

# The Human Milk Metabolome Reveals Diverse Oligosaccharide Profiles<sup>1–3</sup>

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## Abstract

Breast milk delivers nutrition and protection to the developing infant. There has been considerable research on the high-molecular-weight milk components; however, low-molecular-weight metabolites have received less attention. To determine the effect of maternal phenotype and diet on the human milk metabolome, milk collected at day 90 postpartum from 52 healthy women was analyzed by using proton nuclear magnetic resonance spectroscopy. Sixty-five milk metabolites were quantified (mono-, di-, and oligosaccharides; amino acids and derivatives; energy metabolites; fatty acids and associated metabolites; vitamins, nucleotides, and derivatives; and others). The biological variation, represented as the percentage CV of each metabolite, varied widely (4–120%), with several metabolites having low variation (<20%), including lactose, urea, glutamate, *myo*-inositol, and creatinine. Principal components analysis identified 2 clear groups of participants who were differentiable on the basis of milk oligosaccharide concentration and who were classified as secretors or nonsecretors of fucosyltransferase 2 (*FUT2*) gene products according to the concentration of 2'-fucosyllactose, lactodifucotetraose, and lacto-N-fucopentaose I. Exploration of the interrelations between the milk sugars by using Spearman rank correlations revealed significant positive and negative associations, including positive correlations between fucose and products of the *FUT2* gene and negative correlations between fucose and products of the fucosyltransferase 3 (*FUT3*) gene. The total concentration of milk oligosaccharides was conserved among participants (%CV = 18%), suggesting tight regulation of total oligosaccharide production; however, concentrations of specific oligosaccharides varied widely between participants (%CV = 30.4–84.3%). The variability in certain milk metabolites suggests possible roles in infant or infant gut microbial development. This trial was registered at clinicaltrials.gov as NCT01817127. J. Nutr. 143: 1709–1718, 2013.

## Introduction

For the developing infant, breast milk provides specific physiologic advantages over other sources of nutrition. Milk contains proteins, fats, carbohydrates, electrolytes, water, micronutrients, and organic molecules, that when ingested support infant growth by acting upon specific metabolic, immunologic, physiologic, and bacterial targets (1). The mammary gland synthesizes and transports many components including oligosaccharides both free and conjugated to lipids and proteins, intact immune and immunomodulatory cells, toxin-binding and growth factors, and a wide range of small molecule metabolites, all of which are presumed to promote health, growth, development, and protection of the neonate (2–5).

Furthermore, breast milk is proposed to promote immunologic programming of infants through the delivery of viable microbes (6). Understanding both the composition and function of the components of milk is vital to infant health. However, whereas much research has been performed on the highly abundant components of human milk, a complete understanding of all of its components is lacking, particularly of the metabolites present in milk.

Metabolites in milk come from both maternal blood and de novo synthesis in the mammary gland (7) and are influenced by the metabolic states of both the mammary gland and the lactating mother (8). This influence is so acute that milk has been used to monitor bovine health through measurement of acetone and acetoacetate concentrations as indicators of subclinical ketosis (9).

Human milk oligosaccharides (HMOs)<sup>8</sup> have been associated with diverse benefits to infants by promoting specific bacterial

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<sup>3</sup> Supplemental Tables 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

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<sup>8</sup> Abbreviations used: *FUT*, fucosyltransferase; HMO, human milk oligosaccharide; LDFT, lactodifucotetraose; LNFP, lacto-N-fucopentaose; LNnT, lacto-N-neotetraose; LNT, lacto-N-tetraose; PAL, physical activity level; PCA, principal components analysis; PLS-DA, partial least squares-discriminant analysis; UC-Davis, University of California, Davis; VIP, variable importance to the projection; 2'-FL, 2'-fucosyllactose; 3'-FL, 3'-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-SL, 6'-sialyllactose.

colonization of the infant gastrointestinal tract. These HMOs are complex sugars containing a lactose core bound to  $\geq 1$  glucose, galactose, N-acetylglucosamine, fucose, or sialic acid residues (5). Fucose residues may be attached to HMOs by an  $\alpha 1,2$ -linkage, which is catalyzed by a fucosyltransferase genetically encoded by the secretor gene (*FUT2*) or via  $\alpha 1,3$ - or  $\alpha 1,4$ -linkages, which are catalyzed by the Lewis gene (*FUT3*) family (10). In some individuals, allelic variation in the *FUT2* gene results in low or undetectable concentrations of  $\alpha 1,2$ -linked fucosylated HMOs such as 2'-fucosyllactose (2'-FL), lactodifucotetraose (LDFT), lacto-N-fucopentaose (LNFP I), or lacto-N-difucohexose I; and these individuals are referred to as "nonsecretors" (11–17). Milk produced from secretor mothers has been shown to be protective against diarrhea in their infants (18), including diarrhea caused by campylobacter and calicivirus (19). In adults, secretor status was shown to be associated with the diversity and composition of the gastrointestinal microbiota (20) and implicated in protection against celiac disease (21). With the growing realization of the abundant functions and health consequences of oligosaccharides, studies are beginning to address the diversity and function of metabolites of human milk.

Here we report the composition of abundant human milk metabolites measured by NMR of mature milk collected at day 90 postpartum from 52 healthy, lactating women who gave birth to singleton term healthy infants. We hypothesized that interindividual variation in the milk metabolome is largely explained by maternal secretor status, phenotype, and diet. We discuss the impact of maternal phenotype and diet on the milk metabolome and postulate the role that various milk metabolites may play in the developing infant.

## Participants and Methods

**Participants.** Milk samples were obtained from 52 healthy women enrolled in the Foods for Health Institute Lactation Study at the University of California, Davis (UC-Davis). Participants were enrolled at 34- to 38-week gestation and asked to fill out detailed health history questionnaires regarding demographic and anthropometric characteristics, pregnancy history, current and prior health history, dietary habits and restrictions, physical activity level (PAL), as well as medication and supplementation intake history. Reported physical activity, intensity, and frequency were converted into metabolic equivalents (22), which were used to calculate the impact of each reported physical activity on energy expenditure ( $\Delta$  PAL). The PAL was calculated as follows:  $1.1 + \text{sum of } \Delta \text{PAL}_i$ , where  $\Delta \text{PAL}_i$  is the list of each reported activity affect on energy expenditure ( $\Delta \text{PAL}$ ) (23). Half of the participants also completed an online Muldoon Omega-3 FFQ that contains 444 items (Modified Block 2006–Bodnar FFQ, 2006; NutritionQuest/Block Dietary Data Systems). Participants reported the mode of delivery of their infants (C-section vs. vaginal) and infant sex, weight, length, and gestational age at birth; and filled out questionnaires regarding their health and the health of their infants as well as their diet throughout the study. Participants received lactation support and training on proper sample collection from the study's lactation consultant. At day 60 postpartum, participants visited the UC-Davis Ragle Human Nutrition Center to provide a fasting blood sample, and heart rate, blood pressure, weight, and height were measured. BMI measured at day 60 postpartum was used as a surrogate for maternal weight at day 90 because maternal weight loss between 1 to 3 mo postpartum has been shown to be steady (24). The UC-Davis Institutional Review Board approved all aspects of the study, and informed consent was obtained from all participants.

