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Glycosylation of Human Milk Lactoferrin Exhibits Dynamic Changes During Early Lactation Enhancing Its Role in Pathogenic Bacteria-Host Interactions*[S]

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Human milk lactoferrin (hmLF) is the most abundant glycoprotein present in human milk and displays a broad range of protective functions in the gut of newborn infants. hmLF is N-glycosylated, but little is known about the lactation stage-related development of the glycosylation phenotype. hmLF glycosylation from milk samples from five donors during the first 10 weeks of lactation was assessed and observed to be more diverse than previously reported. During this period dynamic changes in glycosylation were observed corresponding to a decrease in glycosylation in the second week followed by an increase in total glycosylation as well as higher order fucosylation thereafter. Gene expression analysis was performed in milk somatic cells from a sixth subject. It was found that fucosyltransferase expression increased during entire period, whereas expression of genes for the oligosaccharyl transferase complex decreased in the second week. The effect of hmLF glycosylation was examined for the protein's ability to affect bacterial binding to epithelial cells. hmLF significantly inhibited pathogen adhesion and purified hmLF glycans significantly reduced *Salmonella* invasion of colonic epithelial cells to levels associated with non-invasive deletion mutants. This study indicates that hmLF glycosylation is tightly regulated by gene expression and that glyco-variation is involved in modulating pathogen association. ***Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.015248, 1–10, 2012.***

Human milk constitutes the first source of nutrients for the newborn infant, but it has also evolved to endow several key physiological advantages to the neonate. Other than to provide the neonate with energy and amino acid building blocks, proteins possess a wide range of biological activities that promote the normal development and maturation of specific organs in the newborn, specifically, the functions of the gut mucosa and the growth of gut microbiota (1). Human milk proteins also display a protective effect against infectious diseases via antimicrobial and immuno-modulatory activities that confer passive immunity to the breast-fed infant (1–3). Many of these proteins are post-translationally modified and the possible roles of such modifications in mediating demonstrated bioactivities are largely unexplored.

Lactoferrin (LF)[1] is an iron-binding glycoprotein found in milk from most species, but human milk LF (hmLF) is the most abundant glycoprotein present in colostrum and mature milk (6–8 mg/ml and 2–4 mg/ml, respectively) (1, 4). The presence of glycans on hmLF is long known (5), but so far, the only role identified is to protect the molecule from proteolysis (6).

Glycosylation is a common but complex type of post-translational modification of proteins, directly affecting glycoprotein structure, trafficking, recognition, and biological functions (7–10). Carbohydrate structures attached to proteins play key roles in mediating cell signaling and cell-cell recognition events (11, 12). Changes in protein glycosylation have been related to the onset and/or progression of several diseases such as different types of cancer, immunological disorders as well as congenital disorders (13–19). Additionally, glycosylation and glycan diversity are directly related to modulating

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[1] The abbreviations used are: LF, Lactoferrin; BSSL, bile-salt stimulated lipase; DHB, 2,5-dihydroxybenzoic acid; FUT, fucosyltransferase; hmLF, human milk lactoferrin; IRMPD, infrared multiphoton dissociation; MWCO, molecular weight cut off; OST, oligosaccharide transferase; PNGase F, peptide N-glycosidase F; RPKM, reads per kilo base per million mapped reads; SEM, standard error of the mean; SWIFT, stored-waveform inverse Fourier transform; TFA, trifluoroacetic acid; FTICR, Fourier Transform Ion Cyclotron Resonance.

microbial adhesion and invasion during infection (9). Indeed, the first step in bacterial infection is the recognition of host glycans by bacterial lectins or *vice versa*. Thus, glycans attached to human milk glycoproteins may act to block or modulate pathogen association to the epithelial surface, which in turn may also be a key role in the protection of breast-fed infants against gastrointestinal tract infections.

Despite numerous studies establishing the variation in total protein concentration as well as composition of human milk, there has been little effort characterizing the variation in glycosylation of milk glycoproteins over the course of lactation. Only one protein, namely bile-salt stimulated lipase (BSSL) was shown to have a dynamic glycosylation pattern over the course of lactation (20). The biological significance of these findings is, however, so far unclear and unexplored.

Given the central role of lactoferrin in infant development and health and its status as the most abundant glycoprotein in milk, we examined the changes in glycosylation during the first months of lactation with the hypothesis that glycan variation is common over the course of lactation as a mechanism to block pathogen association during breastfeeding. hmLF binds several pathogenic Gram-positive (21, 22) and Gram-negative (23, 24) bacteria to exert antimicrobial activity because of either iron-depletion and/or bacterial membrane disruption; the latter being triggered by a short sequence of amino acids found in the N-terminal domain of the protein, known as lactoferricin (25, 26). In addition, hmLF may inhibit infections caused by viruses, yeast, fungi, parasites, and other eukaryotic microbes (27). However, to date, the role of glycosylation of hmLF in these antimicrobial, antiviral, antifungal and antiparasitic activities has not been elucidated. We determined the *N*-glycan profile of human milk lactoferrin by mass spectrometry analysis in individual samples of hmLF purified from five donors during the first 72 days of lactation. The expression of genes associated with glycosylation in milk somatic cells was assessed to evaluate the regulation of the dynamic glycosylation. The biological and/or functional significance of glycans found in hmLF was determined using *in vitro* studies of host-microbe interactions with colonic epithelial cells and gastrointestinal bacterial pathogens in the presence of hmLF glycoforms and released N-glycans.

EXPERIMENTAL PROCEDURES

hmLF N-Glycan Analysis—A purified human milk lactoferrin standard was obtained from Sigma Aldrich (St. Louis, MO), Heparin-Sepharose 6 fast flow was purchased from GE Healthcare (Pittsburgh, PA), and 10 ml econopack columns were purchased from Bio-Rad (Richmond, CA). Glycerol free peptide N-glycosidase F (PNGase F) was purchased from New England Biolabs (Ipswich, MA). α-1–3/4 fucosidase (from Xantomonas sp.) was obtained from Calbiochem (San Diego, CA), and β-1–4 galactosidase from Glyco (Novato, CA). Recombinant α-2–3/6 sialidase was a kind gift from Dr. David Mills (Department of Viticulture and Enology, UC Davis). Solid-phase-extraction graphitized-carbon and C8 cartridges were purchased from Glygen corporation (Columbia, MD) and Supelco (Bellefonte, PA), respectively and Microcon centrifugal filter devices (ultracel YM-10) were from Millipore Corporation (Bedford, MA). Acetonitrile and trifluoroacetic acid were ACS quality or higher.

Human Milk Samples—Samples were donated by five healthy women from Reno, NV, who gave birth to term infants (> 38 weeks). Overall, human milk samples collected on days 1, 5, 10, 15, 30, 44, 58, and 72 postpartum were interrogated in this study. All milk samples were manually expressed and immediately frozen. Samples were then transferred to a −80 °C freezer within 3 h and stored until analysis.

Lactoferrin Purification from Human Milk Samples—LF purification from individual milk samples was performed in parallel following a procedure described by Lonnerdal *et al.* (37) with slight modifications, as follows. Briefly, whole human milk samples (0.5 ml) were centrifuged at max speed, for 30 min, at 4 °C. The lower aqueous phase was recovered in a new tube and a $CaCl_2$ solution (pH 4.6) was added to a final concentration of 60 mM. The mixture was incubated 1 h at room temperature (~25 °C), and further centrifuged at 6750 × *g* for 20 min at room temperature. Empty columns were packed with 1 ml of heparin-Sepharose resin and equilibrated with 50 mM Tris HCl pH 8.0 (running buffer). The whey fractions obtained were loaded onto the columns and the flow-through was collected and reloaded onto the column twice. Columns were closed and the samples were allowed to interact with the resin for 3 h at room temperature. Upon washing with 15 ml of running buffer, weakly bound proteins were eluted using five column volumes of 50 mM Tris HCl pH 8, 0.3 M NaCl (EBI), and 1 ml fractions were collected. LF bound to heparin-Sepharose and was eluted with five column volumes of 50 mM Tris HCl pH 8, 1 M NaCl (EBII). Fractions were collected, dialyzed against 10 mM ammonium bicarbonate, concentrated and stored at −20 °C. Protein concentration was determined using the Bradford assay and 5 μl aliquots were assayed by SDS-PAGE.

