RESEARCH ARTICLE

Milk Protein

Molecular Nutrition & Food Research

www.mnf-journal.com

Selective Proteolysis of α-Lactalbumin by Endogenous Enzymes of Human Milk at Acidic pH

*Junai Gan, Jingyuan Zheng, Nithya Krishnakumar, Elisha Goonatilleke, Carlito B. Lebrilla, Daniela Barile, and J. Bruce German**

Scope: The use of human milk products is increasing for high-risk infants. Human milk contains endogenous enzymes that comprise a dynamic proteolytic system, yet biological properties of these enzymes and their activities in response to variations including pH within infants are unclear. Human milk has a neutral pH around 7, while infant gastric pH varies from 2 to 6 depending on individual conditions. This study is designed to determine the specificity of enzyme–substrate interactions in human milk as a function of pH.

Methods and results: Endogenous proteolysis is characterized by incubating freshly expressed human milk at physiologically relevant pH ranging from 2 to 7 without the addition of exogenous enzymes. Results show that the effects of pH on endogenous proteolysis in human milk are protein-specific. Further, specific interactions between cathepsin D and α-lactalbumin are confirmed. The endogenous enzyme cathepsin D in human milk cleaves α-lactalbumin as the milk pH shifts from 7 to 3.

Conclusions: This study documents that selective proteolysis activated by pH shift is a mechanism for dynamic interactions between human milk and the infant. Controlled proteolysis can guide the use of human milk products based on individual circumstance.

1. Introduction

The global incidence of preterm birth (<37 weeks of gestation) is 15 million per year.[1,2] Preterm birth is the leading cause of infant death,[3] and it causes a range of long-term adverse outcomes in survivors.[1,4] High-risk infants, especially those born extremely preterm (<28 week of gestation) or with very low birth weight (<1500 g), have an urgent need for adequate growth coupled with satisfactory functional development.[5,6] Despite advances in medicine and nutrition, meeting the demands of high-risk infants remains challenging. Feeding intolerance, necrotizing enterocolitis, growth faltering, and neurodevelopmental impairment are common problems in neonatal intensive care.[6–8] To address these challenges, the use of pasteurized donor human milk and human milk fortifiers is increasing for high-risk infants when breastfeeding is unavailable or their mothers' own milk is insufficient.[6,9–11] Compared to formula, human milk products have provided better health outcomes, especially in decreasing rates of necrotizing enterocolitis.[9,10,12] However, the effects of human milk products on long-term metabolic and neurodevelopmental outcomes are debatable.[6,10,12] Differences in composition and efficacy exist between mothers' own milk and donor human milk.[10] In addition to nutrients, mothers' milk contains bioactive factors that provide functional benefits for the infant.[10,13,14] Processing techniques affect nutrients and bioactive proteins of donor milk.[15] Mother's milk is not only a nutritional supply, but also a personalized biofluid that dynamically interacts with the infant.[14] To better understand these interactions for targeted health management, it is necessary to consider individual circumstances and physiological diversity during milk digestion.

One variable that exhibits considerable diversity across infants during digestion is pH.[16,17] Human milk is neutral with a pH of around 7.[18,19] Infant gastric pH varies from pH 2 to 6 depending on individual situations, such as gestational age at birth, developmental stage, and medical indications.[20–28] Preterm infants with an earlier gestational age have a higher gastric pH in the first days of postnatal life than those more mature (median pH of 3.7 for infants born at 24–25 weeks of gestation versus median pH of 1.8 for infants born at 28–29 weeks of gestation).[27,28] Gastric

Dr. J. Gan, N. Krishnakumar, Prof. D. Barile, Prof. J. B. German
Department of Food Science and Technology
University of California
Davis, CA 95616, USA
E-mail: jbgerman@ucdavis.edu
J. Zheng
Department of Nutrition
University of California
Davis, CA 95616, USA
Dr. E. Goonatilleke, Prof. C. B. Lebrilla
Department of Chemistry
University of California
Davis, CA 95616, USA
Prof. C. B. Lebrilla, Prof. D. Barile, Prof. J. B. German
Foods for Health Institute
University of California
Davis, CA 95616, USA

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mnfr.201900259

DOI: 10.1002/mnfr.201900259

acid output increases as preterm infants grow older[24,27]; after 4 weeks, gastric acid secretion has been shown to be comparable between healthy preterm infants and term infants.[27] Among infants with very low birth weight, those with feeding intolerance have gastric pH around 5, which is significantly higher than pH 3 for those without feeding intolerance.[21] For preterm infants with gastroesophageal reflux disease, inhibitors of gastric acid such as omeprazole and H2-blockers that are used to treat the complications of acid reflux result in an increased gastric pH.[29,30] Although methods of measurements may contribute to discrepancies,[25] these studies documented the variations of pH among infants. The complex biogeography of the infant gut, including variations in pH, can lead to distinct molecular changes of human milk, which subsequently stimulate different physiological responses. Deciphering the health impacts of such complex physiological processes requires a mechanistic understanding of how infant conditions affect these molecular changes within human milk.

Human milk contains endogenous enzymes that comprise a dynamic proteolytic system.[31] These proteolytic enzymes, such as plasmin (serine protease) and cathepsin D (aspartic protease), have distinct regulations, catalytic mechanisms, and substrate specificities.[31] Yet, the biological properties of these enzymes and their activities in response to variations including pH within infants are unclear. Human-milk-derived peptides exert various bioactivities, including antimicrobial, immunomodulatory, and opioid activities.[32] Bioactivities of these peptides, mostly encrypted in protein sequences, are latent until proteolytic activation.[32] Prior studies have used digestive enzymes to release peptides from milk proteins; however, the endogenous milk enzymes have been neglected. Upon ingestion, the proteolytic system of human milk may respond to the pH change and decrypt specific bioactive peptides that exert influence on the infant.

This study was designed to determine the specificity of enzyme-substrate interactions in human milk as a function of pH. Endogenous proteolysis was characterized by incubating freshly expressed human milk at physiologically relevant pH ranging from 2 to 7 without the addition of exogenous enzymes. Further, purified proteins were used to confirm the specific enzyme–substrate interactions. Results show that the endogenous enzyme cathepsin D in human milk cleaves α-lactalbumin as the milk pH shifts from 7 to 3, supporting that selective proteolysis activated by the pH shift is a mechanism for dynamic interactions between human milk and the infant.

2. Experimental Section

The overall workflow and experimental design are shown in **Figure 1**.