**Human milk samples.** Participants were instructed to write on all sample tubes the time, date of collection, time of last meal before collection, and contents of the meals. Samples were considered fasted if milk collection occurred  $\geq 10$  h after the participant consumed her last meal and postprandial if milk collection occurred within 4 h of the last meal.

From all participants delivering full-term infants, milk samples were collected in the morning on day 90 postpartum according to a modified published method (25) involving milk collection from 1 breast by using a Harmony Manual Breast pump (Medela Inc.) by the participant 2–4 h after feeding her infant. Participants fully pumped 1 breast into a bottle, inverted 6 times, separated into 12-mL aliquots into a 15-mL polypropylene tube, and subsequently froze the sample in the kitchen freezer ( $-20^\circ\text{C}$ ). Samples were picked up, transported to the laboratory on dry ice, and stored at  $-80^\circ\text{C}$  until processing.

**Sample preparation and analytical repeatability.** The methodologic precision associated with sample preparation and NMR measurement were determined by preparing and analyzing 8 milk samples that were separated into aliquots in triplicate for a total of 24 samples. The researchers were blinded to the experiment, and the samples were prepared and analyzed in a random order. The identity of each sample was unblinded only after analysis was completed.

**Sample preparation.** Milk samples were prepared for NMR analysis by removing them from  $-80^\circ\text{C}$  storage and defrosting at room temperature. Each sample (1 mL) was centrifuged, and the aqueous layer carefully removed and filtered through a 3000 molecular weight cutoff filter (Amicon Ultra-0.5; Millipore) composed of low-protein-binding regenerated cellulose to remove lipids and proteins. To 585  $\mu\text{L}$  of filtrate, 65  $\mu\text{L}$  of internal standard containing 5 mmol/L 3-(trimethylsilyl)-1-propanesulfonic acid- $d_6$  and 0.2%  $\text{NaN}_3$  in 99.8%  $\text{D}_2\text{O}$  was added. The pH of each sample was adjusted to  $6.8 \pm 0.1$  by adding small amounts (1  $\mu\text{L}$  at a time) of NaOH or HCl after adding the internal standard to minimize pH-based peak movement and to ensure easier compound identification and quantification. A 600- $\mu\text{L}$  aliquot was transferred to a 5-mm Bruker NMR tube and stored at  $4^\circ\text{C}$  until NMR acquisition (within 24 h of sample preparation).

**Data acquisition and analysis.**  $^1\text{H}$  NMR spectra were acquired as previously described (26) on a Bruker Avance 600-MHz NMR equipped with a SampleJet autosampler using a NOESY-presaturation pulse sequence (noesypr) at  $25^\circ\text{C}$ . Water saturation was achieved during the prescan delay (2.5 s) and mixing time (100 ms). Spectra were acquired with 8 dummy scans and 32 transients over a spectral width of 12 ppm with a total acquisition time of 2.5 s. Once acquired, all spectra were zero-filled to 128-k data points, Fourier transformed with a 0.5-Hz line broadening applied, and manually phased and baseline corrected by using an NMR Suite v7.1 Processor (Chenomx). Metabolite quantification was achieved as previously described (27) by using a combination of the 600-MHz library from a Chenomx NMR Suite v7.1 Profiler and an in-house library of metabolites prepared by analytically weighing out pure material, dissolving in a precise concentration of 5 mmol/L potassium phosphate buffer (pH 6.8), acquiring NMR data as described above, and creating a new compound by using a combination of Spin Simulator and Compound Builder in Chenomx NMR Suite v7.1. Final reported metabolite concentrations were obtained after correcting for dilution by the added internal standard. The correction factor used to calculate the actual metabolite concentration was the final sample volume (650  $\mu\text{L}$ ) divided by the initial volume of milk filtrate (585  $\mu\text{L}$ ). Unless otherwise indicated, all concentrations are presented as  $\mu\text{mol/L}$  and reported as means  $\pm$  SDs.

**Statistical analyses.** Statistical procedures were conducted by using a combination of Simca v13 (MKS Umetrics AB), SPSS version 20.0 for Windows, Prism v6.0 (GraphPad Software Inc.) and R (R Development Core Team, 2013; <http://www.R-project.org>). Means  $\pm$  SDs are reported for participant characteristics, diet activity, and metabolite concentration, as well as percentage CV (%CV) for metabolites. For multivariate analysis, all metabolite variables were  $\log_{10}$  transformed before analysis. Methodologic precision associated with sample preparation and NMR measurement combined were determined as described above and reported as the means  $\pm$  SDs of the %CV for each of 8 different triplicate samples. Measurement error by NMR has previously been published and shown to be low for metabolites that are not volatile and do not interact with the sample matrix, with citrate being the least accurate ( $\sim 20\%$  error) of all metabolites due to the effects of pH and ionic strength (particularly

divalent cation concentration) altering its spectral characteristics and hence quantitative accuracy (26).

Principal components analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were used to explore the relations within the milk metabolome. Variable importance to the projection (VIP) values were used to identify discriminating metabolites. To predict the postprandial state of the participant, forward logistic regression was performed by using a set of metabolites from various biofluids associated with the postprandial and fasted states (28–34). To explore relations between milk metabolites, Spearman rank correlations were performed on milk sugars and subsets of metabolites reported in the literature to be associated with diet, physical activity, and BMI (Table 1) (35–47). A heat map was generated on the Spearman rank correlations ( $\rho$ ) of milk sugars by using R (package “corrplot”). The  $\alpha$  level was set at 0.05.

## Results

Self-reported participant demographic and pregnancy history data are shown in Supplemental Table 1. Most women were white and delivered their infant vaginally. Approximately equal numbers of male and female infants were born. Maternal habitual diet at enrollment, reported activity level before pregnancy and at enrollment, clinical characteristics measured on day 60 postpartum, and infant gestational age, weight, and length at birth are reported in Supplemental Table 2. Throughout the study, all infants were healthy and did not experience any illnesses related to feeding.

A total of 65 metabolites were identified from NMR spectra of human milk samples. These metabolites were classified as follows: sugars, amino acids and derivatives, energy metabolites, fatty acids and associated metabolites, vitamins, nucleotides and derivatives, and others (Table 2). Of these metabolites, 14 were classified as sugars, including monosaccharides (glucose, galactose, and fucose), disaccharides (lactose), and oligosaccharides [2'-FL, 3'-fucosyllactose (3'-FL), 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), LDFT, LNFP I, LNFP II, LNFP III, lacto-N-neotetraose (LNnT), and lacto-N-tetraose (LNT)]; 21 were relevant to amino acid metabolism and nitrogen balance (2-aminobutyrate, acetylcarnitine, alanine, asparagine, aspartate, betaine, carnitine,

glutamate, glutamine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, taurine, threonine, tryptophan, tyrosine, urea, valine); 11 were associated with glycolysis, tricarboxylic acid cycle intermediates, and products of energy metabolism (2-oxoglutarate, acetone, *cis*-aconitate, citrate, creatine, creatine phosphate, creatinine, fumarate, lactate, pyruvate, succinate); and the rest were linked with metabolic processes involving fatty acids, vitamins or nucleic acids, and microbial metabolism (acetate, azelate, butyrate, caprate, caprylate, choline, ethanolamine, glycerol-3-phosphate, *myo*-inositol, phosphocholine, ascorbate, niacinamide, pantothenate, cytidine, hypoxanthine, uridine, formate, hippurate, methanol). Accurate quantitation of several metabolites by NMR, including glycine, serine, cysteine, cystine, arginine, methionine, and sialic acid, was difficult due to their low concentrations or severe spectral overlap and was thus not reported. In addition, several oligosaccharides were not measured because commercial standards were not available.