N-glycan Release and Purification—Commercially available LF and LF purified from single samples from all donors during the course of lactation (20 μg) were reduced and alkylated in 50 mM NH_4CO_3. PNGase F (1 μl, or 500 NEB units) was added to all samples and glycans were released by incubation at 37 °C for 16 h. Released glycans were purified by solid phase extraction using porous graphitized carbon cartridges. The cartridges were conditioned with three volumes of deionized water, followed by three volumes of 80% acetonitrile in 0.1% aqueous trifluoroacetic acid (v/v) and another three volumes of deionized water. The oligosaccharide samples were loaded onto the cartridge, incubated for 10 min at room temperature, and washed with three volumes of deionized water. Elution was performed to fractionate the oligosaccharides mixture into two fractions previous to MS analysis in order to minimize suppression. Glycans were eluted with three volumes of 20% acetonitrile in water (v/v), followed by three volumes of 40% acetonitrile, 0.1% trifluoroacetic acid in water (v/v) and dried *in vacuo*. Glycans were reconstituted in 10 μl of deionized water.

Matrix-Assisted Laser Desorption Ionization Fourier Transform Ion Cyclotron Resonance FTICR-MS Analysis—All mass spectra were acquired on an HiRes MALDI-FTICR MS instrument with an external MALDI source, a 355-nm pulsed Nd:YAG laser, a quadrupole ion guide, and a 7.0 Tesla superconducting magnet (IonSpec, Irvine, CA). The analyte-matrix deposit was prepared with 1 μl of sample being spotted on the MALDI plate, followed by the addition of 0.1 μl of 0.1 mM NaCl as a dopant and 1 μl of 0.4 M DHB. The spots were allowed to dry under vacuum before analysis. For detection, ion excitation was performed through an arbitrary waveform with amplitude of 150.0 V (base to peak) at a rate of 2 MHz for a scan range of *m/z* 216–4500 and 1024K data points. Five acquisition scans were performed on each sample in the positive ion mode. The instrument was externally calibrated with a malto-oligosaccharide mixture, and glycans were identified by accurate mass with a mass tolerance < 5 ppm.

For MS/MS analysis, individual glycan ions were selected within the ICR cell using stored-waveform inverse Fourier transform (SWIFT) isolation prior to collision-induced-dissociation (CID), and infrared multiphoton dissociation (IRMPD). For IRMPD experiments, the infrared radiation was supplied by a 10.6 μm, 20 W CO_2 laser (Parallax Laser, Inc., Waltham, MA). Fragmentation was optimized by varying the IRMPD laser pulse time between 500 and 1500 ms. Irradiation time was increased until the majority of the precursor ion was dissociated.

Creation of hmLF Glycoforms—Sialic acid-, fucose-, and galactose-free hmLF glycoforms were created by treatment of standard hmLF with the corresponding exoglycosidases, followed by ultrafiltration. For each treatment 2 mg of hmLF was reconstituted in 1 ml of appropriate exoglycosidase digestion buffer. Digestion with α-2–3/6-sialidase (recombinant, gift from Dr. David Mills, UC Davis) was carried out in 50 mM ammonium phosphate pH 6, treatment with α-1–3/4-fucosidase (Calbiochem, San Diego, CA) in 50 mM sodium phosphate pH 5.5, and digestion with β-1–4-galactosidase (Glyco, Novato, CA) was performed in 50 mM Tris-HCl pH 7. The corresponding enzymes (3 μl) were added to each reaction tube and incubated with agitation for 24 h at 37 °C in a dry oven. After digestion, the samples were frozen to inactivate the enzymes and released monosaccharides were removed from hmLF glycoforms by ultrafiltration using deionized water and Microcon centrifugal devices with a MWCO of 10 kDa. LF glycoforms were recovered in 1 ml of water and protein concentration was determined using Bradford. Free hmLF N-glycans were prepared from 2 mg of reduced and alkylated LF with PNGase F as described above and subsequently separated from the protein by solid phase extraction using a C8 cartridge conditioned with six volumes of acetonitrile followed by six volumes of water. Sample was loaded and glycans were recovered in 9 ml of water, followed by drying *in vacuo*.

RNA Extraction, Sequencing and Data Analysis for Gene Expression Studies

Fresh Milk Sample Collection—Fresh milk samples were obtained from a healthy female on days 4, 15, 30, and 40 postpartum who gave birth to a term infant (> 38 weeks). In the early morning period, the donor manually pumped one breast until emptied into a collection bag, and immediately delivered on cold-packs to the lab for processing. Samples were divided into two aliquots of ~20 ml for oligosaccharide profile analysis and RNA extraction from somatic cells. The Institutional Review Board of University of California, Davis, approved the project. The research was conducted in accordance with the ethical standards outlined in the Helsinki Declaration, with all participants providing written informed consent.

RNA Extraction for Gene Expression Studies—Somatic cells were pelleted by adding 50 μl of 0.5 M EDTA to 20 ml of fresh milk and centrifuged at 1800 rpm at 4 °C for 10 min. The pellet of cells was washed with 10 ml of phosphate-buffered saline at pH 7.2 and 10 μl of 0.5 M EDTA (final conc. 0.5 mM) and filtered through sterile cheesecloth to remove any debris. The cells were then centrifuged again at 1800 rpm, 4 °C for 10 min. The supernatant was decanted and RNA was extracted from the milk somatic cell pellet using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was quantified by an ND-1000 spectrophotometer (Fisher Thermo, Wilmington, MA) and the quality and integrity was assessed by the spectrophotometer 260/280 ratio, gel electrophoresis and by capillary electrophoresis with an Experion bio-analyzer (Bio-Rad, Hercules, CA).

RNA Sequencing and Data Analysis—Gene expression analysis was conducted on fresh milk samples collected on days 4, 15, 30, and 40 postpartum by RNA sequencing (RNA-Seq). Messenger RNA was isolated and purified using RNA-Seq sample preparation Kit (Illumina, San Diego, CA and NuGen, San Carlos, CA). Subsequently, mRNA was fragmented to ~200 bp fragments and first and second strand cDNA were synthesized, followed by end repair and adapter ligation. The fragments were purified and sequenced at the UC Davis Genome Center DNA Technologies Core Facility using the Illumina Genome Analyzer (GAII). Short sequence reads of 36–40 bp were assembled, and analyzed in RNA-Seq and expression analysis application of CLC Genomics workbench 3.7 (CLC Bio, Aarhus, Denmark). Human Genome, GRCh37.1 (http://www.ncbi.nlm.nih.gov/genome/guide/human/index.html) was utilized as the reference genome for the assembly. Data were normalized by calculating the "reads per kilo base per million mapped reads" (RPKM) for each gene (38) and annotated with NCBI human genome assembly (35,489 unique genes).

In Vitro Host-Microbe Interactions Assays in the Presence of Lactoferrin Glycoforms

In Vitro Cell Culture—Intestinal epithelial cells (Caco-2; ATCC HTB-37) were grown as per the manufacturer's instructions in T-25 flasks at 37 °C with 5% CO_2. Subsequently, for compound treatment, cells were seeded to a density of 105 cells/cm^2 in a 96-well plate using Dulbecco's modified Eagle's medium/High Modified (Thermo Scientific, Rockford, IL) with 16.6% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), nonessential amino acids (Thermo Scientific), 10 mM 3-(*N*-morpholino)propanesulfonic acid (Sigma, St. Louis, MO), 10 mM TES (Sigma), 15 mM HEPES (Sigma), and 2 mM NaH_2PO_4 (Sigma). Cells were incubated at 37 °C in 5% CO_2 atmosphere for 14-days post confluence to allow differentiation (39). The epithelial cells were washed once with 150 μl of PBS just prior to the treatment with hmLF preparations and bacterial addition.