2.1. Study Subjects and Human Milk Collection

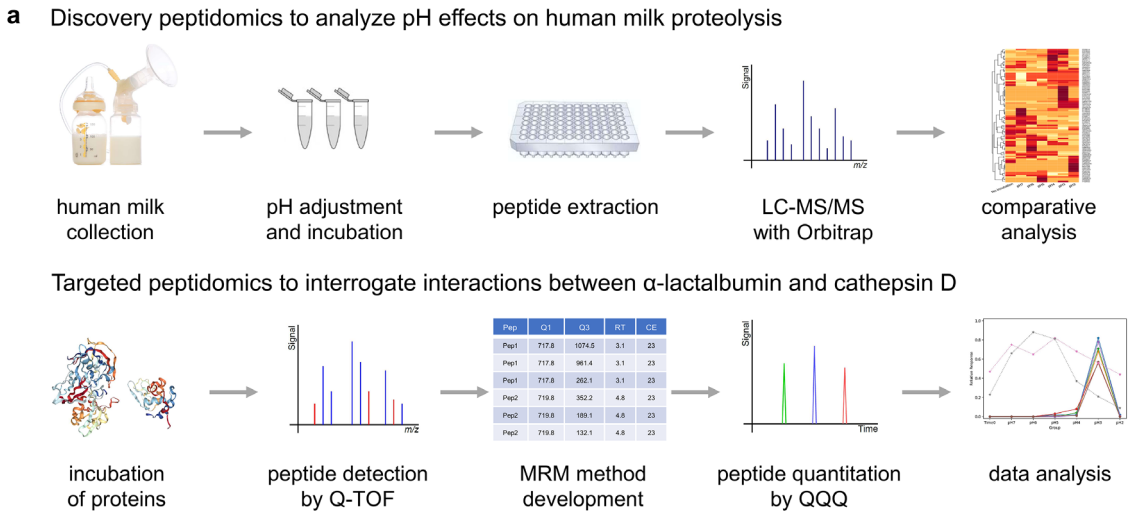
The study protocol was approved by the University of California Davis Institutional Review Board. Informed consent was obtained from five healthy lactating mothers in this study (Table S1, Supporting Information). Subjects were instructed to fully express fore and hind milk from one breast by hand or breast pump after 2 h since last feed/expression from that breast. Freshly expressed milk was gently mixed, and at least 20 mL of the milk sample was collected into sterile container and immediately transported to the laboratory on ice. Then, 500 µL aliquots of human milk were used immediately for pH adjustment and incubation. The time from milk expression to pH adjustment was within 30 min. The remaining milk was stored at −80 °C until compositional analysis.

2.2. Compositional Analysis of Human Milk

Human milk composition was analyzed by a Delta LactoScope FTIR Advanced Mid-Infrared Spectroscopy (Advanced Instruments, Norwood, MA, USA). The instrument was calibrated for human milk analysis prior to sample measurement.[33] Human milk samples were thawed at 4 °C, warmed to 38 °C, and vortexed for 20 s to ensure homogenization for the analysis. Then, 4.8 mL milk was added to 19.2 mL milli-Q water and vortexed for 20 s. The first 8 mL was used to rinse the instrument, and the subsequent two measurements were taken for each sample.

2.3. Proteolysis of Human Milk at Different pH

Proteolysis of human milk was performed as described previously with slight modifications.[34] For the first 500 µL aliquot of human milk, 500 µL of 2× protease inhibitor cocktail (Roche, 05892970001) was added and kept on ice to prevent proteolysis as the no-incubation control. Six treatment groups were incubated at 37 °C for 2 h under different pH conditions in this study. One treatment group remained at its natural pH of approximately 7; the pH of other treatment groups was adjusted by 1M HCl to 6 (6.0 ± 0.1), 5 (5.0 ± 0.1), 4 (4.0 ± 0.1), 3 (3.0 ± 0.1), and 2 (2.0 ± 0.1), respectively. After incubation, 500 µL of 2× protease inhibitor cocktail was added to stop proteolysis. Mixtures of 1M NaOH and 1M HCl were added to neutralize samples and to adjust sample volumes. Final amounts of materials in each tube were the same before the subsequent peptide extraction and analysis (500 µL milk, 500 µL protease inhibitor cocktail, 20 µL of 1M HCl, and 20 µL of 1M NaOH). Each sample was centrifuged at 15 000 × *g* at 4 °C for 15 min and the aqueous layer was collected. An equal volume of 20% w/v trichloroacetic acid (TCA) was added and the mixture was kept at 4 °C for 10 min for protein precipitation. After centrifugation at 15 000 × *g* at 4 °C for 10 min, peptides from the supernatant were purified by solid-phase extraction with C18 96-well plate (GlySci, FNSC18). Peptidomic profiling was performed by LC-MS/MS with Q Exactive Plus Orbitrap Mass Analyzer (Thermo Scientific) as previously described.[34] Briefly, samples were resuspended with 0.1% formic acid in 2% acetonitrile. Peptide concentration was measured with the Pierce Quantitative Fluorescent Peptide Assay (Thermo Scientific, Rockford, IL, USA). One microgram of peptides was loaded onto a 100 µm × 25 mm C18 100 Å trap column before being separated on a 75 µm × 150 mm C18 200 Å column over 120-min run with a gradient from 0 to 100% solvent B (A: 0.1% formic acid in water, B: 0.1% formic acid in 80%