To determine the extent of methodologic precision, 8 random milk samples were separated into aliquots in triplicate and frozen before analysis (for a total of 24 samples). Each triplicate milk sample was individually prepared and analyzed blinded. The average %CV for the triplicate sample analysis was ~5% (Table 2). Metabolites with the highest methodologic %CV (10–15%) were galactose, histidine, fumarate, and hypoxanthine. These results support that NMR spectroscopy has good reproducibility and provides a reference point to allow comparison of biological variability.

The most abundant category of milk metabolites was sugars, of which lactose dominated at a concentration of ~170 mmol/L. The next most abundant metabolites were also sugars and included the oligosaccharides: 2'-FL, 3'-FL, and LNT. Other abundant metabolites in human milk included urea (~4 mmol/L), citrate (~3 mmol/L), and glutamate (~2 mmol/L) (48–50). The biological variation among this cohort of women estimated by the %CV for each metabolite was relatively high (>30%). Yet, a handful of metabolites had moderate to low %CVs ( $\leq 25\%$ ) and included lactose (4%), urea (18%), creatinine (20%), glutamate (20%), *myo*-inositol (20%), valine (23%), and citrate (25%).

**TABLE 1** Targeted variables to determine the relations between milk metabolites and maternal habitual diet, lifestyle, and metabolic status<sup>1</sup>

Maternal characteristics	Potential milk metabolites
Age	Alanine, citrate, creatine, creatinine, hippurate, histidine, isoleucine, lactate, phenylalanine, succinate, taurine, tyrosine, valine
Blood pressure	Alanine, formate, fumarate, hippurate
BMI	Acetone, alanine, choline, citrate, creatine, creatinine, hippurate, isoleucine, lactate, tyrosine, valine
Daily protein intake	Alanine, carnitine, creatine, succinate, taurine
Daily servings of meat	Acetone, alanine, carnitine, citrate, creatine, creatinine, formate, glutamine, hippurate, histidine, hypoxanthine, leucine, lysine, o-acetylcarnitine, phenylalanine, succinate, taurine, threonine, urea, valine
Daily servings of vegetables	Creatinine, hippurate
Weekly servings of dairy foods	Alanine, choline, creatine, glutamate, glutamine, hippurate, histidine, lactose, leucine, lysine, proline, tyrosine, urea, valine
Physical activity	Alanine, creatinine, glutamine, isoleucine, lysine, phenylalanine, taurine, threonine, tryptophan, tyrosine, urea, valine
Postprandial state <sup>2</sup>	2-Oxoglutarate, acetone, alanine, galactose, glucose, hippurate, histidine, isoleucine, leucine, lysine, o-acetylcarnitine, phenylalanine, proline, tryptophan, valine

<sup>1</sup> Spearman rank correlations were performed on maternal age; blood pressure; BMI; daily protein intake (% of energy); number of daily servings of meat, vegetables, and dairy foods; physical activity; and secretor status.  $n = 52$ .

<sup>2</sup> Predictor variables were used in logistic regression to predict maternal postprandial and fasted states.

**TABLE 2** Human milk metabolites measured by using <sup>1</sup>H NMR spectroscopy<sup>1</sup>

Metabolite	Value	Range		Biological CV <sup>2</sup>	Methodologic precision <sup>3</sup>
		Minimum	Maximum		
	$\mu\text{mol/L}$		$\mu\text{mol/L}$	%	%
<b>Sugars</b>					
2'-FL	$2.50 \times 10^3 \pm 1.70 \times 10^3$	BDL	$6.90 \times 10^3$	66.8	$4.6 \pm 2.4$
3'-FL	$2.10 \times 10^3 \pm 1.20 \times 10^3$	480	$5.60 \times 10^3$	56.8	$5.2 \pm 2.7$
3'-SL	$144 \pm 43.7$	70.0	346	30.4	$5.2 \pm 2.5$
6'-SL	$119 \pm 54.9$	43.3	289	46.1	$4.9 \pm 3.2$
Fucose	$182 \pm 135$	BDL	615	74.1	$4.6 \pm 2.5$
Galactose	$92.3 \pm 49.1$	14.2	239	53.2	$15.1 \pm 10.5$
Glucose	$1.50 \times 10^3 \pm 530$	430	$3.30 \times 10^3$	34.3	$5.7 \pm 3.0$
LDFT	$266 \pm 199$	BDL	826	74.9	$2.9 \pm 2.3$
LNnT	$121 \pm 67.5$	51.4	379	55.7	$6.8 \pm 3.5$
LNFP I	$189 \pm 159$	BDL	703	84.3	$2.6 \pm 1.2$
LNFP II	$210 \pm 168$	3.8	717	80.0	$5.2 \pm 4.3$
LNFP III	$233 \pm 74.0$	109	454	31.8	$5.1 \pm 4.2$
LNT	$506 \pm 284$	115	$1.64 \times 10^3$	56.0	$7.5 \pm 3.2$
Lactose	$170 \times 10^3 \pm 7.30 \times 10^3$	$150 \times 10^3$	$180 \times 10^3$	4.3	$4.3 \pm 2.6$
<b>Amino acids and derivatives</b>					
2-Aminobutyrate	$17.7 \pm 6.9$	3.0	38.4	38.9	$7.1 \pm 2.3$
Acetylcarnitine	$10.5 \pm 4.7$	5.0	36.7	45.0	$6.8 \pm 4.3$
Alanine	$231 \pm 67.2$	109	379	29.1	$4.9 \pm 2.6$
Asparagine	$16.6 \pm 10.5$	BDL	41.1	63.3	$8.2 \pm 2.7$
Aspartate	$52.9 \pm 33.6$	11.3	221	63.6	$4.5 \pm 2.6$
Betaine	$30.3 \pm 8.4$	16.6	49.4	27.6	$8.0 \pm 2.6$
Carnitine	$26.2 \pm 10.1$	5.7	63.1	38.7	$4.4 \pm 4.1$
Glutamate	$1.60 \times 10^3 \pm 320$	$1.00 \times 10^3$	$2.50 \times 10^3$	20.2	$4.3 \pm 2.6$
Glutamine	$616 \pm 314$	27.1	$1.41 \times 10^3$	51.0	$8.3 \pm 4.6$
Histidine	$20.3 \pm 6.9$	7.8	40.9	33.8	$11.9 \pm 6.0$
Isoleucine	$7.9 \pm 3.3$	3.1	18.7	42.3	$6.0 \pm 3.8$
Leucine	$24.4 \pm 6.3$	11.8	39.8	25.9	$4.3 \pm 2.5$
Lysine	$11.1 \pm 8.8$	2.8	64.0	78.8	$6.9 \pm 3.7$
Phenylalanine	$13.1 \pm 3.9$	6.2	29.8	30.1	$6.9 \pm 1.8$
Proline	$27.2 \pm 20.0$	8.3	162	73.7	$7.9 \pm 3.8$
Taurine	$190 \pm 66.6$	54.6	334	35.1	$3.7 \pm 2.9$
Threonine	$96.5 \pm 31.4$	49.0	91	32.5	$6.0 \pm 4.5$
Tryptophan	$3.2 \pm 1.3$	0.7	6.3	40.6	$7.4 \pm 3.3$
Tyrosine	$15.0 \pm 6.6$	4.2	36.3	44.3	$5.4 \pm 3.3$
Urea	$4.50 \times 10^3 \pm 830$	$3.10 \times 10^3$	$6.30 \times 10^3$	18.5	$5.6 \pm 2.6$
Valine	$48.7 \pm 11.2$	21.9	74.1	23.1	$5.0 \pm 3.4$
<b>Energy metabolites</b>					
2-Oxoglutarate	$41.0 \pm 16.1$	22.2	112	39.2	$5.4 \pm 3.1$
Acetone	$10.3 \pm 6.4$	2.8	34.0	62.4	$4.8 \pm 2.4$
cis-Aconitate	$10.1 \pm 3.1$	5.6	18.0	30.2	$8.1 \pm 6.1$
Citrate	$2.70 \times 10^3 \pm 680$	$1.50 \times 10^3$	$4.70 \times 10^3$	24.9	$5.0 \pm 2.9$
Creatine	$51.6 \pm 24.5$	6.4	115	47.4	$5.5 \pm 2.4$
Creatine phosphate	$22.9 \pm 17.1$	1.2	72.2	74.8	$7.9 \pm 5.3$
Creatinine	$39.9 \pm 7.9$	23.7	54.8	19.7	$4.2 \pm 3.4$
Fumarate	$2.4 \pm 1.5$	0.4	9.2	60.8	$10.6 \pm 5.3$
Lactate	$69.6 \pm 41.1$	20.2	242	59.0	$4.1 \pm 2.3$
Pyruvate	$5.7 \pm 3.3$	2.0	16.1	57.9	$8.8 \pm 3.8$
Succinate	$8.4 \pm 2.9$	4.1	20.0	34.5	$5.1 \pm 2.6$
<b>Fatty acids and associated metabolites</b>					
Acetate	$27.5 \pm 28.2$	5.6	146	103	$4.6 \pm 3.1$
Azelate	$101 \pm 72.5$	5.9	271	71.7	$5.6 \pm 1.9$
Butyrate	$192 \pm 149$	6.8	689	77.3	$4.3 \pm 1.4$
Caprate	$53.7 \pm 19.3$	6.8	95.1	35.9	$7.0 \pm 1.9$
Caprylate	$267 \pm 154$	26.1	597	57.9	$5.8 \pm 3.1$
Choline	$155 \pm 103$	29.1	477	66.1	$5.1 \pm 2.9$