Bacterial Adhesion and Invasion Assay—Prior to the adhesion assay, each bacterial culture was thawed from −70 °C stock cultures, transferred twice after growth for 16–18 h at 37 °C shaking at 250 rpm, and collected for use in the adhesion assay. Bacterial cells were collected from 15 ml of the respective medium after growth for 16 h, washed twice with an equal volume of PBS, and resuspended to an OD600 nm of 0.2 in Dulbecco's modified Eagle's medium/High Modified containing nonessential amino acids, 10 mM MOPS, 10 mM TES, 15 mM HEPES, and 2 mM NaH_2PO_4 without FBS. Each bacterial suspension (50 μl), was mixed with hmLF, hmLF glycoforms (500 μg/ml), or the purified *N*-glycans (250 μg/ml), mixed by vortexing for 1 min, and added to the washed, differentiated Caco-2 cells to a final multiplicity of infection of 1000. The Caco2 cells treated with bacteria and hmLF were incubated at 37 °C in an atmosphere containing 5% CO_2 for 60 min to let the bacteria interact the epithelial cells. Supernatants were then aspirated and the Caco2 monolayer was washed thrice with 200 μl of Tyrodes buffer (pH 7.2) (40, 41) to remove nonadhered bacteria from the monolayer. Adhered and invaded bacterial counts were performed as described by Elsinghorst (42), except qPCR was used to determine the bacterial count, and that DNA extraction was performed using 50 μl of a commercial lysis buffer (AEX Chemunex, France) as described by Desai (43). Quantitative bacterial analysis was performed using qPCR with a CFX 96 Real Time System (BioRad, Hercules, CA). Reactions were performed in a total volume of 25 μl containing 1 μl of cell lysate, 100 nM of PCR primers (Table I), and iQ SYBR Green Supermix (BioRad, Hercules CA) as per the manufacturer's instructions. The thermocycling parameters consisted of a denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation, annealing and extension at 95 °C for 15 s, 56 °C for 30 s, 72 °C for 30 s, respectively, and a final extension at 72 °C for 1 min. The amplified product was verified using melt curve analysis from 50 °C to 95 °C with a transition rate of 0.2 °C/s.

Statistics—Host-microbes interaction experiments were performed using three biological replicates. Bacterial analysis was performed

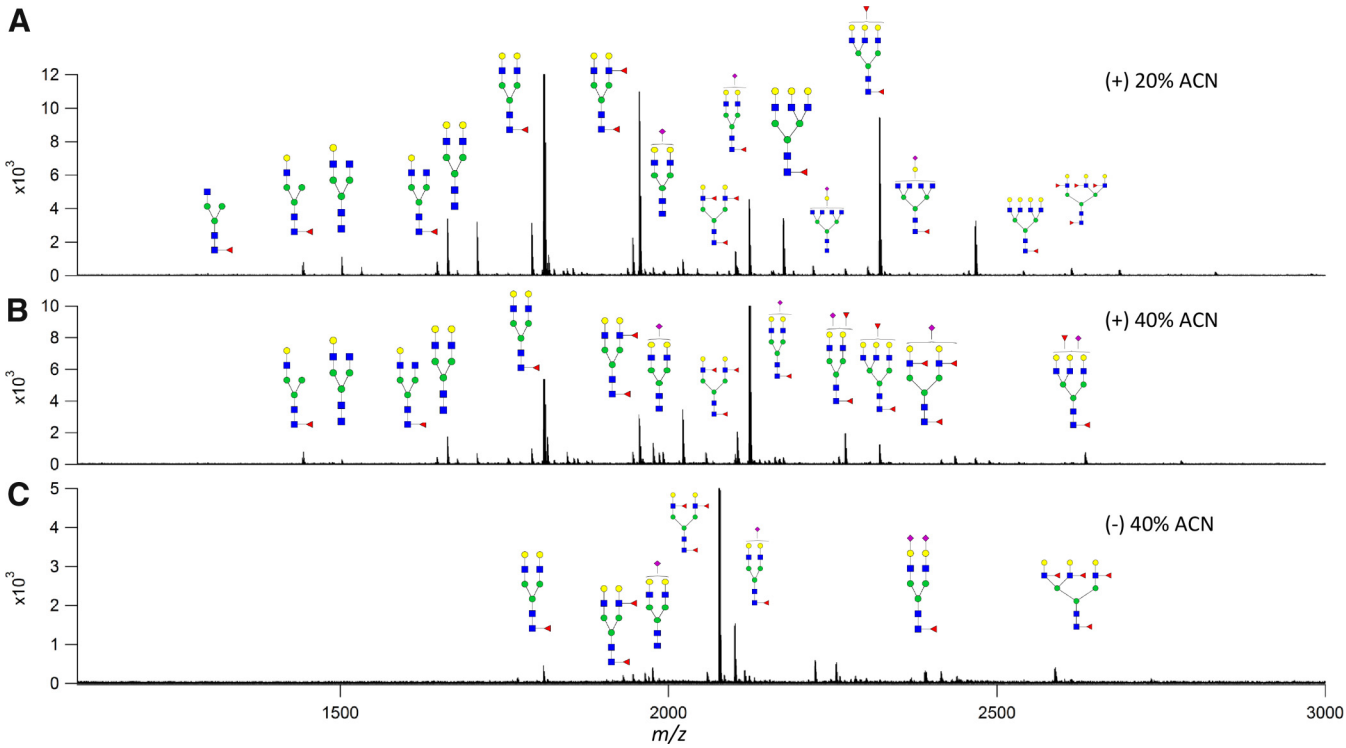

FIG. 1. **N-Glycoprofile of commercial human milk Lactoferrin.** *A*, Positive ion mode MALDI-FTICR mass spectrum of *N*-glycans recovered in 20% AcN:water fraction. *B*, Positive ion mode MALDI-FTICR mass spectrum of *N*-glycans recovered in 40% acetonitrile/water fraction, and *C*, Negative ion mode MALDI-FTICR mass spectrum of *N*-glycans recovered in 40% AcN:water.

using three technical replicates within each biological replicate. The number of adhered bacteria was determined by subtracting mean of invaded bacteria (*B*) from mean of total host associated bacteria (*A*). The error (ΔZ) was calculated as $(\Delta Z)^2 = (\Delta A)^2 + (\Delta B)^2$ where, ΔA is S.E. associated with the *A* and ΔB is S.E. associated with *B*. Significant differences between treatments were determined using Student's *t* test and were considered significant if $p < 0.05$.

RESULTS

Human Milk Lactoferrin N-glycome—To address the diversity of *N*-glycans found in human LF, oligosaccharides were released from a commercially available LF standard obtained commercially from pooled human milk. Upon glycan release, the mixture of oligosaccharides was fractionated into two fractions prior to MS analysis in order to minimize suppression. MALDI-FTICR-MS analysis was performed in positive and negative ion modes and showed the presence of neutral and acidic complex oligosaccharides with a diverse degree of fucosylation (Fig. 1). Positive ion mode analysis of the 20% acetonitrile/water fraction showed biantennary fucosylated oligosaccharides containing from one to three fucose residues as the most abundant species with nominal *m/z* values 1809, 1956, and 2101, respectively. Less abundant signals corresponded to complex fucosylated oligosaccharides containing three lactosaminic units and 1 to 4 fucose residues (nominal *m/z* 2174, 2320, 2463, and 2606). Among the less abundant signals, nonfucosylated biantennary complex (*m/z* 1663), biantennary complex glycans carrying four fucose residues (*m/z* 2248) and oligosaccharides containing four lactosaminic units substituted with two and three fucose residues were also observed (nominal *m/z* 2685 and 2831). Analysis of the 40% acetonitrile/water fraction rendered a less diverse profile. However, signals of anionic, sialylated glycans were observed corresponding to nonfucosylated monosialylated biantennary glycans and monosialylated, mono-, and difucosylated biantennary complex oligoasaccharides with nominal *m/z* values 1977, 2122, and 2267, respectively. Analysis of the 40% acetonitrile/water fraction in negative ion mode rendered no signals of neutral species, but a diversity of sialylated oligosaccharides and the most abundant signals corresponded to the sialylated species observed in positive ion mode (nominal *m/z* 1931, 2077, and 2223). In addition, among the less abundant signals, disialylated nonfucosylated biantennary glycans were observed as well as trilactosaminic (fucosylated tri-antennary) oligosaccharides carrying 2, 3, and 4 fucose residues, as monosialylated species (*m/z* 2588, 2734, and 2880).

