed “caseinophosphopeptides.” When supplemented in infants, they were shown to improve calcium resorption and regression of rickets without the use of vitamin D

(125). Peptides released from pepsin-digested casein with opioid-receptor binding activity were next characterized in the late 1970s by two parallel groups (126, 127). An important development in milk bioactive peptide research was the discovery of angiotensin-converting enzyme (ACE)-inhibitory peptides from pepsin-digested casein in 1982 by Maruyama and Suzuki (128), as the first developed consumer products based upon bioactive milk peptide technology were lactotripeptide supplements intended to lower blood pressure (129). The first antimicrobial peptide released from a milk protein was identified by Bellamy et al. in 1992 from the N-terminal domain of both human and bovine lactoferrin (130).

Improvements in fractionation, sequencing, and mass spectrometry over the years have greatly expanded the field, and milk bioactive peptides have since been discovered with a wide breadth of activities, including immunomodulatory, antioxidant, bifidogenic, anticancer, and anticholesterolemic. However, decades of decentralized data on the known sequences and activities of milk bioactive peptides has led to growing inefficiency in the discovery of new peptides and an inability to effectively sift through the massive peptidomic datasets that modern mass spectrometers are capable of generating. To resolve this issue, I assisted in the creation of the Milk Bioactive Peptide Database (MBPDB) (131). The MBPDB is a comprehensive database of known milk bioactive peptides that allows users to search for peptide sequences or activities, contribute their own validated sequences, and compare their peptidomic data with what those that have already been discovered. Other compiled peptide databases with distinct focuses include the Collection of Antimicrobial Peptides (132) and BIOPEP-UWM (133).

Peptidomic surveys of human milk

Before the development of modern LC-MS/MS techniques that enable sequence identification of a huge array of peptides in milk or milk digesta, rudimentary surveys of milk peptides were performed using tools such as SDS-PAGE gels and other separation techniques (134-137). Though such methods did not provide sequence information, they displayed the breadth of physical and chemical characteristics achievable in the milk peptidome. The use of tandem mass spectrometry and database comparison to identify just the peptide content of a food matrix is a relatively recent development. The challenges in identifying peptides versus proteins using proteomic techniques, despite their biochemical similarity, arise from several instrumentation and technological causes (138). Unlike tryptic peptides, natural peptides are derived from non-specific cleavage and have an N- and C-terminus that can lie anywhere within the parent protein sequence. For a program to match a peptide's mass spectrum to its sequence, it needs to compare the spectrum to hundreds of thousands of potential peptides that can be generated from a user-inputted protein library. For example, a protein with 200 amino acids (aa) has 197 possible 4-aa peptides, 196 5-aa peptides, 195 6-aa peptides, etc. Furthermore, though tryptic peptides are generally of medium length (7–25 aa), natural peptides can range anywhere from dipeptides to near the entire protein sequence, imposing additional challenges in separating on the column and fragmenting in the mass spectrometer. Relative quantitation based on measuring the ion intensity is affected by how efficiently the peptide is ionized coming off the column, which is dependent on the peptide's sequence and the presence of co-eluting compounds; and absolute quantitation is financially prohibitive, as each peptide requires its own isotope-labeled standard.

However, with recent advances in mass spectrometry technology, we have begun to understand the HM peptidome and the factors that influence it.

The natural peptidome of HM as it is expressed by the mammary gland, i.e., milk without any digestion, was first characterized in 2013 by two parallel groups (139, 140). Both found peptides on the order of a few hundred, with peptides from β-casein making up the largest fraction. Peptidomic analyses of undigested HM have been repeated several times since then with distinct focuses, such as comparing preterm and term milk (141), foremilk and hindmilk (142), and pasteurized and raw milk (143-145); measuring differences over the stages of lactation (146); the fate of specific milk proteins (147); and establishing the optimum experimental parameters for HM peptide identification (148). Though all results concurred in establishing β-casein as the largest contributor to the early undigested peptide profile with the other caseins, lactoferrin, α-lactalbumin, and osteopontin frequently being just below, there exists significant variation in the number and species of peptides among mothers. Furthermore, the number of peptides being identified from milk has increased over time, likely as mass spectrometry technology improved and peptidomic methods were refined.

Studies on HM peptides released after digestion are rarer but growing in number. *In vitro* digestions of HM were often performed to identify novel bioactive peptides (149), but the goal of those studies was not to profile the entire peptidome of HM after digestion but to focus on a few specific peptides through reverse-phase fractionation of the milk. The first wide-view profiling of *in vitro* digested HM was not performed until 2015 by Wada and Lönnerdal (145) and Dall'Asta et al. (150). As HM is primarily meant for infants, creating an *in vitro* digestive model that represents the limited infant GI

system was necessary before such studies could be performed. Though some models have since been developed and applied (143, 151), there is not yet a consensus for a standardized model to represent term infant digestion, let alone preterm (152). Characterizing peptides from HM-fed infant GI digesta, though more invasive to generate samples, is thus the golden standard for representing how proteins are digested by the infant. Such studies have previously been performed by Dallas et al. (153) and Nielsen et al. (154) using gastric samples collected from preterm infants in the NICU.

Until now, the stomach is the deepest region of the infant GI system we have been able to probe, and those few studies have been limited to a comparison of peptides from a few preterm milk and gastric samples. The present work has built upon everything that has come previously before it to measure peptide release across the entire duration of preterm infant gastric digestion, to identify milk peptides that have survived the entirety of infant digestion to be excreted in stool, to compare preterm and term peptide release in the milk, stomach, and stool, and finally to be the first to profile milk peptides released in the preterm infant intestinal tract, including the identification of novel milk antimicrobial peptides.

Chapter 2 – Peptidomics analysis of milk protein-derived peptides released over time in the preterm infant stomach

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Abstract

Over the course of milk digestion, native milk proteases and infant digestive proteases fragment intact proteins into peptides with potential bioactivity. This study investigated the release of peptides over three hours of gastric digestion in 14 preterm infant sample sets. The peptide content was extracted and analyzed from milk and gastric samples via Orbitrap tandem mass spectrometry. The relative ion intensity (abundance) and count of peptides in each sample were compared over time and between infants fed milk fortified with bovine milk fortifier and infants fed unfortified milk. Bioactivity of the identified peptides was predicted by sequence homology to known bioactive milk peptides. Both total and bioactive peptide abundance and count continuously increased over three hours of gastric digestion. After accounting for infant weight, length, and post-conceptual age, fortification of milk limited the release of peptides from human milk proteins. Peptides that survived further gastric digestion after their initial release were structurally more similar to bioactive peptides than non-surviving peptides. This work is the first to provide a comprehensive profile of milk peptides released during gastric digestion over time, which is an essential step in determining which peptides are most likely to be biologically relevant in the infant. Data are available via ProteomeXchange with identifier PXD012192.

Keywords: Bioactive; Digestion; Gastric; Human milk; Peptide; Preterm

Introduction

Milk has evolved as the sole source of nutrition for the human infant. Milk contains the ideal balance of macronutrients, micronutrients, and other bioactive factors to fuel infant growth and aid in development (155). In addition to providing amino acids for protein synthesis, intact milk proteins have multiple functions within the infant, including supporting the infant immune system (156, 157), preventing pathogen growth (158), serving as growth factors (159), and regulating sequestration and delivery of iron (116). Digestion of milk proteins occurs by the action of a combination of proteases native to the milk and digestive proteases secreted by the infant gastrointestinal tract (160). Our previous work determined that even before expression of milk from the mammary gland, native milk proteases initiate the hydrolysis of milk proteins (161). However, beyond milk proteins' role as a source of amino acids and as bioactive molecules, digestion of the proteins also releases protein fragments (peptides) that have specific biological activities, including antimicrobial (162), antihypertensive (163), immunomodulatory (164), anti-inflammatory (165), and opioid agonistic and antagonistic effects (166). The evolutionary role of milk proteins in the development of the human infant thus might not be limited to intact function and amino acid release but may also encompass the release and function of bioactive peptides.

The release of these bioactive milk peptides varies by stage of digestion. As different proteases with different cleavage specificities are secreted throughout the gastrointestinal tract, more of the parent protein sequence is broken apart to release peptides with diverse sequences. As the amino acid sequence of the released peptides determines the type of activity they can exert (167), peptides released in the infant

stomach may have different functions and sites of action than those already present in human milk, and peptides that survive gastric digestion intact might be released only to act further downstream in the intestine. As such, identifying the release of peptides from milk proteins across time will provide a more complete picture of gastric protein digestion that can aid in the identification of potential bioactive peptides that are most biologically relevant.

Previous work has measured the proteolysis of intact milk proteins in real or simulated infant stomach conditions without examining the resulting peptides (168, 169). Other studies have examined *in vitro* and *in vivo* gastric milk peptide release, but samples were only collected at a single time point (149, 153, 170). These types of profiles of human milk peptides are vital for efforts to identify promising peptide sequences for determination of further bioactivities that are most relevant to the human infant. However, *in vitro* studies do not adequately represent how digestion occurs in the infant stomach, and the previous *in vivo* peptidomic analysis study (153) only analyzed gastric samples collected two hours after feeding. As such, there is limited knowledge on which peptides are released and at what time during gastric digestion, and the types of functions these peptides may have in the body.

The present study aimed to identify the peptidomic profile of fortified and non-fortified human milk from preterm-delivering mothers and in gastric samples at three time points (one, two, and three hours post-feeding) from their preterm infants. Peptides from the milk and gastric contents were isolated and identified via Orbitrap Fusion Lumos mass spectrometry analysis to assess the change in their release over time.

Bioactivity of the peptides was assessed based on sequence homology to known functional peptides in the literature.

Experimental procedures

Materials

HPLC-grade acetonitrile (ACN) was obtained from Fisher Scientific (Waltham, MA), trifluoroacetic acid (TFA) and HPLC-grade formic acid (FA) were obtained from EMD Millipore (Billerica, MA), and trichloroacetic acid was obtained from Sigma-Aldrich (St. Louis, MO*).*

Participants and samples

This study was approved by the Institutional Review Boards of the University of California, Davis and Oregon State University. Preterm infants were enrolled if they were inpatients in the UC Davis Children's Hospital Neonatal Intensive Care Unit in Sacramento, California, had an indwelling nasogastric or orogastric feeding tube, and could tolerate full enteral feeding. Most of the enrolled infants required a feeding tube because of uncoordinated or immature capacity to suck and swallow. Infants with anatomic or functional gastrointestinal disorders were excluded from enrollment. Samples were collected from 10 preterm-delivering mother-infant pairs ranging in gestational age (GA) at birth from 23 to 32 weeks and postnatal age of 7 to 98 days. Infant characteristics are described in **Table 2.1**. Enrolled preterm infants had medical conditions typical of premature infants, including lung immaturity (respiratory distress syndrome), bronchopulmonary dysplasia, intraventricular hemorrhage, retinopathy of prematurity, patent ductus arteriosus, and sepsis/meningitis but no overt gastrointestinal

tract issues. None of the infants sampled received medications known to affect gastric pH or gastric digestion capacity, including prokinetics, H2 blockers/antagonists or proton-pump inhibitors. The enrolled infants were clinically stable at the time of sample collection.

Table 2.1. Infant characteristics. Post-conceptual age is the time at which samples were collected, and includes infant GA plus infant lifespan. Weight and Length were measured at the post-conceptual age.

Infant	Sex	GA at birth (wk)	Post-conceptual age (wk)	Weight (kg)	Length (cm)	Fortification	Twins with
1[1]	F	23	28	0.95	30.5	None	
1[1]	F	23	30	1.2	32	Gastric Only	
1[1]	F	23	32	1.67	39	Gastric Only	
1[1]	F	23	35	1.8	41.5	All	
1[1]	F	23	37	2.3	42	All	
2	F	29	30	1.1	38.5	None	3
3	F	29	30	1.1	39.4[2]	None	2
4	F	26	32	1.4	37.5	Gastric Only	
5	F	32	32	1.6	42.5	None	6
6	F	32	32	1.5	40	None	5
7	F	29	32	1.5	39	All	8
8	F	29	32	1.4	39	All	7
9	F	26	34	2	40.5	All	
10	M	26	34	2.3	43.8	All	

[1] Samples were collected from infant 1 at five different post-conceptual ages.
[2] Length value represents birth length rather than post-conceptual age length.

The mothers pumped their breast milk into sterile plastic containers at home and froze it in home freezers. Mothers transported their milk to the NICU on ice where it was stored at –20 C. At time of feeding, a 1.5–2 mL sample of the breast milk was collected into a sterile Eppendorf tube after it had been thawed and fortified, or just thawed if no fortification was added. Nine preterm infants (25.2 wk GA ± 0.7, 50 ± 7 days of postnatal age) were fed their mother's milk (not pasteurized) fortified with 1 packet of Human

Milk Fortifier (Similac Human Milk Fortifier Powder, Abbot Park, IL, USA) per 25 mL of milk, and five preterm infants (25 ± 3 wk GA, 29 ± 9 days of postnatal age) were fed their mother's milk (not pasteurized) unfortified. The fortifier contained nonfat bovine milk, whey protein concentrate, corn syrup solids, medium-chain triglycerides, calcium phosphate, and potassium citrate. Three sets of twins were included in the study and shared milk samples (11 total milk samples).

The human milk feedings were delivered via the nasogastric tubes over 30 min. A volume of 2 mL was collected from the initial milk feed, and 0.5–2 mL of each preterm infant's gastric contents was collected in a 3-mL syringe via suction through the feeding tube at 1, 2, and 3 hr after the initiation of feeding. Only infants from whom samples were successfully collected at all three time points were included in the study. Milk and gastric samples were placed into sterile plastic vials and stored immediately at –20°C to prevent further proteolysis. Human milk and gastric samples were transported to OSU on dry ice and stored at –80°C.

Sample preparation

Samples were prepared as in our previous study (142) with the following changes. Fifteen microliter aliquots of the skimmed samples were collected and dissolved in 85 µL of 0.1% FA solution to reduce the viscosity of the gastric samples. Milk proteins were precipitated from the samples by addition of 100 µL of 24% trichloroacetic acid. After mixing for 10 s with a vortex mixer, the samples were centrifuged at 10,000 × *g* for 20 min at 4 °C, and the supernatant containing the peptides was collected. Peptides were loaded onto C18 reverse-phase preparative chromatography 96-well plates (Glygen, Columbia, MD). Trichloroacetic acid, salts, oligosaccharides, and lactose were washed

out with 1% ACN, 0.1% TFA, and peptides were eluted in 80% ACN, 0.1% TFA. The peptide solutions were frozen at –80 °C and lyophilized using a freeze dry system (Labconco FreeZone 4.5 L, Kansas City, MO). After drying, the samples were rehydrated in 15 µL of 0.1% FA.

Liquid chromatography nano-electrospray ionization mass spectrometry

Peptides were analyzed with an Orbitrap Fusion Lumos (Thermo Scientific, Waltham, MA) mass spectrometer connected to a Waters Nano Acquity UHPLC (Water Corporation, Milford, MA). One µL of peptides was loaded onto a C18 180 µm × 20 mm, 5-µm bead nanoAcquity UPLC Trap Column (Waters) for enrichment and desalting, and separated with a 100 µm × 100 mm, 1.7-µm bead Acquity UPLC Peptide BEH C18 column (Waters) over 120 min. The mobile phase consisted of 0.1% FA in water (Solvent A) and 0.1% FA in ACN (Solvent B). The separation gradient consisted of 3−10% solvent B over 3 min, 10−30% solvent B over 99 min, 30−90% solvent B over 3 min, 90% solvent B for 4 min, 90−3% solvent B over 1 min then held at 3% solvent B for 10 min. A 30-min column wash was performed after each sample run.

Mass spectra were collected in positive ionization mode and with data-dependent acquisition. Peptides were ionized with an electrospray voltage of 2,400V and ion transfer tube temperature of 300 °C. The mass spectrometer scanned masses between 400 and 1,500 *m/z*. Full scan MS data were acquired in the orbitrap at 120 K resolution at 200 *m/z*. The automatic gain control target was 4.0×10^5 over a maximum injection time of 50 ms. Precursor ions were selected for most intense peaks with an ion-intensity threshold of 5.0×10^3 and charge state 2–7. Precursor ions were fragmented using

collision-induced dissociation with a collision energy of 35%. Following fragmentation, precursors were excluded (10 ppm mass error) for 1 min.

Raw files were analyzed in Thermo Proteome Discoverer (v2.1.0.81), and a SequestHT search engine identified peptides using an in-house human and bovine milk protein sequence database (**Supplemental Table 2.1**). Potential modifications allowed included phosphorylation of serine and threonine, and oxidation of methionine. Only peptides identified with high confidence were included ($P < 0.01$), and peptide sequences with multiple modifications were grouped into a single peptide for counts. Counts measured the number of unique peptide sequences identified in a sample. Abundance measured the area under the curve of the eluted peak (ion intensity), as determined by Proteome Discoverer. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (171) partner repository with the dataset identifier PXD012192.

Data analysis

A workflow of sample analysis is included as **Supplemental Figure 2.1**. Identified peptides were examined for homology with literature-identified bioactive peptides using our recently created Milk Bioactive Peptide Database (MBPDB, http://mbpdb.nws.oregonstate.edu/) (131). The MBPDB is a comprehensive source for all milk bioactive peptides. The search was performed as a sequence search that searches for bioactive peptides matching the input peptide sequence. The similarity threshold was set to 80%, with the amino acid scoring matrix set to identity. “Get extra output” was selected to obtain the specific percentage similarity between the query sequence and the database sequence.

The total abundances of peptides were summed and mapped to the parent sequence of human milk proteins using an in-house tool (PepEx), which can be accessed at http://mbpdb.nws.oregonstate.edu/pepex/.

Statistical methods

For all statistical analyses, twin samples were considered as separate sample sets (14 complete milk/gastric sample sets). Repeated measures ANOVA followed by Tukey's honest significant difference post hoc test (GraphPad Prism software, version 7.04) were applied to compare human milk and gastric samples at the three times post-ingestion for peptide abundance and count. Bonferroni-corrected *t*-tests were used to compare amino acid percentage between surviving and non-surviving peptides. Chi-squared test with Yates correction was used to compare proportion of peptides with modifications.

A multiple linear regression model (RStudio, version 3.3.2) was used to assess the impact of meal fortification to peptide content over time, starting with the initial model:

$$Y = fortification + time + fortification \times time + body\ weight$$
$$+ body\ length + post\text{–}conceptual\ age$$

Body weight (kg) refers to the infant's weight at time of sample collection, body length (cm) refers to the infant's length at time of collection, and post-conceptual age (days) is the age of the infant plus its gestational period. Model selection was performed among the covariates for body weight, body length, and post-conceptual age using the function "regsubsets" in the package "leaps." *P*-values were determined for the coefficients for fortification, time, and fortification × time. Differences were designated significant at $P \leq 0.05$.

Results and discussion

Peptidomic profile of milk peptides during digestion

This research is the first study in which *in vivo* proteolysis and peptide release were tracked over time in the preterm infant stomach. Both *in vitro* (149, 170) and *in vivo* (153) studies have been performed in order to measure milk protein digestion and identify the peptides released in the infant stomach. Because these were "snapshot" studies, there is no information on whether the identified peptides represent the endpoint of gastric digestion, or some time in between the beginning of feeding and the completion of gastric emptying. The inclusion of time as a variable in the study parameters allowed us to gain a deeper understanding of when milk proteins are digested and peptides are released and which peptides survive further digestion. In addition, the use of a state-of-the-art Orbitrap Fusion Lumos mass spectrometer identified a larger number of identified peptides in each sample than identified in previous studies, thus improving the accuracy and comprehensiveness of the final peptide profile.

The peptidomic data from all 56 samples included 11,592 unique peptides (13,545 when counting different post-translational modifications as unique peptides) derived from 299 different milk proteins (**Supplemental Table 2.2**). Of these peptides, 8,037 were human milk peptides from 202 human milk proteins, and 3,304 were bovine milk peptides from 97 bovine milk proteins. Another 251 identified peptides may have derived from either human or bovine milk proteins due to shared sequences between the species. **Table 2.2** lists the proteins with the highest abundance and count of peptides from all samples. A majority of peptides (8,747) were not present in human milk, and were identified in at least one of the three gastric timepoints (**Figure 2.1**). Compared with a

previous *in vivo* digestion study by Dallas et al. that identified 661 unique peptides in milk or after two hours of digestion (153), this study identified 8,916. Most (88.4%) of the peptides in the Dallas study were also identified in the present study. A likely reason for the large difference in number of peptides identified is due to our use of the Orbitrap Fusion Lumos mass spectrometer, which is able to acquire high-accuracy mass spectra much faster than the Q-TOF used in the previous study.

Table 2.2. Proteins with the highest abundance and count averaged across relevant samples; human protein *n*=14; bovine protein *n*=6.

Protein	Average abundance ± SD	Protein	Average count ± SD
Human β-casein	$8.85x10^{10} \pm 4.49\ x10^{10}$	Human β-casein	285.4 ± 86.8
Bovine β-casein	$4.18x10^{10} \pm 1.60x10^{10}$	Bovine β-casein	185.2 ± 27.4
Human α_{s1}-casein	$2.34x10^{10} \pm 1.63x10^{10}$	Bovine α_{s1}-casein	138.9 ± 18.6
Bovine κ-casein	$2.30x10^{10} \pm 1.50x10^{10}$	Bovine κ-casein	90.2 ± 18.6
Bovine α_{s1}-casein	$2.17x10^{10} \pm 1.28x10^{10}$	Human α_{s1}-casein	88.8 ± 39.3
Human lactoferrin	$2.12x10^{10} \pm 1.67x10^{10}$	Human osteopontin	64.7 ± 35.5
Human α-lactalbumin	$2.06x10^{10} \pm 1.65x10^{10}$	Human PIgR	63.7 ± 19.5
Bovine GLYCAM1	$1.58x10^{10} \pm 8.65x10^{9}$	Human lactoferrin	61.2 ± 37.8
Bovine β-lactoglobulin	$1.54x10^{10} \pm 1.47x10^{10}$	Bovine β-lactoglobulin	57.2 ± 13.7
Human osteopontin	$1.33x10^{10} \pm 1.25x10^{10}$	Bovine α_{s2}-casein	54.3 ± 9.6
Human PIgR	$7.30x10^{9} \pm 3.77x10^{9}$	Bovine GLYCAM1	43.8 ± 6.4
Bovine α_{s2}-casein	$5.94x10^{9} \pm 2.91x10^{9}$	Bovine PIgR	32.4 ± 5.1
Human serum albumin	$5.19x10^{9} \pm 4.79x10^{9}$	Human bile salt-activated lipase	31.8 ± 14.2
Human bile salt-activated lipase	$5.06x10^{9} \pm 4.56x10^{9}$	Human tenascin	21.7 ± 21.7
Human κ-casein	$2.86x10^{9} \pm 2.13x10^{9}$	Human κ-casein	20.4 ± 7.1

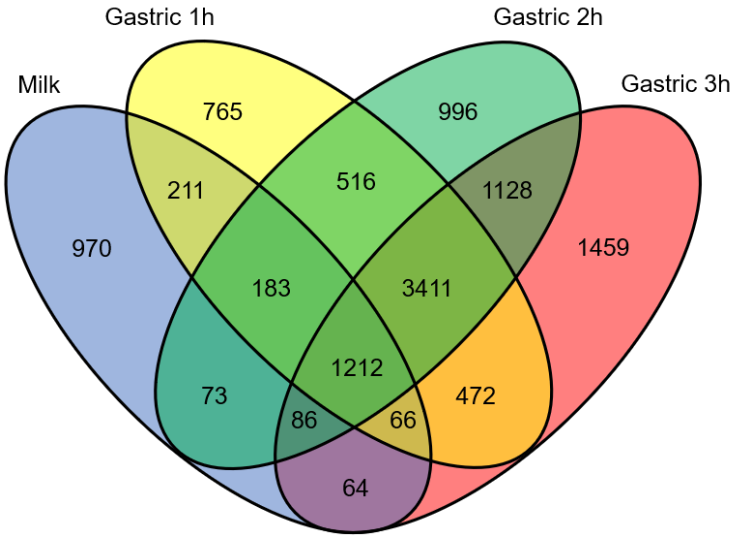

Figure 2.1. Venn diagram of the unique peptides identified in each sample and combination of samples.

Both milk peptide count and abundance continuously increased over three hours of gastric digestion. With milk samples representing the zero time point of gastric digestion for the statistical analyses, the average abundance of peptides increased after each hour of digestion (**Figure 2.2A**). Significant increases in abundance occurred between milk and three-hours gastric digestion, and between one-hour digestion and three-hours digestion. Milk to two-hours digestion trended to increase, but was not significant ($P=0.057$). Milk peptide counts also significantly increased from 671.2 ± 52.7 unique peptides in milk to 1,254.9 ± 142.8 in one-hour gastric samples, 1,490.6 ± 113.2 in two-hour samples, and 1,629.1 ± 102.1 in three-hour samples (**Figure 2.2B**). Interestingly, of the peptides found in both milk and three-hour samples, more peptides were significantly higher in milk than three-hour (**Figure 2.2C**). The majority of the increase in peptide abundance in three-hour samples is thus likely due to the release of additional peptides that were not present in milk.

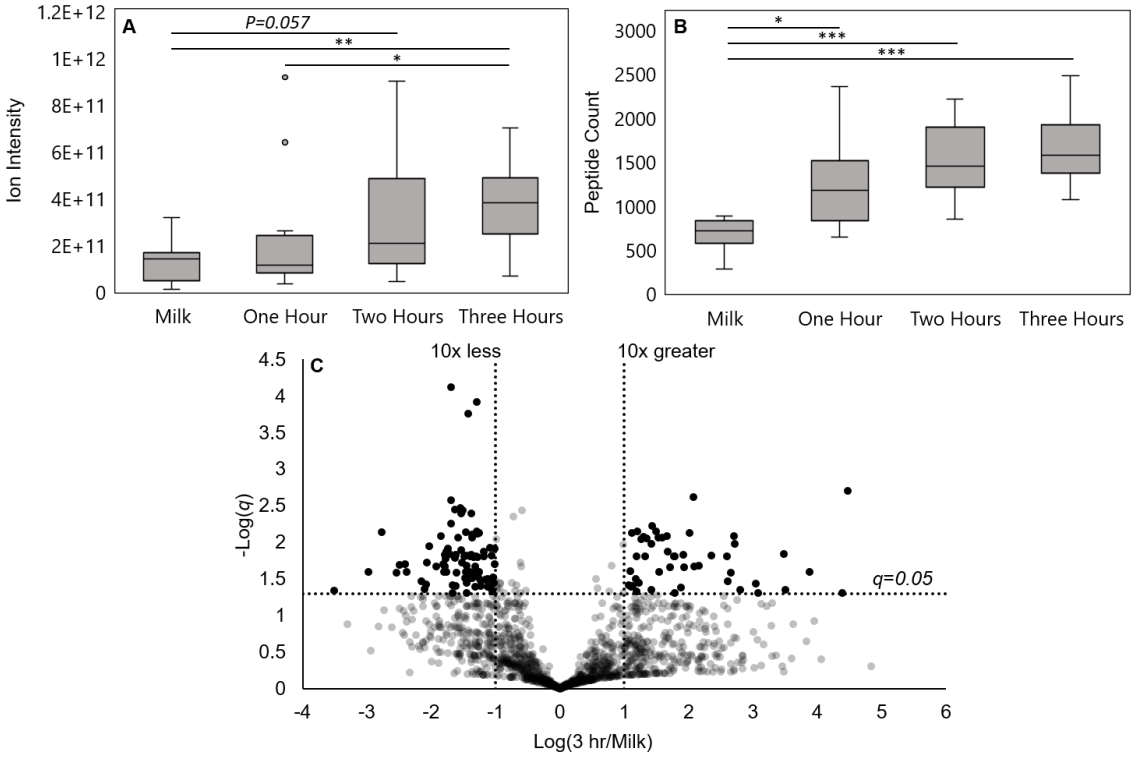

Figure 2.2. Boxplots of the abundance (A) and count (B) of total peptides from all infants by sample type. Boxes are bordered by the first and third quartiles. The center bar represents the median, n=14. ***P<0.001, **P<0.01, and *P<0.05. Volcano plot (C) depicting the fold change in peptide intensity (three-hour gastric divided by milk) vs. the FDR-corrected P-value between peptides found in both milk and three-hour gastric digests. Filled circles indicate significant (q< 0.05) peptides >10-fold higher or lower in gastric samples.

Milk peptides that were present in a majority of the infants (eight or more out of fourteen) for each sample type accounted for the majority of peptide abundance. One hundred and ninety-one peptides were found in a majority of milk samples, 314 in a majority of one-hour gastric samples, 454 in a majority of two-hour samples, and 613 in a majority of three-hour samples. These common peptides accounted for the majority of peptide abundance at 62.9% ± 23.9%, 63.1% ± 15%, 60.5% ± 10.9%, and 69.1% ± 10.7% of sample type abundances, respectively. A small subset of these peptides was found in every infant for each sample type. Sixteen peptides were found in all milk samples, nine

in all one-hour gastric samples, seven in all two-hour samples, and eleven in all three-hour samples. These shared peptides are indicated in **Supplemental Table 2.2**.

These findings support our previous finding of an increase in total proteolysis in the infant stomach over time (172). As average gastric half-emptying time ranges from 45 to 100 minutes for preterm infants (173-175), three hours likely represents the endpoint of gastric digestion and captures all peptides released by pepsin and milk proteases in the stomach before transit to the duodenum. Pepsin is secreted by the infant stomach, and additional proteases, such as cathepsin D and plasmin (160, 176), are naturally present in milk and known to be active even in somewhat acidic gastric conditions in the infant. The combined activity of pepsin and native milk proteases on intact milk proteins and any peptides already released in the mammary gland maximizes the count of unique peptides and peptide abundance prior to milk entering the small intestine. As this study concluded with the end of gastric digestion, it is not known whether three-hour gastric digest samples represent maximum peptide content, or whether the activity of pancreatic proteases continues to increase the count and abundance of milk peptides rather than reduce them through further degradation to their component amino acids.