(Continued)

**TABLE 2** *Continued*

Metabolite	Value	Range		Biological CV <sup>2</sup>	Methodologic precision <sup>3</sup>
		Minimum	Maximum		
Ethanolamine	46.2 ± 18.1	16.6	99.4	39.2	6.9 ± 2.8
Glycero-3-phosphocholine	471 ± 161	177	1.07 × 10 <sup>3</sup>	34.1	4.1 ± 3.2
<i>myo</i> -Inositol	865 ± 177	389	1.50 × 10 <sup>3</sup>	20.5	4.7 ± 3.8
Phosphocholine	355 ± 171	9.9	733	48.3	5.1 ± 2.6
Vitamins					
Ascorbate	96.6 ± 52.5	17.3	305	54.3	4.7 ± 2.9
Niacinamide	5.3 ± 3.3	0.8	13.8	62.8	7.4 ± 2.1
Pantothenate	14.7 ± 7.3	4.9	34.3	49.8	5.0 ± 1.6
Nucleotides and derivatives					
Cytidine	3.4 ± 1.4	0.9	7.1	42.9	6.4 ± 2.1
Hypoxanthine	2.3 ± 1.5	0.1	6.4	65.3	14.5 ± 5.4
Uridine	7.1 ± 3.7	1.0	22.4	52.3	5.3 ± 3.4
Others					
Formate	12.2 ± 14.8	2.0	89.7	121	5.7 ± 2.9
Hippurate	7.8 ± 4.2	1.3	19.4	53.8	5.8 ± 3.7
Methanol	23.6 ± 16.1	6.9	115	68.4	5.9 ± 2.9

<sup>1</sup> Values are means ± SDs, ranges (minimum, maximum), or percentages for secretors and nonsecretors combined; *n* = 52. BDL, below the limit of detection; LDFT, lactodifucotetraose; LNFP, lacto-N-fucopentaose; LNnT, lacto-N-neotetraose; LNT, lacto-N-tetraose; 2'-FL, 2'-fucosyllactose; 3'-FL, 3'-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-SL, 6'-sialyllactose.

<sup>2</sup> Values for biological %CVs were calculated as SD/mean × 100.

<sup>3</sup> Values for the methodologic precision for each metabolite were calculated as the mean ± SD (%) of the %CV for 8 biological milk samples that were individually prepared and analyzed in triplicate by <sup>1</sup>H NMR.

The concentrations of these metabolites measured by NMR agreed with other reports (48–54). The most variable metabolites (%CV >60%) included 2'-FL, fucose, LDFT, LNFP I, asparagine, aspartate, lysine, proline, acetone, creatine phosphate, fumarate, acetate, azelate, butyrate, choline, niacinamide, hypoxanthine, formate, and methanol (Table 2).