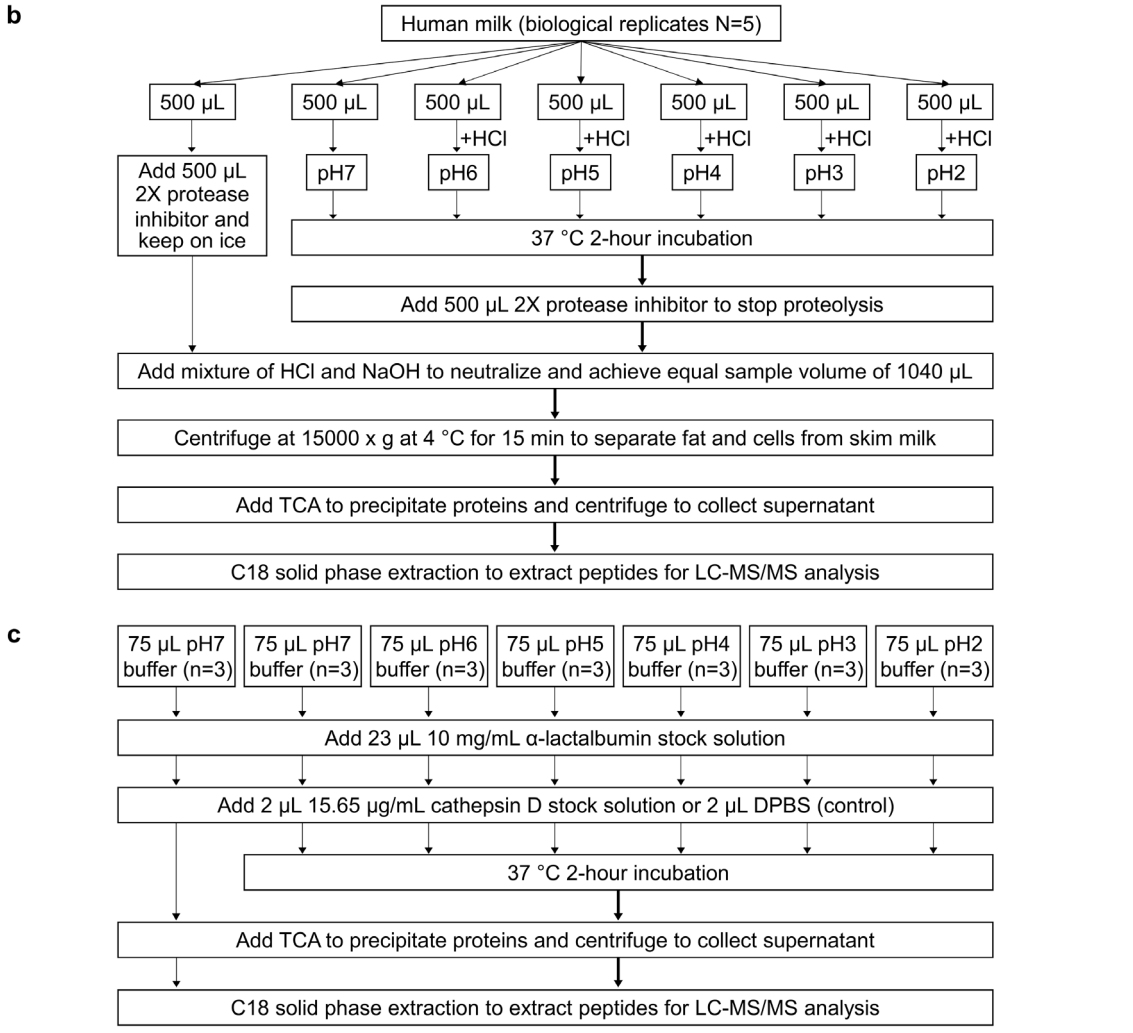


Figure 1. Overall workflow and experimental design. a) Discovery and targeted peptidomics approaches in this study. b) Experimental design for studying pH effects on human milk proteolysis. c) Experimental design for studying interactions between α-lactalbumin and cathepsin D.

acetonitrile). Mass spectra were collected in data-dependent mode with one precursor scan followed by 15 MS/MS scans. Scan ranges were 350–1600 m/z for MS spectra and 200–2000 m/z for MS/MS spectra. Precursors with unassigned, 1, or >4 charge states were excluded from fragmentation.

2.4. Incubation of α-Lactalbumin and Cathepsin D

To study the interactions between α-lactalbumin and cathepsin D, α-lactalbumin from human milk, ≥95% (SDS-PAGE) (Sigma-Aldrich, L7269) and cathepsin D from human liver, ≥250 units mg^{-1} protein (E1%/280) (Sigma-Aldrich, C8696) were purchased. Buffer solutions of pH ranging from 2.0 to 7.0 were prepared with phosphoric acid/monosodium phosphate and citric acid/disodium phosphate.[35] Samples were prepared in triplicate. For each sample in the set with cathepsin D addition, 2 µL of 15.7 µg mL^{-1} cathepsin D stock was added to 98 µL of 0.23 mg α-lactalbumin in buffer solution. The final concentrations of cathepsin D and α-lactalbumin in the mixture were 0.3 µg mL^{-1} and 2.3 mg mL^{-1}, respectively, comparable to their concentrations in human milk.[36,37] For sample in the other set without cathepsin D addition, the cathepsin D stock was replaced by 2 µL of Dulbecco's phosphate-buffered saline (DPBS). Samples in pH treatment groups were incubated at 37 °C for 2 h to model gastric conditions. Peptide extraction was applied to control groups immediately after the mixing of all materials without incubation. Peptides were extracted by C18 solid phase extraction following TCA protein precipitation as described above. Samples were analyzed by Q-TOF LC-MS/MS.

2.5. Discovery Peptide Analysis by Nano-LC-Q-TOF-MS/MS

Samples were reconstituted with 0.1% formic acid in 3% acetonitrile prior to analysis. The analysis was performed with an Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS instrument equipped with a Chip Cube interface (Agilent Technologies, Santa Clara, CA, USA). The mobile phase solvents were 0.1% formic acid in 3% acetonitrile (A) and 0.1% formic acid in 90% acetonitrile (B). Peptides were separated on a nano-LC chip containing a 40 nL enrichment column and a 75 µm × 150 mm analytical column packed with ZORBAX 300SB-C18 30Å material. The gradient employed for a 65-min run was 0–2.3% B from 0 to 0.1 min, 2.3–8% B from 0.1 to 2 min, 8–37% B from 2 to 40 min, 37–48% B from 40 to 45 min, 48–100% B from 45 to 45.1 min, followed by 100% B for 5 min and 0% B for 15 min. The flow rate was 0.3 µL min^{-1}. Mass spectra were collected in positive mode with scan ranges of 275–2000 m/z (MS) and 100–2000 (MS/MS). Fragmentation was performed using collision-induced dissociation. Collision energy was set by formula: $(0.031 \times m/z + 1)$ V for precursors with charge states =2, and $(0.036 \times m/z - 4.8)$ V for precursors with charge states ≥3.

2.6. Human Milk Proteome Construction

Raw data were obtained from the paper published by van Herwijnen et al.,[38] which identified human milk proteins from extracellular vesicles and compiled files from previously published studies. In total, our human milk proteome compiled information from 39 individual studies. Inconsistent entry formats were converted to the UniProtKB accession number (AC) using the UniProt[39] Retrieve/ID mapping tool. Duplicates were removed, and obsolete entries were updated. Only the entries mapped to the human proteome (Proteome ID: UP000005640) were kept. The final human milk proteome consists of 3451 protein entries (October 11, 2018) (Table S2, Supporting Information).

2.7. Peptide Identification by Database Search

Raw MS spectra from Orbitrap were converted to the MGF format by ProteoWizard 3.0[40]; data files from Q-TOF were exported as MGF files with Agilent MassHunter Qualitative Analysis B.06.00. Sequences of peptides were identified using X!Tandem.[41] The SwissProt *Homo sapiens* (Human) proteome was used as the database for the human milk proteolysis dataset, and the in-house constructed human milk proteome was employed for the α-lactalbumin and cathepsin D dataset to account for potential milk proteins that occur in the simpler system. A nonspecific enzyme cleavage pattern ([X]|[X]) was set, and 50 missed cleavage sites were allowed. No complete modifications were defined. Oxidation of methionine, deamidation of asparagine and glutamine, as well as phosphorylation of serine and threonine were selected as potential modifications as they are commonly observed on endogenous human milk peptides. Mass error tolerance was ±20 ppm for precursor ions and 20 ppm for fragment ions. Individual spectra were accepted when the e-value of peptide match was below 0.01.