Comparison between fortified and non-fortified samples

Preterm infant milk is often fortified with additional milk protein to provide for the infant's increased protein requirements. However, fortification is typically sourced from bovine milk, thus altering the total protein composition of the milk. Human milk is the ideal source of nutrition for preterm infants as it reduces risk of sepsis, necrotizing enterocolitis, and other infections compared with formula (177, 178). Human milk provides the infant with bioactive proteins such as immunoglobulins, cytokines, growth

factors, and antimicrobial proteins that are not active in bovine milk-based fortifier (87). It is therefore possible that human milk peptides are also more beneficial to the infant than other peptide sources. To determine the effect of fortification on peptide release, fortified milk-fed infants were compared with non-fortified milk-fed infants. Three sets of infant samples were removed from these comparisons, as the milks collected from these infants' mothers was reported as non-fortified, yet the infant gastric samples reflected fortified milk feeding. The final sample sets included five infants in the non-fortified group and six infants in the fortified group. Peptides that could not be distinguished as either human or bovine milk protein-derived were included in the total peptide analysis but excluded from the human- and bovine-specific analyses.

Neither total peptide abundance nor human milk peptide abundance changed significantly over time, but non-fortified infants had significantly greater total and human milk peptide abundance than fortified infants (**Figure 2.3A**). Bovine milk peptide abundance significantly increased during digestion, but no comparisons were made between fortified and non-fortified samples, as non-fortified infants were fed only human milk. Human milk peptide count, bovine milk peptide count, and total peptide count all significantly increased across three hours of gastric digestion (**Figure 2.3B**). Non-fortified total and human milk peptide counts were also significantly higher than fortified counts. Since the non-fortified samples generally were collected from infants at a lower corrected gestational age (**Table 2.1**), it is unlikely that maturity or development of the GI tract (capacity for efficiently releasing milk peptides) is the explanation for the observed differences. A more likely justification for the observed differences is that pepsin activity in fortified infants is divided between digesting human milk proteins and

fortifier proteins during the same time of digestion, thus releasing fewer human milk peptides than infants who only receive human milk.

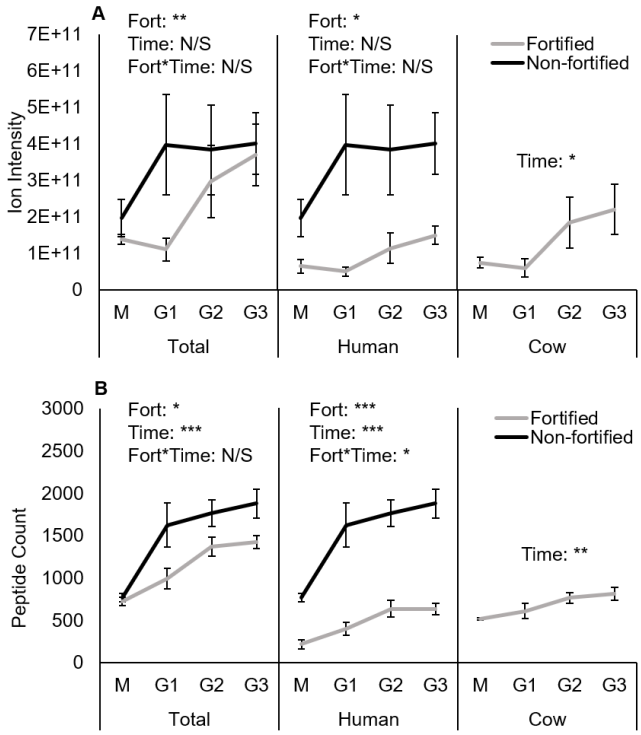

Figure 2.3. Comparison of average peptide abundance (A) and count (B) between fortified and non-fortified infants, grouped by milk protein source species. Data are shown as means ± SE, fortified *n*=6 infants, non-fortified *n*=5 infants. ****P*<0.001, ***P*<0.01, and **P*<0.05. M, milk samples; G1, G2, and G3 are gastric samples after one, two, and three hours of digestion, respectively.

We identified additional significant differences between fortified and non-fortified infants concerning how peptides were released from individual milk proteins. **Figure 2.4** compares the release of peptides from the five highest abundance and count of human milk proteins. Fortified infants had significantly lower β-casein, α_{s1}-casein, and osteopontin peptide abundance and count, and lower polymeric immunoglobulin count. Only lactoferrin peptide release did not differ by fortification status. These results are notable, as every bioactive peptide identified from human milk was identified from either

β-Casein, α_{s1}-casein, lactoferrin, or κ-casein (which also had significantly lower count in fortified infants, results not shown) (131). These differences likely did not impact total protein nutrition to fortified infants, as bovine milk peptides accounted for the difference in protein digestion. However, the impact to infant health and development could potentially arise from the reduced presence of human milk protein-derived bioactive peptides in fortified infants.

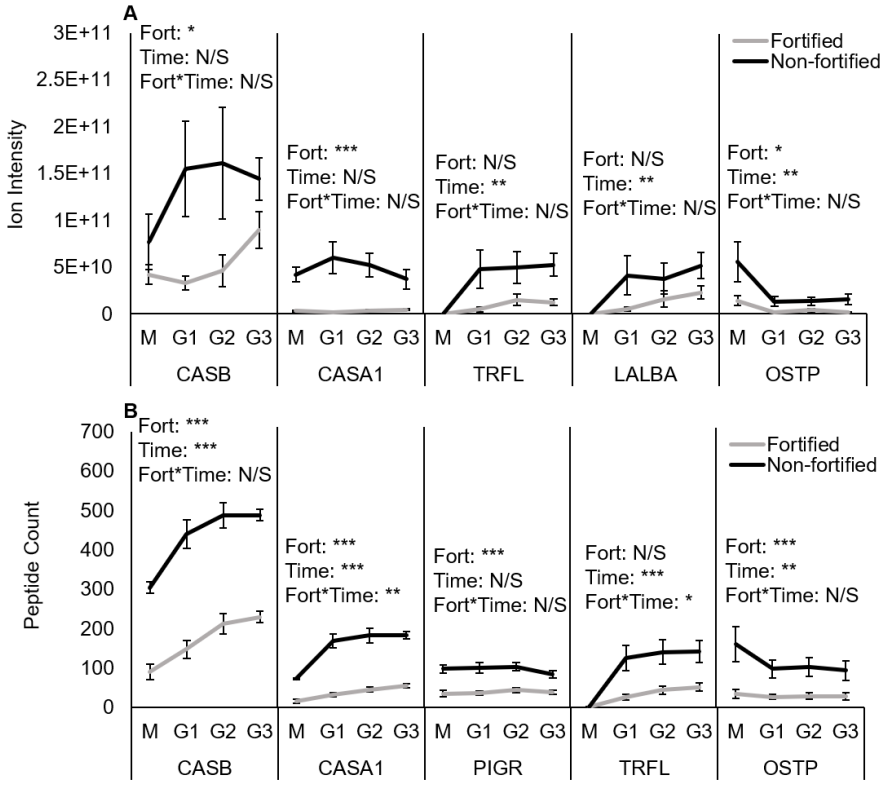

Figure 2.4. Comparison of average peptide abundance (A) and count (B) between fortified and non-fortified infants, grouped by human milk proteins. Data are shown as means ± SE, fortified n=6 infants, non-fortified n=5 infants. ***P<0.001, **P<0.01, and *P<0.05. M, milk samples; G1, G2, and G3 are gastric samples after one, two, and three hours of digestion, respectively; CASB, human β-casein; CASA1, human α_{s1}-casein; TRFL, human lactoferrin; LALBA, human α-lactalbumin; OSTP, human osteopontin; PIGR, human polymeric immunoglobulin receptor.

Bioactive peptides in the stomach

Milk proteins have been a highly-studied source of bioactive peptides due to milk's evolution as a source of nutrition for mammals. Bioactive peptides have been shown to be released from milk proteins by natural milk proteases (161), bacterial proteases (179, 180), and *in vitro* digestive proteases (149, 170, 181), but few studies have measured the bioactivity of peptides released by *in vivo* human digestive proteases. The purpose of this study was not to determine the bioactivity of gastric milk peptides, but it does serve as a launch pad for future studies of bioactive peptides. Homology search of total identified peptides revealed 92 unique peptides identical to a known bioactive peptide, and 678 unique peptides with ≥80% sequence homology (770 total) (**Supplemental Table 2.2**). Of the 770 matching peptides, 127 were human milk protein-derived, 631 were bovine milk protein-derived, and 12 could be sourced to either species. Most of the potential bioactive peptides identified were bovine milk peptides, and thus only present in fortified infants. This finding is despite the fact that there were fewer total bovine milk peptides than human milk peptides; and is most likely a result of our use of a homology search for identification of potential bioactivity. As bovine milk peptides have been studied far more than human milk peptides, a larger pool of bioactive bovine milk peptides is available for comparison. Continued progress in human milk peptide discovery will increase the number of homologous matches that can be identified in milk digestive samples.

Table 2.3 lists the breakdown of proteins from which the peptides were found and the types of bioactive function they were identified with. One hundred forty-nine peptides were matched to more than 1 function: 32 had 4 different functions, 32 had 3 functions,

and 85 had 2 functions. A range of potential bioactivities was determined in peptides across all sample types. However, it is likely that only a few of the peptides may be functional in the stomach. Opioid-receptors in the stomach can bind milk-derived opioid peptides and delay gastric emptying time (182), and increase mucin production (183). Some bacteria susceptible to antimicrobial peptides have been found in the stomach, although whether these bacteria inhabit the stomach or are merely passing through remains unknown (184).

Table 2.3. Milk proteins from which homologous bioactive peptides were identified.

Protein	Homologous peptides	Antihypertensive	Antimicrobial	Antioxidant	Cell-proliferative	Immuno-modulatory	Mucin-stimulatory	Other
Bovine β-casein	227	150	73	21	21	23	22	84
Bovine α_{s1}-casein	146	71	64	5	--	6	5	15
Bovine β-lactoglobulin	118	29	75	5	11	--	--	41
Human β-casein	118	51	46	1	20	--	1	1
Bovine α_{s2}-casein	66	9	59	6	--	--	--	1
Bovine κ-casein	56	5	41	--	--	--	2	11
Both β-casein	12	12	--	--	--	--	--	1
Bovine α-lactalbumin	11	3	--	--	--	--	--	8
Bovine lactoferrin	7	--	2	--	5	--	--	--
Human lactoferrin	6	--	4	--	--	--	2	--
Human κ-casein	2	1	--	1	--	--	--	--
Human α-lactalbumin	1	--	--	--	--	--	--	1

Peptides homologous to bioactive peptides in the MBPDB increased over time of gastric digestion. The average abundance of all ≥80% homologous peptides significantly increased from milk to one hour of gastric digestion (**Figure 2.5A**). Homologous peptide abundance tended to increase after two and three hours of gastric digestion, but changes were not significant. Homologous peptides in the gastric samples represented 18.9%, 18.1%, and 19.9% of the total abundance of one-, two-, and three-hour gastric samples, respectively. Homologous peptides in milk only represented 3.4% of total milk peptide abundance. Peptide count followed a similar pattern of increase as abundance (**Figure 2.5B**). Milk contained an average of 52 ± 9.2 homologous peptides, which significantly increased to 129 ± 14.5 in one-hour gastric samples, 147.9 ± 16.7 in two-hour samples, and 158.2 ± 18.3 in three-hour samples. Unlike for total peptide abundance, homologous peptides did not continue to increase across gastric digestion. The only significant increase occurred from milk to one-hour gastric digestion. It seems that the initial introduction of milk proteins to gastric conditions catalyzed the majority of potentially bioactive peptide release, with only small increases thereafter.

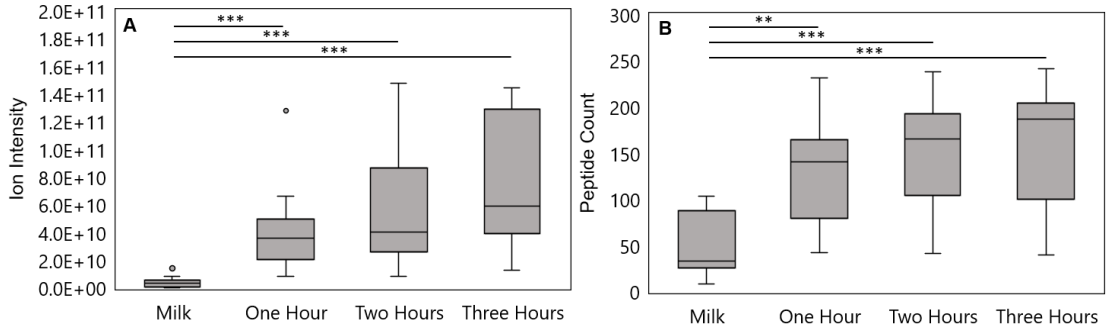

Figure 2.5. Boxplots of the abundance (A) and count (B) of peptides homologous to bioactive peptides by sample type. Boxes are bordered by the first and third quartiles. The center bar represents the median, n=14. ***P<0.001, **P<0.01, and *P<0.05.

Structural characteristics of peptide survivability

Though the relative abundance and count of bioactive peptides increased over digestion, a remaining question was whether bioactive milk peptides were more likely to survive gastric proteolysis than non-bioactive peptides. Consideration of these factors is important, as many identified bioactive peptides are functional for enterocytes specifically (185, 186), or against bacteria that primarily inhabit the gut (179), whereas others are functional only when they are absorbed across the intestinal membrane (187). Peptides that survive longer in gastric proteolytic conditions are more likely to reach the intestine and be biologically relevant. We therefore identified factors that may contribute to a peptide's survival in the stomach by comparing peptides that survived further digestion after their appearance with peptides that were absent from one or more time point after their appearance. However, as gastric emptying is continuous during digestion (173), it is possible that some of the differences were due to peptides progressing into the intestine early rather than being further cleaved.

With milk considered to be digestion time=0 hr, 153 of the 770 homologous peptides were present in at least one infant at all time points, 242 were present after one hour of gastric digestion onward, 80 from two hours onward, and 46 only appeared after three hours of digestion. The remaining 244 peptides were not identified in any infant for at least one of the time points after their initial appearance, thus likely being further digested after release. In the total peptidome, 7,210 peptides survived digestion after their appearance time and 4,402 did not. A slightly larger percentage of homologous peptides survived compared with total peptides (67.9% and 62.1%, respectively).

Homologous peptide amino acid composition had more similarities with surviving peptides than non-surviving peptides (**Figure 2.6A**). Surviving peptides had significantly higher percentages of F, L, M, P, Q, V, W, and Y residues, and lower D, E, R, and S residues than non-surviving peptides. Surviving peptides were thus more likely to be hydrophobic and contain more proline residues. Though pepsin has low amino acid specificity with slight preference for bulky hydrophobic side-chains (188), these bulky side-chains made up a larger percentage of surviving peptides than non-surviving peptides, indicating an alternate determining factor for survivability. Proline, due to its nitrogen being covalently bound into a ring structure, forms bends in a protein's secondary structure (189). These bends might be enough to prevent efficient interaction of the active site of pepsin and other proteases with substrate peptide bonds. Proline has been known to reduce protease efficiency when substituted for other amino acids (190, 191), thus, the relatively higher percentage of proline in surviving peptides could be a contributing factor to their survival. The high ratio of proline in homologous peptides could indicate their tendency to survive further gastric digestion.

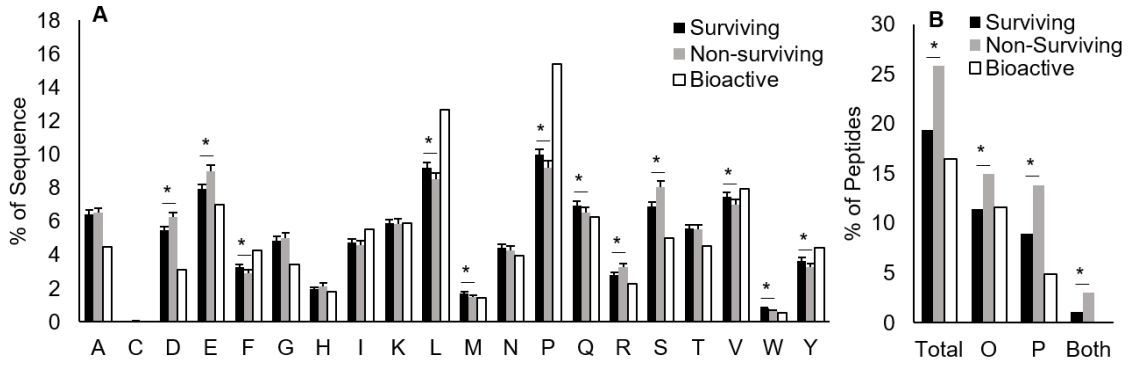

Figure 2.6. Comparison of peptide characteristics. (A) Average percentage of amino acids in surviving, non-surviving, and bioactive peptides. Data are means ± 95% CI, surviving *n*=7,210 peptides, non-surviving *n*=4,402, bioactive *n*=770. Asterisks (*) indicate significant differences. (B) Percentage of total peptides with modifications for surviving, non-surviving, and bioactive peptides; surviving *n*=7,210 peptides, non-surviving *n*=4,402, bioactive *n*=770. Asterisks (*) indicate significant differences. O, oxidation; P, phosphorylation.

As with amino acid composition, bioactive peptide post-translational modification (PTM) rates more closely resembled surviving peptide modification rates than those of non-surviving peptides (**Figure 2.6B**). Surviving peptides had fewer oxidized Met and phosphorylated Ser and Thr residues than non-surviving peptides. There are examples of substrate phosphorylation-enhancing proteolytic activity of proteases or marking substrates for digestion (192, 193), but phosphorylation can also impart proteolytic resistance to the substrate (194). The present study only examined oxidation and phosphorylation as potential PTMs. Future identification of other types of PTMs in peptidomic data can allow further determination of the effect of specific PTMs on peptide resistance or susceptibility to proteolysis. As homologous peptide PTMs more closely

resembled surviving peptide PTMs, it is possible that bioactive peptides are more likely to survive gastric digestion.

In addition to analyzing factors that contributed to overall peptide survivability, we also examined rates of peptide release over time across the sequence of three human milk proteins. β-Casein (**Figure 2.7A)**, α_{s1}-casein (**Figure 2.7B**), and lactoferrin (**Figure 2.7C**) released the most abundant human milk peptides. Regions of protein digestion appeared to be consistent over time within the protein sequences. For β-casein and α_{s1}-casein, the N-terminal regions (β-casein f(1–42) and α_{s1}-casein f(1–36)) were most highly abundant in milk, then decreased during gastric digestion; whereas the peaks in regions β-casein f(52–146), β-casein f(181–211), α_{s1}-casein f(97–126), and α_{s1}-casein f(150–167) consistently increased in abundance as digestion progressed. Though lactoferrin had major abundance peaks at f(65–93) and f(271–299), abundance was only distinctly different between milk and gastric samples, and not over time.

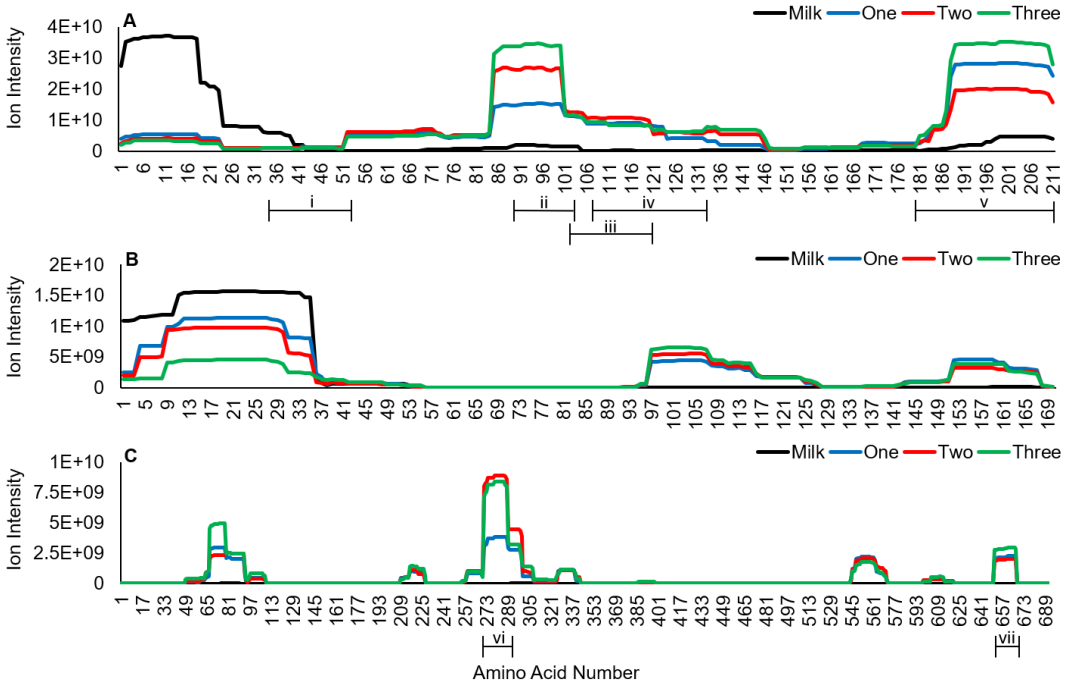

Figure 2.7. Abundance of peptides released from human milk β-casein (A), α_{s1}-casein (B), and lactoferrin (C) mapped to the protein sequence by amino acid. Results are shown as means, *n*=14. Bracketed lines indicate regions of homologous bioactive peptides. i, Antihypertensive (20 peptides); ii, antihypertensive (8); iii, cell-proliferative (20); iv, antihypertensive (31); v, antimicrobial (46); vi, antimicrobial (4); vii, mucin-stimulatory (2).

We identified seven regions in these three proteins that released multiple peptides with similar bioactivity based on homology. The regions β-casein f(35–52), β-casein f(90–103), and β-casein f(107–133) were antihypertensive, β-casein f(179–211) and lactoferrin f(269–288) were antimicrobial, β-casein f(102–120) was cell-proliferative, and lactoferrin f(660–667) was mucin-stimulatory. Four of these regions corresponded to peaks of peptide release that increased during digestion, suggesting their release may be controlled inside the stomach.

Conclusions

In summary, total milk peptide release increased in both count and abundance over three hours of preterm gastric digestion, though individual milk proteins had varying rates of increase and decrease. Furthermore, several known bioactive peptides are released in the stomach, and some are capable of surviving gastric proteolysis after which they have opportunity to enter the intestinal tract. Several limitations of this study include the use of mass spectrometry to measure peptide release and protein digestion over time. Peptide abundance is an incomplete estimation of protein digestion as the ionization efficiency of peptides differs based on amino acid side chains and PTMs (195, 196). Furthermore, the parameters of this study's mass spectrometry analysis did not allow for identification of low *m/z* peptides, which may significantly contribute to the total peptide content. The identification of these peptides will be necessary in future studies to complete the peptide profile of milk protein digestion. This study also lacked information on the protein composition of the milk and fortifier, which would improve the analysis of relative peptide release from individual milk proteins. Human milk proteins can vary by the individual, so it will be important to understand how initial milk protein composition affects peptide release (108).

The results from the present study contribute to a comprehensive view of milk protein digestion and bioactive peptide release from milk in the stomach of infants. Some remaining questions that future studies will need to address include how protein digestion and peptide release differ between term and preterm infants, the degree of peptide release in the infant intestinal tract, and what bioactive peptides remain undiscovered from the less well-studied regions of the milk proteome, particularly those known to release a large

amount of milk peptides. In addition, questions related to clinical practice that deserve study include the impact of released peptide profiles on measures of intestinal health, including inflammation, permeability, motility, the composition and functional capacity of the microbiota, and on gut-related health outcomes such as sepsis and necrotizing enterocolitis. It also will be valuable to determine the impact of continuous vs bolus feeding on milk peptide release and to determine whether some infants would benefit from exogenous proteases (e.g., infants with short gut syndrome or other forms of protein malabsorption).

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Chapter 3 – Milk peptides survive *in vivo* gastrointestinal digestion and are excreted in the stool of infants

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Abstract

Background: Human milk peptides released by gastrointestinal proteases have been identified with bioactivities that can benefit the infant but must first reach their respective sites of activity. Peptides in the stool either survived to or were released inside the intestinal tract, and thus had the opportunity to exert bioactivity there. However, it is unknown whether any milk peptides, bioactive or not, can survive into the stool of infants.

Objective: The aim of this study was primarily to identify milk peptides in infant stool samples and secondarily test the hypotheses that the milk peptide profiles of stools are different between preterm infants at different days of life and between preterm and term infants.

Methods: Infant stool samples were collected from sixteen preterm infants (<34 wk gestational age) at days 8 or 9 and 21 or 22 of life, and from ten term infants (>34 wk gestational age) at days 8 or 9 of life. Milk peptides were isolated from the stool samples and identified using tandem mass spectrometry. The peptide counts and abundances were compared between infant groups.

Results: One hundred and eighteen exclusively milk-derived peptides from the caseins and α-lactalbumin were present in the stool samples, including some peptides with known or potential bioactivity. The remaining 8,014 identified peptides could be derived either from milk or endogenous proteins. Though many individual milk peptides were significantly different between preterm infants at 8/9 and 21/22 days of life and between preterm and term infants, total peptide abundance and count were similar for all three groups.

Conclusions: This is the first study to confirm the survival of milk peptides to the stool of infants. Some of the peptides had potential bioactivities that could influence infant gut development. These results are important to understanding the physiological relevance of human milk peptides to the infant.

Keywords: peptides, human milk, stool, infant, bioactive

Introduction

Over the course of digestion, human milk proteins are broken down by the action of milk, gastric, intestinal, and bacterial proteases. These proteases release amino acids that the infant can absorb and utilize for protein synthesis and growth, but the first compounds cleaved from proteins are larger peptides. Many milk peptides have been identified with bioactivities that may be useful to the developing infant by killing pathogenic microbes (197, 198), stimulating the growth of commensal bacteria (199, 200), stimulating the immune system (201), stimulating mucin secretion (202), and extending gastrointestinal (GI) transit time (203). In many instances, the released peptides have distinct and more potent bioactivity than their intact parent proteins (204, 205). However, most bioactive peptides have been identified from *in vitro* digested milk and milk products. Only one study has identified novel bioactive peptides from undigested human milk (140), whereas others have confirmed the *in vivo* release of bioactive peptides in human milk and infant gastric digesta through homology searches (154, 206). As milk and gastric digesta represent only the beginning of protein digestion, little is known about how bioactive peptides are released and survive digestion throughout the entire gastrointestinal tract.

For bioactive milk peptides to be relevant to the developing infant, they must first be released from their parent protein by digestion and then survive until they reach their site of action. For many of the bioactivities, this site is the upper intestinal tract and colon, where they either can be absorbed into the bloodstream to act systemically, or can act locally on bacteria, immune cells, and intestinal epithelial cells. The different proteolytic environments of the GI tract alter the peptide profile at each site by cleaving

new peptides and breaking down those already released (154, 207). Milk proteins are exposed to different proteases with different cleavage site specificities at each site of digestion. In human milk within the mammary gland, proteases such as plasmin, thrombin, elastase, and kallikrein initiate the breakdown of milk proteins into peptides (161, 208, 209). In the stomach, pepsin is secreted, and cathepsin D from milk is activated by the high acidity (160). Protein digestion continues in the intestinal tract with the addition of pancreatic proteases like trypsin and chymotrypsin and brush border exopeptidases. In the colon, microbes can contribute to further proteolysis. Indeed, fecal proteolysis experiments on pancreatectomized and healthy subjects show that the gut microbiota contributes to digestive proteolysis (210). Any peptides that are present in the stool must therefore either have been released in the colon or survived proteolysis long enough to make it to the colon. These peptides would have had the potential to exert site-specific bioactivities in the colon and upper intestinal tract relevant to the infant's health, such as antimicrobial or immunomodulatory activity.

There have been few studies on the survival of milk proteins to stool. Human whey proteins such as lysozyme, lactoferrin, and IgA have been identified in both preterm and term infant stool through immunoblotting (169, 211, 212) and proteomics (213). Intact casein proteins have not been identified in the stool. Although lactoferrin and many other milk proteins can be produced endogenously by humans, human lactoferrin and its fragments were identified only in the stool of infants fed human milk and not bovine milk, suggesting that no endogenous lactoferrin is secreted by the infant (214). No studies have yet been performed on the survival of milk protein-derived peptides.

The primary objective of this study was to determine whether any human milk peptides survive GI digestion to reach the stool of infants. Secondary objectives were to compare the milk peptides in the stools of preterm infants between 8 or 9 days of life (8/9 DOL) and 21 or 22 days of life (21/22 DOL), and between preterm and term infants at 8/9 DOL. Peptidomics analysis was used to identify milk peptides from the stool of term and preterm infants. The data were analyzed for similarities and differences in the peptide profiles of stools from the infants and to identify the presence of bioactive peptides.

Methods

Materials

Ammonium bicarbonate and HPLC-grade acetonitrile were obtained from Thermo Fisher Scientific (Waltham, MA), trifluoroacetic acid and HPLC-grade formic acid were obtained from EMD Millipore (Billerica, MA), HPLC-grade ethanol, iodoacetamide, and trichloroacetic acid were obtained from Sigma-Aldrich (St. Louis, MO), and dithiothreitol was obtained from Promega (Madison, WI).