Tandem MS analysis using collision-induced-dissociation and infra-red multi-photon-dissociation (IRMPD) was performed on the most abundant fucosylated oligosaccharides to confirm compositional assignment. Results of the difucosylated biantennary glycan at *m/z* 1955.67 and the trifucosylated biantennary glycan at *m/z* 2101.79 are depicted in supplementary Fig. S1. Attachment of the second and third

TABLE I

hmLF glycosylation changes over the course of lactation. A summary of the changes observed in each mother regarding total glycosylation content and levels of fucosylation are presented for each individual mother

	Mother 1	Mother 2	Mother 3	Mother 4	Mother 5
Days sampled	1; 5; 10; 15; 30	1; 5; 15; 30; 58; 72	1; 5; 10; 15; 30; 44; 58; 72	3; 5; 10; 15; 30; 44; 57; 72	1; 5; 10; 15; 30; 44; 58; 72
Glycan diversity	decrease	early decrease, later recovery	constant	early decrease, later recovery	constant
Monofucosylated	variable	later decrease	decrease	constant	decrease
Difucosylated	progressive increase	progressive increase	progressive increase	progressive increase	progressive increase
Trifucosylated	variable	later increase	constant	constant	later increase

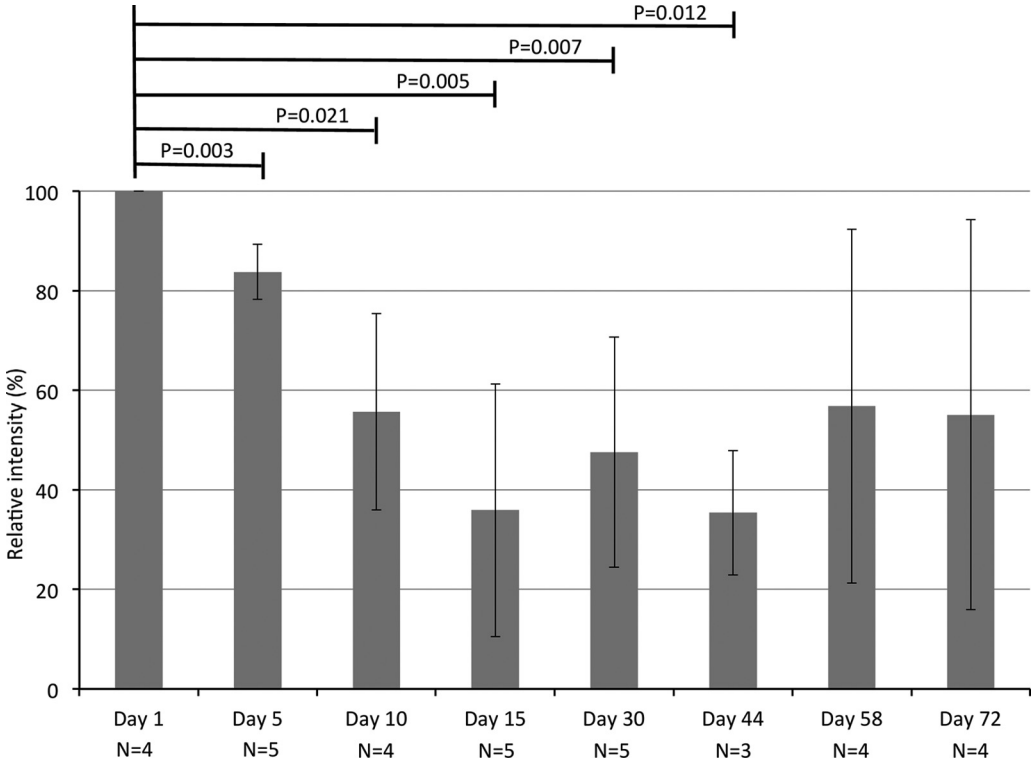

FIG. 2. **Degree of glycosylation of human milk LF from individual donors across lactation.** All glycan signals were summed and the abudances normalized relative to Day 1, which was regarded as 100%. Unfortunately, mother 4 did not produce milk on Day 1 and therefore was normalized to Day 5. A paired *t* test was performed to determine statistically significant differences between Day 1 and other days.

fucose to the *N*-acetylglucosamine residues of the outer antenna(e) was confirmed by enzymatic digestion with α-1–3/4 fucosidase, followed by MALDI-FTICR analysis. These results suggest the presence of Lewis^{X} epitopes in hmLF, however it does not rule out the possibility of other Lewis epitopes.

Dynamic Changes in Glycosylation of Human Milk LF Over the Course of Lactation—To address changes in glycosylation, hmLF was purified from milk samples obtained from five mothers during early to late lactation stages (days 1, 5, 10, 15, 30, 44, 58, and 72). Glycans were released from the purified hmLF and SDS-PAGE was performed to determine the efficacy of the enrichment. As an example, supplementary Fig. S2 shows the purification from mother 1 along the course of lactation.

Equal amounts of isolated hmLF from each donor and time point were deglycosylated, and the resulting glycan mixtures were analyzed by MALDI MS analysis. Supplementary Fig. S3 shows the intensities of the most abundant N-glycan signals in the profiles obtained, and results are summarized in Table I. Interestingly, all donors showed very similar glycoprofiles. The most abundant signals corresponded to biantennary complex type glycans, with up to three fucose residues. These results correspond to the profiles obtained from commercial hmLF standard; however, unlike the latter, monofucosylated biantennary complex oligosaccharides were the most abundant species in all donors.

Glycans obtained from milk collected on day 1 of lactation showed the highest intensities in all mothers as compared

TABLE II

Gene expression of the genes associated with glycosylation at different time points during lactation. Values are given in "reads per kilo base per million mapped reads" (RPKM)

Gene	Annotation	Day 4	Day 15	Day 30	Day 40
STT3A	Oligosaccharyltransferase complex subunit A	101.9	44.84	35.94	36.53
STT3B	Oligosaccharyltransferase complex subunit B	26.34	21.76	32.38	28.23
DDOST	dolichyl-diphosphooligosaccharide glycosyltransferase	17.08	94.68	70.31	88.97
RPN1	Ribophorin I	35.55	105.4	80.91	102.1
RPN2	Ribophorin II	9.33	91.35	72.28	81.54
DDA1	Cross-immunization reaction protein	2.11	6.44	5.27	6.73
OSTC	oligosaccharyltransferase complex subunit	28.60	47.55	43.01	44.64
KRTCAP2	keratinocyte associated protein 2	17.00	106.7	94.4	83.35
OST4	oligosaccharyltransferase 4	361.1	175.3	139.9	169.2
FUT1	**Fucα2**Galβ4GlcNAc-R	0	0	0.06	0.01
FUT2	**Fucα2**Galβ3GlcNAc-R	3.85	12.48	8.56	6.37
FUT3	Galβ4[**Fucα3**]GlcNAc-R	4.01	8.99	9.79	6.08
	Siaα3Galβ4[**Fucα3**]GlcNAc-R				
	Fucα2Galβ4[**Fucα3**]GlcNAc-R				
	Galβ3[**Fucα4**]GlcNAc-R				
	Siaα3Galβ3[**Fucα4**]GlcNAc-R				
	Fucα2Galβ3[**Fucα4**]GlcNAc-R				
FUT4	Galβ4[**Fucα3**]GlcNAcβ3Galβ4GlcNAc-R	0.38	0.28	0.65	1.74
	Galβ4]GlcNAcβ3Galβ4[**Fucα3**GlcNAc-R				
	Galβ4[**Fucα3**]GlcNAcβ3Galβ4[**Fucα3**]GlcNAc-R				
	Siαα Galβ4GlcNAcβ3Galβ4[**Fucα3**]GlcNAc-R				
	Siαα3Galβ4[**Fucα3**]GlcNAc-R				
FUT5	Galβ4[**Fucα3**]GlcNAc-R	0	0	0	0
	Siaα3Galβ4[**Fucα3**]GlcNAc-R				
FUT6	Galβ4[**Fucα3**]GlcNAc-R	0.11	11.1	9.77	7.29
	Siaα3Galβ4[**Fucα3**]GlcNAc-R				
FUT7	Siaα3Galβ4[**Fucα3**]GlcNAc-R	0.02	0.04	0.02	0.02
FUT8	GNGNManβ4GlcNAcβ4[**Fucα6**]GlcNAc-Asn	0.48	0.11	0.22	0.22
FUT9	Galβ4[**Fucα3**]GlcNAc-R	0.15	0.08	0.17	0.05
FUT10	Unknown	1.03	0.71	1.34	0.85
FUT11	Unknown	0.39	3.20	3.16	5.06

with later time points. To determine the extent of hmLF glycosylation in each mother, intensities of all glycan signals at each time point were summarized and normalized to the total intensity of ions observed on day 1. Fig. 2 shows the degree of glycosylation of hmLF in each donor across the course of lactation relative to day 1. A decrease in glycosylation was observed in all mothers from day 1 to day 15, during the transition from colostrum to transitional milk. Overall glycosylation then seems to increase at day 30 with the beginning of production of mature milk. After day 30, however, no consistent trends could be observed: Mother 2 and mother 3 showed an increase in glycosylation close to or equal to the original levels; whereas mothers 4 and 5 varied between 20 and 40% of the initial degree of glycosylation.