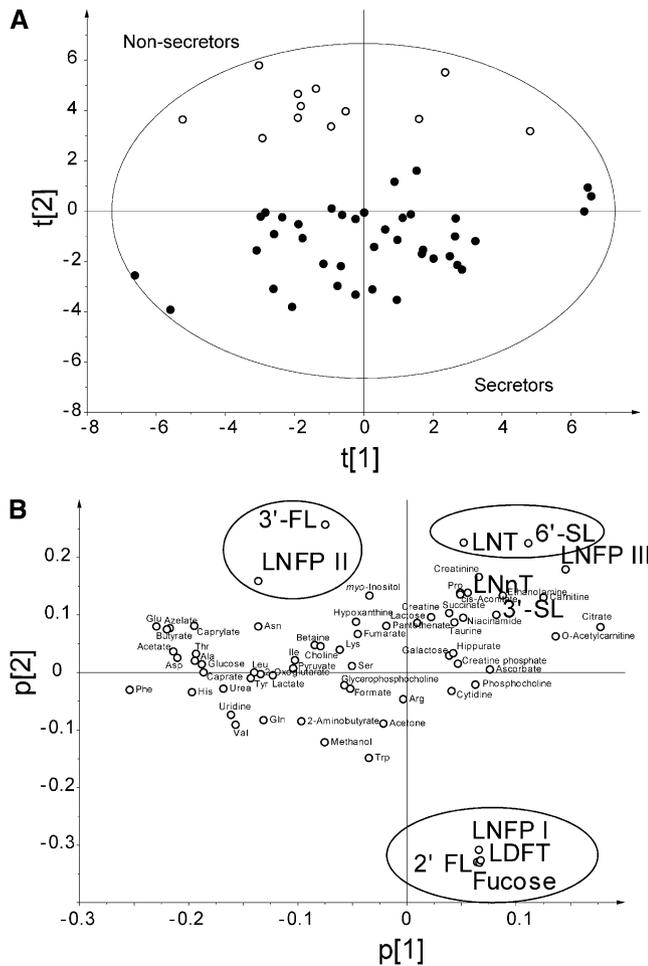
Analysis of all metabolite concentrations by using unsupervised PCA revealed that several sugars appeared to separate individuals across principal component 2 (Fig. 1). Examining the scores (Fig. 1A) and loadings (Fig. 1B) plots, 2 clear groups were distinguishable: those with measurable concentrations (*n* = 40) and those with concentrations that were low or below the detection limit (*n* = 12) of 2'-FL, LNFP I, LDFT (Fig. 2), and fucose (Fig. 3A). Notably, 2'-FL, LNFP I, and LDFT are synthesized through the action of the product of the *FUT2* gene, and the consequence of variation in gene expression or a low-functioning enzyme is low or undetectable concentrations of these oligosaccharides in milk (11–17). These individuals were therefore classified as nonsecretors. Additional examination of the loadings plot revealed that several sugars appeared to group, with LNFP I, LDFT, 2'-FL, and fucose composing 1 group in the lower right-hand quadrant; 3'-FL and LNFP II forming another group in the upper left-hand quadrant; and 6'-SL and LNT forming a distinct third group in the upper right-hand quadrant of the PCA loadings plot (Fig. 1B). Notably, 3'-FL and LNFP II are synthesized through action of the Lewis gene *FUT3* and are higher in concentration in nonsecretors (Fig. 3, B, C) (*P* < 0.001), consistent with previous studies (12,13). In addition, LNT (*P* < 0.001) and 6'-SL (*P* < 0.05) were higher in concentration in the milk of nonsecretors compared with secretors (Fig. 3, D, E), which also agrees with previous research (12).

On the basis of on the concentration of 2'-FL in milk, participants were classified as either secretors or nonsecretors and further exploration of metabolite differences by using PLS-DA was undertaken (data not shown). By using a VIP cutoff of 1.0, metabolite variables found to be most important for separation of secretors and nonsecretors included the following: 2'-FL,

LNFP I, LDFT, fucose, 3'-FL, LNFP II, 6'-SL, and LNT. Summation of the concentration of all oligosaccharides for each participant revealed a total concentration between 5 and 6 mmol/L (slightly higher for secretors) and a %CV of 18% regardless of secretor status. These data indicate that the interindividual variation in total oligosaccharide concentration is low, unlike for individual milk sugars, which showed moderate to high interindividual variation. Because not all oligosaccharides were measured due to a lack of availability of oligosaccharide standards, the exact concentration is not reported.

To explore the interrelations between milk sugars, Spearman rank correlations were performed and are presented in a heat map (Fig. 4). When data from secretors and nonsecretors were included, 2'-FL was positively correlated with LDFT (*P* < 0.0005) and LNFP I (*P* < 0.0005), both of which contain an α1,2-linked fucose, and negatively correlated with LNT (*P* < 0.001). Moreover, 2'-FL was negatively correlated with 3'-FL (*P* < 0.0005) and LNFP II (*P* < 0.0005), which contain α1,3-linked and α1,4-linked fucose molecules, respectively. LDFT was also positively correlated with LNFP I (*P* < 0.0005) and negatively correlated with LNFP II (*P* < 0.01) and LNT (*P* < 0.0005). LNFP I was negatively associated with LNFP II (*P* < 0.01). Furthermore, 3'-FL was negatively associated with fucose (*P* < 0.001) and LNFP I (*P* < 0.0005) and positively associated with glucose (*P* < 0.05) and LNFP II (*P* < 0.0005). Nonsecretors also had higher concentrations of 6'-SL (Fig. 3E), which was positively correlated with LNFP III (*P* < 0.01) and LNT (*P* < 0.05). The relations between concentrations of α1,2-linked fucosyloligosaccharides with α1,3/4-linked fucosyloligosaccharides in our study are supported by observations made by Chaturvedi et al. (14).

Other correlations between HMOs were found between LNnT and LNFP II (*P* < 0.05), LNFP III (*P* < 0.05), and LNT (*P* < 0.05) and between LNT and LNFP II (*P* < 0.0005), and LNFP III (*P* < 0.001). Fucose, the core monosaccharide of fucosylated oligosaccharides, was also related to the concentrations of milk oligosaccharides: 2'-FL (*P* < 0.0005), 3'-FL (*P* < 0.001), glucose



**FIGURE 1** Principal components analysis scores plot of milk samples from 52 participants (A). Those with 2'-FL concentrations at low or undetectable concentrations are labeled as "nonsecretors" and are indicated as open circles. Those with high concentrations of 2'-FL are labeled as "secretors." Principal components are indicated [t(1) and t(2)]. A corresponding [p(1) and p(2)] loadings plot illustrating the variables responsible for separation of the observations in A is depicted in panel B. Metabolite variables contributing the most to separation are circled. LDFT, lactodifucotetraose; LNFP, lacto-N-fucopentaose; LNnT, lacto-N-neotetraose; LNT, lacto-N-tetraose; 2'-FL, 2'-fucosyllactose; 3'-FL, 3'-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-SL, 6'-sialyllactose.

( $P < 0.01$ ), LDFT ( $P < 0.0005$ ), LNFP I ( $P < 0.0005$ ), LNFP II ( $P < 0.0005$ ), and LNT ( $P < 0.0005$ ). Other mono- and disaccharides that correlated with HMOs included 3'-SL and galactose ( $P < 0.05$ ) or lactose ( $P < 0.05$ ) and glucose with the following oligosaccharides: 2'-FL ( $P < 0.0005$ ), LDFT ( $P < 0.001$ ), LNFP I ( $P < 0.0005$ ), and LNFP III ( $P < 0.01$ ) (Fig. 4).

To further investigate the link between the overall milk metabolome and maternal health or dietary status, PLS-DA was performed on subsets of women, comparing the top and bottom 20% of individuals with respect to age, blood pressure, diet, and physical activity. In addition, individuals who reported fasting were compared with those who consumed a meal within 4 h of providing a sample. Using all metabolite variables, or subsets of metabolite variables (Table 1), multivariate models could not be developed for any of the potential classifiers, indicating that the human milk metabolome is relatively insensitive to fluctuations in health or diet for relatively healthy individuals. Lower concentrations of acetone in milk were observed in women with higher BMI ( $\rho = -0.41$ ,  $P < 0.01$ ), but no other correlations were observed.