Participants and enrollment

This study was approved by Institutional Review Boards at Legacy Health Systems and Oregon State University. Infant subjects were enrolled at Randall Children's Hospital and were grouped as either preterm (preterm infants of <34 wk gestational age) or term (late preterm and full-term infants of >34 wk gestational age). As this study was primarily exploratory as to the presence of milk peptides in stool, sample size of the groups was not calculated. Clinical data for each infant were collected upon enrollment and at each feeding and are listed in **Table 3.1**. Eligibility criteria for enrollment included

having an indwelling naso/orogastric feeding tube, tolerating <60 min bolus feeding and ≥4 mL feed volumes, and mothers that could produce a volume of milk for one full day of feeding. Exclusion criteria included life-threatening diagnoses, GI anomalies or major GI surgeries, genitourinary anomalies, and any significant metabolic or endocrine diseases.

Table 3.1. Characteristics for infants included in this study.

Infant	Maturity	8/9 DOL	21/22 DOL	Gender	Post-menstrual age (wk)	Birth weight (g)
1	Preterm	X	X	F	30.3	1205
2	Preterm	X	X	F	30.3	1105
3	Preterm	X	X	M	31.7	2070
4	Preterm	X		M	26.6	695
5	Preterm	X		M	27.9	1165
6	Preterm		X	M	31.4	1843
7	Preterm	X		F	31.9	1920
8	Preterm		X	F	26.4	1140
9	Preterm	X		F	26.4	880
10	Preterm		X	F	27.7	1080
11	Preterm		X	F	27.7	1050
12	Preterm	X	X	F	26	900
13	Preterm	X	X	M	26	900
14	Preterm	X	X	F	31	1340
15	Preterm	X		M	31	1220
16	Preterm	X	X	M	32	1245
17	Term	X		F	38.7	1930
18	Term	X		M	34.7	1625
19	Term	X		M	35.9	3657
20	Term	X		M	39.6	2455
21	Term	X		F	34	2105
22	Term	X		F	35.7	2785
23	Term	X		M	37.3	3040
24	Term	X		F	34.4	2280
25	Term	X		F	34.4	2135
26	Term	X		M	34	2570

Stool was collected from preterm infants once during a two-day period at 8/9 DOL and/or 21/22 DOL and from term infants at 8/9 DOL. As all enrolled term infants

were discharged from the hospital prior to reaching 21 DOL, no stool was able to be collected for the 21/22 time point for term infants. Additionally, several preterm infants did not stool over the course of one or the other of the two-day periods and only had one stool sample collected. Feedings were prepared at Randall Children's Hospital, Portland, OR using aseptic techniques. Frozen human milk was thawed at 37°C and delivered to the infant through the naso/orogastric feeding tube over a time period of 30–60 min. Nurses attempted to collect all stool produced over 48 hr by the infant after their first feeding on day 8 and 21. In total, sixteen preterm infants and ten term infants were included in this study. After stool was collected, it was immediately frozen at –80°C and transported to Oregon State University on dry ice for sample analysis.

Sample preparation

Initial stool preparation

Stool samples were thawed on ice, and a small portion (~500 mg) was collected, weighed, and dissolved in water to a concentration of 10% m/v. The samples were thoroughly agitated with a vortex mixer and sonicated for 10 s at 60 amps to ensure homogenization of the mixture. The samples were centrifuged at 4,000×g for 10 min to precipitate remaining large solids, and the supernatant was centrifuged at 12,000×g for 20 min to remove cellular matter and lipids. The infranatant was pipetted from below the lipid layer and stored at –80°C until analysis.

Protein and peptide concentration determination

The combined protein and peptide concentrations and peptide isolate concentrations of the stool samples were determined in duplicate with the Pierce™

Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific, Waltham, MA) based on the reduction of Cu^{2+} to Cu^{1+} by peptide bonds. Two aliquots of 40 μL were removed from the stool infranatants. The first aliquot was analyzed for combined protein and peptide following the protocol for the kit. The concentration of only the peptide (peptide isolate) was determined in the second aliquot after ethanol precipitation of intact proteins. The samples were mixed with 160 μL of ice-cold ethanol and incubated for 2 hr at –20°C. Samples were centrifuged at 12,000×g for 30 min and the pellet was discarded. The supernatant was lyophilized, and the peptides were reconstituted in 40 μL of water for concentration determination.

Total peptide extraction

Peptides were extracted from 100 μL of the infranatant as described in our previous peptidomic publication, with some modifications (161). To prevent milk peptides from potentially being precipitated with intact proteins, any disulfide bonds between the peptides and proteins were reduced and alkylated. The samples were mixed with 100 μL of 200 mM ammonium bicarbonate. Dithiothreitol was added to the samples to a final concentration of 40 mM, and the samples were incubated at 56°C for 45 min. Iodoacetamide was added to a final concentration of 100 mM and the samples were incubated at room temperature in the dark for 1 hr. Intact proteins were precipitated as described previously (161). The peptides in the supernatant were treated by C18 reverse-phase extraction as described previously (161). After elution from the C18 column, the peptides were lyophilized and rehydrated in 100 μL of nanopure water prior to mass spectrometry analysis.

Liquid chromatography mass spectrometry

Peptides were analyzed with mass spectrometry as described in our previous publication (206) with some modifications as follows. The liquid chromatography phase was condensed so that the peptides were eluted from the UPLC column over a period of 60 min. The separation gradient was 3–10% solvent B over 3 min, 10–30% solvent B over 42 min, 30–90% solvent B over 3 min, held at 90% solvent B for 4 min, 90–3% solvent B over 1 min, and held at 3% solvent B for 7 min. A 30-min column wash was included between sample separations. The mass spectra were collected and analyzed with the following altered parameters. Peptides were ionized with an electrospray voltage of 2,300 V. Scanned masses were between 375 and 1,500 *m/z* and with a charge state of 2–8. Precursor ions were fragmented with high-energy collisional dissociation with a collision energy of 35%. Peptides were detected from the raw files using Thermo Proteome Discoverer 2.2.0.388. Dynamic peptide modifications allowed were phosphorylation of serine and threonine, oxidation of methionine, and carbamidomethylation of cysteine.

Data analysis

Stool samples were analyzed for the combined protein and peptide concentration, peptide isolate concentration, peptide abundance, and peptide count. Peptide abundance is unitless and represents the summed ion intensities from the mass spectra, and peptide count is the number of unique peptide sequences. Milk peptides from like proteins were grouped for analysis into the following groups: immunoglobulins, antiproteases, proteases, nutrient-binding proteins, caseins, mucins, and other milk proteins. The protein compositions of the groups are defined in **Supplemental Table 3.1**.

Statistical comparisons were performed with RStudio 1.2.1335. Stool peptides were grouped into preterm infants at 8/9 DOL, preterm infants at 21/22 DOL, and term infants at 8/9 DOL. Paired *t*-tests were used to compare stool peptide isolate concentrations and stool protein and peptide concentrations within each group. ANOVA followed by post-hoc Tukey-Kramer test was used to compare peptide abundance, count, and concentration between preterm 8/9, 21/22, and term 8/9 groups. Significance was determined by a *P*-value of <0.05. Data are presented as mean ± standard error.

Milk peptides were analyzed for sequence homology with bioactive peptides in the Milk Bioactive Peptide Database (MBPDB) (131). The search type was "Sequence" with a similarity threshold of 80%. PepEx (http://mbpdb.nws.oregonstate.edu/pepex/) was used to map the identified milk peptides to their location in the parent protein sequence.

Results

Milk peptide profile of infant stool

The overall peptide profile of all stool samples was composed of 8,132 peptides divided amongst 169 unique proteins previously identified in human milk. While the majority of the peptides are derived from proteins that could be from either milk or endogenous sources (hereafter referred to as "potential milk peptide"), 118 peptides were derived from proteins that are exclusive to breast milk: 73 peptides from α-lactalbumin, 42 from β-casein, 2 from α_{s1}-casein, and 1 from κ-casein. Of the remaining peptides that are potentially derived from milk, lactoferrin was the single largest contributor with 1,863 peptides (**Figure 3.1**). There were 2,077 combined peptides from all Ig proteins, and the largest Ig protein contributors were Ig heavy constant alpha (IGHA) 1 and 2, which

encode the constant segment of the heavy chain of IgA. IGHA1 had 360 peptides and IGHA2 had 197; an additional 408 were indistinguishable between IGHA1 and IGHA2. IgM and IgG are the remaining antibodies present in breast milk, and 166 and 118 peptides derived from these two Igs' heavy chains, respectively. The full list of proteins and how many peptides were identified from each is included as **Supplemental Table 3.2**.

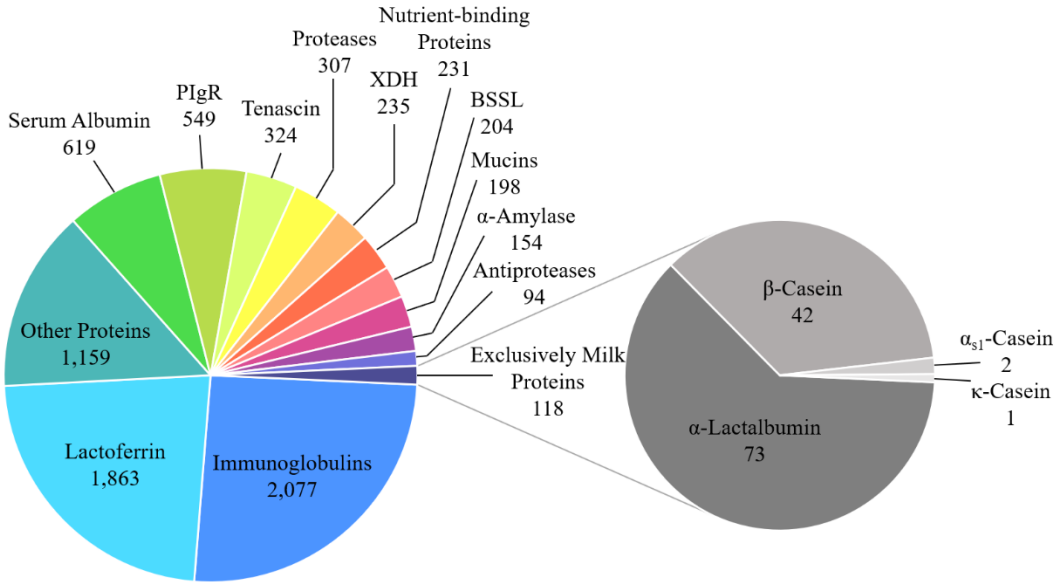

Figure 3.1. Pie chart of the breakdown of milk peptides identified in the infant stool samples. Numbers underneath the pie section labels represent the number of unique peptides identified from all infant stool samples, *n*=33. PIgR, polymeric Ig receptor; XDH, xanthine dehydrogenase; and BSSL, bile salt-stimulated lipase.

The combined protein and peptide concentrations and peptide isolate concentrations of the stool samples were measured with the bicinchoninic acid assay, and are included in **Table 3.1** for each infant. The mean concentrations of combined proteins and peptides in the stool samples were 28.2 ± 4.3 μg/mg for preterm infants at 8/9 DOL,

20.6 ± 2.6 μg/mg for preterm infants at 21/22 DOL, and 17.8 ± 2.7 μg/mg for term infants at 8/9 DOL (**Supplemental Figure 3.1**). The mean concentrations of peptide were less than half of the combined protein and peptide, at 9.6 ± 2.2 μg/mg, 7.7 ± 1.1 μg/mg, and 7.6 ± 1.4 μg/mg for preterm 8/9, preterm 21/22, and term 8/9, respectively. All infant groups had significantly different stool peptide and protein concentrations. There were no significant differences between preterm 8/9, 21/22, and term 8/9 for peptide concentration or combined protein and peptide concentration.

Bioactive milk peptides in the infant stool

To predict the bioactivity of identified milk peptides, the peptides were compared for sequence homology with known bioactive peptides in the MBPDB (131). There were 26 peptides that matched with ≥80% sequence homology with the database, but as some query peptides matched with multiple known peptides, only 19 of the matches were unique peptides (**Table 3.2**). All homologous peptides derived from lactoferrin and β-casein, which is unsurprising as the majority of bioactive peptides in the MBPDB are from these two proteins. Two peptides, VVPYPQR and DLENLHLPLPL, matched with peptides derived from bovine milk β-casein; the rest matched with peptides from human milk proteins. Antimicrobial activity was the most prevalent function with 12 homologous peptides, followed by DNA synthesis-stimulatory with 4 peptides, antioxidant with 2 peptides, antihypertensive with 2 peptides, and opioid with 1 peptide. Only one query peptide, RETIESLSSSEESITEYK from β-casein, was 100% homologous with a known bioactive peptide. This peptide was identified as one that stimulates DNA synthesis and cell proliferation of BALB/c3T3 mice fibroblasts (215) and was previously identified in milk and gastric samples (154, 206).

Table 3.2. Milk peptides from stool that matched with ≥80% sequence homology with a known bioactive milk peptide from the MBPDB.

Known peptide	Query peptide	Protein	% Sequence Homology	Function	No. of Infants
AVPYPQR[1]	VVPYPQR	β-casein	85.7	Antihypertensive	31
AVPYPQR[1]	VVPYPQR	β-casein	85.7	Antimicrobial	31
AVPYPQR[1]	VVPYPQR	β-casein	85.7	Antioxidant	31
ENLHLPLPLL[1]	DLENLHLPLPL[2]	β-casein	81.8	Antihypertensive	13
LENLHLPLP	DLENLHLPLPL[2]	β-casein	81.8	Antihypertensive	13
LLNQELLLNPTHQIYPV	NQELLLNPTHQIYPV[2]	β-casein	88.2	Antimicrobial	6
LLNQELLLNPTHQIYPV	QALLLNQELLLNPTHQIYP[2]	β-casein	85	Antimicrobial	25
QELLLNPTHQIYPVTQPLAPVHNPISV	NQELLLNPTHQIYPVTQPLAPVHNPISV[2]	β-casein	96.4	Antimicrobial	29
QELLLNPTHQIYPVTQPLAPVHNPISV	LLNPTHQIYPVTQPLAPVHNPISV[2]	β-casein	88.9	Antimicrobial	21
QELLLNPTHQIYPVTQPLAPVHNPISV	LLLNQELLLNPTHQIYPVTQPLAPVHNPISV[2]	β-casein	87.1	Antimicrobial	24
QVVPYPQ	QVVPYPQR	β-casein	87.5	Antioxidant	16
QVVPYPQ	VVPYPQR	β-casein	85.7	Antioxidant	31
RETIESLSSSEESITEYK	RETIESLSSSEESITEYK[2]	β-casein	100	Stim. DNA synthesis	30
RETIESLSSSEESITEYK	ETIESLSSSEESITEYK[2]	β-casein	94.4	Stim. DNA synthesis	33
RETIESLSSSEESITEYK	RETIESLSSSEESITEYKQK[2]	β-casein	90	Stim. DNA synthesis	20
RETIESLSSSEESITEYK	RETIESLSSSEESIT[2]	β-casein	83.3	Stim. DNA synthesis	16
VENLHLPLPLL[1]	DLENLHLPLPL[2]	β-casein	81.8	Antihypertensive	13
EATKCFQWQRNMRKVR	SQPEATKCFQWQRNMR	Lactoferrin	81.3	Antimicrobial	7
FFSASCVPGADKGQFPNLCRLCAGTGENKCA	FFSASCVPGADKGQFPNLCRLCAGTGENK	Lactoferrin	93.6	Antimicrobial	6
KYLGPQY	KYLGPQYV	Lactoferrin	87.5	Opioid	23
PEATKCFQWQRNMRKVR	SQPEATKCFQWQRNMR	Lactoferrin	82.4	Antimicrobial	7
QPEATKCFQWQRNMRKVR	AVSQPEATKCFQWQRNMR	Lactoferrin	83.3	Antimicrobial	17
QPEATKCFQWQRNMRKVR	SQPEATKCFQWQRNMR	Lactoferrin	83.3	Antimicrobial	7
TKCFQWQRN	ATKCFQWQR	Lactoferrin	88.9	Antimicrobial	21
TKCFQWQRN	TKCFQWQR	Lactoferrin	88.9	Antimicrobial	23
TKCFQWQRN	EATKCFQWQR	Lactoferrin	80	Antimicrobial	15

[1] Known peptide was derived from a bovine milk protein.

[2] Peptide has been previously identified in human milk or infant gastric samples.

Twelve homologous peptides were identified in ≥50% of the infants' stools, and four in ≥80% of the infants. RETIESLSSSEESIT, with potential DNA synthesis-stimulating activity, was the only homologous peptide identified in every infant stool. Each infant had a mean of 11.4 ± 3.6 homologous peptides in their stool samples (between 4 and 17 homologous peptides per infant). Every peptide was present in at least one infant stool from each of the three groups (preterm at 8/9 DOL, preterm at 21/22 DOL, and term at 8/9 DOL).

Comparison of peptides from preterm and term infant stools

The total abundance and count of the peptides were highly similar among stools from preterm infants at 8/9 DOL, preterm infants at 21/22 DOL, and term infants at 8/9 DOL (**Figure 3.2**). There were no significant differences in peptide abundance or count between preterm 8/9, 21/22, and term 8/9 infants.

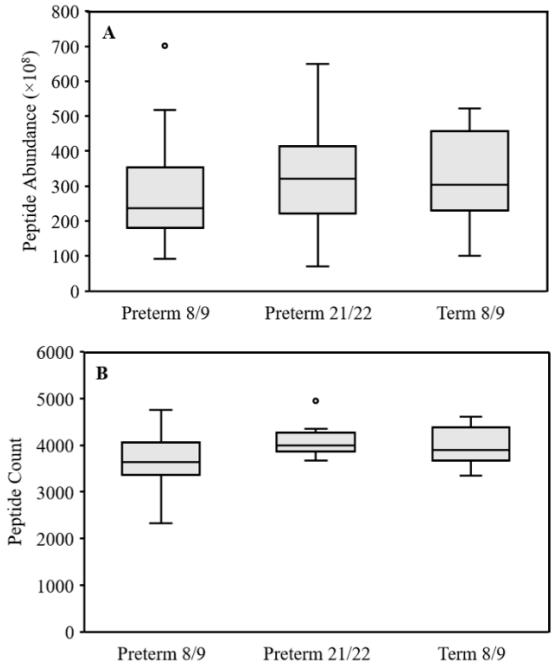

Figure 3.2. Boxplots of the abundance (A) and count (B) of total potential milk peptides from the stool of preterm infants at 8/9 DOL and 21/22 DOL and term infants at 8/9 DOL. Preterm 8/9 *n*=12, preterm 21/22 *n*=11, and term *n*=10.

The three infant groups remained similar when peptides from individual potential milk proteins or groups of related milk proteins were analyzed for abundance (**Table 3.3**) and count (**Table 3.4**). There were no significant differences for peptide abundances in individual proteins, only for peptide counts. Preterm 8/9 infant peptide counts were significantly lower than preterm 21/22 for lactoferrin, polymeric Ig receptor (PIgR), xanthine dehydrogenase, and mucins, and were significantly lower than term 8/9 for PIgR. The peptides from individual milk proteins and potential milk proteins were thus highly similar in stools among the three infant groups for both count and abundance.

The peptide profiles of each group were highly conserved, with peptides common to all groups accounting for the majority of the peptide abundance and count. One

hundred and twenty-five peptides were identified in 100% of the samples in each group and accounted for 18.8% of the mean abundance of peptides in the samples, 550 peptides were identified in ≥80% of the samples in each group and accounted for 32.5% of the mean abundance, 1,716 peptides were identified in ≥50% of the samples in each group and accounted for 33.5% of the mean abundance, and 5,742 peptides were identified in <50% of the samples in at least one group and accounted for only 15.2% of the mean abundance. On an individual protein level, peptides present in ≥50% of the samples in each group made up the majority of the mean peptide abundance for all proteins except bile salt-stimulated lipase and the caseins (**Figure 3.3**). These results suggest that although each group had different peptide profiles, those peptides that were the same between the groups were present at much higher relative amounts than peptides that were different.

Table 3.3. Comparison of the mean abundance of peptides from select milk proteins and potentially milk proteins between preterm infants at 8/9 DOL, preterm infants at 21/22 DOL, and term infants at 8/9 DOL.[1]

	Preterm 8/9[2] n=12	Preterm 21/22[2] n=11	Term[2] n=10
Immunoglobulins	$7.13\times10^9 \pm 1.08\times10^9$	$6.78\times10^9 \pm 1.12\times10^9$	$7\times10^9 \pm 9.55\times10^8$
Lactoferrin	$6.15\times10^9 \pm 1.33\times10^9$	$7.7\times10^9 \pm 1.23\times10^9$	$7.16\times10^9 \pm 1.03\times10^9$
PIgR	$1.9\times10^9 \pm 3.92\times10^8$	$2.35\times10^9 \pm 3.05\times10^8$	$2.45\times10^9 \pm 3.74\times10^8$
Serum Albumin	$1.82\times10^9 \pm 2.8\times10^8$	$2.08\times10^9 \pm 2.37\times10^8$	$2.18\times10^9 \pm 3.28\times10^8$
Antiproteases	$1.08\times10^9 \pm 2.28\times10^8$	$1.18\times10^9 \pm 1.62\times10^8$	$1.31\times10^9 \pm 2.46\times10^8$
Proteases	$1.11\times10^9 \pm 3.24\times10^8$	$1.27\times10^9 \pm 2.34\times10^8$	$9.54\times10^8 \pm 1.69\times10^8$
Tenascin	$9.05\times10^8 \pm 1.38\times10^8$	$1.09\times10^9 \pm 1.39\times10^8$	$1.12\times10^9 \pm 1.83\times10^8$
Nutrient-binding Proteins	$1.06\times10^9 \pm 2.2\times10^8$	$1.02\times10^9 \pm 1.71\times10^8$	$1\times10^9 \pm 1.72\times10^8$
Xanthine Dehydrogenase	$7.58\times10^8 \pm 1.71\times10^8$	$9.78\times10^8 \pm 1.38\times10^8$	$1.12\times10^9 \pm 2.13\times10^8$
α-Amylase	$7.04\times10^8 \pm 1.77\times10^8$	$7.45\times10^8 \pm 1.46\times10^8$	$9.68\times10^8 \pm 1.82\times10^8$
Bile Salt-Stimulated Lipase	$6.02\times10^8 \pm 1.57\times10^8$	$7.53\times10^8 \pm 1.71\times10^8$	$6.01\times10^8 \pm 1.57\times10^8$
Mucins	$4.15\times10^8 \pm 1.03\times10^8$	$6.94\times10^8 \pm 8.66\times10^7$	$7.43\times10^8 \pm 1.21\times10^8$
α-Lactalbumin	$3\times10^8 \pm 7.45\times10^7$	$4.05\times10^8 \pm 7.94\times10^7$	$5.42\times10^8 \pm 1.31\times10^8$
Caseins	$3.01\times10^8 \pm 9.19\times10^7$	$4.74\times10^8 \pm 1.26\times10^8$	$3.56\times10^8 \pm 7.6\times10^7$

[1] Data are represented as mean ± standard error.
[2] Abundance units are the summed ion intensities for each peptide from the mass spectra.

Table 3.4. Comparison of the mean count of peptides from select milk proteins and potentially milk proteins between preterm infants at 8/9 DOL, preterm infants at 21/22 DOL, and term infants at 8/9 DOL.[1]

	Preterm 8/9[2] n=12	Preterm 21/22[2] n=11	Term[2] n=10
Immunoglobulins	916 ± 45	987 ± 29.6	972 ± 30.3
Lactoferrin	789 ± 58.5	945 ± 26.6*	916 ± 38.6
Serum Albumin	269 ± 14.7	309 ± 10	299 ± 8.7
PIgR	256 ± 16	302 ± 8.1*	300 ± 9.4*
Proteases	136 ± 7.9	159 ± 5	146 ± 6.4
Tenascin	136 ± 8.9	159 ± 5.6	144 ± 6.8
Xanthine Dehydrogenase	103 ± 8	128 ± 5.6*	125 ± 4.1
Nutrient-binding Proteins	111 ± 5.7	115 ± 3.9	120 ± 4.4
Mucins	89.1 ± 7.7	111 ± 4.1*	106 ± 4.9
Bile Salt-Stimulated Lipase	87.2 ± 5.1	90.3 ± 4	89 ± 3.19
α-Amylase	70.2 ± 3.6	78.7 ± 3.5	74.5 ± 3.5
Antiproteases	59.3 ± 2.6	60 ± 2.2	58.5 ± 1.8
α-Lactalbumin	28.3 ± 3	32.9 ± 1.7	34.6 ± 1.7
Caseins	21.2 ± 2.7	26.5 ± 1.5	26.6 ± 1.4

[1] Data are represented as mean ± standard error.
[2] Count units are the number of unique peptide sequences.
* Indicates the mean is significantly different from the mean for preterm 8/9 in the same row, $P<0.05$.

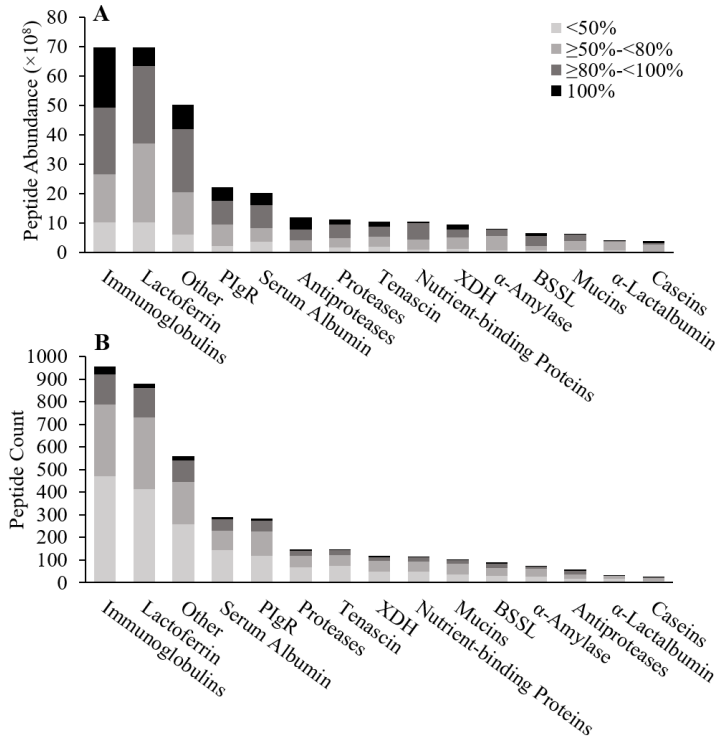

Figure 3.3. Stacked column charts of the abundance (A) and count (B) of peptides from individual milk proteins and protein groups. Stacking is based on the percentage of infants in each group that the peptides were identified in. Data are represented as means, *n*=33. PIgR, polymeric Ig receptor; XDH, xanthine dehydrogenase; BSSL, bile salt-stimulated lipase.

Only once individual peptides were analyzed did many of the differences between groups begin to appear. Both preterm 21/22 and term 8/9 infants had many more individual stool peptides that were in significantly higher abundance (>5-fold increase) than preterm 8/9 (**Figure 3.4A, 3.4B**). This pattern was conserved for both lactoferrin peptides (**Figure 3.4C, 3.4D**) and combined α-lactalbumin and casein peptides (**Figure 3.4E, 3.4F**). Three homologous peptides also were significantly different among the groups. DLENLHLPLPL from β-casein, with potential antihypertensive activity, was significantly higher in preterm 21/22 and term 8/9 infant stools compared with preterm 8/9, and LLNPTHQIYPVTQPLAPVHNPISV from β-casein, with potential antimicrobial

activity, was significantly higher in term than in preterm 8/9. Only ATKCFQWQR from lactoferrin, with potential antimicrobial activity, was significantly higher in the preterm 8/9 infants than in the term 8/9.

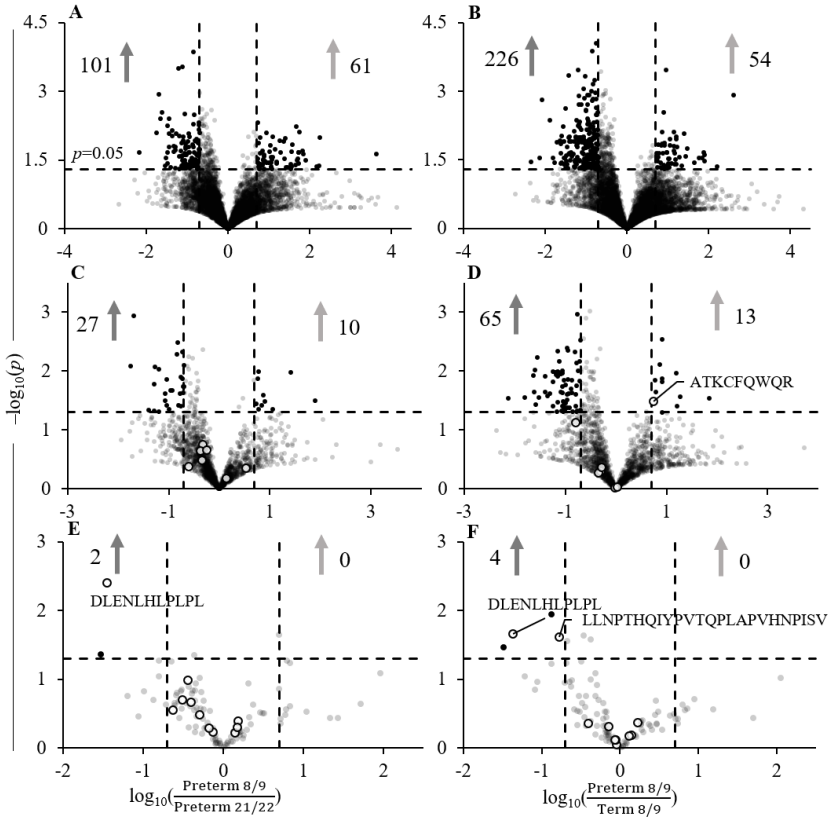

Figure 3.4. Volcano plots of total peptide abundance for preterm 8/9 and preterm 21/22 (A) and preterm 8/9 and term 8/9 (B), lactoferrin peptide abundance for preterm 8/9 and preterm 21/22 (C) and preterm 8/9 and term 8/9 (D), and combined α-lactalbumin and casein peptide abundance for preterm 8/9 and preterm 21/22 (E), and preterm 8/9 and term 8/9 (F). Filled dots represent peptides that were both significantly different (P<0.05) and present in one of the groups at 5-fold increased abundance. For lactoferrin and α-lactalbumin/casein, white dots with black borders represent peptides with ≥80% sequence homology to a known bioactive peptide from the MBPDB. Light grey arrows represent the number of peptides increased in the numerator, and dark grey arrows represent the number of peptides increased in the denominator. Preterm 8/9 n=12, preterm 21/22 n=11, and term n=10.