All together these results demonstrate dynamic glycosylation of hmLF from individual donors across the course of lactation. Although each mother showed individual variations there were common trends:

(1) A decrease in the extent of glycosylation in all mothers during the first 2 weeks of lactation, and a decrease in glycan diversity at day 15 in 3 out of 5 mothers (M2, M4, and M5, see Fig. 2).

(2) A moderate decrease in the relative abundance of monofucosylated biantennary complex oligosaccharides (11–18%) were observed at day 30 in three out of four mothers (M1, M3, and M4); however, a stronger effect (decrease of 20–51%) was observed later, at days 58 and 72 in 4 out of 5 donors (M2, M3, M4, and M5, see Table I).

(3) An increase in difucosylated biantennary complex glycans was observed in all mothers. At day 30, an increase of 78–100% was found for four out of five mothers (M1, M2, M3, and M4). This effect was greater as the lactation course progressed showing increases of up to 100%, 165 and 235% in four mothers (M3 and M5, M2, and M4, respectively, see Table I).

Glycan Gene Expression Analysis of Glycosylation in Milk—Protein *N*-glycosylation is an enzymatic process, and the changes observed in glycosylation of hmLF can be related to two sets of genes: those belonging to the oligosaccharide transferase (OST) complex, which transfers the nascent oligosaccharide chain from its lipid anchor to the synthesized protein (28), and the fucosyl transferase genes (29). Gene expression levels were examined in somatic cells in milk from mother 6 (M6) at days 4, 15, 30, and 40 postpartum. The

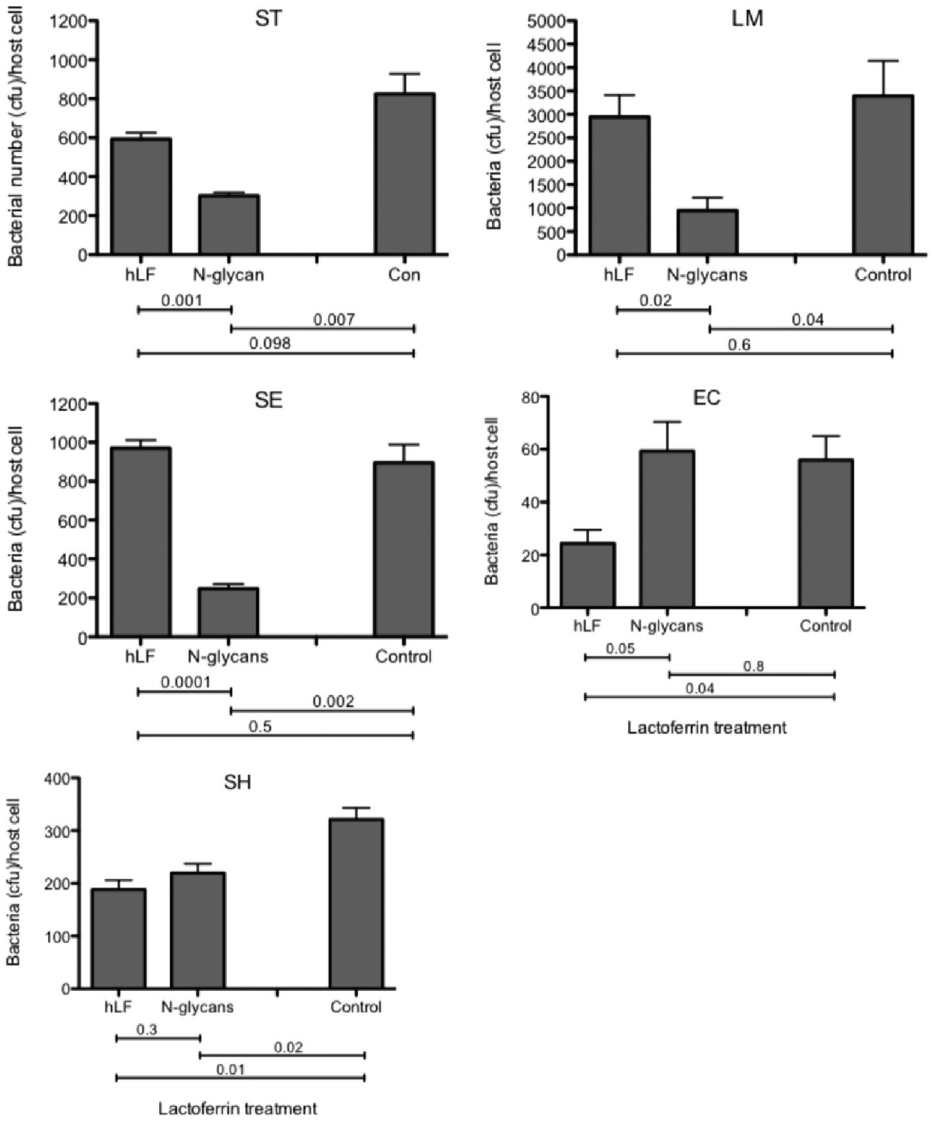

FIG. 3. **Changes in bacterial adhesion with hmLF addition and the purified glycan from hmLF.** Bars below the *x* axis indicate the *p* value between the respective treatments. Control is adhesion of the respective bacterium without glycan addition. Bacterial isolates used were ST = *Salmonella enterica* ssp *enterica* typhimurium; SE = *Salmonella enterica* ssp *enterica* enteritidis; SH = *Salmonella enterica* ssp *enterica* heidelberg; LM = Listeria monocytogenes; EC = *Escherichia coli* O157:H7.

observed decrease in overall glycosylation of lactoferrin is most likely because of alterations in expression or activity of subunits of the OST complex. The key step to *N*-glycosylation of protein is catalyzed by this complex, which is known to involve a small number of genes including STT3A, STT3B, DDOST (OST48), RPN1, RPN2, DDA1 (OST2), OSTC, KRT-CAP2, and OST4 (30). Gene expression data was obtained for the genes STT3A, STT3B, DDOST, RPN1, and RPN2 and is summarized in Table II. The expression of genes representing the OST changes mainly during the early lactation period: STT3A showed a 2.3-fold decrease from day 4 to day 15 and then remained constant; DDOST, RPN1, and RPN2 increased between three- and ninefold during the same period. Although the net effect of individual genes on *N*-glycosylation of hmLF is not known, the large changes observed by Day 15 are consistent with the changes in glycosylation observed for lactoferrin.

The percentage of fucosylated glycans on hmLF increases during early lactation. The data obtained for the different fucosyltransferases, which are summarized in Table II, are consistent with the observations of increasing fucosylation during lactation. The expression of 11 fucosyltransferase genes were monitored, of which the expression of four genes increased during lactation and seven genes remained constant over the lactation period. The observed increased expression of the FUT genes in somatic cells from human milk corresponds with the increased levels of fucosylation that were observed on hmLF.