2.8. Targeted Peptide Quantitation by LC-QQQ-MS/MS

Targeted peptide analysis was performed with an Agilent 1290 infinity LC system coupled to an Agilent 6495A triple quadrupole (QQQ) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The mobile phase solvents were 0.1% formic acid in 3% acetonitrile (A) and 0.1% formic acid in 90% acetonitrile (B). Peptides were separated on an Agilent ZORBAX Eclipse Plus C18 2.1 × 100 mm, 1.8 µm column by an optimized 16-min run with the following gradient: 2–50% B from 0 to 11 min, 50–100% B from 11 to 12 min, followed by 100% B for 2 min and 2% B for 2 min. Peptides were chosen based on the Q-TOF discovery results. The chosen peptides were consistently identified (more than once in triplicate) and without potential modifications. Fragmentation patterns of those peptides were evaluated to select the precursor and product ions. The chosen product ions were b- or y-ions with the highest signal intensities. For each peptide, 3–5 transitions were selected to confirm the retention time. Collision energy (CE) was optimized by monitoring transition intensity at 6–7 CE points of 5 V steps above and below the theoretical value calculated as $(0.036 \times m/z - 4.8)$ V. Dynamic multiple reaction monitoring (MRM) method with optimized parameters was used to monitor the peptides (Table S3, Supporting Information). Peak areas were integrated in Agilent MassHunter Quantitative Analysis B.08.00 and results were exported (Table S4, Supporting Information).

2.9. Data Analysis

Analysis on the study of human milk proteolysis was performed in R v3.5.1 (https://www.R-project.org). Data points for each human milk from the compositional analysis and their mean and SD ($n = 5$) were plotted. To characterize the pattern of proteolysis, unique peptide sequences identified in samples were counted and plotted by groups. ANOVA for randomized block design with milk as a blocking variable and Tukey's post hoc test were performed. To reduce variation and focus on pH effects, peptide sequences that appeared <3 under each condition among five biological replicates were dropped. To further analyze the proteolysis at the protein level, unique peptide sequences were counted by proteins and groups. Proteins that did not yield peptide sequences in any of the treatment groups were dropped. A heatmap was generated with z-scores of sequence occurrence centered and standardized by each protein, and the clustering of proteins was based on Pearson correlation. The index of dispersion or variance-to-mean ratio (VMR) of sequence occurrence in different groups was calculated for each protein. Peptides derived from α-lactalbumin (protein with the highest VMR score) were further analyzed. The sequence information of α-lactalbumin was retrieved from UniProt.[39] The 3D structure of α-lactalbumin (PDB ID: 1A4V)[42] was visualized in Jmol v14.29 (http://www.jmol.org). Analysis of the QQQ targeted peptide quantitation results was performed with Python 3.6.5.

2.10. Data Availability

The mass spectrometry discovery peptidomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository[43] with the dataset identifier PXD012748 and 10.6019/PXD012748. The targeted MRM datasets have been submitted to PeptideAtlas (http://www.peptideatlas.org/) with the dataset identifier PASS01342.

3. Results

3.1. Effects of pH on Proteolysis are Distinct among Human Milk Proteins

To study the endogenous proteolysis of human milk, freshly expressed human milk was collected from five healthy mothers. Human milk collected from each mother served as a biological replicate; one control group without incubation and pH adjustment, and six treatment groups with 2-h incubation under pH levels ranging from 2 to 7 were randomly assigned to aliquots of each human milk; 5 biological replicates and 7 groups yielded 35 samples in total (Figure 1a,b). Compositional results of milk samples in this study (**Figure 2**a and Table S5, Supporting Information) were similar to data reported from previous studies.[13] Protein and lactose contents exhibited less variations than fat content. Using LC-MS/MS with high-resolution, accurate-mass Orbitrap mass analyzer, a total of 4850 peptide sequences were identified from 35 samples. The numbers of unique peptide sequences in samples after incubation at pH 4 and pH 3 were higher compared to the control group without incubation ($p < 0.01$, ANOVA for randomized block design and Tukey's post hoc test) (Figure 2b).

To reduce variation and focus on pH effects, peptide sequences were filtered by frequency among five biological replicates in each group. Peptide sequences that were identified more than twice out of five replicates were further analyzed. There were 528 validated sequences in the no-incubation control group, and 591, 569, 566, 724, 718, 552 validated sequences in the pH7, pH6, pH5, pH4, pH3, pH2 treatment groups, respectively. Z-scores of sequence occurrence for 90 proteins were plotted in a heatmap to visualize the pattern of proteolysis (Figure 2d). Sequence occurrence of different proteins exhibited peak values under different pH conditions. For example, α-lactalbumin (P00709) had the highest numbers of sequences when incubated at pH 3, while bile salt-activated lipase (P19835) and parathyroid hormone-related protein (P12272) had the highest sequence occurrence when incubated at pH 7. The effects of pH on proteolysis varied across different human milk proteins.

3.2. pH Shift Activates the Selective Proteolysis of α-Lactalbumin in Human Milk

Ranked by the index of dispersion (or variance-to-mean ratio, VMR) of sequence occurrence across groups, α-lactalbumin (P00709), osteopontin (P10451), β-casein (P05814), and polymeric immunoglobulin receptor (P01833) were most sensitive to the changes of pH (Figure 2c). In human milk, α-lactalbumin displayed the largest difference in sequence occurrence across pH groups, and its peptides were detected only when the pH dropped below 5 (Table S6, Supporting Information). To further analyze the specificity of its proteolysis, the frequency of sequences derived from α-lactalbumin was plotted (**Figure 3**a). Most of these peptides were released at pH 3, and a few of them were found at pH 4 and 2. All of the 14 identified sequences were located between residues 32 and 52 of α-lactalbumin (Figure 3b). The pH-optimum and the restricted region for peptide release indicate that the proteolysis of α-lactalbumin is selective and mediated by specific enzymes in human milk.

3.3. Cathepsin D Cleaves α-Lactalbumin to Release Specific Peptides Corresponding to the pH Change

Within the milk proteolytic system, cathepsin D is the major aspartic protease with an acidic pH optimum.[36] Cathepsin D proteolytically prefers hydrophobic residues around the scissile bond, especially Leu and Phe.[44] Common cleavage sites of α-lactalbumin in human milk include 52–53 between a Leu residue and a Phe residue, matching the cleavage pattern of cathepsin D.[44] To test whether cathepsin D is responsible for the pH-dependent selective proteolysis of α-lactalbumin in human milk, purified α-lactalbumin was incubated at pH 2–7 with and without the addition of cathepsin D (Figure 1c). Peptides were extracted and identified by nano-LC-Q-TOF MS/MS. Ten sequences derived from α-lactalbumin were consistently found in samples