Preterm 8/9 and 21/22 and term 8/9 infants had similar counts and abundances of peptides, but there was considerable variation in the peptide profiles of each individual

infant's stool. Compared with the mean peptide abundance in stools across all infants, the stools of infants 3, 16, 19, and 20 had higher peptide abundances for nearly all major proteins and protein groups, whereas stools of infants 2, 12, 13, 21, and 26 had much lower peptide abundances (**Figure 3.5A**). There were no clear patterns of increase or decrease in peptide abundance in preterm infant stools from 8/9 to 21/22 DOL. Some infants, such as infant 2, had decreased stool peptide abundance between measurements, whereas the abundance in stools of others, such as infants 12 and 13, was increased. The percentage composition of each infant's stool peptide profile also varied between and within groups (**Figure 3.5B**). Each group had variation in the relative abundance of peptides from the measured milk proteins and potential milk proteins. Ig and lactoferrin peptides dominated the profiles in stools from infants 4 and 13 at 8/9 DOL compared with the other infants, and stools from infants 9 at 8/9 DOL, 12 at 21/22 DOL, and 23 had increased percentages of lower abundant peptides such as α-lactalbumin, caseins, α-amylase, and nutrient-binding proteins.

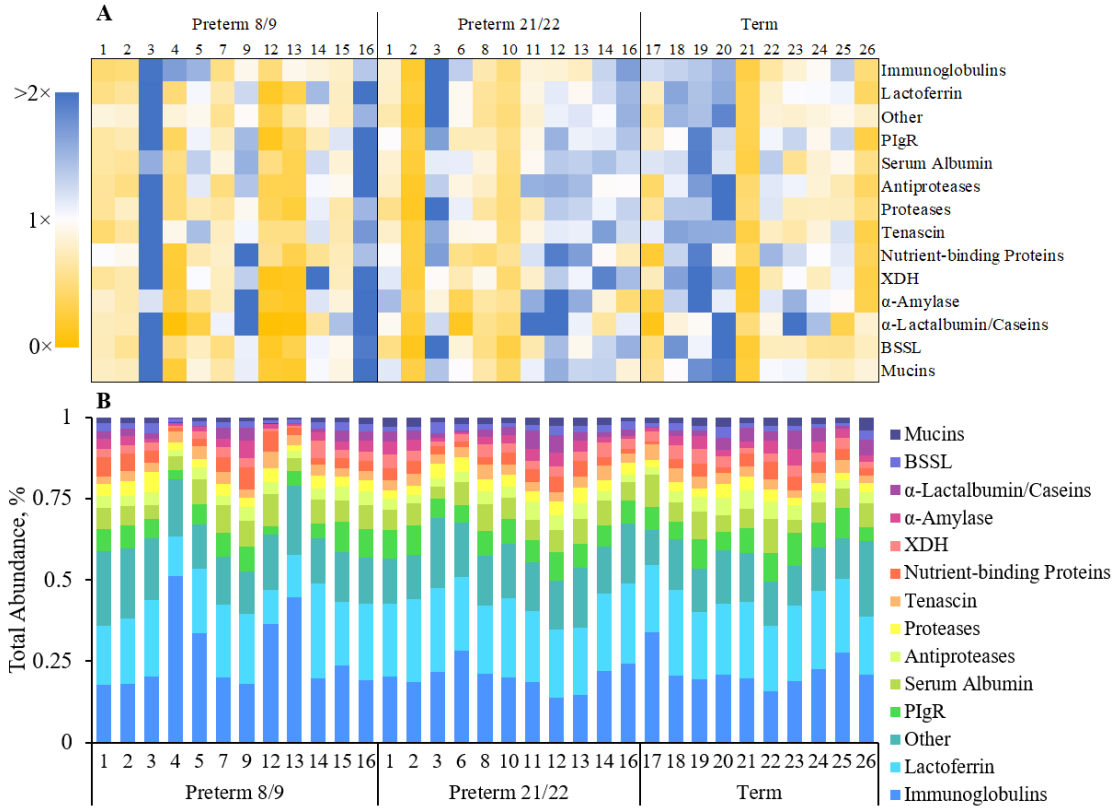

Figure 3.5. Variation in the peptide profiles of each infant. (A) Heatmap of the fold-change in peptide abundance from a specific protein or group of proteins for each infant compared to the mean. (B) Percent contribution of each protein or group of proteins to the total peptide profile of each infant. PIgR, polymeric Ig receptor; XDH, xanthine dehydrogenase; BSSL, bile salt-stimulated lipase.

Mapping the stool peptides to their parent protein

Peptide abundances were mapped to the parent protein sequence for lactoferrin, α-lactalbumin, and β-casein (**Supplemental Figure 3.2**). Peptides came from nearly identical regions of the proteins for all three groups. There were clearly defined regions of peptides at α-lactalbumin f(42–64) and f(71–93), and β-casein f(1–24) and f(161–211). Lactoferrin peptides were more spread out across the lactoferrin sequence, however. There were distinct peaks of peptide abundance in lactoferrin, but there was a nearly continuous abundance of peptides across the entire sequence. For peptide count, the

pattern of release was similar to that of abundance but with more similar peptide counts across the sequences of the proteins for the three groups (**Supplemental Figure 3.3**). For both count and abundance, stools from term 8/9 infants had slightly more peptides at each of the sites in the protein except for some regions in lactoferrin and the N-terminus of β-casein, where stools from preterm infants at 21/22 DOL had the most.

Discussion

This study is the first to profile the milk peptidome of infant stool. Previous investigations have profiled the peptidomes of human milk and infant gastric samples (153, 154, 206). Whereas those samples represent the beginning of protein digestion in the infant, stool represents the endpoint. Any milk proteins and peptides remaining in the stool are unavailable to the infant for nutrition but may be important bioactive factors in milk by influencing the gut environment through interactions with intestinal epithelial cells, immune cells, or the microbiota. The identification of remaining milk peptides in stool, some with known bioactivity or potential for bioactivity, represents another step along the path to determining whether the release and survival of milk peptides is coordinated to provide physiological benefit to the infant.

The peptide profile of stools differs significantly from the peptide profiles previously identified in undigested human milk and preterm gastric samples. The peptides in undigested human milk are primarily sourced from the caseins, with some from whey proteins such as osteopontin, PIgR, and butyrophilin subfamily 1 member a1 (140, 161). Almost no lactoferrin nor α-lactalbumin peptides are in human milk, suggesting these proteins are relatively undigested. In the infant stomach, casein peptides still dominate the profile, but lactoferrin and α-lactalbumin begin to release small

amounts of peptides at select regions in their sequences (153, 154, 206). The peptides in stool are completely different from those in milk and gastric digesta, with a predominance of lactoferrin peptides from across the entire sequence and little to no casein peptides. As the casein proteins are rapidly digested in the stomach and intestine (216), it is likely they are almost entirely broken down and absorbed as amino acids and di- and tripeptides before they reach the stool, with only the N- and C-termini of β-casein remaining in significant quantities. On the other hand, while lactoferrin is cleaved at many more sites along its sequence as it progresses through the GI tract, the released peptides persist into the stool. Lactoferrin, especially iron-saturated lactoferrin, is highly resistant to pepsin, trypsin, and chymotrypsin proteolysis (217, 218), so the presence of its peptides in stool provides evidence that the peptides may retain some of this resistance.

Many of the peptides detected in this study could be derived from either milk proteins or endogenous proteins. However, there was evidence that at least the peptides from lactoferrin, the caseins, and α-lactalbumin were from milk. β-Casein, αs1-casein, κ-casein, and α-lactalbumin are only secreted into milk, so any peptides from these proteins must have derived from the ingested milk. Lactoferrin is produced endogenously by mucosal linings and neutrophils in the intestine as part of the innate immune system (219), but a previous study comparing human lactoferrin in the stool of both human milk-fed and bovine milk-fed infants discovered no human lactoferrin from the bovine milk-fed infants (214). Therefore, the majority of the lactoferrin peptides identified in this study were also likely from the milk, rather than from the infants' endogenous production. IgA, IgM, and IgG, bile salt-stimulated lipase, and PIgR are found in large

quantities in breast milk, but these are also produced endogenously so it remains unclear whether their peptides derive from milk or from the infant (220-222).

There were very few differences in the stool peptide profiles between the preterm infants at 8/9 DOL, 21/22 DOL, and the term infants at 8/9 DOL. The majority of the peptide abundance in stool was composed of peptides that were conserved in both preterm and term infants. Similar results were reported for peptides in human milk, where a minority of shared peptides accounted for the majority of milk peptide abundance (154, 161). The overall peptide content and the protein-specific peptides were similar between groups, although there was a large degree of variation in the stool peptide profiles of individual infants within groups. Only at the individual peptide level did many of the differences become apparent. The results show that while similar numbers of milk peptides and potential milk peptides survived to the stool, the specific peptides differed by infant age and birth maturity. However, as no sample size was calculated for this study, the significance of the comparisons between infant groups are considered exploratory in nature. Due to the high amount of variation between the peptides in the infant stools, a larger sample size may be required to more accurately determine the differences or similarities in peptides between infants.

Previous peptidomics studies comparing term and preterm milk peptides were performed on only human milk (141, 146). Milk peptide abundances are higher in preterm milk during early lactation up to 41 days but then decrease to match term levels. The infants in this study were all 8–22 days old, so would have been at the stage of life when preterm infants are consuming larger quantities of milk peptides than term infants. However, as the milk and potential milk peptide abundance in term and preterm infant

stool were similar, something may have occurred during digestion to equalize the peptide levels. Compared with term infants, preterm infants have lower gastric proteolysis (208), and some studies report higher gastric pH (223), which could inhibit further milk protein digestion and peptide release in the stomach. In the duodenum, preterm infants produce less trypsin than term infants, which could also impact milk peptide release (224), but little else is known about infant intestinal digestion. Thus, it could be that preterm infants start with a higher peptide abundance in milk (141, 146) but are unable to cleave milk peptides as efficiently as term infants, leading to a similar milk peptide content in stool. Studies that clarify the digestive differences in the stomach and intestines of preterm and term infants are necessary for a full understanding of milk peptide release inside the infants.

Compared with the peptides identified in stool, many more homologous peptides have been identified in human milk and infant gastric samples. Previous searches with the MBPDB identified between 37 and 55 ≥80%-homologous peptides in undigested human milk samples, with 2 being identical to known bioactive peptides (153, 154, 206). Infant gastric samples contained 85 and 123 homologous human milk peptides, with up to 8 being identical to known bioactive peptides (154, 206). None of the homologous lactoferrin peptides identified in the present study has previously been identified, but nine of the twelve homologous β-casein peptides were identified in both milk and gastric samples (154, 206). Only VVPYPQR (antihypertensive, antimicrobial, antioxidant), QVVPYPQR (antioxidant), and QALLLNQELLLNPTHQIYPV (antimicrobial) from β-casein are homologous peptides that are for the first time identified in stool but not milk

or gastric samples, although peptides that differ by the addition or subtraction of one residue were previously identified (154, 206).

Although many peptides homologous to known bioactive peptides are released at the beginning of digestion, the present data show that most are further degraded until no longer bioactive by the time they reach the stool. However, some peptides with possible bioactivities relevant to infant health do persist. Many of the query peptides matched bioactive peptides with antimicrobial activity against bacteria and fungi that can infect the infant gut, such organisms as *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Candida albicans* (225, 226). Antioxidant milk peptides scavenge reactive-oxygen species and prevent lipid hydroxylation to reduce oxidative stress (170). The only peptide that matched 100% with a known bioactive peptide had DNA synthesis-stimulating properties for mouse fibroblasts (215). Human milk naturally contains peptide growth factors that stimulate intestinal development (20), so it could be that this DNA synthesis-stimulating peptide and others like it contribute to the trophic effects of milk. As stool represents the endpoint of digestion, it is unknown if other bioactive peptides are present inside the small intestine and gut that are broken down to amino acids before excretion. The majority of the peptides in stool, as in milk and gastric digesta, did not match a known bioactive peptide, but it may be that some of these peptides possess a bioactivity that is currently undetermined. Furthermore, it is unknown whether the identified peptides are present in high enough quantities to have an appreciable effect on the infant. Further studies are needed to answer these remaining questions if the full relevance of milk peptides to infant health is to be determined.

This study is the first to identify that milk peptides of both whey and casein origin survive GI digestion to reach the stool of infants. Several of these peptides have sequences homologous to bioactive milk peptides with antimicrobial and DNA-stimulating activity. These peptides are present in the gut of the infant, where they have the opportunity to influence intestinal cells and the microbiota. The majority of the peptides were not homologous to a known bioactive milk peptide, but they should be investigated for bioactivity due to their confirmed presence in the infant gut. This study contributes to the developing map of milk peptide release across infant digestion and can provide directions for future studies that will determine the relevance of milk peptides to infant health.

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Chapter 4 – Differences in human milk peptide release along the gastrointestinal tract between preterm and term infants

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In Press

Abstract

Background and aims: Preterm infants are born with a gastrointestinal tract insufficiently developed to digest large quantities of human milk proteins. Peptides released from the digestion of human milk proteins have been identified with bioactivities that may be beneficial to the developing infant. However, it is unknown how prematurity affects total and bioactive peptide release along the gastrointestinal tract. The aim of this study was to compare milk peptide release from milk to stomach to stool between preterm and term infants.

Methods: Milk, gastric, and stool samples were collected from preterm infants as early collection (days 8 and 9 of life) and late collection (days 21 and 22 of life), and from term infants as early collection. Milk peptides were extracted from the samples and identified using Orbitrap mass spectrometry. Peptide abundance and count were compared across digestion and among the three infant groups at each stage of digestion.

Results: Total milk peptide count and abundance increased from milk to stomach then decreased in stool. Total peptide release was similar among the three infant groups for milk and stool samples. In the stomach, preterm early collection had significantly higher peptide abundance and count than the other two groups. Patterns for peptide release from individual milk proteins were distinct from total peptide release both across digestion and among the infant groups. When analyzing single peptides, term early collection gastric samples had significantly higher peptide abundance than preterm early collection for a known antimicrobial peptide, QELLLNPTHQIYPVTQPLAPVHNPISV.

Conclusions: Preterm and term infants digest milk proteins differently along their gastrointestinal tracts. Preterm infants released more total peptides in the stomach, amd

term infants released specific bioactive peptides at higher abundance. We identified a region at the C-terminus of β-casein that is conserved from milk through stool and from which are released known and potential antimicrobial peptides.

Keywords: Preterm, Term, Peptide, Bioactive, Human milk, Digestion

Introduction

Over 10% of all infants born in the US each year are premature, or born <37 weeks gestational age (2). Premature infants face a variety of nutritional challenges due to their restricted development time. In order to match intrauterine rates of growth, the enteral energy and protein needs of preterm infants are far greater than those of term infants, at up to 135 kcal/kg/day and 4.5 g protein/kg/day (227, 228). Furthermore, the preterm gastrointestinal system is underdeveloped at birth and is not optimized for the complete digestion of large quantities of macronutrients. In the stomach, preterm infants produce less gastric acid, have lower pepsin activity (25, 26), and have increased gastric emptying time (229, 230), all of which can impact protein digestion.

Human milk with fortification is widely accepted as the best source of nutrition for the preterm infant. Human milk proteins contain the optimal balance of amino acids to meet infant growth requirements (231). Many of the proteins, such as lactoferrin, immunoglobulins, and growth factors, can remain undigested to the benefit of the infant's health and development (232). Upon being fed to the infant, most milk proteins are cleaved into smaller peptides by a variety of proteases either present in the milk or secreted by the infant's gastrointestinal tract. These peptides may also benefit the infant, as hundreds of milk peptides have been identified with antimicrobial, antihypertensive, antioxidant, immunomodulatory, cell proliferative, and nutrient-delivery activity (131, 233). Peptidomics, an offshoot of proteomics, has evolved as a method to identify and quantify these smaller peptides as they are released during digestion and assess how the proteins are digested at various stages (234, 235). Peptidomics research on milk and milk products has found that bioactive peptides are released from milk proteins during *in vitro*

digestions (143, 145), digestion in animal models (236), and *in vivo* gastric digestion in preterm infants (153, 154, 237). However, only a few comparisons between preterm and term infants using peptidomic methods have been performed, and only on undigested milk samples (139, 141, 146). No research has yet investigated the release of human milk peptides in the intestinal tract.

The full impact of prematurity on protein digestion in the stomach and along the gastrointestinal tract remains unknown. Impaired protein digestion may not only affect the quantity of amino acids available to the infant for protein synthesis but also the release of bioactive peptides from the milk proteins. If bioactive peptides are to have an effect on infant health, they must be released in significant quantities and survive to their site of activity. For most activities, the peptides must reach the intestinal tract and either cross into the bloodstream to have systemic effects (238), or act locally on gut bacteria and intestinal tissues or immune cells (239). Infants who are unable to digest milk proteins in a manner that releases and preserves bioactive peptides are thus unable to take advantage of the full benefits of human milk.

The aim of this research was to test the hypothesis that there are differences in human milk peptide release between preterm and term infants across digestion. Peptidomics analysis was used to identify peptides from the milk, gastric fluid, and stool samples of preterm and term infants. Total and individual peptides were compared between the infants at each phase of digestion. Milk protein cleavage and peptide release were mapped across digestion to gain a deeper understanding of where bioactive peptides first appear in the infant.

Methods

Materials

Ammonium bicarbonate and HPLC-grade acetonitrile were obtained from Thermo Fisher Scientific (Waltham, MA), trifluoroacetic acid and HPLC-grade formic acid were obtained from EMD Millipore (Billerica, MA), HPLC-grade ethanol, iodoacetamide, and trichloroacetic acid were obtained from Sigma-Aldrich (St. Louis, MO), and dithiothreitol was obtained from Promega (Madison, WI).

Participants and enrollment

Ethical approval for this study was granted by the Institutional Review Boards at Legacy Health Systems and Oregon State University. Milk, gastric, and stool samples were collected from term and preterm infants enrolled at Randall Children's Hospital. Informed consent was obtained for all subjects. Stool samples from this infant population were previously analyzed for peptidomics in our previous study (237).

Infant enrollment criteria and sample collection procedures were identical to those described in our previous publications (237, 240). Briefly, collections of milk and gastric samples from preterm and term infants were attempted at 8, 9, 21, and 22 days of life (DOL). However, we were unable to complete the full four days of sample collection for many of the infants due to insufficient gastric volume or removal of infants from the NICU. Mother's or donor milk was prepared by the nurses at Randall Children's Hospital using aseptic techniques. Milk was prepared without human milk fortifier only for the sample feeding. A sample of the milk was collected upon thawing and prior to feeding and immediately frozen at –80°C. Gastric residual was removed and the volume

recorded, and the milk was administered to the infants through naso/orogastric feeding tubes over 30–60 min. Thirty minutes after completion of the feed, a gastric sample was collected from the infant and immediately frozen at –80°C. The volume of gastric fluid collected was recorded and replaced with fresh feed. Stool samples were collected from each infant over each two-day period (8/9 and 21/22 DOL). Nurses attempted to collect all stool produced by the infant after the sample milk feeding. Each stool sample was immediately frozen at –80°C. All samples were transported from Randall Children's Hospital to Oregon State University on dry ice for sample analysis. The number of infants and samples collected at each DOL are listed on **Supplemental Table 4.1**.

Sample preparation

Milk and gastric samples were prepared as previously described (206) with the following modifications. A 20-μL aliquot was taken from each sample and mixed with 80 μL of nanopure water. Lipids were skimmed by centrifugation at 4,000 × *g* for 10 min at 4°C after mixing. To reduce disulfide bonds, 100 μL of 200 mM ammonium bicarbonate was mixed with each sample, and dithiothreitol was added to a final concentration of 40 mM. The samples were then incubated at 56°C for 45 min. Iodoacetamide was added to a final concentration of 100 mM, and the samples were incubated at room temperature in the dark for 1 hr. Protein precipitation with 200 μL of 24% TCA and peptide enrichment via C18 reverse-phase extraction were performed as previously described (142). The data from the stool samples were taken from a previous study (237).

Liquid chromatography mass spectrometry

Peptides were analyzed with mass spectrometry as previously described (237). The order of sample loading on the mass spectrometer was randomized to minimize bias

caused by instrumental drift over time. UPLC and mass spectrometry settings were as previously described (237).

Peptides were identified from the raw files with Thermo Proteome Discoverer 2.2.0.388 using a Sequest HT search on an in-house human milk protein database. Samples were grouped for analysis based on their sample type (milk, gastric fluid, or stool), DOL (8/9 or 21/22), and infant maturity (preterm or term). Allowed peptide modifications were serine and threonine phosphorylation, methionine oxidation, and cysteine carbamidomethylation. Peptide identification was validated using a decoy database search strategy with a false-discovery rate of $P < 0.01$. Only peptides with high confidence were included in the final data analyses.

Data analysis

Milk, gastric, and stool samples were analyzed for peptide abundance (the summed area under the ion-intensity curve from the mass spectra) and peptide count (the number of unique peptide sequences). All statistical comparisons were carried out in GraphPad Prism 8.4.1. Peptide abundances and counts were averaged for the milk and gastric samples for each infant across days 8/9 (Early Collection, EC) and days 21/22 (Late Collection, LC) of life. Stool samples were collected only once over each two-day period, so no averaging was necessary. Significant differences in peptide abundance and count between sample types, and infant age and maturity status were determined by two-way ANOVA and post-hoc multiple comparisons corrected with the Tukey-Kramer method. Significance was determined by a *P*-value of <0.05.

R version 3.6.1 was used for peptide density and hierarchical clustering analyses. Peptide isoelectric point was determined using the calculator at http://isoelectric.org/

(241). Peptide grand average of hydropathy (GRAVY) score (242) was determined using the calculator at http://www.gravy-calculator.de/. Hierarchical clustering analysis was performed with the *pheatmap* package. Perseus version 1.6.14.0 was used to calculate permutation-based false discovery rate *q*-values for comparisons between infant groups of the individual peptides.

Milk peptides were analyzed for sequence homology with bioactive peptides in the Milk Bioactive Peptide Database (MBPDB) (131). The search type was "Sequence" with a similarity threshold of 80%. PepEx (http://mbpdb.nws.oregonstate.edu/pepex/) was used to map the identified milk peptides to their location in the parent protein sequence (243).

Results

Human milk peptide profiles across digestion

Across all milk, gastric, and stool samples analyzed, 19,612 peptides with unique sequences were identified. The number of peptides unique to the milk, gastric, or stool samples and the number of peptides shared among the samples are shown in **Figure 4.1A**. Although all sample types were unique in terms of the milk peptides present in each, there was more crossover for peptide sequences from milk to gastric fluid (4,928 peptides in common) than from milk to stool (935 in common) or gastric fluid to stool (1,900 in common). There were 849 peptides that were present in all three samples; however, the present study was not designed to determine whether these peptides were first released in milk and survived through the entirety of digestion or were released multiple times by distinct proteolytic events in the milk, stomach, and colon.

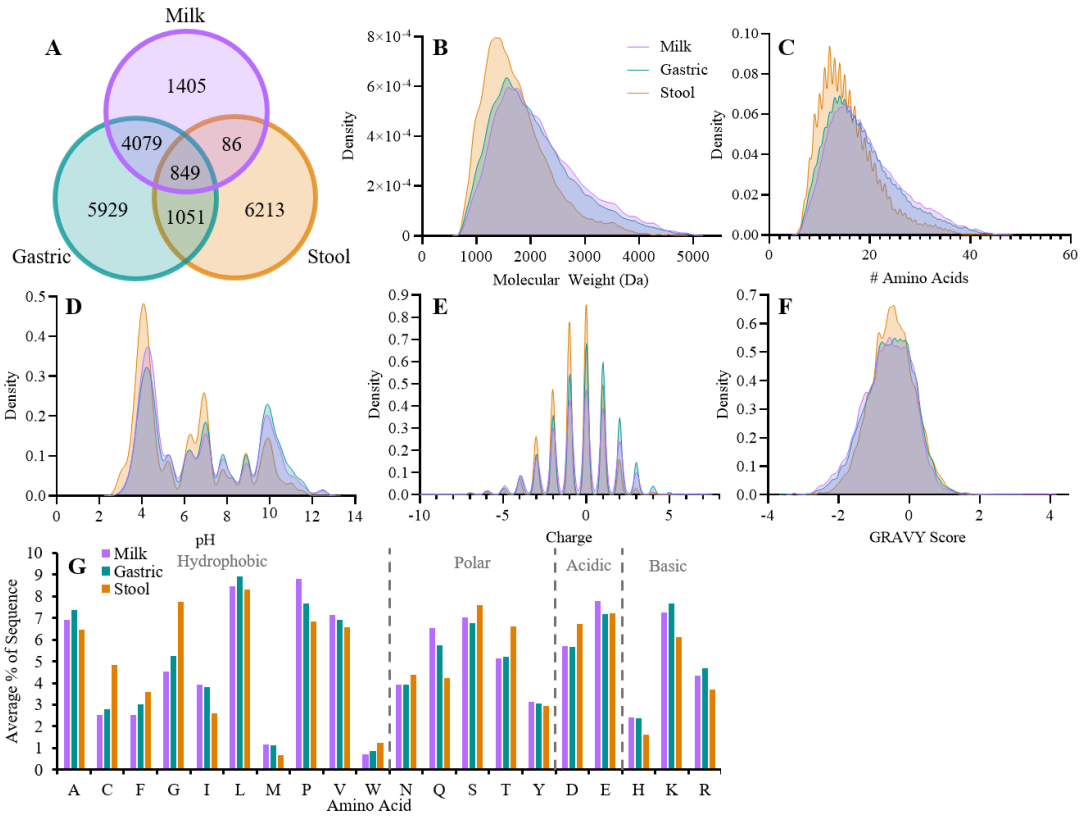

Figure 4.1. Differences in human milk peptides from infant milk, gastric, and stool samples. (A) Venn diagram of the number of peptides in each sample. Density plots of peptides in each sample for the physicochemical characteristics: (B) molecular weight, (C) length, (D) peptide isoelectric point, (E) net charge at pH 7, and (F) GRAVY score. (G) Average percentage composition of each amino acid in the total milk peptidome of the milk, gastric, and stool samples. Amino acids are represented by their one-letter code.

Peptides from milk and gastric samples were more similar physicochemically than either was to stool. Milk and gastric peptides were distributed across higher molecular weights than were those in stool (**Figure 4.1B**). Although the differences in molecular weight among milk, gastric, and stool peptides may be in part explained by differences in the amino acid makeup of the peptides, the larger contributing factor was the shorter peptide lengths in the stool samples (**Figure 4.1C**). Milk and gastric peptides were also distributed at higher isoelectric points and thus were more basic, whereas stool peptides

had more peptides distributed at lower isoelectric points and thus were more acidic (**Figure 4.1D**). The higher acidity of stool peptides is further supported by the shift to lower net charges at neutral pH compared with those of the milk and gastric peptides (**Figure 4.1E**). The GRAVY score predicts the hydropathy of a peptide, with a negative value indicating hydrophilicity and a positive value indicating hydrophobicity. There was a large amount of overlap between peptide GRAVY scores, but stool peptides were slightly more hydrophobic than milk and gastric peptides, which had longer tails in the negative direction (**Figure 4.1F**).

The differences in isoelectric point, net charge, and GRAVY score of the peptides can be explained by differences in the primary structure of the peptides. Histidine, lysine, and arginine (positively-charged amino acids) all comprised a larger percentage of milk and gastric peptides on average, whereas stool peptides had a larger percentage of aspartate (negatively-charged amino acid) (**Figure 4.1G**). Stool peptides also had more cysteine and glycine and slightly more phenylalanine and tryptophan, which contributed to their higher GRAVY scores.

Differences between preterm and term infants at 8/9 and 21/22 DOL

Total milk peptide count and abundance of each sample type were compared among preterm infants at 8/9 DOL (PEC), preterm infants at 21/22 DOL (PLC), and term infants at 8/9 DOL (TEC). No term infants remained enrolled in the study long enough to collect samples at 21/22 DOL. For milk and stool samples, there were no significant differences among the infant groups (**Figure 4.2A–B**). In the gastric samples, PEC had the highest peptide abundance and count, whereas PLC and TEC were not significantly different. Peptide abundance and count were also compared across the three samples for

each infant group. For all infants, both average peptide abundance and count significantly differed within the milk, gastric, and stool samples. Average peptide abundance increased from milk to gastric samples, then decreased back to levels equivalent to milk in the stool. Average peptide count similarly increased from milk to gastric samples, then decreased in stool to a level higher than in milk.