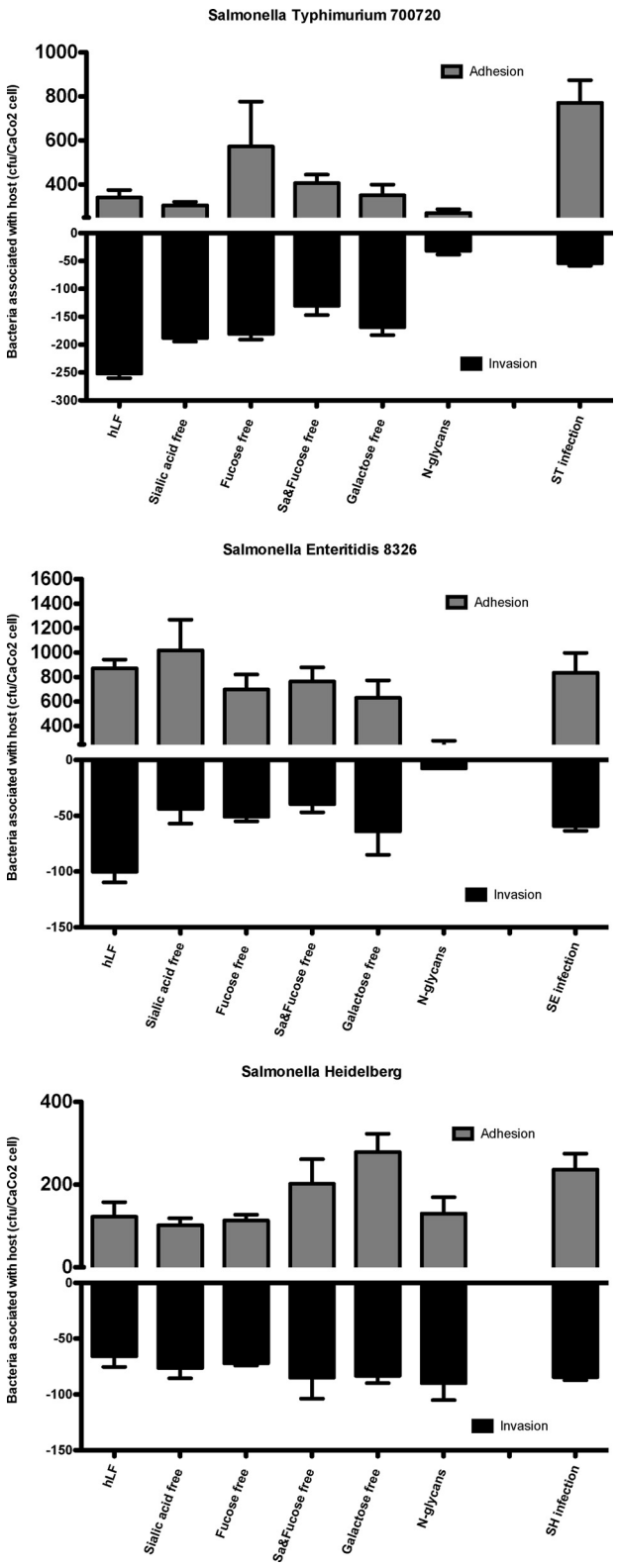

FIG. 4. **Bacterial association changes with modification of the hmLF glycosylation.** Bacteria were incubated with CaCO-2 cells in the presence of hmLF (hLF), hmLF treated with sialidase (Sialic acid free), hmLF treated with fucosidase (Fucose free), hmLF treated with sialidase and fucosidase (Sia and Fucose free) hmLF treated with galactosidase (Galactose free) and *N*-glycans released from hmLF (N-glycans). Baseline infection (infection) was determined without any additives. Gray bars above the horizontal line represent the number of bacteria adhered to the epithelial surface during 60 min of incubation, whereas black bars below the horizontal line represent the amount of bacteria invaded into the epithelial cell during the same incubation period.

Host/Microbe Interaction with hmLF Glycans—To examine the influence of hmLF glycosylation on host/microbe interactions, assays with differentiated human colonic intestinal epithelial cells (Caco-2) and five enteropathogenic bacteria were performed with addition of hmLF, free hmLF N-glycans, or hmLF glycoforms in which terminal monosaccharides were removed. *Listeria monocytogenes*, *Escherichia coli* O157:H7, and three serotypes of *Salmonella enterica* (serotypes Typhimurium, Enteritidis, and Heidelberg) were examined, because these bacteria are enteropathogens usually found in the infants gut during the first weeks of their lives. To determine the role of the hmLF protein backbone as well as the hmLF glycans, organisms were examined for total association (Fig. 3). Surprisingly, the association of only two organisms was significantly reduced with addition of hmLF - *E. coli* O157:H7 ($p = 0.04$) and *S. enterica* Heidelberg ($p = 0.01$). Conversely, addition of only the released hmLF *N*-glycans significantly reduced ($p < 0.04$) association for each organism tested, except *E. coli*. Thus, *E. coli* was influenced by the protein backbone, whereas binding of all the other organisms was significantly reduced by the hmLF N-glycans.

Considering that the *Salmonella* used in this study are serotypes of a single species and yet were influenced differently by hmLF and its glycans, the influence of specific hmLF glycosylation on binding was examined by enzymatic removal of terminal residues of fucose, sialic acid, and galactose from the intact protein. Additionally, association was differentiated into adhesion and invasion to specifically examine the role of individual residues (Fig. 4). Localization of the bacterium was significantly altered by hmLF and each of the glycovariants. Presence of hmLF unexpectedly resulted in a significant shift in location of *S. enterica* typhimurium and *S. enterica* enteritidis to the inside of the cell. Enzymatic removal of fucose from intact lactoferrin resulted in a significant increase in adhesion of *S. enterica* typhimurium (>300%, $p < 0.05$), whereas it had no influence on *S. enterica* Heidelberg and it reduced invasion of *S. enteritidis*. Removal of galactose increased adhesion, but not invasion of *S. enterica* Heidelberg, but had no impact on the other two organisms.

DISCUSSION

Despite numerous studies that identified the variation in total protein concentration and protein composition in human milk, little progress has been made in determining the glycosylation and variations in glycosylation of milk glycoproteins among individuals and throughout the course of lactation. The

glycosylation profile of bile-salt-stimulated-lipase (BSSL) was studied in 1997 (31), and later increased levels of fucosylation on BSSL *N*-glycans, together with altered levels of sialylated *O*-glycans were observed with progression of lactation (20). More recently, we reported dynamic changes in glycosylation in a limited proteomic study. Using SDS-PAGE with Coomassie Blue as well as carbohydrate staining (32) several proteins including hmLF were found to vary in their glycosylation over the course of lactation. However, actual glycan structures were not addressed.

Previous studies have shown that the overall concentration of hmLF, and the total protein content as a whole, decreases during lactation (32). Nonetheless, even with the extensive studies published on hmLF, surprisingly little is known regarding the structure and function of the glycosylation of human milk lactoferrin. Thus far, the glycosylation pattern of hmLF has only been studied once in 1982 (5). Using NMR, it was observed that lactoferrin carries biantennary glycans that are mono- or bisialylated as well as mono- or bifucosylated (5). Such structures were also observed in both the 20 and 40% fractions of each of the individual mothers in the present study. In this study, however, significantly more glycans were observed using MS analysis, which reveals that the glycoprofile of hmLF is more diverse than previously described. Furthermore, remarkable dynamic changes in glycosylation are observed in both the extent and types for hmLF. An initial overall decrease in levels of glycosylation is observed from birth to day 15 followed by an increase in the levels of higher order fucosylation. This period, interestingly, also corresponds to the transition between colostrum and mature milk.

Glycan biosynthesis is an enzymatic process with well-established enzymes. However, studies describing *in vivo* relations between expression of glycosyltransferase genes and actual glycosylation patterns are scarce. The transfer of a "glycan precursor" ($HexNAc_2Man_9Glc_3$) to the substrate protein, which is facilitated by the oligosaccharyl transferase complex (OST), produces the nascent glycoprotein. The gene expression data suggests that this step is regulated through gene expression resulting in the decrease of lactoferrin glycosylation. Furthermore, expression of many of the 11 fucosyltransferases increased during the course of lactation consistent with the increased levels in fucosylation reported earlier for BSSL (20) and here for hmLF (Table II). We thus observe some correlation between gene expression and hmLF glycosylation, indicating potentially direct genetic control of protein glycosylation in human milk. Dynamic glycosylation and bacterial binding studies support a pathogen deflection role for hmLF. Lactoferrin appears to be a multifaceted molecule to modulate bacterial binding through different fucosylated and sialylated glycoforms but also the polypeptide backbone. Glycans therefore appear to have differential effects on each organism that altered adhesion. Indeed, many human gastrointestinal pathogens bind glycan structures in the gut mucosa via fimbrae, flagella, and pili (also used to bind other bacteria) for adherence during gut transit, such as *H. pylori* in the stomach and *Salmonella*, *Clostridium*, and *E. coli* among others to intestinal cells, causing peptic ulcers and infectious diarrhea, respectively. More specifically, fucosylated oligosaccharides found in mammalian glycoproteins are involved in a wide range of mechanisms of cell adhesion during fertilization, development (33), leukocyte trafficking, and inflammatory responses (34).