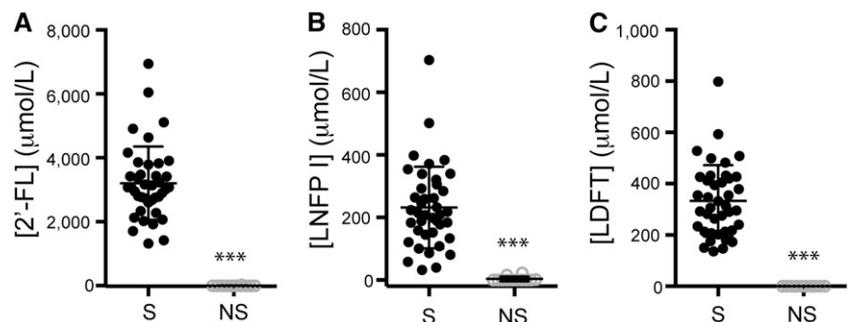
The fact that multivariate models could not be generated is likely due to the fact that feature selection can be compromised with large numbers of variables when using the PLS-DA-based VIP method (55). Therefore, forward logistic regression was used with subsets of metabolites (Table 1). On the basis of forward logistic regression, galactose (OR: 5.5;  $P < 0.05$ ) and alanine (OR: 3.2;  $P = 0.05$ ) were found to be higher when comparing postprandial with fasted states ( $P < 0.01$ ; Fig. 5).

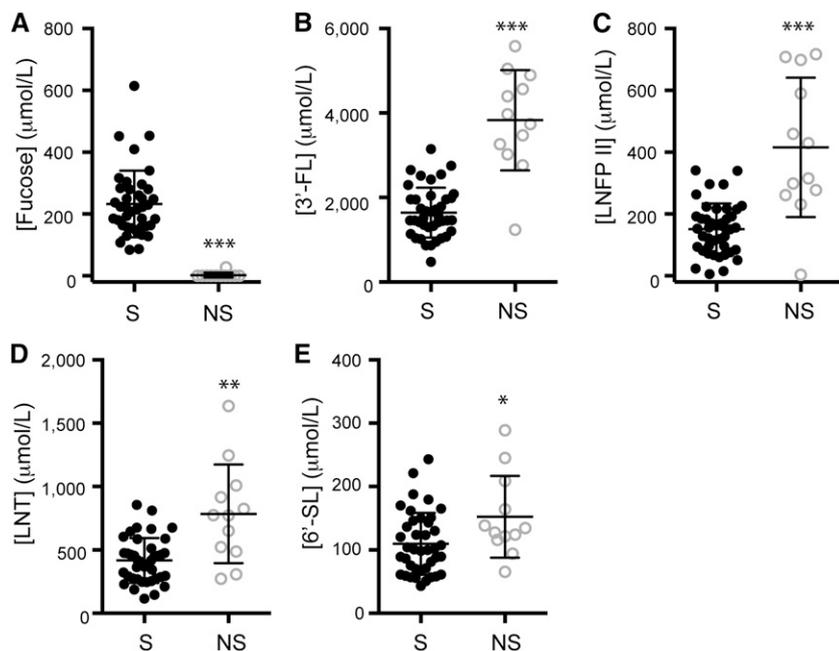
## Discussion

For more than a decade, the use of NMR spectroscopy for metabolomics has led to the successful identification and measurement of metabolite concentrations in serum and urine that reflect metabolic phenotype, health, disease, lifestyle, and diet (44,46,56). Only a few metabolomics studies using NMR have been conducted on milk, which includes bovine (9), human preterm (57), and recently, rhesus macaque milk (58). Studies have shown high interindividual variation in human milk in terms of fat, protein, lactose, glucose, citrate, creatinine, and mineral content (59,60). Several factors contribute to this variation including time of day, collection before or after infant feeding, breast used to collect the milk, length of pregnancy, stage of lactation, and mother's diet (60–63). In this study, we quantified the concentrations of 65 metabolites in human milk collected on day 90 postpartum from 52 women. We identified metabolites whose abundances were highly conserved across the cohort and thus likely represent metabolites with specific functional roles for the developing neonate. Moreover, relations between several oligosaccharides that have not previously been reported were discovered.

Concentration analysis of milk metabolites suggested several highly conserved metabolites with low %CV ( $\leq 25\%$ ), including lactose, urea, creatinine, glutamate, *myo*-inositol, citrate, and valine, as well as total oligosaccharide content. The presence and concentration of metabolites in mammalian milks have been proposed to reflect their evolutionary history (64). Lactose (4% CV),

**FIGURE 2** Comparison of the concentration of HMOs synthesized by the product of the *FUT2* (fucosyltransferase 2) gene. 2'-FL (A), LNFP I (B), and LDFT (C). Secretors (S;  $n = 40$ ) and nonsecretors (NS;  $n = 12$ ) are shown. \*\*\* $P < 0.0005$ . HMO, human milk oligosaccharide; LDFT, lactodifucotetraose; LNFP, lacto-N-fucopentaose; 2'-FL, 2'-fucosyllactose.





**FIGURE 3** Comparison of the concentration of sugars not produced by the product of the *FUT2* (fucosyltransferase 2) gene. Fucose (A), 3'-FL (B), LNFP II (C), LNT (D), and 6'-SL (E). Secretors (S;  $n = 40$ ) and nonsecretors (NS;  $n = 12$ ) are shown. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0005$ . LNFP, lacto-N-fucopentaose; LNT, lacto-N-tetraose; 3'-FL, 3'-fucosyllactose; 6'-SL, 6'-sialyllactose.

the most abundant component in milk after water (~7% wt:v), comprising ~40% of total energy intake of the infant, is the major osmotic component regulating milk volume (65). That lactose concentration in milk is closely conserved among mothers suggests that there is value in consistent delivery to the neonate, and furthermore that osmotic balance in milk is important. Another osmolyte with low %CV was *myo*-inositol (20%). This metabolite, which is also relatively high in concentration, is important in infant development as an osmoregulator (66) and a precursor of phospholipids (67). A previous study reported that *myo*-inositol supplementation during the early neonatal period decreases the likelihood of injury to the retina and lung in premature infants with respiratory distress syndrome (68).