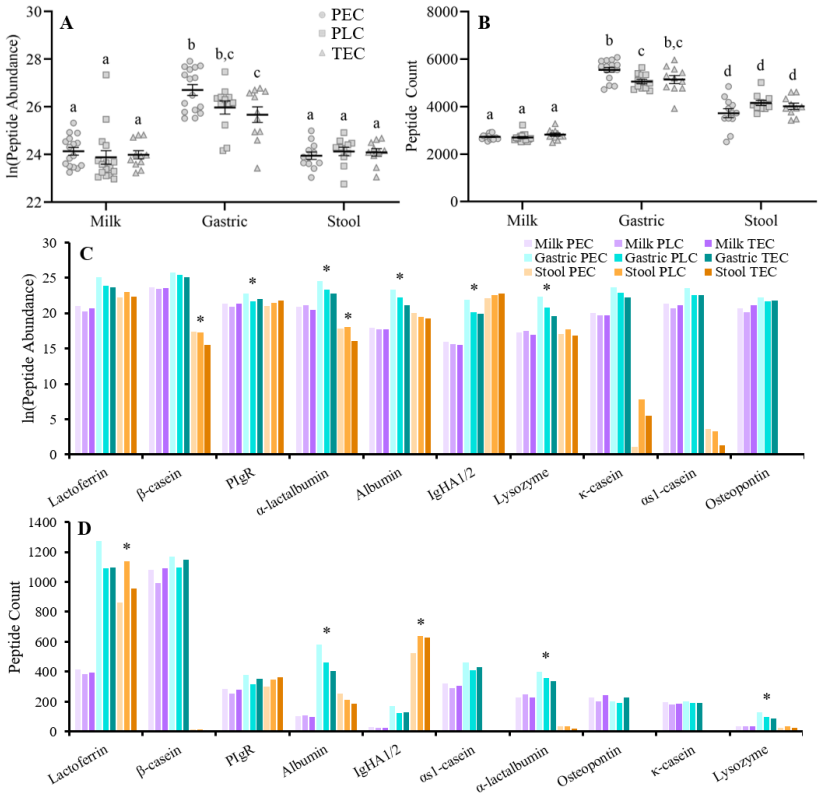

Figure 4.2. Comparison of milk peptide abundance and count across digestion and among PEC, PLC, and TEC infants. (A) Total peptide abundance and (B) total peptide counts were compared. Individual peptide abundances and counts were summed for each sample, and sample types of different infant maturity and age were compared using two-way ANOVA with post-hoc Tukey-Kramer corrected multiple comparisons. Data with different overhead letters represent significant differences among the groups. (C) Peptide abundance and (D) peptide count from individual milk proteins were similarly derived from the summed individual peptide abundances and counts for each protein and compared using two-way ANOVA and multiple comparisons. *Indicates significant differences between infant groups for the sample type and protein. PEC, preterm early collection; PLC, preterm late collection; TEC, term early collection. For milk, PEC *n*=16, PLC *n*=16, TEC *n*=11; for gastric, PEC *n*=16, PLC *n*=12, TEC *n*=11; and for stool, PEC *n*=12, PLC *n*=11, TEC *n*=10.

As differences in the total milk peptide abundance and count among PEC, PLC, and TEC infants were only found in the gastric samples, we next determined whether these results remained constant for peptides from individual milk proteins (**Figure 4.2C–D**). All proteins had similar peptide abundance and count among PEC, PLC, and TEC infants in the milk samples. Due to overlap in the sequences of peptides, Ig heavy constant α (IgHA) 1 and 2 were combined for the analysis. Polymeric Ig receptor (PIgR), α-lactalbumin, serum albumin, IgHA1/2, and lysozyme had significantly different gastric abundance among PEC, PLC, and TEC, whereas β-casein and α-lactalbumin had significantly different stool abundance. Serum albumin, α-lactalbumin, and lysozyme had significantly different gastric count, and lactoferrin and IgHA1/2 had significantly different stool count. As with the total peptides, each individual protein was significantly different from milk to the infant stomach to the stool for both peptide abundance and count.

Finally, to identify differences among the PEC, PLC, and TEC infants at an individual peptide level, we performed hierarchical clustering on the peptide abundances scaled across all the samples and generated a heatmap of the peptides (**Supplemental Figure 4.1**). As with the total abundance, there were no clear distinctions among the peptide profiles of the infant groups, even for the gastric samples. Rather than clustering under one or two branches, the PEC, PLC, and TEC samples were scattered over the entire dendrogram. However, despite heterogeneity within each of the milk, gastric, or stool samples, the peptide profiles of each phase of digestion were distinct from each other, and the samples clustered mostly within their own branches.

Bioactive milk peptides

Peptides were compared with the Milk Bioactive Peptide Database to identify bioactivities or potential bioactivities based on sequence homology. There were 271 homologous milk peptides that had ≥80% similarity of sequence with a known bioactive peptide. Sixteen of these were identical to a known bioactive milk peptide and are listed in **Table 4.1**. Of the 271 peptides, 139 peptides were homologous to peptides with antimicrobial activity, 83 with antihypertensive activity, 42 with cell-proliferative activity, 7 with antioxidant activity, 6 with DPP-IV inhibitory activity, and one peptide each with opioid, anticancer, and antithrombin activity.

Table 4.1. Known bioactive peptides in the milk, gastric, or stool samples.

Peptide	Protein	Activity	Milk	Gastric	Stool
TVYTKGRVMP	β-casein (107–116)	ACE-inhibitory	X	X	
LTDLENLHLP	β-casein (133–142)	ACE-inhibitory		X	
LENLHLPLP	β-casein (136–144)	ACE-inhibitory	X	X	
ENLHLPLP	β-casein (137–144)	ACE-inhibitory		X	
ENLHLPLPLL	β-casein (137–146)	ACE-inhibitory		X	
NLHLPLP	β-casein (138–144)	ACE-inhibitory		X	
NLHLPLPLL	β-casein (138–146)	ACE-inhibitory	X	X	
QVPQPIP	β-casein (152–158)	ACE-inhibitory		X	
WSVPQPK	β-casein (169–175)	ACE-inhibitory Antioxidant	X	X	
WLAHKAL	α-lactalbumin (123–129)	ACE-inhibitory DPP-IV inhibitory		X	
YANPAVVRP	κ-casein (81–89)	ACE-inhibitory		X	
LLNQELLLNPTHQIYPV	β-casein (197–213)	Antimicrobial	X	X	
QELLLNPTHQIYPVTQPLAPVHNPISV	β-casein (200–226)	Antimicrobial	X	X	
YPVTQPLAPVHNPIS	β-casein (211–225)	Antimicrobial	X	X	
RETIESLSSSEESITEYK	β-casein (16–33)	Cell-proliferation	X	X	X
SPTIPFFDPQIPK	β-casein (120–132)	Cell-proliferation	X	X	

Homologous peptides were disproportionately abundant in the milk and gastric samples compared with non-homologous peptides. Averaged across the milk samples, homologous peptides comprised 31.9% of the total peptide abundance despite comprising only 5.1% of identified peptides. In the gastric samples, homologous peptides comprised 28.9% of peptide abundance and 3.3% of peptide count. Only in the stool samples was the abundance and count of homologous peptides proportional, comprising 0.15% and 0.19%, respectively.

There were no significant differences in homologous peptide abundance among PEC, PLC, and TEC infants for any sample, and only between PLC and TEC for milk and gastric samples for homologous peptide count (**Supplemental Figure 4.2**). Though the combined abundance of homologous peptides was similar among the infant groups, we wanted to compare individual peptides to see whether any homologous peptides were more abundant in the TEC or PLC infants than the PEC infants (**Figure 4.3**). In the milk and stool samples, no homologous peptides were significantly different, although TEC infants had two non-homologous peptides significantly higher than PEC in both samples. In the gastric samples, 121 peptides were significantly higher in abundance in PEC infants than TEC, and only 24 were higher in TEC. Both TEC and PEC had four significantly higher homologous peptides, but only TEC had a known antimicrobial peptide. There were no differences in any of the samples between the PEC and PLC groups.

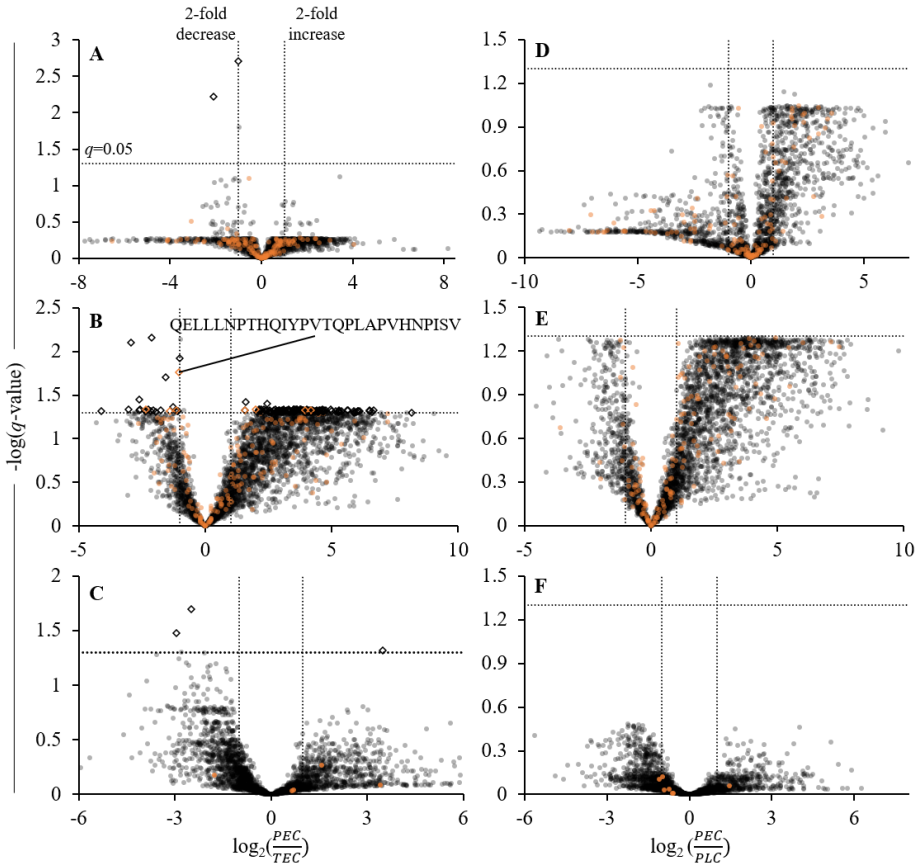

Figure 4.3. Volcano plots for comparisons of individual peptide abundances within milk, gastric, and stool samples. Peptides were compared between (A) PEC and TEC milk, (B) gastric, and (C) stool samples; and between (D) PEC and PLC milk, (E) gastric and (F) stool samples. Only peptides present in ≥50% of the samples for each comparison were included. Diamonds represent peptides that were both significantly different between groups (q<0.05) and with >2-fold change in abundance. Orange data represent peptides homologous with a known bioactive peptide. PEC, preterm early collection; PLC, preterm late collection; TEC, term early collection.

Changes in peptide release across the protein sequences

Peptides were differentially released from distinct regions of the milk proteins as they progressed from milk to gastric fluid to stool. The mean abundance of peptides that contained the amino acids at each position across the full sequences of nine of the most abundant milk proteins is shown in **Figure 4.4**. **Supplemental Figures 4.3–4.5**, respectively, show the milk, gastric, and stool peptides broken down by PEC, PLC, and

TEC infants. **Figure 4.4A–D** are proteins exclusively found in human milk: β-casein, α-lactalbumin, κ-casein, and α_{s1}-casein, respectively. From the milk to the infant stomach, peptides were released from almost identical regions of the proteins, with the only difference being a higher peptide abundance in some of the proteins in the gastric samples. In the stool, the pattern of release was distinct from milk or gastric fluid. The regions of peptide abundance in the stool overlapped regions in milk and gastric fluid for β-casein, α-lactalbumin, and α_{s1}-casein, indicating that some peptides released early on could have resisted complete degradation. However, the sole peptide in κ-casein was released from a region that was not digested in the milk or gastric fluid, so this peptide could only have come from additional proteolysis in the intestine.

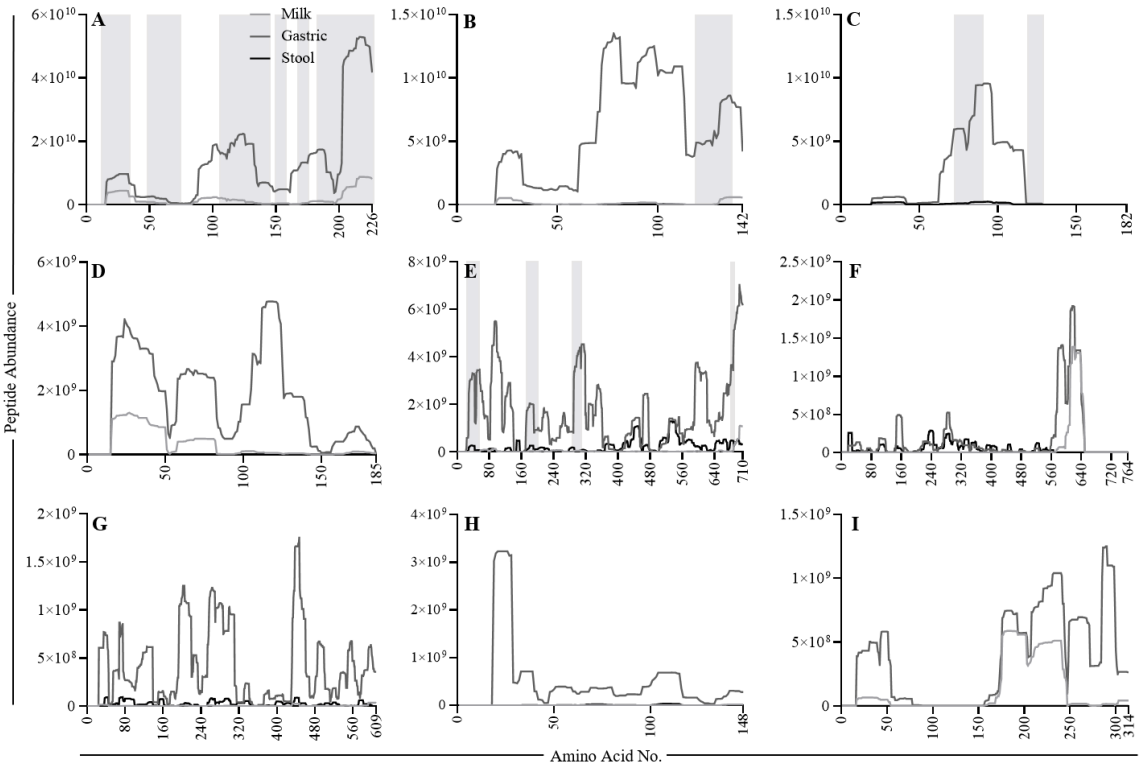

Figure 4.4. Peptide release across the sequence of individual milk proteins during different phases of digestion. For all figures, the *y*-axis is the mean peptide abundance and the *x*-axis is the linear amino acid sequences for the proteins (A) β-casein, (B) α-lactalbumin, (C) κ-casein, (D) α_{s1}-casein, (E) lactoferrin, (F) PIgR, (G) serum albumin, (H) lysozyme, and (I) osteopontin. Grey shaded boxes represent regions of homology to bioactive peptides. PIgR, polymeric immunoglobulin receptor.

Shown in **Figure 4.4E–I** are proteins that are found in human milk but can also be secreted endogenously by the infant: lactoferrin, PIgR, serum albumin, lysozyme, and osteopontin. Like the exclusively milk proteins, there was conservation in the regions of peptide abundance from milk to gastric samples, although with a greater magnitude difference. Peptides from lactoferrin, serum albumin, and PIgR were highly abundant in the stool and dispersed across the entire protein sequences, potentially due to contributions from endogenous secretions. Osteopontin had no identifiable peptides

surviving in the stool, thus it was likely completely digested or absorbed within the intestinal tract.

Peptides conserved across digestion

For a milk peptide to be bioactive in the infant, it must first be released from its parent protein and resist digestion long enough to reach its site of activity. Thus, we next searched for peptides that were identified in the milk, gastric, and stool samples from the same infant on the same DOL, i.e., milk at 8 DOL, gastric fluid at 8 DOL, and stool at 8/9 DOL. From 60 complete sample sets, there were 591 total peptides that fit these criteria, with a mean of 114 ± 29 (mean ± S.D.) peptides (**Figure 4.5A**). When considering only gastric fluid to stool conservation, which represented peptides that appeared in the stomach and survived or were re-released in the intestine, there were 1,803 total peptides with an average of 486 ± 117 peptides. For conservation from only milk to gastric fluid, which represented peptides in milk that were able to survive gastric but not intestinal digestion, there were 70 sample sets that had both samples from the same infant at the same DOL. These sample sets had 4,415 total peptides present in both milk and gastric fluid with an average of 1,525 ± 272. Finally, for conservation only in the milk and stool, which represented peptides that did not survive gastric digestion but were released by another proteolytic event at a later point, there were 734 peptides with an average of 167 ± 40.

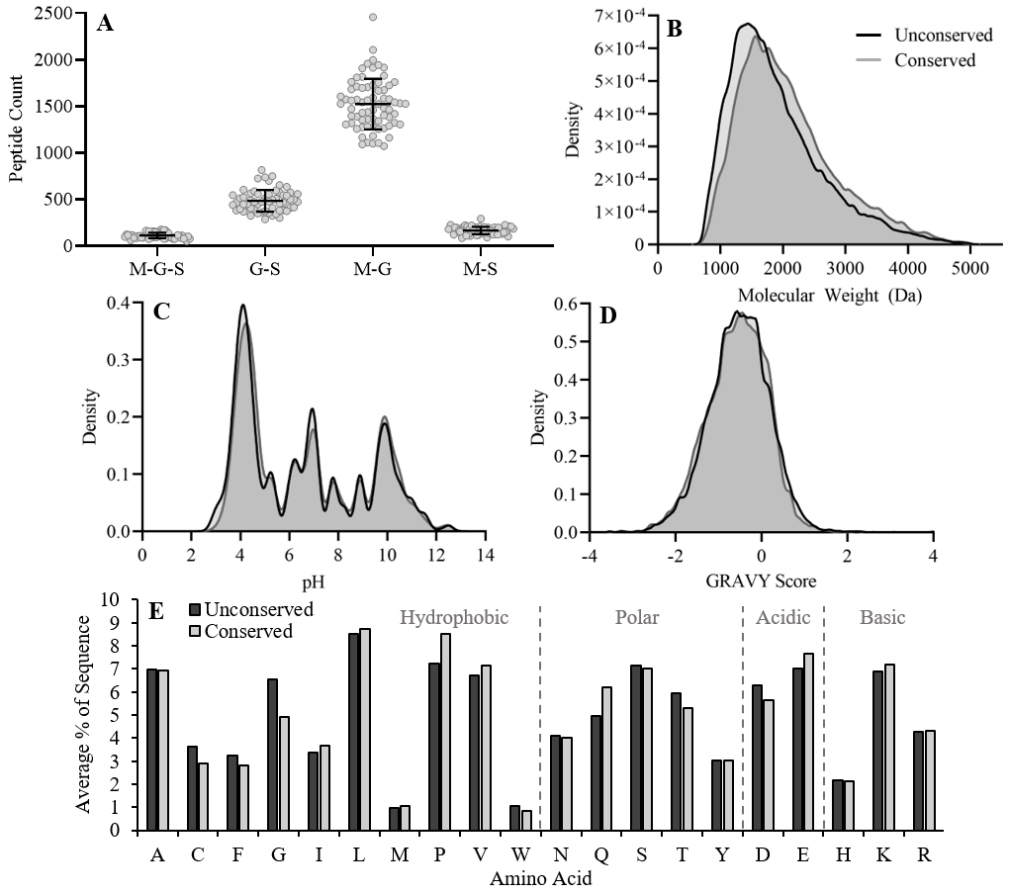

Figure 4.5. Milk peptides conserved across infant digestion. (A) Count of peptides conserved across two or more samples in one infant at one time point. Data are shown as mean ± S.D. Density plots of peptides conserved between samples and peptides that were not conserved for (B) molecular weight, (C) isoelectric point, and (D) GRAVY score. (E) Average percentage composition of each amino acid in the conserved and unconserved milk peptides. M, milk; G, gastric; S, stool.

We next compared the chemical and structural characteristics of the peptides that were conserved across two or more samples with those that were not to determine whether there were any unique properties to the conserved peptides that could explain their apparent increased resistance to digestion. The isoelectric point, hydropathy, and molecular weight distributions of the two groups of peptides are highly similar (**Figure 4.5B–D**). The peptide groups were also similar in terms of average amino acid

composition in the primary sequence with three differences: peptides that were conserved across digestion had a higher percentage of proline and glutamine residues and a lower percentage of glycine residues (**Figure 4.5E**).

Across digestion, 168 conserved peptides were homologous with bioactive peptides, including 9 peptides with 100% sequence similarity. Nine of the homologous peptides were identified in the milk, gastric, and stool samples–four with antimicrobial activity, three with cell-proliferative activity, and one each with antioxidant and antihypertensive activity. Of the remaining conserved homologous peptides, 156 were conserved from milk to gastric fluid, and 3 were conserved from gastric fluid to stool. No homologous peptide was identified in the milk and stool only.

Discussion

To our knowledge, this is the first time peptidomics has been applied to compare in preterm and term infants the digestion of milk proteins as they pass through the gastrointestinal tract. Peptidomics has previously been used to profile human milk (140, 142, 148, 244), infant gastric and stool samples (153, 154, 206, 237), and *in vitro* digestions of human milk (144, 145). Compared with previous investigations, the present study identified greater numbers of milk peptides in both the milk and gastric samples. The count of peptides in undigested milk has consistently been reported to be within a range of several hundred per sample. In gastric samples, the earliest study reported a range of several hundred, but later studies reported counts in the thousands. The average peptide count of the undigested milk samples in the present study are one order of magnitude higher than previously reported. This higher count more closely aligns with the results of Dingess et al. (148), where the investigators used a combination of TCA

precipitation and high-energy collisional dissociation fragmentation to identify thousands of unique milk peptides.

Importantly, the observed peptide profiles of the milk, gastric, and stool samples in the present study may differ from the true peptide profiles. Modifications to the peptide extraction procedure, liquid chromatography and mass spectrometry settings, and peptide identification software could be applied to target peptides of different physicochemical characteristics.-Comparisons of peptide abundance between studies are currently impossible as label-free quantification of the spectra is relative to each experiment based on instrumentation, methods, and software (245). It is clear that the gradual refinement of the extraction protocols and mass spectrometry instrumentation over time have increased the number of peptides identified in milk and other samples, but additional methodologies are needed to unearth the "hidden" peptidome and to compare results between different studies.

Although peptidomic surveys of various human milk samples and digesta have often been performed, differences in the peptide profiles of preterm and term infants have only been analyzed a few times for human colostrum (139) and milk samples (141, 146). The three studies were aligned in identifying differences in the levels of individual milk peptides between the term and preterm infants but conflicted in the cumulative peptide difference. Wan et al. (139) and Dingess et al. (146) found no difference in the total number of peptides, whereas Dallas et al. (141) found preterm infant milk to have higher peptide count from <14–41 DOL and higher abundance from 29–41 DOL. The results of the present study once more confirmed differences in the individual peptide levels of preterm and term milks at 8/9 DOL but not in total peptide count or abundance. Although

preterm milk has a higher concentration of protein than term milk in the first weeks of life (246), this did not appear to correspond with a greater release of milk peptides.

In the gastric samples, preterm infants at 8/9 DOL had significantly higher total, protein-specific, and individual peptide abundance than term infants. These results seem contrary to expected results if preterm infants had reduced capacity to digest proteins than term infants, which the current literature supports. Gastric acid secretion is lower in preterm than term infants (26, 28, 247). Preterm infants have lower pepsin activity than term infants (25, 26), and lower total proteolysis and protease activity in the stomach (25). Less pepsin activity and less proteolysis would lead to less peptide content from cleaved proteins, indicating that there must be other factors to consider.

The stomach secretes water and mucus that can dilute the feed proteins and peptides, and term infant secretions are greater in volume than preterm infants in the unstimulated stomach (248). In the present study, any residual gastric fluid was removed prior to feeding, but differences in secretion volume during digestion could explain the lower peptide abundance in term infants. This study did not control for feed dilution, but future studies will address this matter to make a more accurate determination of digestive differences. An additional factor is that the settings used for mass spectra collection and peptide identification in this study were optimized for a range of 375–1,500 *m/z* and 6–50 amino acids. Due to more active proteases in the term infant stomach, smaller peptides may have made up a larger fraction of the term gastric samples but were unidentified. Other potential factors that could affect gastric peptide abundance include the timing of sample collection after initiation of the feed. Our previous study showed that protein digestion and peptide release in the stomach increases up to three hours after feeding in

preterm infants (206). The present study only measured gastric peptides at one hour after feeding. As gastric digestion and gastric emptying is a dynamic process, there could be changes in the rate of peptide release between preterm and term infants the present study was unable to distinguish.

Though the PEC infants had more peptide abundance in the gastric samples than TEC infants, there were several individual peptides that were more abundant in the term stomach. Only the TEC infants had a known bioactive peptide, QELLLNPTHQIYPVTQPLAPVHNPISV, in significantly higher abundance. This peptide was first identified with antimicrobial activity against a range of pathogenic bacteria (180). It derives from the C-terminus of β-casein, which encodes several peptides with antimicrobial activity (249-251), and weak antihypertensive and antioxidant activity (149, 170, 252). The present study showed that not only were more peptides released from the C-terminus of β-casein than from any region of any other protein, but that peptides from this region were consistently identified in the milk, stomach, and stool. Our previous peptidomic investigations discovered a similar phenomenon (154, 206). It is not clear what the importance of this region of β-casein is to the infant, but if it is able to survive through the intestinal tract in significant amounts, it could inhibit the colonization of pathogenic bacteria or influence the development of the early microbiome.

In the present study, milk proteins were differentially digested along the infant gastrointestinal tract. Digestion of the caseins and α-lactalbumin from milk to stomach increased at similar regions along the sequence, but between the stomach and stool, their peptide abundances were almost entirely depleted as the peptides were likely fully broken down into amino acids or di- and tripeptides and absorbed. The fate of the remaining

whey protein peptides is more difficult to decipher, as the infant can also produce those proteins endogenously. The major whey proteins of human milk are lactoferrin, α-lactalbumin, secretory IgA, serum albumin, and lysozyme (253). Lactoferrin and lysozyme are produced by gastrointestinal tissues (254, 255), whereas IgA and serum albumin are major components of plasma and can be excreted in stool (121). This study also identified a large number of peptides from osteopontin and PIgR, both of which can be secreted into the gastrointestinal tract (256, 257). Distinguishing peptides from these proteins as being milk-derived versus endogenously secreted would require stable isotope labeling. Although that was outside the scope of the present study design, several peptides from these proteins were found in the milk, gastric, and stool samples from the same infant on the same DOL, suggesting some of the peptides in the stool at the end of digestion could be milk-derived.

By identifying peptides in the milk, gastric fluid, and stool from the same infant at the same DOL, we were able to compare peptides present at the beginning, middle, and end of digestion. There were only a few differences in the physical properties of peptides present at more than one site versus peptides that were not. The most interesting difference was the higher percentage of proline residues in the peptides that were conserved. It has previously been noted that peptides with leucine and proline residues, particularly at the C-terminus, are more resistant to intestinal peptidase activity (258, 259). One of the major issues with using food-derived bioactive peptides as a therapeutic or food additive is their susceptibility to gastrointestinal digestion (260). Depending on where the peptide is first released, it can encounter over 20 human proteases and peptidases (261, 262) and an unknown number of microbial proteases (263). Knowing

what inherent factors contribute to a peptide's ability to resist digestion can aid in future bioactive peptide discovery by focusing on specific regions of the proteome or modifying previously discovered peptides to increase resistance.

Conclusion

This paper is the first to track the digestion of human milk proteins from milk to the infant stomach to stool and compare the peptide release between preterm and term infants. Preterm and term infants digested milk proteins differently. At the gastric phase of digestion, preterm infants had higher total peptide count and abundance than term infants of the same age, but term infants released specific bioactive peptides in significantly greater amounts. Many milk peptides were conserved across digestion, including some known bioactive peptides. We pinpointed a region encompassing the C-terminus of β-casein that was released in large amounts in the milk and stomach, survived to the stool, and hypothesized that it may play a protective role against bacterial infection in the infant intestinal tract. Future research should investigate this region of the C-terminus of β-casein to elucidate its effects on infant health, along with the factors that allow it to resist digestion better than the other milk proteins.

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Chapter 5 – Milk peptides in the intestinal tract of breast milk-fed infants have antimicrobial and bifidogenic activity

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Not published

Abstract

Objectives: Many bioactive milk peptides have been identified with health benefits for the infant. However, for these peptides to be relevant to the infant, they must be released from their parent protein and resist further digestion until they reach their site of activity in the small and large intestine. Little is known about the digestion of milk proteins in the infant intestinal tract. We hypothesized that the natural digestion of milk proteins in the small intestine releases peptides with antimicrobial and bifidogenic activities.

Methods: Infants enrolled at Doernbecher Children's Hospital in Portland, OR were fed fortified human milk through a nasogastric tube. Samples from the small intestine were collected through a nasoduodenal or nasojejunal tube. Milk peptides were extracted using sterile methods, and activity of the bulk peptide extracts was determined by measuring growth of *Escherichia coli*, *Staphylococcus aureus*, and *Bifidobacterium longum* spp. *infantis* after incubation with serial dilutions. Milk peptides were identified in each extract, and the peptide profiles of active and inactive samples were compared to identify candidate bioactive peptides. The antimicrobial and bifidogenic activities of candidate peptides were determined.