Interestingly, even within *Salmonella* hmLF and the glycan had a different effect on host association depending on serotype. *Salmonella* is a particular concern with neonates. Disease associated with *Salmonella enterica* is initiated by attachment to and invasion of gastrointestinal tract (GIT) cells that leads to injection of effector molecules via the Type III secretion system that ultimately leads to inflammation of the lamina propia, invasion into the host cell within a lysosome and ultimately to lymph nodes (35).

The decrease in glycosylation during the transition from colostrum to mature milk is possibly related to the protective role of glycosylation for the polypeptide backbone. A well-established role of glycosylation is to protect the protein from protease digestion. The loss of glycosylation could make hmLF more susceptible to protease digestion thereby producing peptides that are antimicrobial such as lactoferricin (25, 26). It should also be remarked that the transition from colostrum to mature milk accompanies extensive change in the gut flora population (36).

CONCLUSION

hmLF can modulate the interactions between specific enteropathogenic bacteria and host cells. These results support a hypothesis that *in vivo* hmLF glycans play a key role in protecting the intestinal mucosa from different pathogens and thus modulate the microbial content of the gut. Furthermore, the glycosylation, which is genetically controlled, may respond to the changing bacterial population in the neonatal gut. Therefore, in infants, hmLF provides an important degree of protection. Glycans may play a key role in this process as they act as selective ligands for enteropathogens and thus deflect them from the gastrointestinal tract.

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[S] This article contains supplemental Figs. S1 to S3 and Table S1.

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Experimental m/z	Calculated m/z	Hex	HexNAc	Fuc	NeuAc	20%+ R. A.	40%+ R. A.	40%- R. A.
1282.453	1282.454	3	3	1	0	0.3		
1444.607	1444.607	4	3	1	0	1.54	1.84	
1501.529	1501.528	4	4	0	0	2.35	0.8	
1647.587	1647.586	4	4	1	0	2.01	1.41	
1663.581	1663.581	5	4	0	0	34.56	0.92	
1809.639	1809.639	5	4	1	0	100	100	2.31
1955.695	1955.697	5	4	2	0	90.32	12.65	
1976.659	1976.659	5	4	0	1	1.4	3.92	0.93
2101.754	2101.755	5	4	3	0	1.87	3.52	25.03
2122.718	2122.717	5	4	1	1	39.01	96.53	100
2174.771	2174.771	6	5	1	0	30.98	3.17	
2220.765	2220.765	4	6	0	1	0.98		
2268.772	2268.775	5	4	2	1		9.34	4.43
2320.829	2320.829	6	5	2	0	83.23	9.76	
2366.822	2366.823	4	6	1	1	0.54		
2466.889	2466.888	6	5	3	0	28.76	3.67	
2539.905	2539.904	7	6	1	0	0.78		
2612.945	2612.946	6	5	4	0	0.11		0.21
2832.019	2832.019	7	6	3	0	0.45		
2414.833	2414.833	5	4	3	1		1.91	
2633.909	2633.907	6	5	2	1		5.13	
2435.794	2435.794	5	4	1	2			2.56

Table S1. N-glycans from human milk Lactoferrin. Experimental and calculated m/z of N-glycans released from lactoferrin, as observed using MALDI-FTICR-MS. Annotation in ferms of hexoses (hex) N-acetylhexoses (HexNAc), Fucoses (Fuc) and NeuAc. Abundances relative to 100% for each of the three measurements (20% and 40% positive mode, 40% negative mode) are depicted.

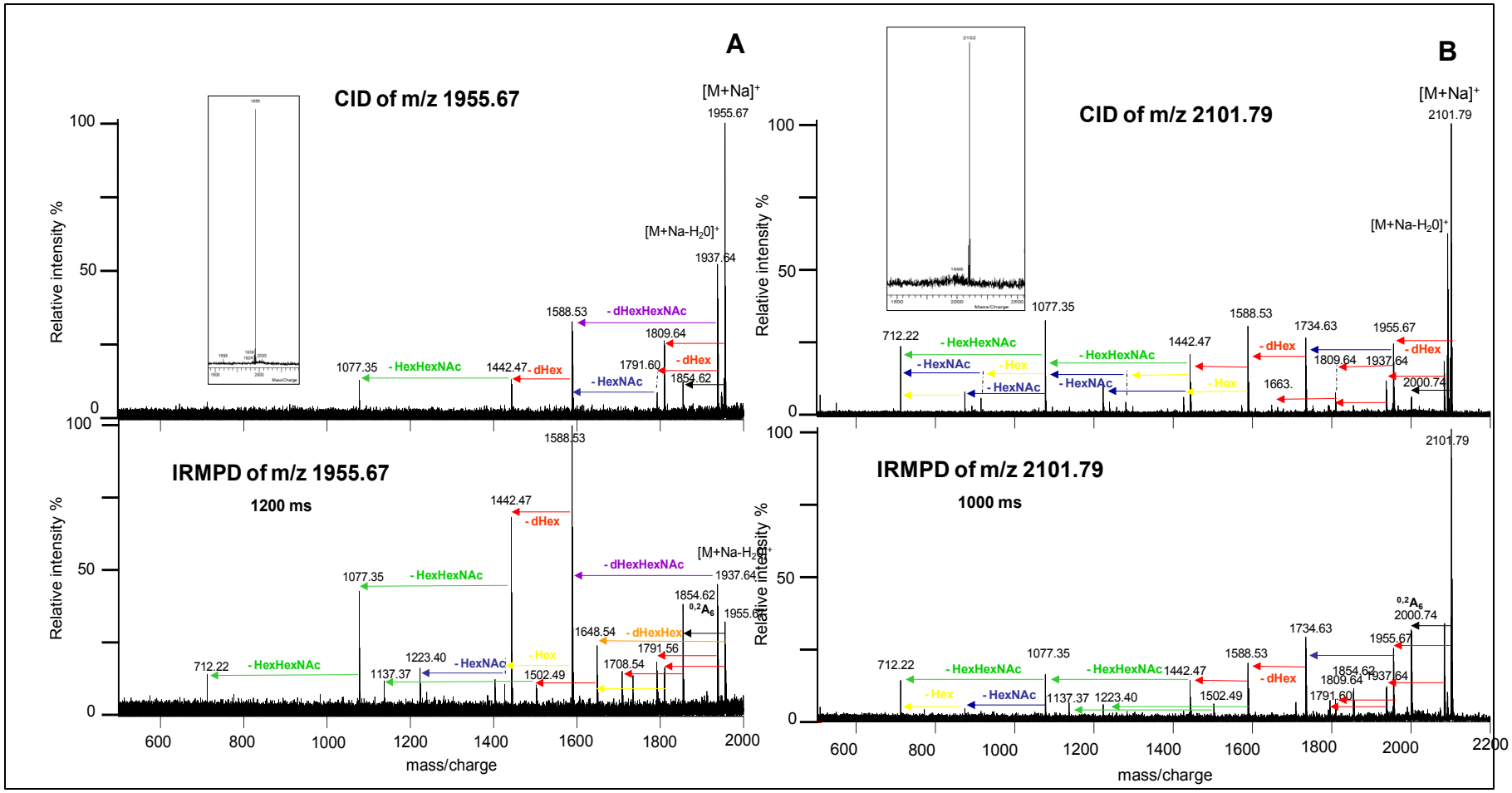


Figure S1. Tandem mass spectra (SORI-CID and IRMPD MALDI-FTICR) of two fucosylated oligosaccharides. A) m/z 1955.67 and B) m/z 2101.79.