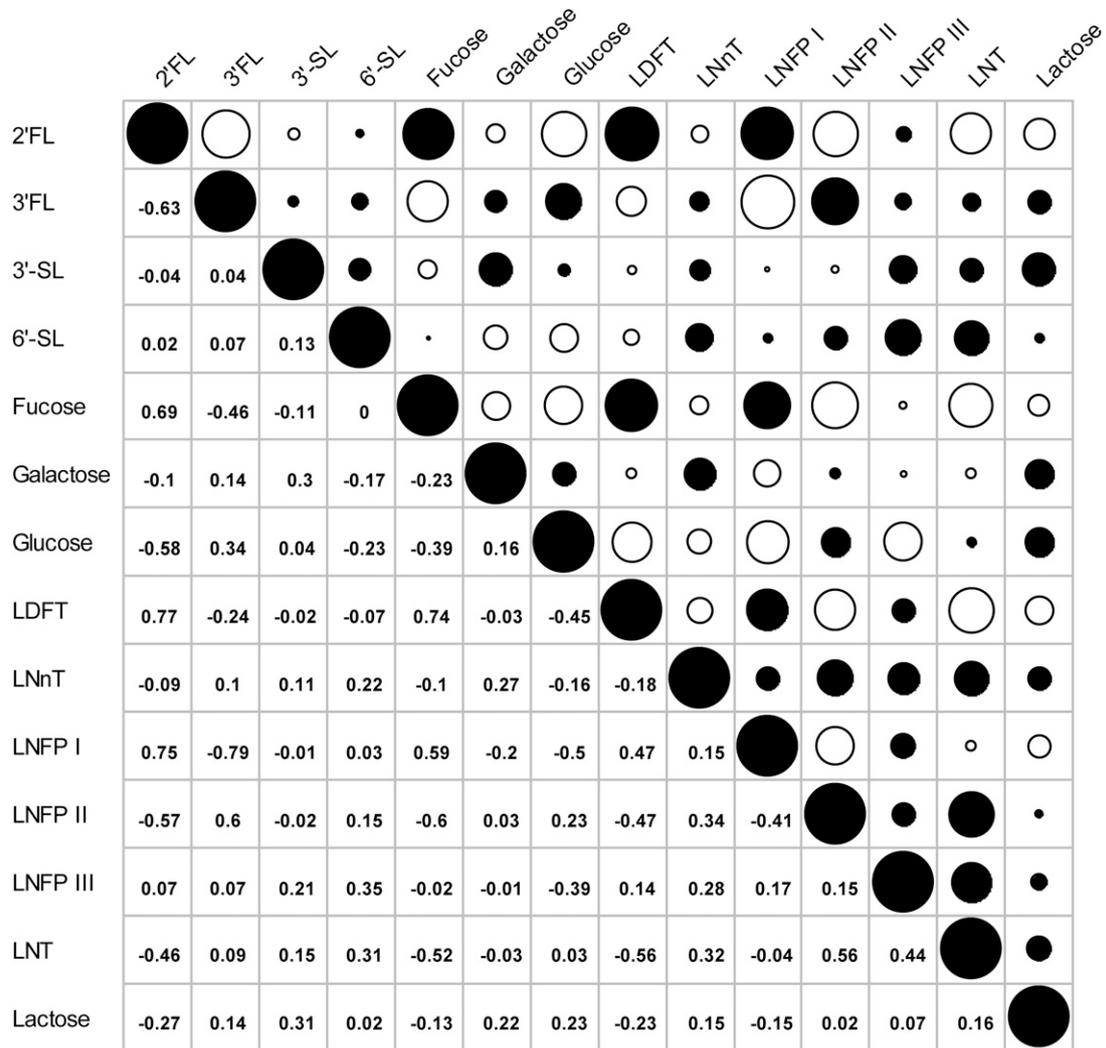
The next highest abundant metabolite in human milk was urea ( $4.5 \pm 0.8$  mmol/L), with the second lowest %CV (18%). This is unlike the high variation in urea concentrations in the urine and serum of infants <6 mo of age (77% and 44%, respectively; unpublished observations) and is higher than urea in serum (1.2–3.5 mmol/L). The low %CV and abundant concentrations of urea in milk suggest regulation at the level of the mammary gland. A study by Mephram and Linzell (69) revealed that 3 times more L-arginine accumulates in the mammary gland than is required for milk protein synthesis. Moreover, infusion of radiolabeled arginine into the lactating goat mammary gland resulted in 18% of it being recovered as urea in milk and none in blood (69). Tight regulation of urea concentration by the mammary gland suggests that urea may have an important role in the developing neonate. One such role could be to provide commensal gut microbiota with a readily available nitrogen source (70,71). Gut microbes use urea and ammonia for protein synthesis, which is driven by the energy produced by carbohydrate fermentation (72). Indeed, ingestion of lactulose for 4 wk resulted in a significant reduction in urinary  $^{15}\text{N}$  accompanied by significant increases in fecal amounts of  $^{15}\text{N}$  and *Bifidobacterium* (73). Because HMOs enrich the growth of bifidobacteria in breastfed infants (74), and total HMOs had a %CV similar to urea (18%), it is interesting to speculate that urea may be an important nitrogen source for gut bacteria.

In agreement with other studies (49,75), the most abundant free amino acids in human milk were the nonessential amino

acids: glutamate, taurine, alanine, and glutamine. The biological variation for glutamate was 20%, which may be explained by its multifunctional effects on gut health, energy metabolism, and signaling. Glutamate is not absorbed at similar rates as other dietary amino acids and is therefore considered to act as a major oxidative fuel for enterocytes. Glutamate is also a signaling molecule that supports neuroendocrine reflexes and gastrointestinal barrier function (76). The low variability in valine in breast milk may be related to ensuring enough glutamate is present in the gastrointestinal tract. Indeed, branched-chain amino acid aminotransferase is found in intestinal cells and in the mammary gland and is the first step in catabolism of branched-chain amino acids, producing glutamate from  $\alpha$ -ketoglutarate (77). Similarly, citrate, which is a key metabolite in the tricarboxylic acid cycle, is important for glutamate production from 2-oxoglutarate. The fact that fumarate and succinate do not have low %CVs suggests that at least part of the function of citrate may be to produce glutamate. These conserved milk metabolites may play essential roles in regulating infant growth and support the development of gut microbial colonization.

The most variable metabolites in milk included the following: 2'-FL, fucose, LDFT, LNFP I, LNFP II, aspartate, lysine, proline, acetone, creatine phosphate, fumarate, acetate, azelate, butyrate, choline, niacinamide, hypoxanthine, formate, and methanol as well as the essential nutrients obtained from maternal diet that include choline, niacinamide, ascorbate, and pantothenate. As previously indicated, 2'-FL, LDFT, and LNFP I reflect maternal secretor status, because their production is a consequence of the fucosyltransferase encoded by the *FUT2* gene. Similarly, the correlation between 3'-FL and LNFP II is expected based on the fact that their production is a consequence of the fucosyltransferase encoded by the *FUT3* gene. Interestingly, positive correlations among 6'-SL, LNT, and LNFP III were also observed. The extensive correlation (both positive and negative) of milk monosaccharides, disaccharides, and oligosaccharides coupled with the conserved total oligosaccharide concentration suggests diverse maternal phenotypes that regulate the concentrations of milk sugars, and in particular milk oligosaccharides.

Fucose was positively correlated with the  $\alpha$ 1,2-linked, and negatively correlated with the  $\alpha$ 1,3/4-linked, fucosyloligosaccharides,

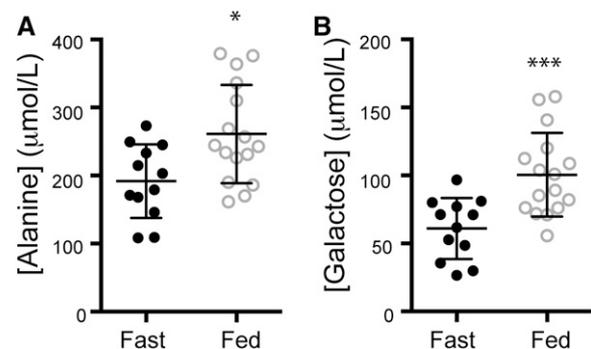


**FIGURE 4** Spearman correlation heatmap of human milk sugars. The size of the circle in the upper right triangle represents the magnitude of the correlation, whereas the color represents the direction of the relationship (black = positively correlated; white = negatively correlated). The values located at the bottom left are the Spearman  $\rho$  values corresponding to the pairwise correlation. LDFT, lactodifucotetraose; LNFP, lacto-N-fucopentaose; LNnT, lacto-N-neotetraose; LNT, lacto-N-tetraose; 2'-FL, 2'-fucosyllactose; 3'-FL, 3'-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-SL, 6'-sialyllactose.

suggesting that free fucose in milk could be a consequence of metabolism of  $\alpha$ 1,2-linked fucosyloligosaccharides. Human milk fucosyltransferase and fucosidase activities vary according to lactation stage (78) and are susceptible to sample handling (79). In addition, human milk contains anaerobic bacteria including bifidobacteria (6) that express fucosidase (74). Whether milk fucose concentrations are reflected by endogenous enzymatic activity in the mammary gland or by bacterial action remains to be clarified.