Results: We extracted peptides from 29 intestinal samples collected from 16 infants. Five samples had significant antimicrobial activity against *S. aureus* and six samples had significant bifidogenic activity for *B. infantis*. From these samples, we narrowed down a list of 6,645 milk peptides to 11 candidate peptides for synthesis, of which 6 fully inhibited *E. coli* and *S. aureus* growth at concentrations of 2,500 and 3,000 μg/mL.

Conclusions: This study provides evidence for the potential activity of bioactive milk peptides *in vivo.* Intestinal fluid peptide extracts from human milk-fed infants can inhibit

the growth of pathogenic bacteria and stimulate commensal bacteria. Significantly, we identified novel human milk antimicrobial peptides that are released within the infant gastrointestinal system.

Introduction

Over 380,000 infants are born prematurely in the US each year (2). Compared with infants born at full term, preterm infants are at heightened risk of developing infections such as sepsis (264) and necrotizing enterocolitis (265), with infection risk increasing as gestational age at birth decreases (5, 266, 267). Due to their reduced development time *in utero*, preterm infants are often born with an underdeveloped gastrointestinal (GI) tract and innate immunity (reduced gastric acidity, looser tight junctions, dysbiotic microbiome) that leave them susceptible to pathogens (268-270). The organisms most commonly responsible for systemic infections vary by hospital but typically include coagulase-negative *Staphylococcus*, group B *Streptococcus*, Gram-negative bacteria, and *Candida* (70, 271-274). The etiology of necrotizing enterocolitis is less well understood but is associated with aberrant colonization of the gut with a predominance of gammaproteobacteria and reduced commensal *Bifidobacteria* and *Bacteroidetes* species (275, 276). Though the causes and locations of infection are disparate among infants, the universal standard of care for risk reduction is early and dedicated enteral feeding with human milk, whether mother's or donor milk (277).

Human milk is the ideal source of nutrition for the preterm infant as it contains a variety of bioactive compounds that provide protection for the infant GI system. Immunoglobulins and antimicrobial proteins such as lactoferrin and lysozyme inhibit bacterial growth (122, 130, 278, 279), human milk oligosaccharides prevent pathogen adhesion (280, 281) and act as a specific source of nutrition for commensal bacteria (282), and growth factors can facilitate the maturation of the intestinal epithelium (283, 284). Additional components of human milk potentially protective for the infant are milk

peptides. Human milk proteins are exposed to a variety of proteases in the mammary gland and the infant GI system that initiate the degradation of the proteins into individual amino acids for infant nutrition. Protein digestion deactivates many of the functional proteins secreted in milk, but it also releases tens of thousands of peptides as an intermediary stage between the intact protein and its component amino acids (206).

Though the majority of these peptides are likely biologically inert, many have been identified with potential bioactive properties both similar to and distinct from their parent proteins through *in vitro* methods (131). Of particular significance for infants are those with antimicrobial (162), immunomodulatory (164, 285), and bifidogenic (199) properties that have the potential to provide additional immunological support as their GI system matures. However, the relevance of these peptides to infant health is dependent upon whether they are released during GI digestion and survive to their sites of activity. Several peptidomic studies have revealed hundreds of bioactive peptides are released in human milk and the stomach of breast milk-fed infants (161, 206). Furthermore, bioactive milk peptides have been identified to survive to infant stool (237), but little is known about the presence or activity of bioactive peptides inside the infant intestinal tract. Bioactive peptides present in the intestinal tract have the highest potential to positively impact infant health, either by absorption into the infant's circulation or by local activity on the intestinal cells and bacteria.

The aim of this study was to identify milk peptides in the intestinal fluids of breast milk-fed preterm and term infants and characterize them for antimicrobial and bifidogenic activity. Peptides were extracted from infant intestinal fluids and assayed for

bioactivity. Liquid chromatography-mass spectrometry (LC-MS) was used to identify peptides that were selected for synthesis and activity testing.

Methods

Materials

Trichloroacetic acid was obtained from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile and phosphate-buffered saline (PBS) were obtained from Thermo Fisher Scientific (Waltham, MA), and trifluoroacetic acid and HPLC-grade formic acid were obtained from EMD Millipore (Billerica, MA). Mueller-Hinton broth (MHB) and Bacto Agar were obtained from BD (Franklin Lakes, NJ). Stock bacteria (*Staphylococcus aureus* ATCC 12600, *Escherichia coli* BL21 (DE3), *Bifidobacterium longum* spp. *infantis* ATCC 15697) were obtained from ATCC (Manassas, VA). Candidate peptides were synthesized to ≥98% purity by GenScript (Piscataway, NJ).

Sample collection

Ethical approval for this study was granted by the Institutional Review Board at Oregon Health and Sciences University (STUDY 00017968). Infants were enrolled at the OHSU NICU following informed consent from the parents. For inclusion in this study, infants had to have an indwelling nasogastric or orogastric feeding tube and had to tolerate full enteral feeding volumes. Infants were excluded from the study if they had anatomic or functional GI disorders that would affect protein digestion, were medically unstable, were nonviable, or had disorders that would be expected to affect normal digestion.

Upon enrollment, a sampling tube was placed into the distal duodenum or proximal jejunum, with the position of the sampling port confirmed by abdominal X-ray. Human milk with and without fortification (Similac Human Milk Fortifier or Neosure® fortifier) was fed to infants via a nasogastric tube over 30 min or less. Samples were collected from the nasoduodenal/jejunal tube via gravity flow as the digesta passed the collection tube port if a post-pyloric tube had been placed, or collected from a jejunostomy bag if present. Intestinal samples were collected into sterile, low-protein binding collection tubes and placed immediately on ice then stored at −80 °C. All samples were transported to Oregon State University on dry ice and stored at −80 °C upon arrival. Infant demographic and anthropometric data were recorded at time of feeding.

Peptide extraction

Intestinal samples were thawed on ice; 1 mL of each sample was centrifuged at 14,000×*g* for 10 min at 4 °C to separate fats and solids, and the infranatant was pipetted into a new tube. To ensure complete extraction of the peptides, the remaining fats and solids were agitated with a vortex mixer with 500 mL of nanopure water and recentrifuged at the same speed and time. The second infranatant was added to the previous infranatant. Each sample was mixed with an equivalent volume of 24% trichloroacetic acid and centrifuged at 12,000×*g* for 20 min at 4 °C to precipitate remaining intact proteins. Peptides were separated from the supernatant via C18 solid-phase extraction following our previous methodology (206). The eluate was freeze-dried and rehydrated in 1 mL of sterilized PBS for bioactivity screening.

Mass spectrometry analysis

Peptides from 20 µL of each intestinal sample were extracted as described above and dissolved in 20 µL of nanopure water after freeze drying for LC-MS analysis. LC-MS was performed as previously described (237) with the following change: as a number of the infants were fed milk with Neosure® bovine-based fortifier, peptides were identified using Proteome Discoverer 2.2.0.388 with a Sequest HT search against a database that contained both human and bovine milk proteins. Dynamic peptide modifications only included phosphorylation of serine and threonine and oxidation of methionine.

Intestinal peptide extract bioactivity screening

The bioactivity of the intestinal peptide extracts was determined via the microdilution method. Antimicrobial activity was screened with *E. coli* and *S. aureus* and growth-promoting activity with *B. infantis*. For the antimicrobial assays, colonies of bacteria were selected and inoculated in 2 mL of MHB and incubated at 37 °C for 24 hr. The inoculum was diluted to 2×10^5 CFU/mL with MHB. For the growth-promoting assays, 100 µL of stock bacteria were inoculated in 10 mL of reinforced clostridial broth supplemented with 0.1% ascorbic acid and incubated under anaerobic conditions (BD BBL™ GasPak™) at 37 °C for 24 hr. Optical density was measured at 600 nm, and the inoculum was diluted to an optical density of 0.05 with reinforced clostridial broth.

The intestinal peptide extracts were serially diluted with PBS to concentrations of 1×, 1/2×, 1/4×, 1/8×, 1/16×, 1/32×, 1/64×, and 1/128×. In a 96-well plate, 50 µL of each dilution was incubated with 50 µL of inoculum in duplicate, along with a negative control of 50 µL of inoculum with 50 µL of pure PBS and a sterility test of 50 µL of peptide with

50 µL of broth. Growth was determined by optical density readings at 600 nm (OD_{600}) taken at 0 hours (T_0) and 18 hours (T_{18}). The following equation was used to determine percent inhibition or promotion of bacterial growth:

$$100 \times \frac{(\mathrm{OD}_{600(\mathrm{Sample})} \text{ at } T_{18} - \mathrm{OD}_{600(\mathrm{Sample})} \text{ at } T_0)}{(\mathrm{OD}_{600(\mathrm{Control})} \text{ at } T_{18} - \mathrm{OD}_{600(\mathrm{Control})} \text{ at } T_0)}$$

Peptide extracts were classified as "growth-inhibiting" if they decreased OD_{600} at any dilution, "growth-promoting" if they increased OD_{600}, and "inactive" if they did not change OD_{600} for all dilutions. The threshold for activity was set as anything greater than the variation of OD_{600} for the bacteria grown without peptide under the same conditions (~15%).

Candidate peptide selection and bioactivity determination

The peptidomic data was compared with the Milk Bioactive Peptide Database (MBPDB) (131) to identify known and potential bioactive peptides. The search type was "Sequence," and a similarity threshold of 80% was used to identify peptides with high sequence homology to known bioactive peptides that may be predictive of bioactivity.

The peptide profiles of active and inactive intestinal samples were compared to identify candidate peptides for synthesis. The percentage abundance of each peptide in a sample was calculated by dividing each peptide's abundance (the ion intensity of the peptide as measured by the mass spectrometer) by the sample's total peptide abundance. Pearson correlation coefficients were determined for the effect of each peptide's percentage abundance within a sample on the sample activity with R version 3.6.1. Candidate peptides were selected from active samples based on high percentage

abundance, Pearson correlation coefficient, and ratio of percentage abundance in active samples to percentage abundance in inactive samples.

Candidate peptides were synthesized and dissolved in sterile nanopure water. Antimicrobial and growth-promoting assays were carried out as described above with serial dilutions ranging from 3,000 μg/mL to 15.6 μg/mL. Minimum inhibitory concentration (MIC) was determined by the concentration at which all visible growth was inhibited.

Results

Infant characteristics

The clinical characteristics for the infant cohort at the time of sample collection is shown in **Table 5.1**. We collected 29 intestinal samples from 16 infants. Infant gestational age at birth ranged from 25–41 weeks, and day of life at enrollment ranged from 6–57 days.

Table 5.1. Clinical characteristics of infants from whom intestinal samples were collected.

Infants	16
Samples	29
Preterm	26 (89.7)[1]
Fortification	16 (55.2)[1]
Ostomy	5 (17.2)[1]
Gestational Age (wk)	33.15 ± 4.12[2]
Day of Life (d)	28.07 ± 15.96[2]
Weight (kg)	2.11 ± 0.51[2]
Length (cm)	44.23 ± 3.79[2]
Feed Volume (mL)	40.03 ± 13.92[2]
Energy Intake (kcal/kg/d)	122.82 ± 26.03[2]

[1]Data in parentheses is the percentage of samples
[2]Data are presented as mean ± standard deviation.

Growth effects of intestinal peptide extracts

The infant intestinal peptide extracts were screened for antimicrobial activity on *S. aureus* and *E. coli* and for growth-promoting activity on *B. infantis* after an 18 hr incubation. Sterility checks of peptide extracts incubated in sterile Mueller-Hinton broth without bacteria confirmed that none had been contaminated during extraction. Optical density readings were compared with a control of bacteria incubated with sterile PBS to determine the percentage change in growth. The results of the screening assays are shown in **Figure 5.1**.

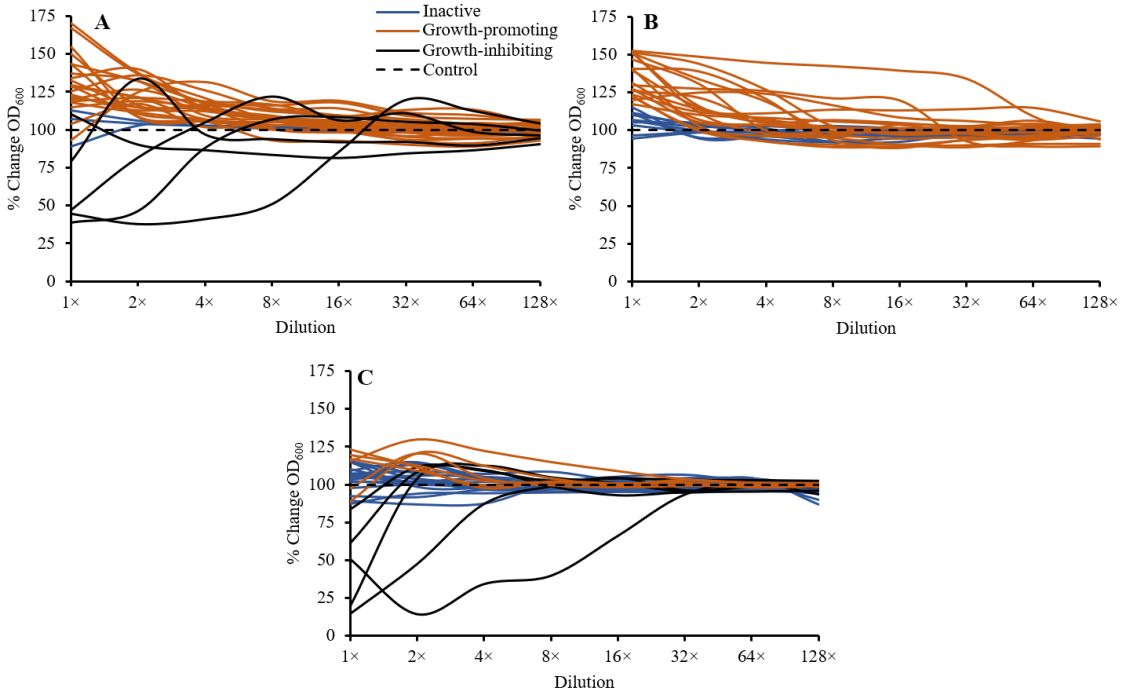

Figure 5.1. The effect of serial dilutions of intestinal peptide extracts on the OD_{600} of *Staphylococcus aureus* (A), *Escherichia coli* (B), and *Bifidobacterium longum* spp. *infantis* (C) after an 18 hr incubation.

Active peptide extracts were identified for only *S. aureus* and *B. infantis*, as no extract inhibited *E. coli* growth to a significant degree. *S. aureus* was the most susceptible to peptide influence on growth, with three of the samples having reduced OD_{600} by >50%

at the highest concentration and continuing to be active up to eight-fold dilution, and another two extracts reducing OD_{600} by ~20% for at least one dilution. Twenty-one peptide extracts promoted *S. aureus* growth and only three were inactive at assayed concentrations.

None of the peptide extracts had growth-inhibiting activity against *E. coli*. Nineteen of the extracts promoted *E. coli* growth, and the remaining ten were inactive at all dilutions. For *B. infantis*, six of the extracts had growth-promoting activity. One extract increased OD_{600} by 30% and another three extracts increased it by >20% at either the highest concentration or two-fold dilution. Five extracts had growth-inhibiting activity against *B. infantis*, and 18 had no activity.

There was no one peptide extract that had simultaneous growth-inhibiting activity against *S. aureus* and growth-promoting activity against *B. infantis* (**Table 5.2**). Extracts 21 and 28 had high inhibitory activity against both *S. aureus* and *B. infantis*, and extracts 12, 26, and 27 promoted the growth of both bacteria. Extracts 1, 4, and 6 had inhibitory activity against *S. aureus* without impacting the growth of *B. infantis*, and similarly, extract 19 promoted *B. infantis* growth without impacting *S. aureus* growth.

Table 5.2. Infant birth information and screening activity for each intestinal peptide extract.

Infant	GA (wk/d)	Collection	Day of Life	Sample ID	Activity[1]		
					S. aureus	*E. coli*	*B. infantis*
1	25/6	1	32	1	-	+	
		2	33	2	+	+	
2	29/4	1	50	3	+	+	
		2	50	4	-	+	
3	30/6	1	37	5	+		-
		2	37	6	-		
4	27/0	1	41	7	+		
		2	42	8	+		
		3	43	9	+	+	-
5	34/2	1	10	10	+	+	
		2	10	11	+		
6	33/1	1	7	12	+		+
		2	8	13	+		
7	31/0	1	25	14	+	+	
8	33/3	1	6	15	+		
		2	8	16	+		
9	36/3	1	33	17		+	+
		2	34	18	+	+	
		3	34	19		+	
10	37/4	1	57	20			
11	34/5	1	55	21	-	+	-
12	35/2	1	8	22	+	+	+
		2	8	23	+	+	
13	34/2	1	24	24	+	+	+
		2	24	25	+	+	-
14	34/3	1	7	26	+	+	+
15	39/2	1	27	27	+	+	+
		2	27	28	-	+	-
16	41/3	1	15	29	+	+	

[1] + Indicates the sample stimulated growth of the bacteria, - indicates the sample inhibited growth, and no symbol indicates the sample was inactive.

Peptide profiles of the intestinal samples

Peptidomic analysis of the intestinal peptide extracts identified 6,645 milk peptides, with 5,251 derived from human milk proteins, 1,233 from bovine milk proteins, and 161 that could come from either due to sequence overlap. Of the total peptides, 814

had an identical primary sequence to one or more other peptides but with different post-translational modifications. The mean peptide count was 2,455.1 ± 727.9, and the mean abundance was $1.53\times10^{11} \pm 1.47\times10^{11}$.

Peptides were identified from 223 proteins, 160 of which were human milk proteins and 63 of which were bovine milk proteins. The relative percentage of peptides from each protein for each intestinal sample is shown in **Figure 5.2**. Most intestinal peptide extracts were primarily composed of human casein peptides except for extracts 1–4 and extract 20, all of which were collected from ostomy output rather than by gravity drip. These extracts had a higher percentage of serum albumin peptides and peptides from human whey or fortifier proteins. These extracts also had much lower overall peptide abundance than the other intestinal extracts, likely as they spent more time sitting in the ostomy bag at room temperature before collection, allowing for additional protease activity (**Supplemental Figure 5.1**). The intestinal peptide extracts that inhibited *S. aureus* growth had diverse peptide profiles, with extracts 1 and 4 being serum albumin-dense, extract 28 having much larger than average levels of perilipin-2 and polymeric Ig receptor peptides, and extracts 6 and 21 having casein-dense profiles. Conversely, the intestinal peptide extracts that stimulated *B. infantis* growth were all similar to each other and composed mostly of human β-casein peptides. All of the *B. infantis*-stimulating extracts except for 22 had higher total peptide abundance than the other extracts (1.25–2.65 times higher than the average).

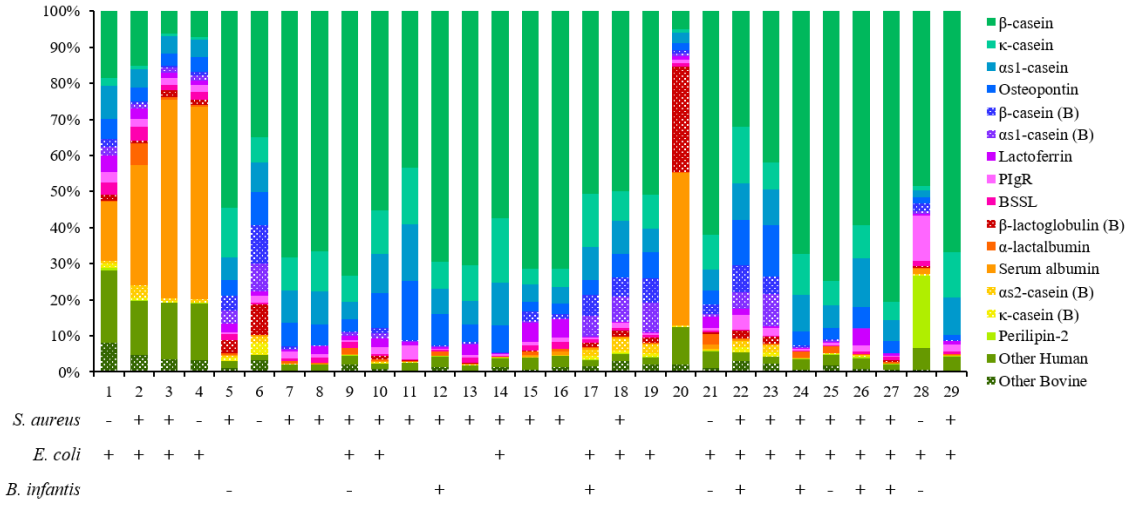

Figure 5.2. Percentage composition of the peptides from each intestinal sample sorted by protein from highest to lowest mean abundance. Bovine milk proteins are labeled with a (B) in the legend and are filled with a dotted pattern. In the rows below the graph, a + indicates the sample stimulated growth of the bacteria, - indicates the sample inhibited growth, and no symbol indicates the sample was inactive. PIgR, polymeric immunoglobulin receptor; BSSL, bile salt-stimulated lipase.

Bioactivity of the candidate synthetic peptides

The identified peptides were first compared to the MBPDB to identify known bioactive peptides in the infant intestine and peptides with highly homologous sequences (≥80% match). From all intestinal samples, there were 73 known bioactive peptides (14 from human milk proteins, 55 from bovine milk proteins, and 4 with shared sequences between human and bovine) and 467 homologous peptides (173 human, 286 bovine, and 8 shared). The sequences and activities of identified known bioactive peptides are presented in **Supplemental Table 5.1**.

Based on each peptide's percentage abundance and Pearson correlation coefficient, the list of 6,645 peptides was narrowed down to 18 with potential antimicrobial activity against *S. aureus* and 13 with potential growth-promoting activity

for *B. infantis* (**Supplemental Figures 5.2 and 5.3**). From these 31 peptides, 11 were selected for synthesis with the aim of choosing from several milk proteins and different regions within a protein.

The results of the growth assays for the peptides incubated with the bacteria are shown in **Table 5.3**. Of the eleven peptides, MIC values within the range of concentrations tested could be determined for six peptides for both *S. aureus* and *E. coli*. The most active antimicrobial peptides were Peptide 5 from α_{s1}-casein and Peptide 11 from serum albumin (**Figure 5.3A**). Peptide 5 had an MIC of 2,500 μg/mL for both *S. aureus* and *E. coli*, but the first signs of growth inhibition were noticeable at concentrations of 500 and 1,000 μg/mL, respectively. Peptide 11 had an MIC of 3,000 μg/mL for both bacteria. At 2,500 μg/mL, peptide 5 inhibited all new colony formation of *S. aureus* and *E. coli* after 8 hr, and peptide 11 inhibited colony formation by ~100-fold (**Figure 5.3B-C**).

Table 5.3. Antimicrobial activity of synthesized human milk peptides.

ID	Sequence	Protein	Position	*S. aureus* MIC[1]	*E. coli* MIC[1]	*B. infantis* MIC[1]
1	HLPLPLLQPLMQQVPQPI	β-casein	140-157	>3000	>3000	>3000
2	LLNPTHQIYPVTQPLAPVHNPIS	β-casein	203-225	>3000	>3000	>3000
3	HQIYPVTQPL	β-casein	208-217	>3000	>3000	>3000
4	LAPVHNPI	β-casein	217-224	3000	3000	>3000
5	EPIPLESREE	α_{s1}-casein	35-44	2500	2500	>3000
6	YANPAVVRPHAQIPQR	κ-casein	81-96	>3000	>3000	>3000
7	RPNLHPS	κ-casein	110-116	3000	3000	>3000
8	EKFGKDKSPKFQ	Lactoferrin	295-306	3000	3000	>3000
9	DMLVVDPK	Osteopontin	283-290	3000	3000	>3000
10	MTSALPIIQK	Perilipin-2	62-71	>3000	>3000	>3000
11	FKDLGEENFK	Serum albumin	35-44	3000	3000	>3000

[1] MIC units are in µg/mL.

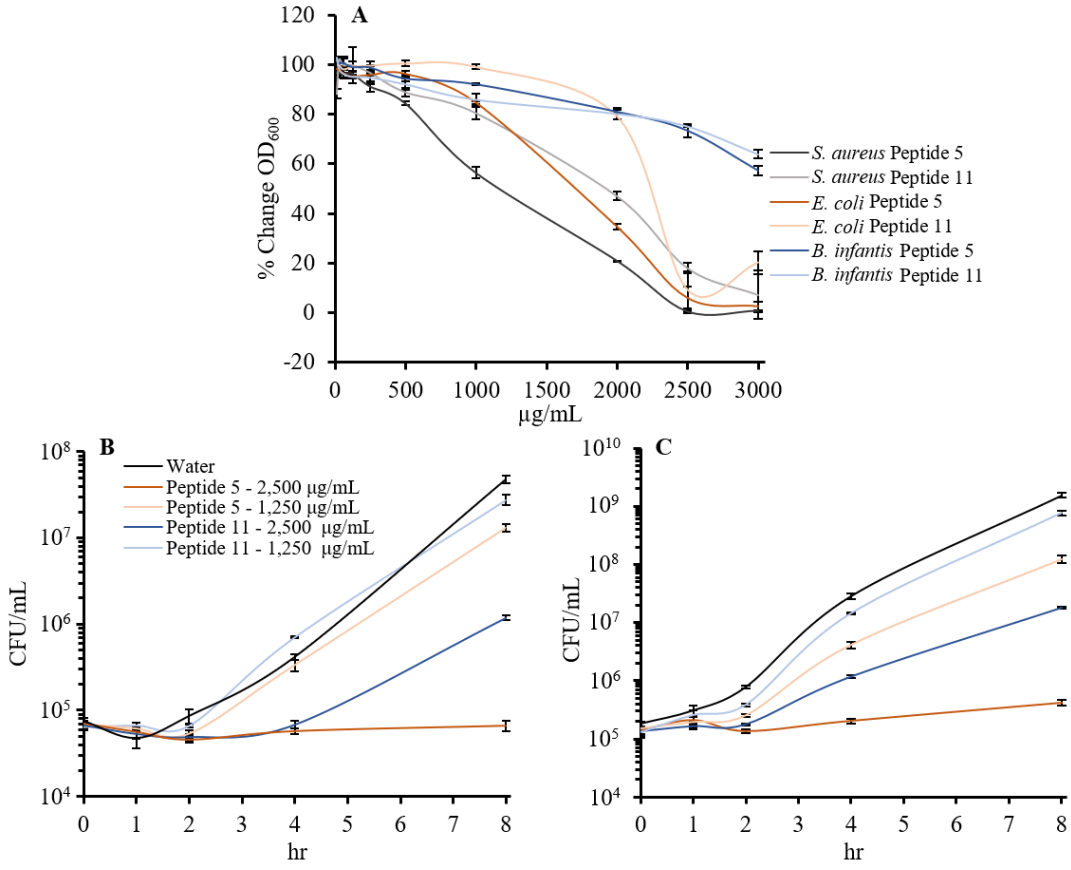

Figure 5.3. Antimicrobial activity of peptides α_{s1}-casein (35-44) and serum albumin (35-44). Percentage change in OD_{600} of *Staphylococcus aureus*, *Escherichia coli*, and *Bifidobacterium infantis* after 18 hr (A). Change in CFU/mL over time of *S. aureus* (B) and *E. coli* (C) after incubation with peptides. Data are shown as mean ± standard deviation.

The only growth-promoting effects of any of the peptides were identified for Peptide 1, Peptide 2 and Peptide 10, which promoted *E. coli* growth at 500, 2,500, and 2,500 μg/mL, respectively. Despite the activity of the intestinal peptide extracts, none of the synthesized peptides promoted *B. infantis* growth at any concentration. None of the peptides fully inhibited *B. infantis* growth either, although many began to show inhibition (>15% reduction in OD_{600}) at concentrations of 2,000 μg/mL and up. At concentrations between 500–2,000 μg/mL, Peptide 5 and Peptide 11 partially inhibited *S. aureus* and *E. coli* growth and had no effect on *B. infantis* growth, and at concentrations of 2,500 and 3,000 μg/mL, fully inhibited *S. aureus* and *E. coli* and only partially inhibited *B. infantis*.

Discussion

Until now, novel bioactive human milk peptides primarily have been identified from undigested milk or *in vitro* digests of milk (286). *In vitro* modeling, however, does not necessarily reflect the range of *in vivo* biology. Peptides that are released by proteolytic digestion of milk are not guaranteed to survive further GI digestion, and it is difficult to create *in vitro* digestion methods that accurately mimic the immature infant GI system (152). Though previous peptidomic studies have found that several species of bioactive peptides from human milk are released after gastric digestion (154, 287), these studies were restricted to identifying only already-known peptides deriving from only a few regions of β-casein, κ-casein, α-lactalbumin, and lactoferrin (287), and limited to an early stage of digestion. Identifying novel bioactive human milk peptides from infant digesta, as done in the present study, improves on previous procedures by immediately establishing the relevance of these peptides and eliminating the question of whether they are released during infant digestion. The major drawback with identifying peptides from

infant digesta is acquiring a sufficient volume of sample with which to perform the necessary screening assays. To overcome this challenge, we assayed the peptide extracts of many individual infant intestinal samples and compared the peptide profiles of those with activity versus those without. Bioactive milk peptides are typically identified through *in silico* analysis (288) or through iterative fractionation (130, 149, 180), which requires a large initial sample volume. The present strategy used less than one milliliter of volume to complete. Furthermore, the peptides were assayed at the same concentration as they were found in the intestinal tract, thus providing evidence for the potential health effects of peptide bioactivity inside the infant GI system, in this instance, potential shaping of the gut microbiota.