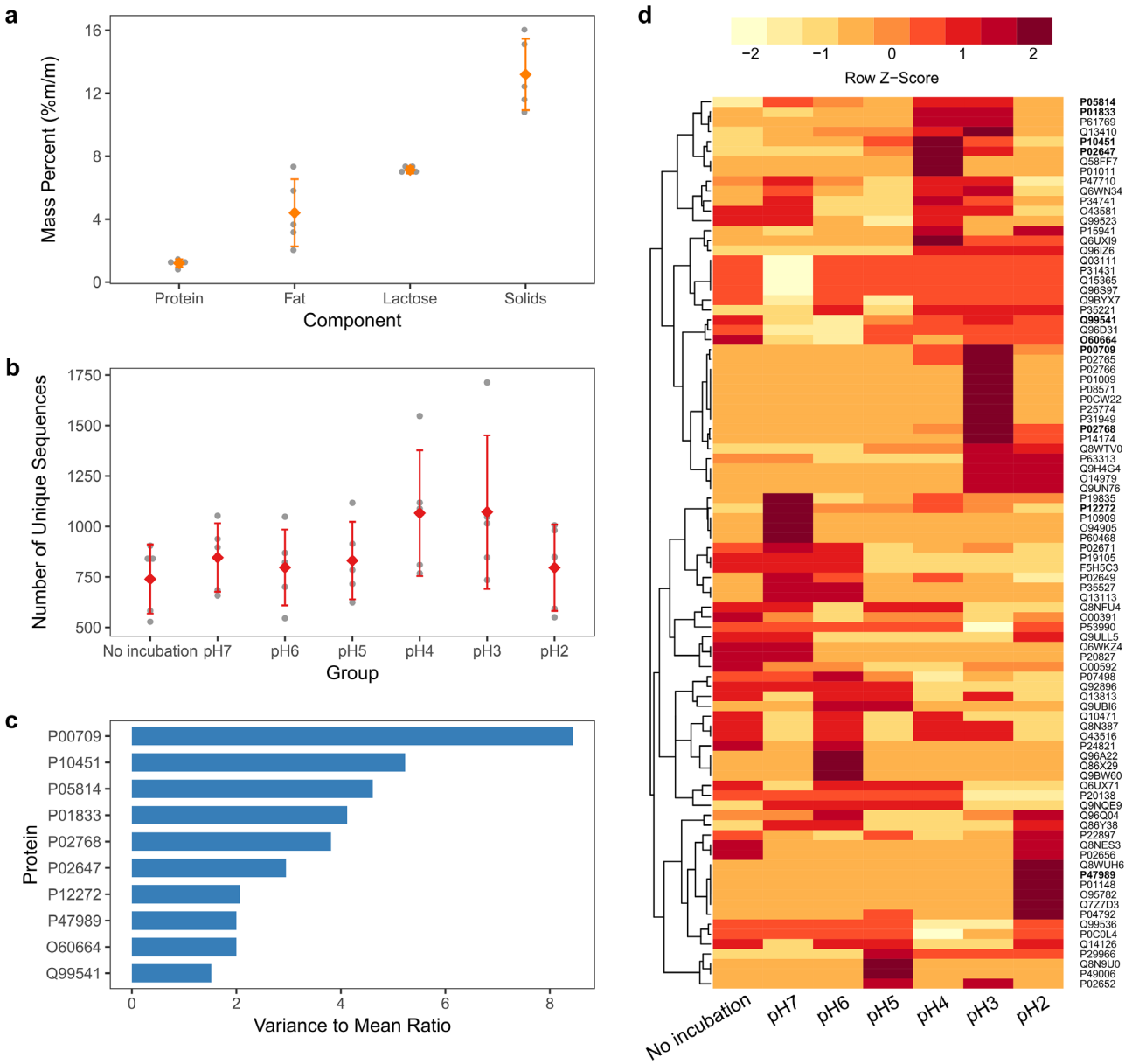


Figure 2. Effects of pH on proteolysis are distinct among human milk proteins. a) Overall composition (protein, fat, lactose, solids) of human milk samples presented as data points for each milk and their mean and SD ($n = 5$). b) Numbers of unique peptide sequences identified presented as data points for each sample and the mean and SD in each group ($n = 5$ per group). c) Top 10 proteins ranked by the variance-to-mean ratio (or index of dispersion) of sequence occurrence across groups. These proteins are α-lactalbumin (P00709), osteopontin (P10451), β-casein (P05814), polymeric immunoglobulin receptor (P01833), serum albumin (P02768), apolipoprotein A-I (P02647), parathyroid hormone-related protein (P12272), perilipin-3 (O60664), xanthine dehydrogenase/oxidase (P47989), and perilipin-2 (Q99541). d) Heatmap of z-scores of sequence occurrence for milk proteins in no-incubation control and treatment groups with incubation at varied pH levels. Red indicates that the value of sequence occurrence under that condition is above the averaged sequence occurrence among all groups, and yellow indicates that the value is below the mean. Rows (proteins) are clustered with Pearson correlation.

incubated at pH 3 (Figure 3c). Peptides from other milk proteins such as κ-casein and β-casein were also found in samples, which was plausibly arising from the impurity of the α-lactalbumin purified from human milk (Sigma-Aldrich, L7269, ≥95% SDS-PAGE). Transitions (precursor/fragment ion pairs) were selected from the MS/MS spectra to develop MRM method for targeted peptide quantitation on a triple quadrupole (QQQ) mass spectrometer. Only the peptides after validation of selectivity and optimization of sensitivity were included in the final analysis.

The highest signal intensity was obtained for the α-lactalbumin-derived peptide IVENNESTEYGL after incubating with cathepsin D at pH 3 (**Figure 4**). This peptide is within the domain of β-strands of α-lactalbumin (Figure 3d). Several α-lactalbumin-derived peptides were also detected at pH 3 in