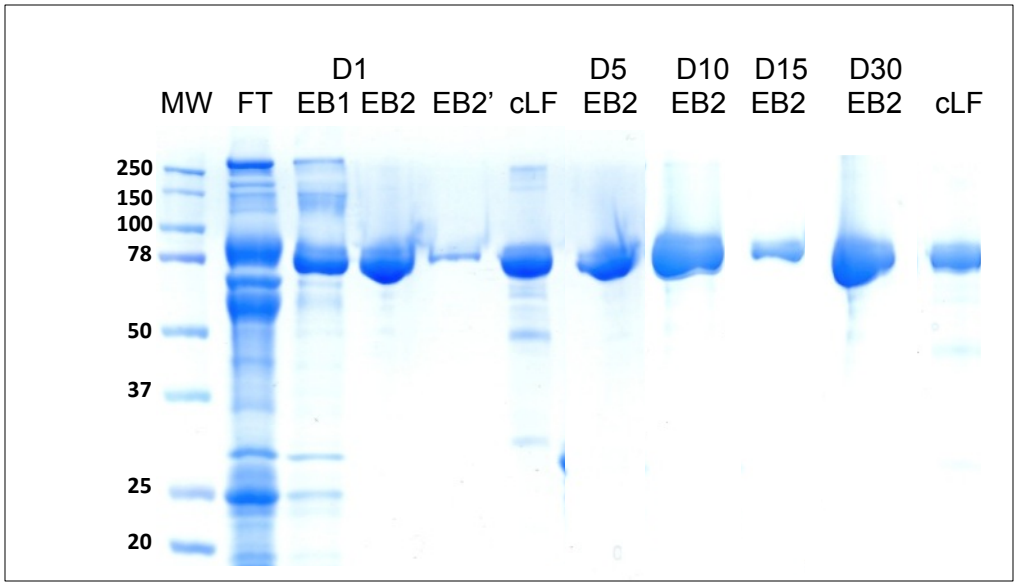
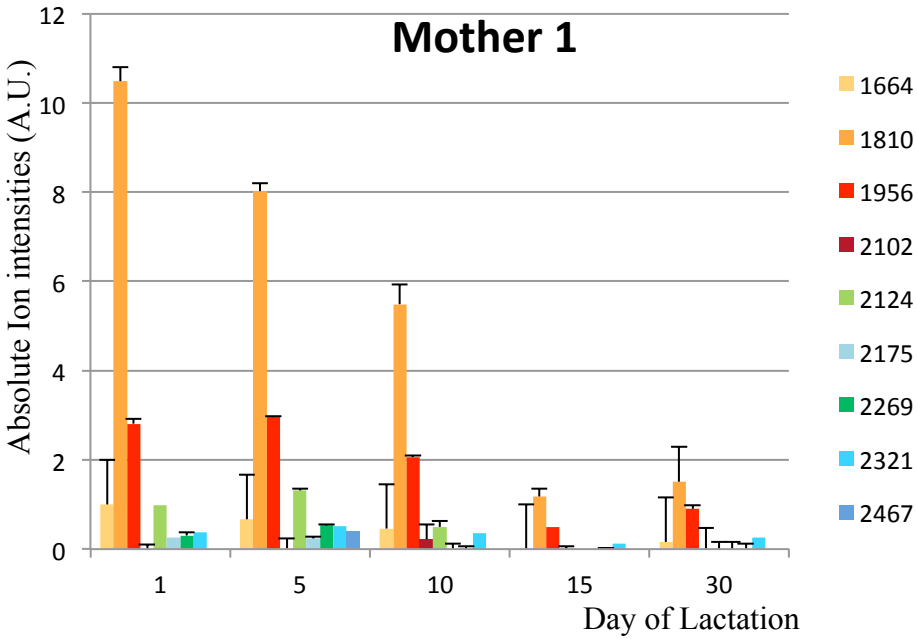


Figure S2. Purification of human milk LF from Mother 1 across lactation course by affinity-chromatograpy to Heparin-Sepharose.



Composition of N-glycans identified in all donors

m/z	Hex	HexNAc	DeoxyHex	NeuAc
1663.582	5	4	0	0
1809.640	5	4	1	0
1955.697	5	4	2	0
2101.755	5	4	3	0
2123.724	5	4	1	1
2174.772	6	5	1	0
2268.761	5	4	0	2
2320.851	6	5	2	0
2466.888	6	5	3	0

Figure S3. N-Glycoprofiles obtained from human milk LF purified from individual donors across lactation course.

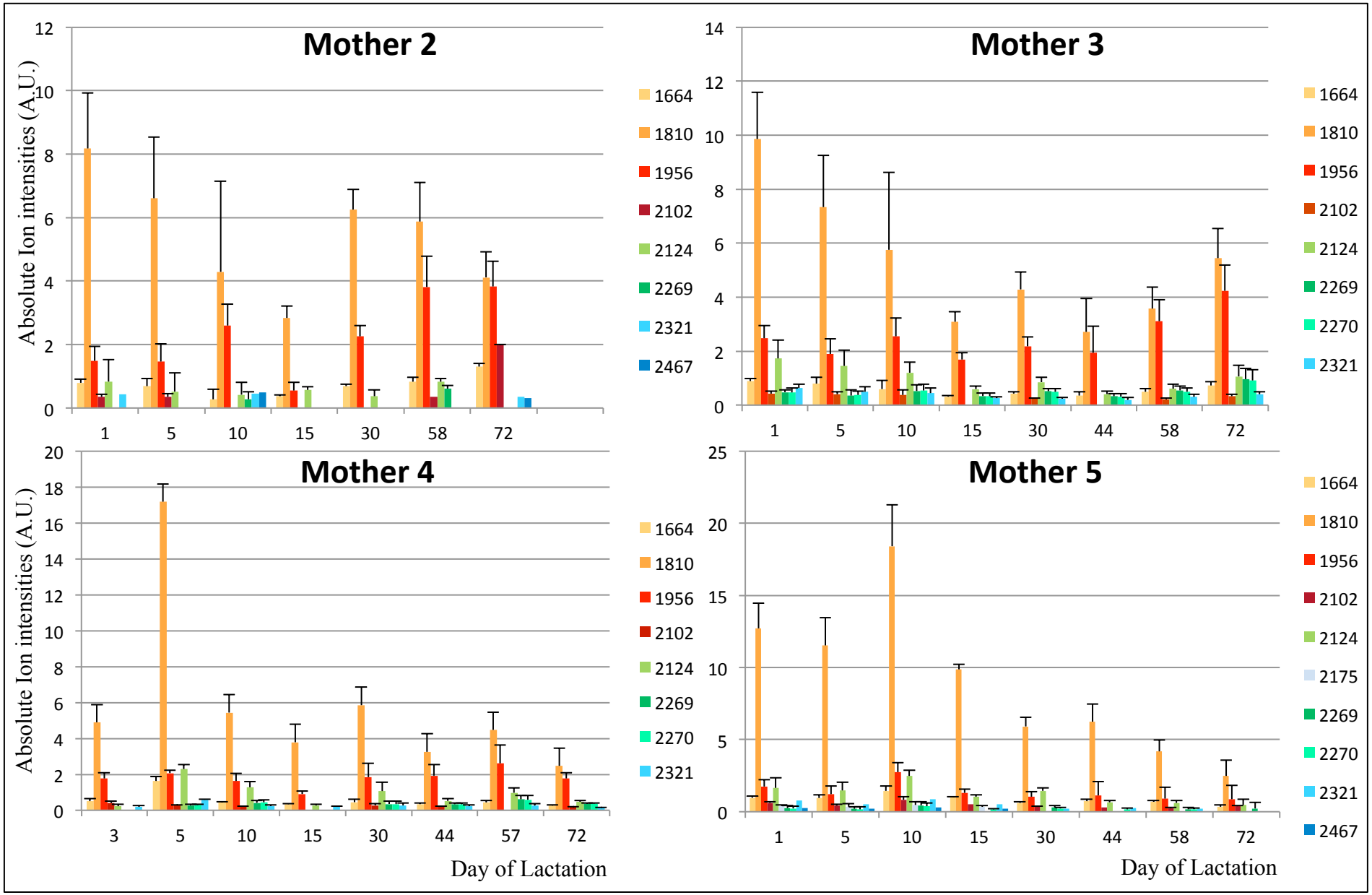


Figure S3. (continued)