From the 29 intestinal peptide extracts assayed, five inhibited *S. aureus* activity and six promoted *B. infantis* activity. None of the samples had both activities, indicating that a peptide profile that can suppress pathogen colonization may be distinct from one that can promote commensal bacteria colonization. These results demonstrate that even within the small number of infant digestive samples available for this investigation, there was notable variation in the antimicrobial or bifidogenic activity of each patient intestinal peptide extract. This variation could arise from differences intrinsic to each infant, e.g., the protein profile of the feed milk (289, 290), protease abundance or activity (208, 291), the extent of digestion at the time of sampling (206, 292, 293), or other factors yet to be discovered. These results are the first confirmation that milk peptides in the intestinal tract have the ability to influence the growth of bacteria.

Human milk contains a variety of intact bioactive factors that protect the infant from enteral infection and promote a healthy gut environment. Secretory IgA is the

principal immunoglobulin in human milk. Secretory IgA resists GI digestion and prevents enteric infection by binding to bacterial adhesion sites (294) and inhibiting bacterial translocation (120). Lysozyme increases the abundance of bacteria associated with a healthy gut and decreases those associated with disease (295), and lactoferrin stimulates intestinal cell development, promotes bifidobacteria and lactobacilli growth, and reduces risk of infectious disease (296). Beyond proteins, human milk oligosaccharides both reduce bacterial adhesion to intestinal cells and are preferentially utilized by bifidobacteria as an energy source (297). Milk peptides are another facet to the suite of immunological factors provided in human milk that protect the infant from disease, and future work on their activity *in vivo* is required to elucidate the magnitude of their contribution.

Though all the individual candidate peptides in the present study had some antimicrobial activity at up to 3,000 μg/mL for *S. aureus* and seven had activity for *E. coli*, MIC values were determined only for six of the candidate peptides. All six peptides are novel antimicrobials from human milk, though three are related to previously identified peptides. Peptide 4 is derived from the C-terminus of β-casein, a region from where several antimicrobial peptides have been identified (131). Peptide 7 is a fragment of a previously identified antimicrobial peptide from κ-casein (181), and Peptide 8 is a fragment of human lactoferrampin from lactoferrin (298). Peptides 5, 9, and 11 are the first antimicrobial peptides to be identified from human α_{s1}-casein, osteopontin, and serum albumin, respectively, and their sequences and activities have been added to the MBPDB. However, the MICs of these peptides are fairly weak, on the range of 20–30 times higher than human lactoferricin (130).

It is unlikely that the MICs determined for the synthetic peptides were achieved in the intestinal peptide extracts. Derivatives of these peptides extended or shortened at either the N- or C-terminus might improve the efficacy, as has been shown with lactoferricin (299). The activity of the peptide extracts may not be due to a high concentration of specific peptides but the accumulated concentration of peptides with similar sequences from the same region of a milk protein, or their individual local concentrations in the digesta and their interactions with bacteria in the gut. Furthermore, it may be that the purpose of antimicrobial milk peptides is not to eliminate bacteria in the infant intestine, like an antibiotic would, but to put non-lethal negative growth pressure on harmful bacteria so that commensal species can flourish. As infants in the NICU receive feeds at a maximum of every three hours (300), their GI system is constantly being replenished with doses of peptides that we have shown can suppress *S. aureus* and *E. coli* growth. In addition to the newly discovered antimicrobial peptides, the intestinal samples also contained 22 previously known antimicrobial peptides: 2 each from human β-casein and bovine κ-casein, 3 from bovine α_{s1}-casein, 4 from bovine α_{s2}-casein, 5 from bovine β-lactoglobulin, and 6 from bovine β-casein. These peptides may have contributed to the overall activity of the growth-inhibitory extracts for *S. aureus* and *B. infantis*, as each active extract contained multiple known antimicrobial peptides.

Several milk peptides have been discovered to possess bifidogenic activity. Caseinomacropeptide, a large glycopeptide from bovine κ-casein, has stimulating activity for several bifidobacterial species (301, 302). The enhanced growth caused by caseinomacropeptide may be due to its multiple fermentable sugars that bifidobacteria can preferentially use (303). Bifidogenic activity was also characterized for three peptides

from human lactoferrin (199), one from polymeric Ig receptor (199), and one from bovine lactoferrin (200). These five peptides all contained a disulfide bond and were identified from pepsin hydrolysates of human or bovine milk that was iteratively fractionated and tested. Bifidobacteria have a surface lactoferrin-binding protein that may play a role in recognizing disulfide-bonded peptides to stimulate growth (117). In the present study, peptide identification was performed with MS/MS conditions that optimized the number of peptides identified but were unable to determine post-translational glycosylation or disulfide bond formation. As none of the unmodified candidate peptides stimulated *B. infantis* growth, it may be that unidentified, modified peptides were responsible for the stimulation caused by the peptide extracts.

Though this was the first study to confirm that milk peptides in the infant intestine have antimicrobial and bifidogenic properties, it was only partially successful in identifying single bioactive peptides that could account for the overall activity of the extracts. The methods utilized can be improved by expanding coverage of the sample peptidomes through identification of peptides outside the optimal size range and peptides with single or multiple post-translational modifications. Further refinements to label-free quantitation or the application of absolute quantitation methods will also indicate which peptide species are truly the most abundant in the samples. In addition, improvements to methods used to select candidate peptides can be made through machine learning algorithms or quantitative structure-activity relationship modeling to the identified peptides.

In conclusion, this paper represents another step in the process of determining the relevance of bioactive human milk peptides in the infant. Though past studies have

focused on identifying bioactive peptides from undigested or *in vitro* digested milk, these peptides may not be present or survive to their sites of activity in the infant GI tract. This is the first study to confirm that bioactive peptides in the infant intestinal tract have antimicrobial and bifidogenic activity. These peptides may play a role in shaping the local microbiota of the region of the intestine in which the peptides are generated, or may have a more general impact if the peptides persist into the larger intestine. This shaping could have significant effects on infant health and represents how products of protein digestion can benefit the infant beyond provision of precursors for anabolism. Future research should investigate whether infant physiological differences can lead to differential peptide release and what health-promoting effects these bioactive peptides may have *in vivo* so that they may be applied to clinical improvements.

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Chapter 6 – Conclusions

For many decades, it was believed that the sole reason for the gastrointestinal digestion of food proteins is to release amino acids that can then be absorbed and utilized in protein synthesis or metabolism, and that peptides are simply a necessary intermediary between the phases of intact protein and individual amino acids. The present research does not debate the matter that the release of amino acids may be the primary reason for gastrointestinal digestion but delves further into the multi-faceted functions of protein digestion and all of its complexities. Many food proteins have beneficial bioactivity in their intact state, but across large regions of the gastrointestinal tract, oligopeptides–not intact proteins or amino acids–are the predominant form of protein. HM proteins in particular, having evolved specifically to nourish the human infant, have the highest opportunity to provide additional functionality after proteolysis has been initiated, for if the bioactivities of HM proteins were limited to only their intact state, most activity would occur only in the stomach and the upper regions of the small intestine. But if the bioactivities of HM proteins extend down into their peptides, they may persist throughout the entire infant GI system, potentially affecting everything from tooth enamel to the microbiome. The present doctoral research provides several key pieces of evidence supporting the specificities of infant protein digestion in releasing HM bioactive peptides that can support health and development in early life.

The scale of human milk peptide species released during digestion

The first two published peptidomic studies of undigested HM identified 328 (140) and 419 (139) unique peptides from 37 and 34 proteins, respectively. The sample sizes of the two studies were five and six milk samples. The first published peptidomic study of

infant gastric digestion of HM identified 784 unique peptides from 36 proteins, with a sample size of three infants (153). In Chapter 2, I identified 1,720 HM peptides from 57 proteins in undigested HM, and up to a maximum of 5,641 HM peptides from 187 proteins after three hours of gastric digestion. I was able to collect 14 milk and 14 gastric samples for this analysis. In Chapter 4, I identified 6,419 HM peptides from 51 proteins in undigested HM, and after two hours of gastric digestion, 11,908 HM peptides from 144 proteins. These peptides were identified from 83 milk samples and 70 gastric samples.

There was a clear increase in the number of identifiable milk peptides and their respective parent proteins from the earliest studies to the present studies. Though a portion of the increase can be explained by advances in mass spectrometry instrumentation and analysis, it becomes clear that as more samples are analyzed, more of the variation between infants is captured, and the total number of peptides identified in milk and milk digesta increases. The peptide profile of each mother's milk sample is not only distinct from profiles of peptides from other mothers, it also differs from over each mother's own course of lactation. So too are infant gastric samples distinct both from other infants and within the same infant over time. However, despite such variation, I identified hundreds of peptide species that were conserved across at least half of the samples in each analysis. Furthermore, it was first thought that HM might contain only a few hundred peptide species; these results show that number can be in the thousands, thus increasing the likelihood of bioactive peptide species being present even before the infant has consumed the milk.

Though milk peptides have previously been identified in HM and infant gastric digesta, this work is the most detailed peptidomic examination of almost the entire infant gastrointestinal tract. In Chapter 5, I was able to identify HM peptides in the infant small intestine for the very first time. In Chapter 3, I was able to identify HM peptides in infant stool for the very first time. I identified 5,251 HM peptides from 160 proteins in the intestine and 8,132 HM peptides from 169 proteins in stool. Making comparisons on the number of "milk" peptides that arise from infant digestion from milk to gastric to intestine to stool proves challenging, however. There are only four proteins that are exclusively found in HM: β-casein, α_{s1}-casein, κ-casein, and α-lactalbumin. The rest are synthesized by various tissues including the small and large intestine, thus obscuring whether the peptides identified truly came from mother's milk or from endogenously-produced proteins. Furthermore, though the same peptide extraction and mass spectrometry methods were used to analyze and compare peptides from the milk, gastric, and stool samples from the Randall Children's Hospital infant cohort in Chapter 4, the methods used were altered for analyzing intestinal samples from the Oregon Health and Sciences University cohort in Chapter 5. This problem extends to comparisons of all peptidomic data. Different clean-up procedures, protein-peptide separation columns, HPLC or UPLC stationary phases, MS/MS fragmentation methods, etc. can all alter the final profile of the peptides that can be identified. Because of the multitude of extraction and identification methods that can select for peptides of differing chemical characteristics, the thousands of peptides identified in this present work likely represent only a fraction of the true HM peptidome.

Mapping the digestion of human milk proteins

Using the PepEx tool developed by our laboratory, I mapped the locations of high peptide abundance along the sequence of several milk proteins at the various stages of digestion. A peptide's abundance in a sample is determined by its relative ion intensity in the mass spectrometer and is a form of relative quantitation. The abundance maps generated by PepEx are displayed in **Figure 2.7**, **Supplemental Figure 3.2** and **3.3**, **Figure 4.4**, and **Supplemental Figures 4.3–4.5**. These maps show where peaks of peptide release occur along the protein sequence, correlating to more proteolytic activity at the amino acids preceding and following the peaks. In several instances, these peaks corresponded to regions where bioactive peptides had previously been identified, and could indicate directed release of these peptides. The best example of this phenomenon is the C-terminus of β-casein, a region that has produced several peptides with known antimicrobial activity (discussed in detail in the Discussion of Chapter 4). Hundreds of peptides with very high abundance were released from this region of β-casein in human milk, and in the gastric and intestinal stages of digestion. A few of these peptides also survived to the stool, indicating they may pervade the entirety of the GI system after feeding. Despite the high degree of variation in peptide profiles between infants, release of this region was conserved across study infant populations. Similar regions in lactoferrin and κ-casein were also discovered. As more bioactive peptides are confirmed, it will be possible to reference back to these maps to determine how likely they are to be released at the various stages of infant digestion.

The peptide maps may also provide insight into the digestive differences between infants. Though it was not the focus of the present dissertation research, these maps may

be used to find specific sites within the protein sequence that are either highly cleaved or not well cleaved by infant proteases. As we develop a deeper understanding of the post-translational modification of milk proteins through phosphorylation, glycosylation, and oxidation, that information can be applied to the maps to identify correlations between modified residues and enzymatic cleavage. Such post-translational modifications could act to preserve specific peptides or direct GI proteases to specific regions of the milk protein.

Bioactive milk peptides in the infant gastrointestinal system

One of the central goals of the present dissertation research was to identify the bioactive potential of milk peptides as they are released during infant digestion. In Chapters 2-4, the identification of bioactive peptides was accomplished through comparing peptidomic data of the milk, stomach, and stool samples to the Milk Bioactive Peptide Database (MBPDB). Our laboratory created the MBPDB in 2017 to be comprehensive of the milk bioactive peptide literature thus far and continue to curate it to keep it up to date with newly published sequences. In Chapter 5, the MBPDB was once more used to identify known bioactive peptides in the infant intestinal tract, but novel antimicrobial peptides were also identified through activity testing.

From comparisons with the MBPDB, I found that bioactive peptides were already present in the milk of mothers before it was fed to the infants (Chapter 2, Chapter 4). These peptides are likely released as a result of native mammary gland proteases, which are known to be active in milk (208). In Chapter 2, only two human milk peptides with known bioactivity were discovered in preterm milk: SPTIPFFDPQIPK, which stimulates cell proliferation (215), and the antimicrobial peptide

QELLLNPTHQIYPVTQPLAPVHNPISV (180), both from β-casein. Both of these peptides were again identified in Chapter 4 and also additional cell-proliferative, antimicrobial, and ACE-inhibitory peptides (Table 4.1). Whether these peptides have a relevant purpose in the milk remains unknown; however, the antimicrobial peptides may aid in preserving the milk and preventing infiltration by bacteria.

Once milk reaches the infant stomach, pepsin initiates the large-scale proteolysis of the human milk proteins, catalyzing the release of many known HM bioactive peptides. From all gastric samples in Chapter 2, there were seven known bioactive peptides: three ACE-inhibitory peptides, and one each of antimicrobial, cell-proliferative, opioid, and dual antioxidant/ACE-inhibitory peptides. The same two bioactive peptides found in the undigested milk were also present in the stomach up to three hours after feeding. Six of these peptides were once more identified in Chapter 4 from a separate cohort of infants, but the opioid peptide KYLGPQY from lactoferrin (304) and the ACE-inhibitory peptide DKIYPSFQPQPLIYP from β-casein (252) were only found in Chapter 2. There were 16 bioactive peptides identified in the gastric samples of Chapter 4. The functions of the bioactive peptides diversify from the milk to the infant stomach, expanding to include opioid and antioxidant activity. Depending on their bioactivity, some of these peptides may have local effects. Opioid peptides can interact with stomach opioid receptors and influence transit time and stimulate the production of mucins (305). Antioxidant peptides can quench reactive-oxygen species present in milk or created during digestion, which can cause oxidative stress, particularly in preterm infants (170). Though the stomach contains fewer microorganisms than the colon, antimicrobial peptides may prevent the growth of pathogens that can take advantage of the low-acidity

conditions of the neonatal stomach. Furthermore, even if the bioactive peptides do not act locally in the stomach, their release may be the first stage for their eventual progression into the infant intestinal tract, where they have more opportunity to exert their activities.

In the intestinal tract, the infant secretes over a dozen proteases that digest the milk proteins further, almost down to their component amino acids for most milk proteins. From my results in Chapter 5, I identified 17 known human bioactive peptides in the infant duodenal/jejunal samples (Supplemental Table 5.1). Most of the sequences overlapped with the bioactive peptides in the stomach in Chapters 2 and 4, with the main differences being an exchange of some β-casein ACE-inhibitory peptides for others and losing the antimicrobial peptide YPVTQPLAPVHNPIS from β-casein while gaining an antioxidant peptide QVVPYPQ from β-casein. The equivalent amounts of known bioactive peptides from the stomach to the intestine suggest that while there may be some flux in which species are present at which amounts, the overall bioactivity may be conserved.

Outside of the use of MBPDB for comparison with the peptidomic data, I identified several new bioactive peptides from the infant intestinal samples based on their *in vitro* growth-inhibitory activity of *S. aureus* and *E. coli*. These peptides were only weakly active but came from milk proteins including α_{s1}-casein, osteopontin, and serum albumin from which no bioactive peptides have yet been identified (Table 5.3). These results suggest that there may be more yet undiscovered regions of the milk proteome capable of producing bioactive peptides. Furthermore, though their individual activities were weak, the cumulative release of multiple peptide species could be enough to reach sufficient concentrations capable of influencing the microbial environment. Previous

research has also shown that bioactive peptides with more or fewer amino acids at their termini either retain or increase their activity (299), and these peptides should be explored through that route to identify whether they can be improved.

Finally, in the stool, only one known bioactive peptide was identified (the cell-proliferative peptide RETIESLSSSEESITEYK from β-casein). The scarcity of bioactive peptides is unsurprising given preterm infant GI transit times, as milk proteins will have undergone between 10–20 hours (306) of proteolysis from a wide catalogue of human and microbial proteases by the time they reach the stool. However, the bioactive milk peptides present at this stage will have had the most opportunities across the entire GI system to activate in their target tissue or be absorbed into the bloodstream.

Final remarks and future opportunities

The peptidomic work I have completed during my dissertation research is only a starting point for what I hope to accomplish in the future. The main area I plan to improve is the mass spectrometry quantitation methods used for measuring peptide concentrations. In the present studies, I used peptide abundance based on ion intensity as a relative correlation of concentration. However, our laboratory has recently undergone method development of applying absolute quantitation through the use of parallel reaction monitoring to determine the concentrations of specific milk proteins along the infant GI system (307). These methods could be applied to measuring the release of bioactive peptides over time as well. Given how dependent many of the peptides' bioactivities are on concentration, it will be crucial to absolutely quantify select peptide species if we are to determine their relevance to infant health and development.

Finally, there are several questions regarding the activity of bioactive peptides *in vivo* and as supplements that will require intervention trials, which are difficult to perform in preterm infants. These questions include whether the absence of any milk bioactive peptides in the GI system correlates with the development of disease, what the fate of bioactive peptides are as they transit the GI system, and whether additional bioactive peptides can be supplemented for additional health benefit. The primary application of milk peptide research is to develop technologies, foods, and feeding practices to improve the health outcome of infants–similar to how research on human milk oligosaccharides has led to improvements in infant formula to better mimic mother's milk–and the above questions will need to be answered before such steps can be taken.

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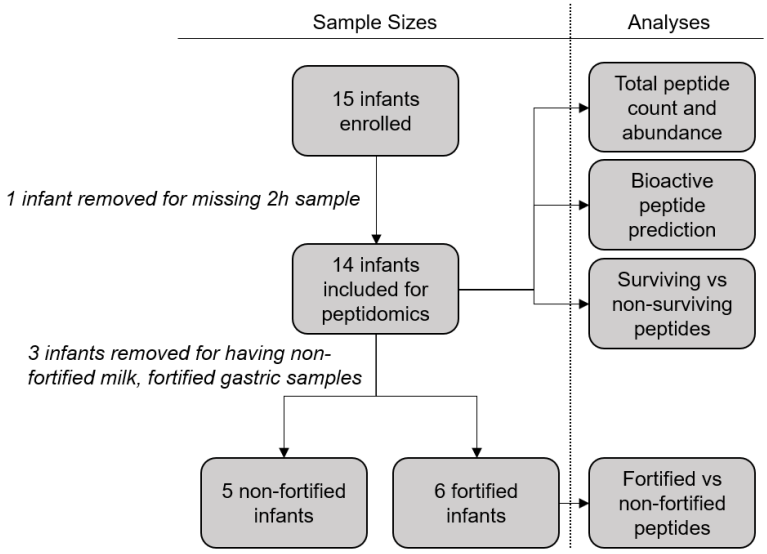
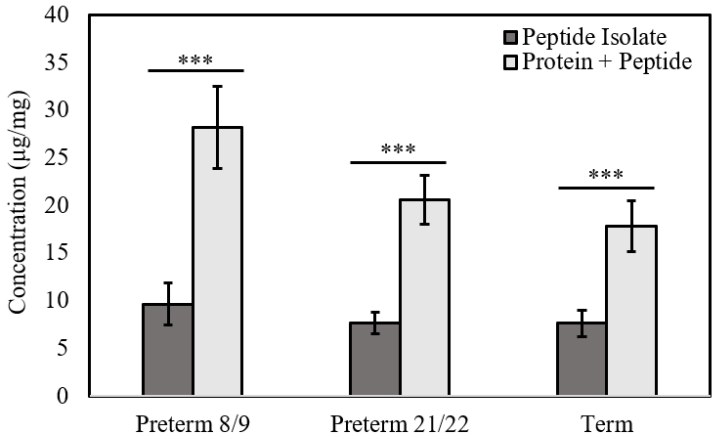
Appendices

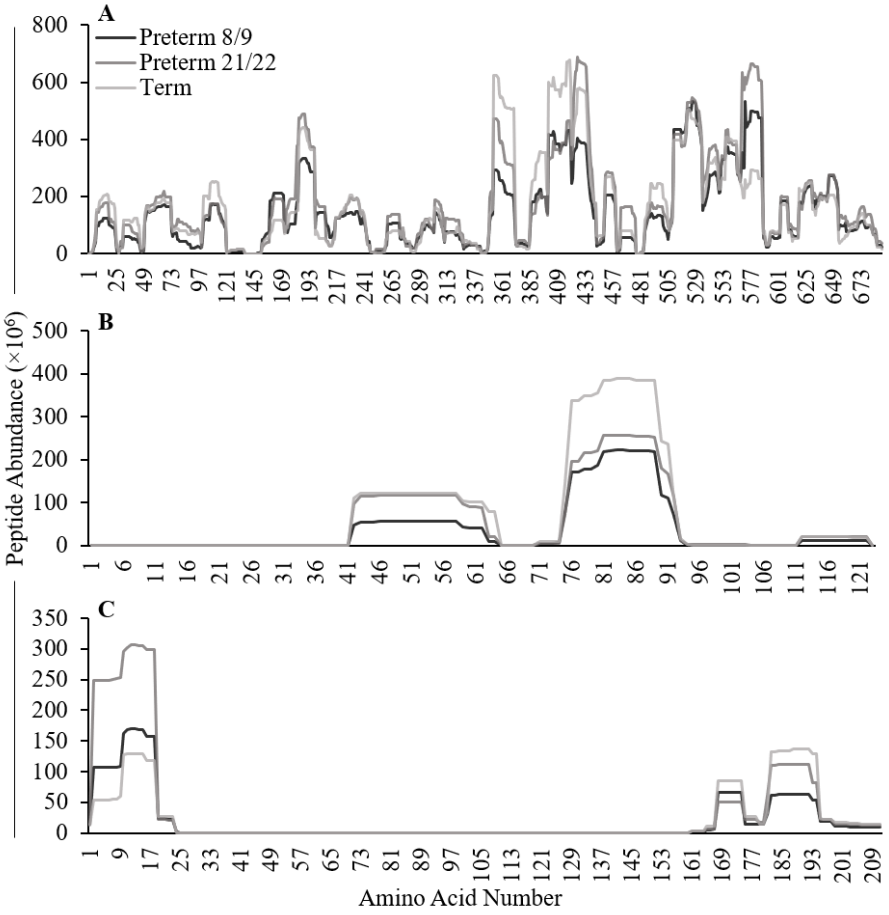
Supplemental figures



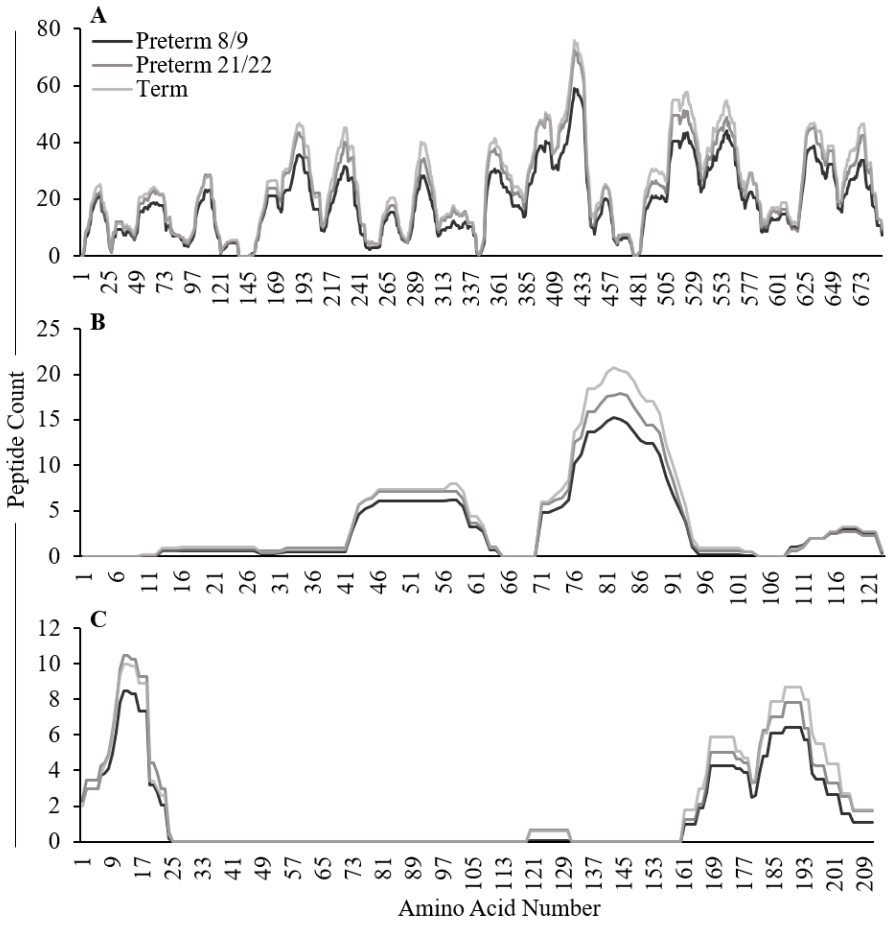
Supplemental Figure 2.1. Workflow of the infant samples and the analyses performed on them. One infant was removed from the initial enrollment of 15 infants as there was insufficient gastric 2-hr sample for peptidomic analysis. Three infants were removed from the fortified vs. non-fortified peptide analysis, as these infants had differing fortification status across their entire sample set.



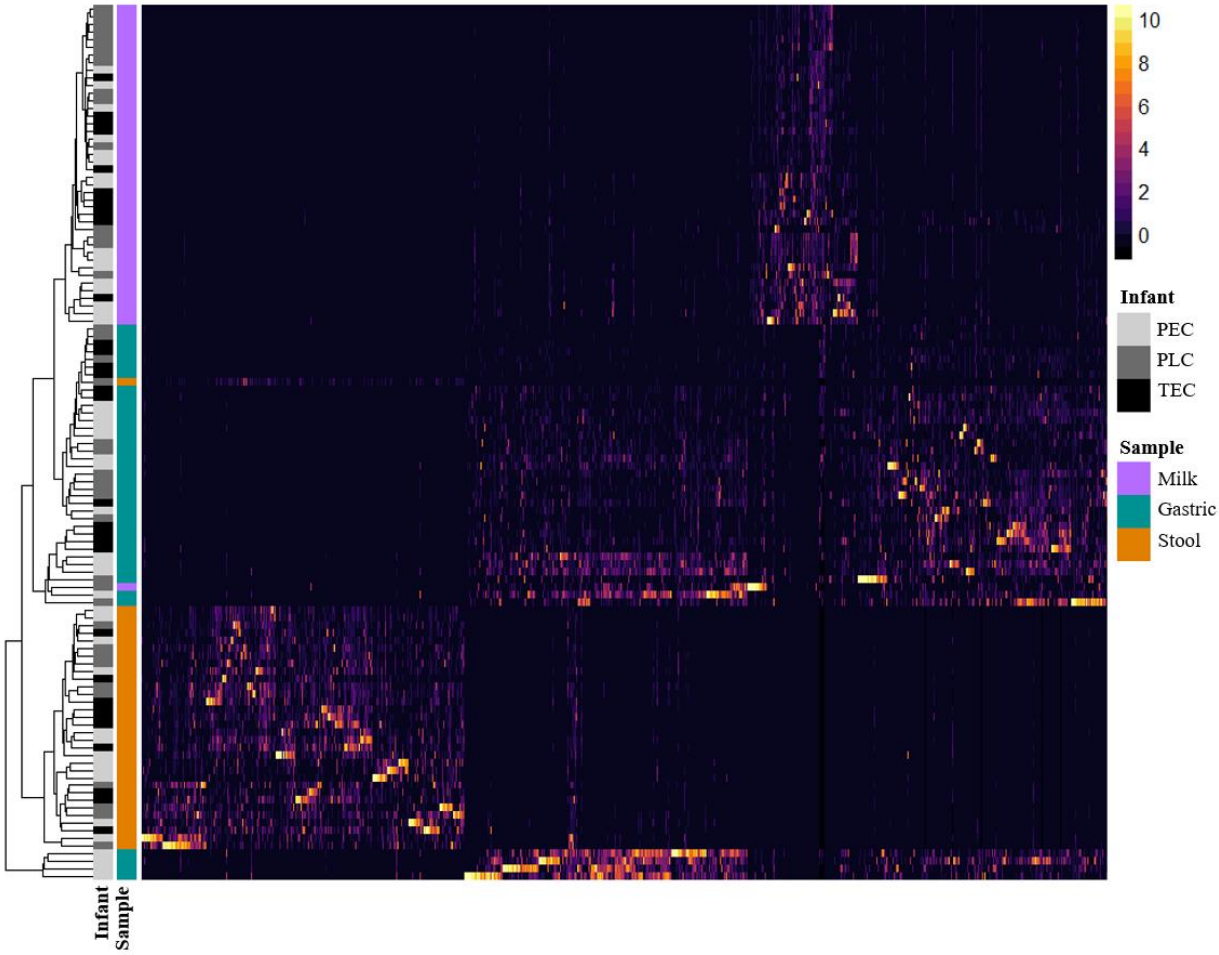
Supplemental Figure 3.1. Comparison of the combined protein and peptide concentration and the peptide isolate concentration. Data are represented as mean ± the standard error. Preterm 8/9 n=12, preterm 21/22 n=11, and term n=10. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.