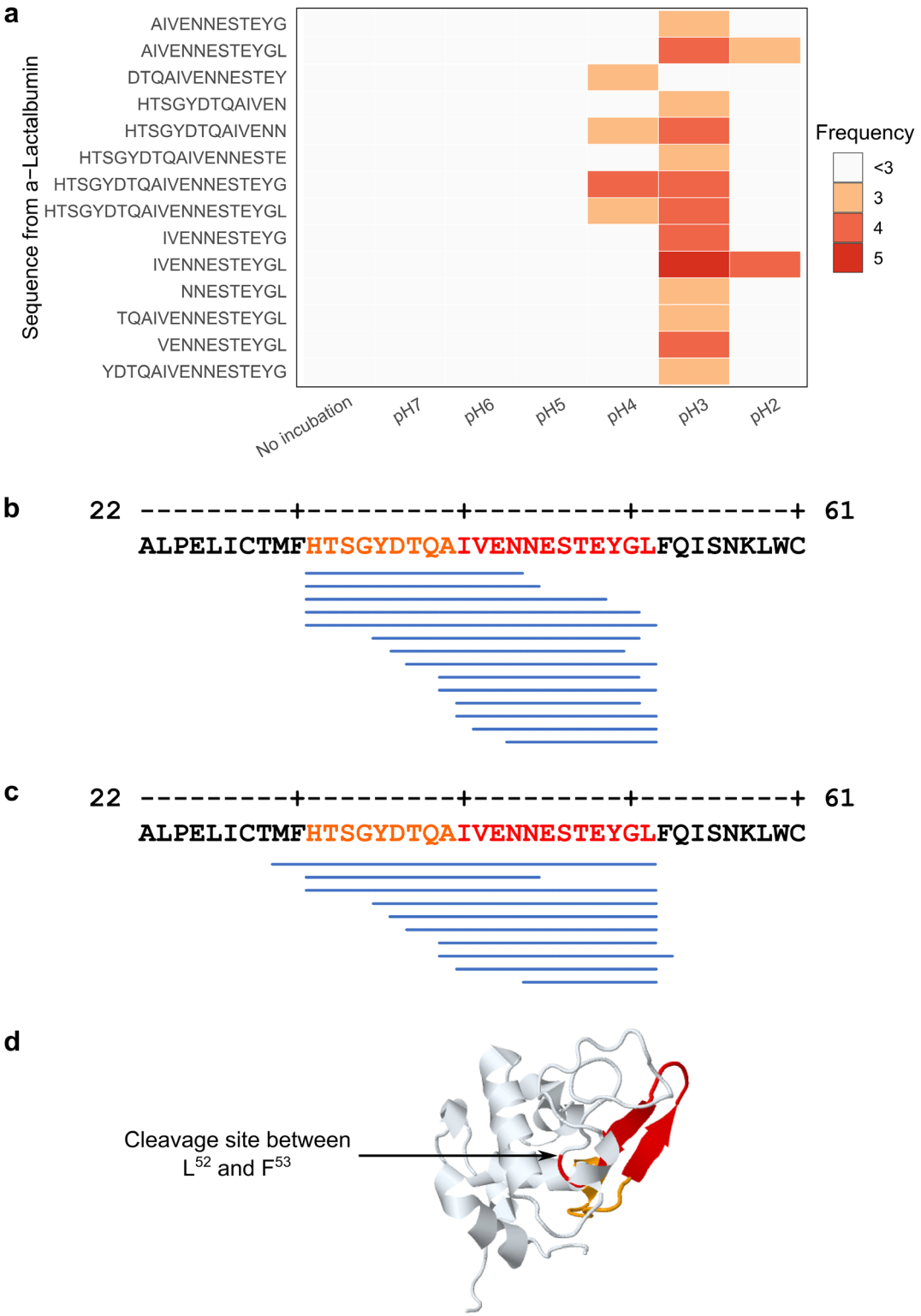


Figure 3. The proteolysis of α-lactalbumin is pH-dependent and selective. a) Frequency of α-lactalbumin-derived sequences in each group out of five biological replicates showing that the pH shift activates the proteolysis of α-lactalbumin in human milk. b) Mapping of peptide sequences from α-lactalbumin identified in human milk ($n = 5$) to the primary structure of α-lactalbumin. The proteolysis of α-lactalbumin in human milk is restricted to the highlighted region of residues 32–52. c) Mapping of peptide sequences from α-lactalbumin when incubated with cathepsin D ($n = 3$) to the primary structure of α-lactalbumin. The same as in (b), the highlighted region of α-lactalbumin covers residues 32–52. d) Sequences mapped to the tertiary structure of α-lactalbumin (PDB ID: 1A4V). Peptide IVENNESTEYGL highlighted in red is in the β-sheet domain of the protein, and HTSGYDTQA highlighted in orange are residues near the β-sheet domain.

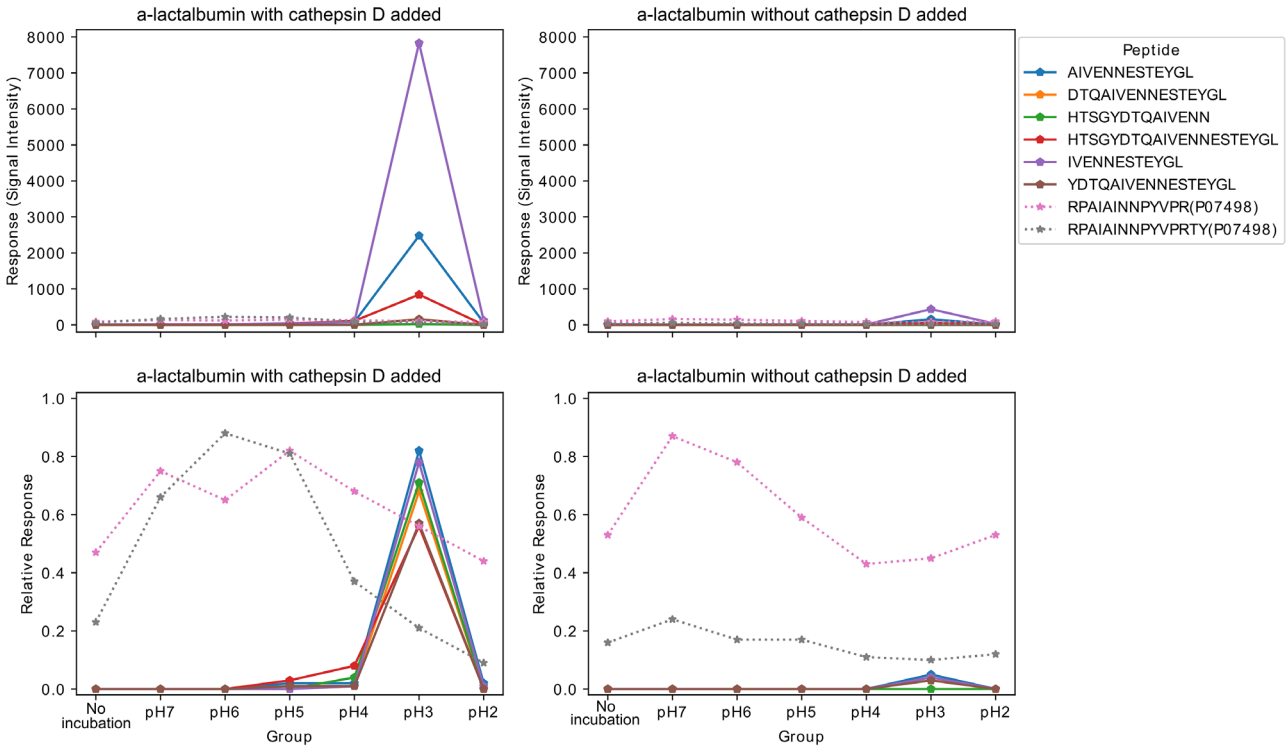


Figure 4. Cathepsin D selectively cleaves α-lactalbumin as pH shifts to 3. Results from the triple quadrupole (QQQ) mass spectrometer for α-lactalbumin incubated with the addition of cathepsin D ($n = 3$) are shown on the left, and results for α-lactalbumin incubated without the addition of cathepsin D ($n = 3$) are shown on the right. Responses of targeted peptides are shown on the top, and relative responses of each peptide are shown at the bottom. Solid lines represent peptides derived from α-lactalbumin and dotted lines represent peptides derived from κ-casein. The release of all peptides derived from α-lactalbumin displayed the same pattern—reaching peak responses at pH 3, while the release of peptides from κ-casein was not pH dependent.

controls (without the addition of cathepsin D), but their intensity was less than 1/10 of that with the addition of cathepsin D, which was plausibly due to trace amounts of cathepsin D remaining in the α-lactalbumin extracted from human milk. Considering differences in quantity and ionization efficiency, relative response was calculated for each peptide to identify the effects of pH on its release. The release of all peptides derived from α-lactalbumin displayed the same pattern—reaching peak responses at pH 3, while the release of peptides from κ-casein was not pH dependent.