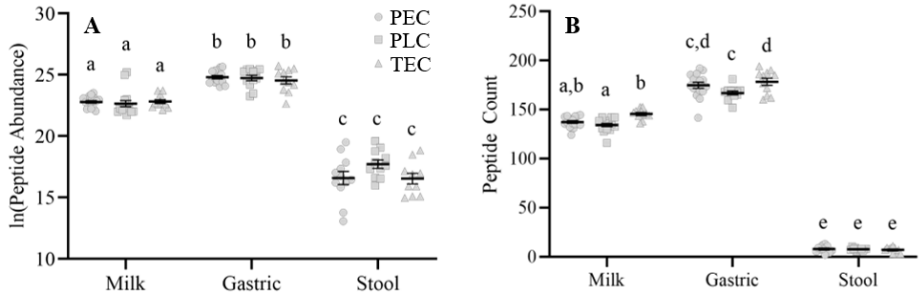
Supplemental Figure 3.2. Map of peptide abundances identified in the stool of preterm infants at 8/9 DOL, preterm infants at 21/22 DOL, and term infants at 8/9 DOL to the sequences of lactoferrin (A), α-lactalbumin (B), and β-casein (C).



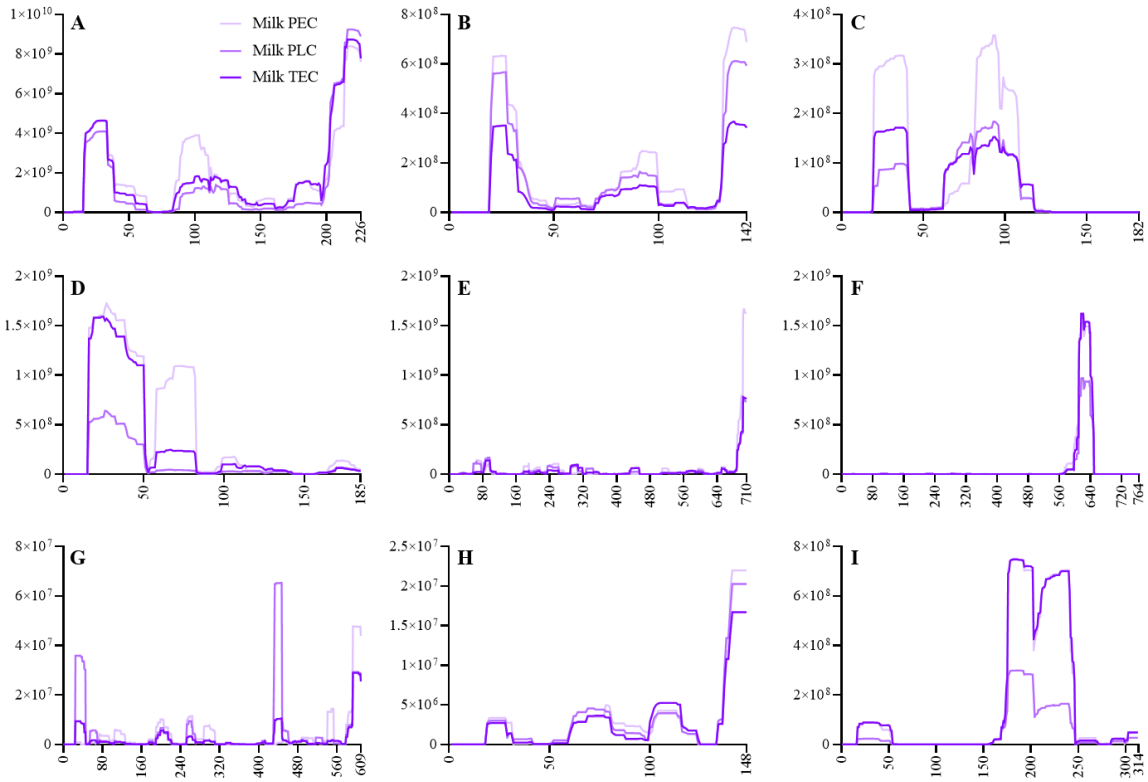
Supplemental Figure 3.3. Map of peptide counts identified in the stool of preterm infants at 8/9 DOL, preterm infants at 21/22 DOL, and term infants at 8/9 DOL to the sequence of lactoferrin (A), α-lactalbumin (B), and β-casein (C).



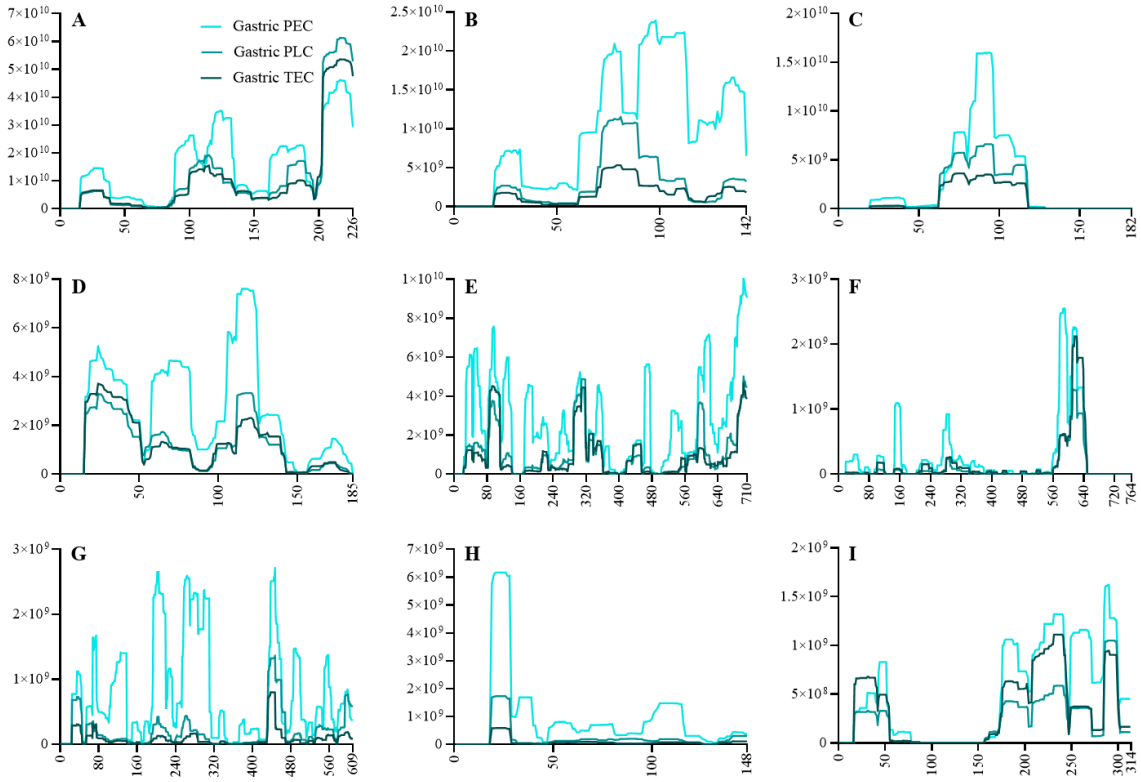
Supplemental Figure 4.1. Hierarchical clustering and heatmap of the scaled milk peptide abundances across all samples. Dendrogram clustering was performed using Ward's method. Milk, gastric, and stool samples from all infants are sorted on the *y*-axis, and individual milk peptides are sorted on the *x*-axis. The colored bar labeled "Infant" denotes the maturity status of the infant the sample came from (PEC, PLC, or TEC), and the colored bar labeled "Sample" denotes the sample phase of digestion (milk, gastric, or stool). PEC, preterm early collection; PLC, preterm late collection; TEC, term early collection.



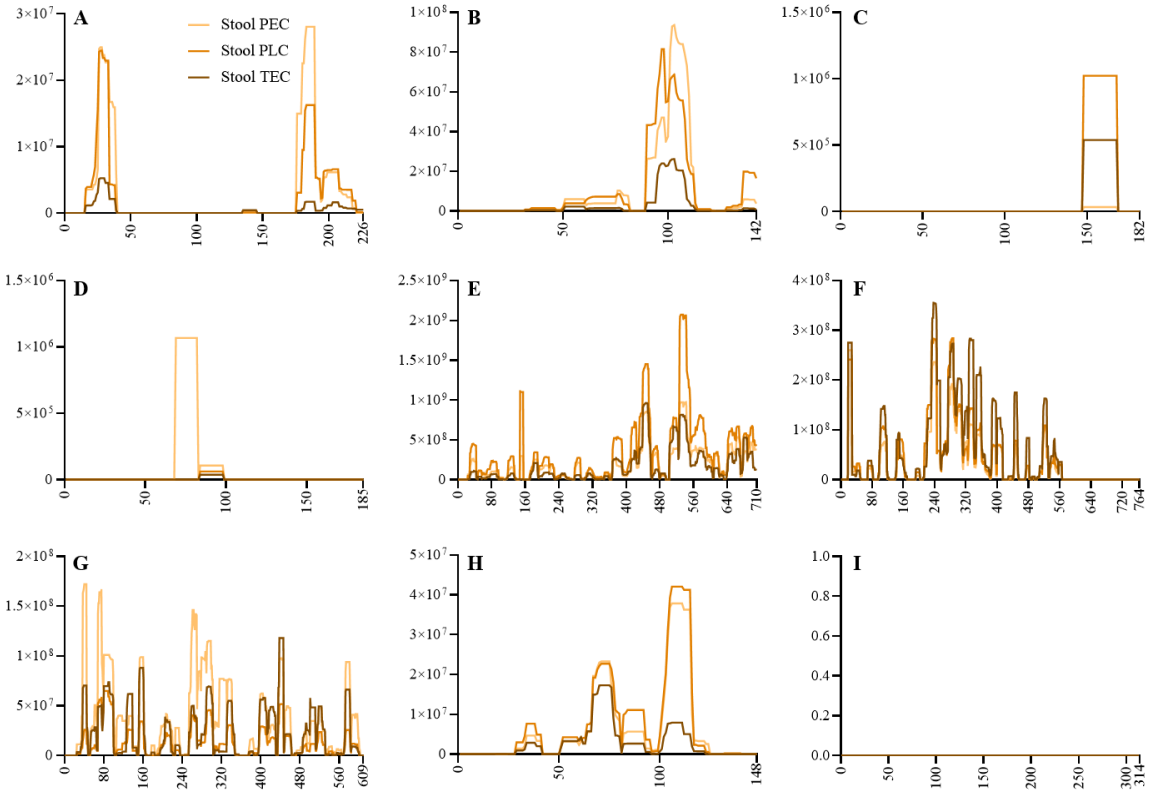
Supplemental Figure 4.2. Comparison of homologous (A) peptide abundance and (B) count across digestion and between PEC, PLC, and TEC infants. PEC, preterm early collection; PLC, preterm late collection; TEC, term early collection.



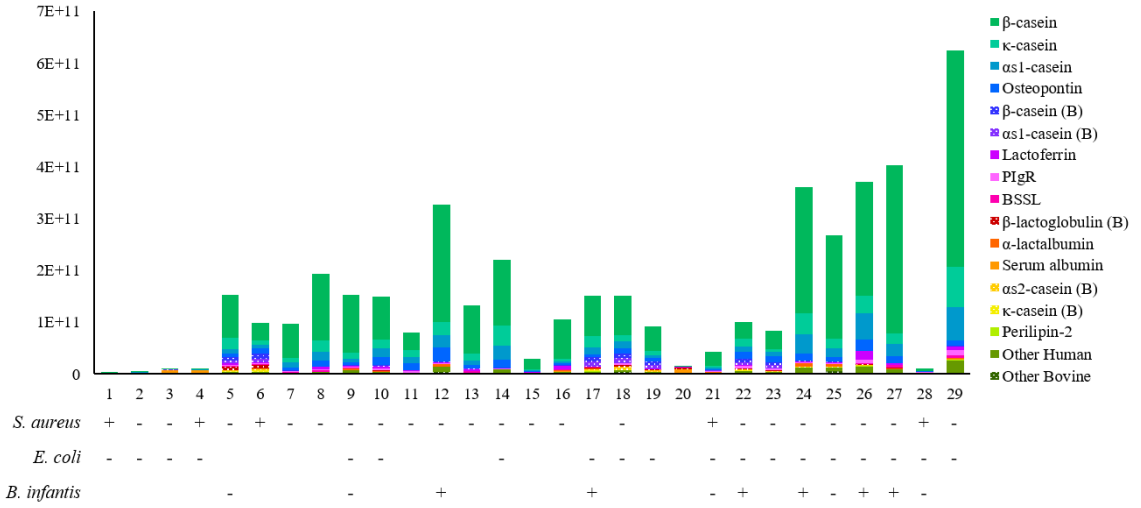
Supplemental Figure 4.3. Peptide release across the sequence of individual milk proteins in human milk. For all figures, the *y*-axis is the mean peptide abundance and the *x*-axis is the linear amino acid sequences for the proteins: (A) β-casein, (B) α-lactalbumin, (C) κ-casein, (D) α_{s1}-casein, (E) lactoferrin, (F) PIgR, (G) serum albumin, (H) lysozyme, and (I) osteopontin. Grey shaded boxes represent regions of homology to bioactive peptides. PIgR, polymeric immunoglobulin receptor.



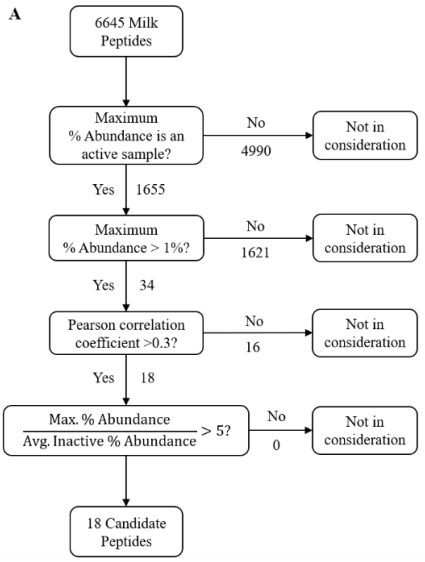
Supplemental Figure 4.4. Peptide release across the sequence of individual milk proteins in infant gastric samples. For all figures, the *y*-axis is the mean peptide abundance and the *x*-axis is the linear amino acid sequences for the proteins: (A) β-casein, (B) α-lactalbumin, (C) κ-casein, (D) α_{s1}-casein, (E) lactoferrin, (F) PIgR, (G) serum albumin, (H) lysozyme, and (I) osteopontin. Grey shaded boxes represent regions of homology to bioactive peptides. PIgR, polymeric immunoglobulin receptor.



Supplemental Figure 4.5. Peptide release across the sequence of individual milk proteins in infant stool samples. For all figures, the *y*-axis is the mean peptide abundance and the *x*-axis is the linear amino acid sequences for the proteins: (A) β-casein, (B) α-lactalbumin, (C) κ-casein, (D) α_{s1}-casein, (E) lactoferrin, (F) PIgR, (G) serum albumin, (H) lysozyme, and (I) osteopontin. Grey shaded boxes represent regions of homology to bioactive peptides. PIgR, polymeric immunoglobulin receptor.



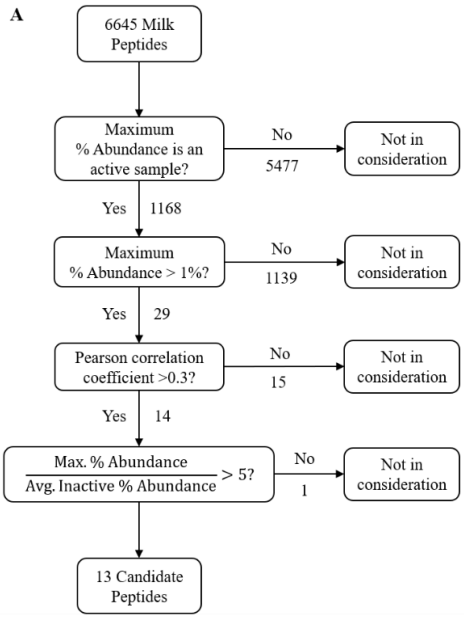
Supplemental Figure 5.1. Total abundance of the peptides from each intestinal sample sorted by protein from highest to lowest mean abundance. Bovine milk proteins are labeled with a (B) in the legend and are filled with a dotted pattern. In the rows below the graph, a + indicates the sample was active against the bacteria, a – indicates the sample was antagonistic, and no symbol indicates the sample was inactive. PIgR, polymeric immunoglobulin receptor; BSSL, bile salt-stimulated lipase.



B

Sequence	Modifications	Protein	Start	Stop	Max % Abundance	Pearson Coefficient	Ratio	Sequence Homology
MTSALPIIQK		Perilipin-2	62	71	20.05	0.41	11515.13	
HLPLPLLQPLMQQVPQPI		β-casein	140	157	12.00	0.40	214.85	
EIENKAIQDPR		PIgR	593	603	11.74	0.41	450.72	
DLENLHLPL		β-casein	135	143	3.87	0.42	124.04	
DETYVPK		Serum albumin	518	524	3.63	0.40	22.86	
FKDLGEENFK		Serum albumin	35	44	2.61	0.54	18.38	
TVDGPSGKLW		GAPDH	187	196	2.38	0.42	870.26	
EKFGKDKSPKFQ		Lactotransferrin	295	306	2.19	0.41	1673.49	
RPNLHPS		κ-casein	110	116	1.88	0.40	151.02	
QYKLGPKTGPGQ	Phospho [T8]	Fibroblast growth factor 2*	132	143	1.67	0.44	30.56	
EPIPLESREE		α_{s1}-casein	35	44	1.50	0.51	25.84	
RETIESLSSSEE		β-casein	16	27	1.41	0.42	19.48	
QLFGSPSGQK		Lactotransferrin	306	315	1.27	0.40	26.28	
LPLRYPERLQNPSESSEPIPLESREEY		α_{s1}-casein	19	45	1.22	0.47	63.47	
ESSSHHPGIAEFPSRGK		Fibrinogen α-chain	559	575	1.17	0.40	340.44	
ADAAPDEKVLDSG		PIgR	578	590	1.16	0.42	65.94	
ILDIKGID		α-lactalbumin	114	121	1.16	0.32	25.92	
HQPHQPLP		β-casein*	160	167	1.13	0.40	115.01	

Supplemental Figure 5.2. Flow diagram of criteria and thresholds by which peptides with potential antimicrobial activity against *Staphylococcus aureus* were excluded from selection for activity testing.



B

Sequence	Modifications	Protein	Start	Stop	Max % Abundance	Pearson Coefficient	Ratio	Sequence Homology
LAPVHNPI		β-casein	217	224	4.66	0.39	207.63	
LLNPTHQIYP		β-casein	203	212	3.44	0.32	20.13	
HQIYPVTQPL		β-casein	208	217	3.33	0.37	48.87	
LLNPTHQIYPVTQPLAPVHNPIS		β-casein	203	225	3.02	0.32	14.42	85.20
YANPAVVRPHAQIPQR		κ-casein	81	96	2.69	0.3	8.94	
VTQPLAPVHNP		β-casein	213	223	2.27	0.41	239.07	
LWSVPQPK		β-casein	168	175	2.06	0.35	18.16	
VTQPLAPVHNPI		β-casein	213	224	1.94	0.37	34.19	
LAPVHNPIS		β-casein	217	225	1.83	0.41	321.29	
DMLVVDPK		Osteopontin	283	290	1.34	0.36	40.67	
LLNPTHQIYPVTQPLAPVHNPI		β-casein	203	224	1.28	0.31	5.22	81.48
PVLKSPTIP		β-casein	116	124	1.14	0.33	8.7	
LMQQVPQPIPQT		β-casein	149	160	1.07	0.44	12.02	

Supplemental Figure 5.3. Flow diagram of criteria and thresholds by which peptides with potential bifidogenic activity for *Bifidobacterium infantis* were excluded from selection for activity testing.

Supplemental tables

Supplemental Tables 2.1–2.2 are available from the *Journal of Proteome Research* here.

Supplemental Tables 3.1–3.2 are available from the *Journal of Nutrition* here.

Supplemental Table 4.1. List of samples that were collected from each infant at each day of life.

	Infant	Milk				Gastric				Stool[1]	
		8	*9*	*21*	*22*	*8*	*9*	*21*	*22*	*8/9*	*21/22*
Preterm	1	X	X	X	X	X	X	X	X	X	X
	2	X	X	X	X	X	X	X	X	X	X
	3	X	X	X	X	X	X	X	X	X	X
	4	X	X	X	X	X	X	–	–	X	–
	5	X	X	X	–	X	X	–	–	X	–
	6	X	X	X	X	X	X	X	–	–	X
	7	X	X	X	X	X	X	–	–	X	–
	8	X	X	X	X	X	X	X	–	–	X
	9	X	X	X	X	X	–	X	–	X	–
	10	X	X	X	X	X	X	X	X	–	X
	11	X	X	X	X	X	X	X	X	–	X
	12	X	X	X	X	X	X	X	X	X	X
	13	X	X	X	X	X	X	X	X	X	X
	14	X	X	X	X	X	X	–	X	X	X
	15	X	X	X	–	X	X	–	–	X	–
	16	X	X	X	X	X	X	X	X	X	X
Term	17	X	X	–	–	X	X	–	–	X	–
	18	X	X	–	–	X	–	–	–	X	–
	19	X	X	–	–	X	–	–	–	–	–
	20	X	X	–	–	X	X	–	–	X	–
	21	X	X	–	–	X	X	–	–	X	–
	22	X	–	–	–	X	–	–	–	X	–
	23	X	X	–	–	X	X	–	–	X	–
	24	X	X	–	–	X	X	–	–	X	–
	25	X	X	–	–	X	X	–	–	X	–
	26	X	X	–	–	X	X	–	–	X	–
	27	X	X	–	–	X	X	–	–	X	–

[1] Stool samples were collected only once for each two-day period.

Supplemental Table 5.1. List of known bioactive peptides in the infant intestinal samples.

Peptide	Species	Parent Protein Location	Function
ENLHLPLP	Both	β-casein (137–144)	ACE-inhibitory
NLHLPLP	Both	β-casein (138–144)	ACE-inhibitory
ENLHLPLPLL	Both	β-casein (146–155)	ACE-inhibitory
NLHLPLPLL	Both	β-casein (147–155)	ACE-inhibitory
FFVAPFPEVFGK	Bovine	α_{s1}-casein (38–49)	ACE-inhibitory
FVAPFPEVFG	Bovine	α_{s1}-casein (39–48)	ACE-inhibitory
FPEVFGK	Bovine	α_{s1}-casein (43–49)	ACE-inhibitory
IGSENSEKTTMP	Bovine	α_{s1}-casein (201–212)	ACE-inhibitory
ALNEINQFYQK	Bovine	α_{s2}-casein (96–106)	ACE-inhibitory
AMKPWIQPK	Bovine	α_{s2}-casein (204–212)	ACE-inhibitory
LVYPFPGPI	Bovine	β-casein (73–81)	ACE-inhibitory
MPFPKYPVEP	Bovine	β-casein (124–133)	ACE-inhibitory
VENLHLPLPLL	Bovine	β-casein (145–155)	ACE-inhibitory
LLYQEPVLGPVRGPFPIIV	Bovine	β-casein (206–224)	ACE-inhibitory
QEPVLGPVRGPFPIIV	Bovine	β-casein (209–224)	ACE-inhibitory
EPVLGPVRGPFP	Bovine	β-casein (210–221)	ACE-inhibitory
VLGPVRGPFP	Bovine	β-casein (212–221)	ACE-inhibitory
LDIQKVAGTW	Bovine	β-lactoglobulin (28–37)	ACE-inhibitory
DAQSAPLRVY	Bovine	β-lactoglobulin (49–58)	ACE-inhibitory
FSDKIAK	Bovine	κ-casein (39–45)	ACE-inhibitory
HPHPHLSF	Bovine	κ-casein (119–126)	ACE-inhibitory
DKIYPSFQPQPLIYP	Human	β-casein (53–67)	ACE-inhibitory
IYPSFQPQPLIYP	Human	β-casein (55–67)	ACE-inhibitory
FQPQPLIYP	Human	β-casein (59–67)	ACE-inhibitory
TVYTKGRVMP	Human	β-casein (107–116)	ACE-inhibitory
LTDLENLHLP	Human	β-casein (133–142)	ACE-inhibitory
LENLHLPLP	Human	β-casein (136–144)	ACE-inhibitory
YANPAVVRP	Human	κ-casein (81–89)	ACE-inhibitory
IKHQGLPQEV	Bovine	α_{s1}-casein (21–30)	Antimicrobial
LRLKKYKVPQL	Bovine	α_{s1}-casein (114–124)	Antimicrobial
SDIPNPIGSENSEK	Bovine	α_{s1}-casein (195–208)	Antimicrobial
TKKTKLTEEEKNRL	Bovine	α_{s2}-casein (163–176)	Antimicrobial
IQPKTKVIPYVR	Bovine	α_{s2}-casein (209–220)	Antimicrobial
TKVIPYVRYL	Bovine	α_{s2}-casein (213–222)	Antimicrobial
TEDELQDKIHPF	Bovine	β-casein (56–67)	Antimicrobial
PVVVPPFLQPE	Bovine	β-casein (96–106)	Antimicrobial
VLPVPQKAVPYPQR	Bovine	β-casein (185–198)	Antimicrobial
YQEPVLGPVRGPFPI	Bovine	β-casein (206–220)	Antimicrobial
GLDIQKVAGT	Bovine	β-lactoglobulin (27–36)	Antimicrobial
AASDISLLDAQSAPLR	Bovine	β-lactoglobulin (41–56)	Antimicrobial

Supplemental Table 5.1 (cont.)

Peptide	Species	Parent Protein Location	Function
IIAEKTKIPAVF	Bovine	β-lactoglobulin (89–100)	Antimicrobial
FSDKIAK	Bovine	κ-casein (39–45)	Antimicrobial
MAIPPKKNQDKTEIPTINT	Bovine	κ-casein (127–145)	Antimicrobial
LLNQELLLNPTHQIYPV	Human	β-casein (197–213)	Antimicrobial
QELLLNPTHQIYPVTQPLAPVHNPISV	Human	β-casein (200–226)	Antimicrobial
QVVPYPQ	Human	β-casein (182–188)	Antioxidant
YLGYLEQLLR	Bovine	αs1-casein (106–115)	Anxiolytic
ALKALPMHIR	Bovine	β-lactoglobulin (155–164)	Cell-proliferative
RETIESLSSSEESITEYK	Human	β-casein (16–33)	Cell-proliferative
SPTIPFFDPQIPK	Human	β-casein (120–132)	Cell-proliferative
LIVTQTMK	Bovine	β-lactoglobulin (17–24)	Cytotoxic
LPQNIPPLT	Bovine	β-casein (85–93)	DPP-IV-inhibitory
LKPTPEGDL	Bovine	β-lactoglobulin (62–70)	DPP-IV-inhibitory
LKPTPEGDLE	Bovine	β-lactoglobulin (62–71)	DPP-IV-inhibitory
INNQFLPYPY	Bovine	κ-casein (72–81)	DPP-IV-inhibitory
VYVEELKPTPEGDLEILLQK	Bovine	β-lactoglobulin (57–76)	Hypocholesterolemic
LYQEPVLGPVRGPFPIIV	Bovine	β-casein (207–224)	Immunomodulatory
VYPFPGPI	Bovine	β-casein (74–81)	PEP-inhibitory
ALPMHIR	Bovine	β-lactoglobulin (158–164)	ACE-inhibitory Cell-proliferative
FALPQYLK	Bovine	αs2-casein (189–196)	ACE-inhibitory Antioxidant
IQKVAGTW	Bovine	β-lactoglobulin (28–35)	ACE-inhibitory DPP-IV-inhibitory
LKALPMH	Bovine	β-lactoglobulin (156–162)	ACE-inhibitory DPP-IV-inhibitory
PYVRYL	Bovine	αs2-casein (217–222)	ACE-inhibitory Antimicrobial Antioxidant
RELEELNVPGEIVESLSSSEESITR	Bovine	β-casein (16–40)	Caseinophosphopeptide Immunomodulatory
TPEVDDEALEK	Bovine	β-lactoglobulin (141–151)	Antimicrobial DPP-IV-inhibitory
VKEAMAPK	Bovine	β-casein (113–120)	Antimicrobial Antioxidant
VLVLDTDYK	Bovine	β-lactoglobulin (108–116)	Antimicrobial DPP-IV-inhibitory
VYPFPGPIPN	Bovine	β-casein (74–83)	ACE-inhibitory Antioxidant
WSVPQPK	Human	β-casein (169–175)	ACE-inhibitory Antioxidant

Supplemental Table 5.1 (cont.)

Peptide	Species	Parent Protein Location	Function
YQEPVLGPVR	Bovine	β-casein (208–217)	ACE-inhibitory Immunomodulatory
YQEPVLGPVRGPFPIIV	Bovine	β-casein (208–224)	ACE-inhibitory Antimicrobial Antithrombin Immunomodulatory
YQKFPQY	Bovine	αs2-casein (105–111)	ACE-inhibitory Antioxidant