4. Discussion

α-Lactalbumin is the dominant whey protein in human milk with a concentration of 2–3 g L^{-1}.[37] This protein appears to have multiple roles in milk synthesis and in infant nutrition. Infant formulas enriched with α-lactalbumin have shown to achieve better outcomes regarding infant growth and metabolism.[45–47] Studies have shown peptides derived from α-lactalbumin have various bioactivities such as immunomodulatory effects and antimicrobial activities.[48,49] Compared to previous studies using exogenous enzymes for peptide production, we investigated the effects of endogenous enzymes in human milk and observed different peptides released from α-lactalbumin. Evaluating bioactivities of these newly identified peptides is the next step to discover their bioactive functions for infants.

The effects of pH on properties of α-lactalbumin and cathepsin D have been studied independently, but their interactions in human milk have been overlooked. The current model allowed us to interrogate the pH effect on the specificity of such enzyme–substrate interaction in human milk within controlled experiments. α-Lactalbumin is a Ca^{2+} binding protein with pI 4–5.[50] As pH shifts from neutral to acidic, protons compete with the bound Ca^{2+} and α-lactalbumin transitions to a molten globule, where the α-helical domain is structured while the β-sheet domain is largely unfolded.[50,51] Meanwhile, cathepsin D becomes most active at pH 3 with changes on the ionization state of its active site residues.[52] In such an environment, cathepsin D is most likely to start cleaving the 52–53 bond between Leu and Phe at the unstructured β-sheet domain of α-lactalbumin and other scissile bonds nearby. At pH 2, cathepsin D is less active than at pH 3, and less peptides are produced by such enzyme–substrate interactions. In vivo data collected from infants who consumed their mother's milk also showed that α-lactalbumin releases peptides in the infant stomach, as peptides derived from α-lactalbumin were not found in milk samples but found in infant gastric samples.[53] In addition to peptides such as IVENNESTEYGL and AIVENNESTEYGL located in the same region as found in our study, several peptides from other domains were also identified in infant gastric samples.[53] Future interdisciplinary approaches to identify the proteolytic processing of human milk during infant digestion and the molecular target of proteolytic products in the infant gut biogeography will be critical to

a better understanding of the impact of human milk on infant health.

Taken together, the pH shift upon infant ingestion of milk activates selective proteolysis of α-lactalbumin by the endogenous enzyme cathepsin D within human milk. Specific interactions between a mother's milk and the infant are important strategies emerging from evolution. For example, human milk oligosaccharides enrich specific bifidobacteria and mediate the microbiome in the infant gut, affecting downstream processes including the infant's metabolism and immunity.[14,54] The pH-dependent selective proteolysis is another mechanism for such dynamic interactions. Differences exist among individuals and along the gastrointestinal biogeography. In addition, processing techniques such as thermal pasteurization and nonthermal treatments of human donor milk contribute to variations in bioactive compounds including proteins and enzymes.[15] Different processing methods may have an impact on the controlled proteolysis of human milk, leading to subsequent changes in physiological interactions in the infant. A mechanistic understanding of how molecules in human milk change dynamically in response to these differences will inform donor milk handling and specific feeding protocols for high-risk infants.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

J.G. and J.B.G. conceived the study. J.G., J.Z., and N.K. conducted the experiments. E.G. assisted in MRM method development. J.G. performed the data analysis and discussed data interpretation with J.B.G., D.B., and C.B.L.; J.G. drafted the manuscript with input from J.Z., N.K., D.B., and J.B.G. All authors contributed to manuscript revisions. This project was financially supported by the United States National Institutes of Health (AT007079, AT008759), the UC Davis Jastro-Shields Research Scholarship (J.G.), and the UC Davis Provost's Undergraduate Fellowship (J.Z. and N.K.).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

bioactivity, human milk, pH, protein, selective proteolysis

Received: March 14, 2019
Revised: May 3, 2019
Published online: July 16, 2019

[1] H. Blencowe, S. Cousens, D. Chou, M. Oestergaard, L. Say, A. B. Moller, M. Kinney, J. Lawn, *Reprod. Health* **2013**, *10*, S2.
[2] S. E. Purisch, C. Gyamfi-Bannerman, *Semin. Perinatol.* **2017**, *41*, 387.
[3] L. Liu, S. Oza, D. Hogan, Y. Chu, J. Perin, J. Zhu, J. E. Lawn, S. Cousens, C. Mathers, R. E. Black, *Lancet* **2016**, *388*, 3027.
[4] J. P. Vogel, S. Chawanpaiboon, A. B. Moller, K. Watananirun, M. Bonet, P. Lumbiganon, *Best Pract. Res. Clin. Obstet. Gynaecol.* **2018**, *52*, 3.
[5] C. Agostoni, G. Buonocore, V. P. Carnielli, M. De Curtis, D. Darmaun, T. Decsi, M. Domellof, N. D. Embleton, C. Fusch, O. Genzel-Boroviczeny, O. Goulet, S. C. Kalhan, S. Kolacek, B. Koletzko, A. Lapillonne, W. Mihatsch, L. Moreno, J. Neu, B. Poindexter, J. Puntis, G. Putet, J. Rigo, A. Riskin, B. Salle, P. Sauer, R. Shamir, H. Szajewska, P. Thureen, D. Turck, J. B. van Goudoever, et al., *J. Pediatr. Gastroenterol. Nutr.* **2010**, *50*, 85.
[6] J. E. Harding, B. E. Cormack, T. Alexander, J. M. Alsweiler, F. H. Bloomfield, *Lancet* **2017**, *389*, 1660.
[7] J. Neu, *Am. J. Clin. Nutr.* **2007**, *85*, 629S.
[8] J. Neu, W. A. Walker, *N. Engl. J. Med.* **2011**, *364*, 255.
[9] Committee on Nutrition;Section on Breastfeeding; Committee on Fetus and Newborn, *Pediatrics* **2017**, *139*, e20163440.
[10] P. Meier, A. Patel, A. Esquerra-Zwiers, *J. Pediatr.* **2017**, *180*, 15.
[11] P. G. Radmacher, D. H. Adamkin, *Semin. Fetal Neonatal Med.* **2017**, *22*, 30.
[12] M. Quigley, N. D. Embleton, W. McGuire, *Cochrane Database Syst Rev.* **2018**, *2018*, CD002971.
[13] O. Ballard, A. L. Morrow, *Pediatr. Clin. North Am.* **2013**, *60*, 49.
[14] C. G. Victora, R. Bahl, A. J. D. Barros, G. V. A. França, S. Horton, J. Krasevec, S. Murch, M. J. Sankar, N. Walker, N. C. Rollins, *Lancet* **2016**, *387*, 475.
[15] M. A. Pitino, S. Unger, A. Doyen, Y. Pouliot, S. Aufreiter, D. Stone, A. Kiss, D. L. O'Connor, *J. Nutr.* **2019**, *149*, 497.
[16] C. Bourlieu, O. Menard, K. Bouzerzour, G. Mandalari, A. Macierzanka, A. R. Mackie, D. Dupont, *Crit. Rev. Food Sci. Nutr.* **2014**, *54*, 1427.
[17] J. Gan, G. M. Bornhorst, B. M. Henrick, J. B. German, *Mol. Nutr. Food Res.* **2018**, *62*, 1700231.
[18] C. Ansell, A. Moore, H. Barrie, *Pediatr. Res.* **1977**, *11*, 1177.
[19] F. H. Morriss, E. D. Brewer, S. B. Spedale, L. Riddle, D. M. Temple, R. M. Caprioli, M. S. West, *Pediatrics* **1986**, *78*, 458.
[20] M. Agunod, N. Yamaguch, R. Lopez, A. L. Luhby, G. B. J. Glass, *Am. J. Dig. Dis.* **1969**, *14*, 400.
[21] L. He, W. Chen, Y. Li, M. Deng, *Am. J. Nurs. Sci.* **2017**, *6*, 366.
[22] H. V. L. Maffei, F. J. Nobrega, *Gut* **1975**, *16*, 719.
[23] S. Mason, *Arch. Dis. Child.* **1962**, *37*, 387.
[24] J. M. Sondheimer, D. A. Clark, E. P. Gervaise, *J. Pediatr. Gastroenterol. Nutr.* **1985**, *4*, 352.
[25] M. R. Palla, S. Harohalli, T. N. Crawford, N. Desai, *Front. Pediatr.* **2018**, *6*, 211.
[26] D. J. Mitchell, B. G. McClure, T. R. J. Tubman, *Arch. Dis. Child.* **2001**, *84*, 273.
[27] P. E. Hyman, D. D. Clarke, S. L. Everett, B. Sonne, D. Stewart, T. Harada, J. H. Walsh, I. L. Taylor, *J. Pediatr.* **1985**, *106*, 467.
[28] E. J. Kelly, S. J. Newell, K. G. Brownlee, J. N. Primrose, P. R. F. Dear, *Early Hum. Dev.* **1993**, *35*, 215.
[29] T. I. Omari, R. R. Haslam, P. Lundborg, G. P. Davidson, *J. Pediatr. Gastroenterol. Nutr.* **2007**, *44*, 41.
[30] K. More, G. Athalye-Jape, S. Rao, S. Patole, *Am. J. Perinatol.* **2013**, *30*, 849.
[31] D. C. Dallas, N. M. Murray, J. Gan, *J. Mammary Gland Biol. Neoplasia* **2015**, *20*, 133.
[32] Y. Wada, B. Lonnerdal, *J. Nutr. Biochem.* **2014**, *25*, 503.
[33] J. T. Smilowitz, D. S. Gho, M. Mirmiran, J. B. German, M. A. Underwood, *J. Hum. Lact.* **2014**, *30*, 180.
[34] J. Gan, R. C. Robinson, J. Wang, N. Krishnakumar, C. J. Manning, Y. Lor, M. Breck, D. Barile, J. B. German, *Food Chem.* **2019**, *274*, 766.
[35] T. C. McIlvaine, *J. Biol. Chem.* **1921**, *49*, 183.
[36] M. J. Hurley, L. B. Larsen, A. L. Kelly, P. L. H. McSweeney, *Int. Dairy J.* **2000**, *10*, 673.

[37] J. G. Jackson, D. B. Janszen, B. Lonnerdal, E. L. Lien, K. P. Pramuk, C. F. Kuhlman, *J. Nutr. Biochem.* **2004**, *15*, 517.
[38] M. J. C. van Herwijnen, M. I. Zonneveld, S. Goerdayal, E. N. M. Nolte-'t Hoen, J. Garssen, B. Stahl, A. F. M. Altelaar, F. A. Redegeld, M. H. M. Wauben, *Mol. Cell. Proteomics* **2016**, *15*, 3412.
[39] The UniProt Consortium, *Nucleic Acids Res.* **2017**, *45*, D158.
[40] M. C. Chambers, B. Maclean, R. Burke, D. Amodei, D. L. Ruderman, S. Neumann, L. Gatto, B. Fischer, B. Pratt, J. Egertson, K. Hoff, D. Kessner, N. Tasman, N. Shulman, B. Frewen, T. A. Baker, M.-Y. Brusniak, C. Paulse, D. Creasy, L. Flashner, K. Kani, C. Moulding, S. L. Seymour, L. M. Nuwaysir, B. Lefebvre, F. Kuhlmann, J. Roark, P. Rainer, S. Detlev, T. Hemenway, et al., *Nat. Biotechnol.* **2012**, *30*, 918.
[41] R. Craig, R. C. Beavis, *Bioinformatics* **2004**, *20*, 1466.
[42] N. Chandra, K. Brew, K. R. Acharya, *Biochemistry* **1998**, *37*, 4767.
[43] Y. Perez-Riverol, A. Csordas, J. Bai, M. Bernal-Llinares, S. Hewapathirana, D. J. Kundu, A. Inuganti, J. Griss, G. Mayer, M. Eisenacher, E. Pérez, J. Uszkoreit, J. Pfeuffer, T. Sachsenberg, Ş. Yılmaz, S. Tiwary, J. Cox, E. Audain, M. Walzer, A. F. Jarnuczak, T. Ternent, A. Brazma, J. A. Vizcaíno, *Nucleic Acids Res.* **2019**, *47*, D442.
[44] N. D. Rawlings, A. J. Barrett, P. D. Thomas, X. Huang, A. Bateman, R. D. Finn, *Nucleic Acids Res.* **2018**, *46*, D624.
[45] A. Davis, B. Harris, E. Lien, K. Pramuk, J. Trabulsi, *Eur. J. Clin. Nutr.* **2008**, *62*, 1294.
[46] J. Trabulsi, R. Capeding, J. Lebumfacil, K. Ramanujam, P. Feng, S. McSweeney, B. Harris, P. DeRusso, *Eur. J. Clin. Nutr.* **2011**, *65*, 167.
[47] E. L. Lien, A. M. Davis, A. R. Euler, M. S. Group, *J. Pediatr. Gastroenterol. Nutr.* **2004**, *38*, 170.
[48] B. Lonnerdal, E. L. Lien, *Nutr. Rev.* **2003**, *61*, 295.
[49] S. M. Kamau, S. C. Cheison, W. Chen, X. M. Liu, R. R. Lu, *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 197.
[50] E. A. Permyakov, L. J. Berliner, *FEBS Lett.* **2000**, *473*, 269.
[51] K. Kuwajima, *FASEB J.* **1996**, *10*, 102.
[52] S. B. P. Athauda, K. Takahashi, *Protein Pept. Lett.* **2002**, *9*, 289.
[53] D. C. Dallas, A. Guerrero, N. Khaldi, R. Borghese, A. Bhandari, M. A. Underwood, C. B. Lebrilla, J. B. German, D. Barile, *J. Nutr.* **2014**, *144*, 815.
[54] A. M. Zivkovic, J. B. German, C. B. Lebrilla, D. A. Mills, *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 4653.