To further elucidate causes of variation in the milk metabolome, associations between maternal diet, lifestyle, and phenotype with milk metabolites were investigated. A negative association between maternal BMI measured at day 60 postpartum and milk concentrations of acetone in this study agree with a previous study that reported higher plasma acetone concentrations in lean compared with obese participants (28). An analysis of fasted compared with fed states by participants at the time of sample collection revealed that galactose and alanine were 65% and 36%, respectively, higher in milk collected from women who had consumed a meal within 4 h of milk collection compared with women who reported collecting milk after at least a 10 h fast. These data are consistent with other reports

illustrating that plasma alanine increases by 40% in adult participants 1 h after consuming a protein-rich meal (80), and 68% of galactose in milk is derived from plasma glucose during the postprandial state compared with 50% in the fasted state (34). Future studies will be aimed at using global metabolomics



**FIGURE 5** Comparison of metabolites that differentiate fasting ( $n = 12$ ) from fed ( $n = 16$ ) states: alanine (A) and galactose (B). Values are means  $\pm$  SEMs, \* $P < 0.01$ , \*\*\* $P < 0.0001$ .

to determine relations between specific analytes derived from the diet and excreted by the mammary gland.

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## Literature Cited

1. Le Huërou-Luron I, Blat S, Boudry G. Breast- v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects. *Nutr Res Rev*. 2010;23:23–36.
2. Brandtzaeg P. The mucosal immune system and its integration with the mammary glands. *J Pediatr*. 2010;156:S8–15.
3. Morrow AL, Ruiz-Palacios GM, Jiang X, Newburg DS. Human milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea. *J Nutr*. 2005;135:1304–7.
4. Lönnerdal B. Bioactive proteins in human milk: mechanisms of action. *J Pediatr*. 2010;156:S26–30.
5. Kunz C, Rudloff S, Baier W, Klein N, Strobel S. Oligosaccharides in human milk: structural, functional, and metabolic aspects. *Annu Rev Nutr*. 2000;20:699–722.
6. Cabrera-Rubio R, Collado MC, Laitinen K, Salminen S, Isolauri E, Mira A. The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *Am J Clin Nutr*. 2012;96:544–51.
7. Anderson SM, Rudolph MC. Secretory activation in the mammary gland: it's not just about milk protein synthesis. *Breast Cancer Res*. 2007;9:204–7.
8. Duffield TF, Lissemore KD, McBride BW, Leslie KE. Impact of hyperketonemia in early lactation dairy cows on health and production. *J Dairy Sci*. 2009;92:571–80.
9. Klein MS, Buttchereit N, Miemczyk SP, Immervoll A-K, Louis C, Wiedemann S, Junge W, Thaller G, Oefner PJ, Gronwald W. NMR metabolomic analysis of dairy cows reveals milk glycerophosphocholine to phosphocholine ratio as prognostic biomarker for risk of ketosis. *J Proteome Res*. 2012;11:1373–81.
10. Oriol R, Pendu J, Mollicone R. Genetics of ABO, H, Lewis, X and related antigens. *Vox Sang*. 1986;51:161–71.
11. De Leoz MLA, Gaerlan SC, Strum JS, Dimapasoc LM, Mirmiran M, Tancredi DJ, Smilowitz JT, Kalanetra KM, Mills DA, German JB. Lacto-N-tetraose, fucosylation, and secretor status are highly variable in human milk oligosaccharides from women delivering preterm. *J Proteome Res*. 2012;11:4662–72.
12. Thurl S, Munzert M, Henker J, Boehm G, Müller-Werner B, Jelinek J, Stahl B. Variation of human milk oligosaccharides in relation to milk groups and lactational periods. *Br J Nutr*. 2010;104:1261–71.
13. Totten SM, Zivkovic AM, Wu S, Ngyuen U, Freeman SL, Ruhaak LR, Darboe MK, German JB, Prentice AM, Lebrilla CB. Comprehensive profiles of human milk oligosaccharides yield highly sensitive and specific markers for determining secretor status in lactating mothers. *J Proteome Res*. 2012;11:6124–33.
14. Chaturvedi P, Warren CD, Altaye M, Morrow AL, Ruiz-Palacios G, Pickering LK, Newburg DS. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. *Glycobiology*. 2001;11:365–72.
15. Grollman EF, Ginsburg V. Correlation between secretor status and the occurrence of 2'-fucosyllactose in human milk. *Biochem Biophys Res Commun*. 1967;28:50–3.
16. Shen L, Grollman EF, Ginsburg V. An enzymatic basis for secretor status and blood group substance specificity in humans. *Proc Natl Acad Sci USA*. 1968;59:224–30.
17. Viverge D, Grimmonprez L, Cassanas G, Bardet L, Solere M. Discriminant carbohydrate components of human milk according to donor secretor types. *J Pediatr Gastroenterol Nutr*. 1990;11:365–70.
18. Newburg DS, Ruiz-Palacios GM, Altaye M, Chaturvedi P, Meinzen-Derr J, Guerrero Mde L, Morrow AL. Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. *Glycobiology*. 2004;14:253–63.
19. Morrow AL, Ruiz-Palacios GM, Altaye M, Jiang X, Guerrero ML, Meinzen-Derr JK, Farkas T, Chaturvedi P, Pickering LK, Newburg DS. Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants. *J Pediatr*. 2004;145:297–303.
20. Wacklin P, Mäkiuokko H, Alakulppi N, Nikkilä J, Tenkanen H, Rabinä J, Partanen J, Aranko K, Mättö J. Secretor genotype (FUT2 gene) is strongly associated with the composition of Bifidobacteria in the human intestine. *PLoS ONE*. 2011;6:e20113.
21. Parmar AS, Alakulppi N, Paaola-Sakki P, Kurppa K, Halme L, Farkkila M, Turunen U, Lappalainen M, Kontula K, Kaukinen K, et al. Association study of FUT2 (rs601338) with celiac disease and inflammatory bowel disease in the Finnish population. *Tissue Antigens*. 2012;80:488–93.
22. Jetté M, Sidney K, Blümchen G. Metabolic equivalents (METs) in exercise testing, exercise prescription, and evaluation of functional capacity. *Clin Cardiol*. 1990;13:555–65.
23. Gerrior S, Juan WY, Basiotis P. An easy approach to calculating estimated energy requirements. *Prev Chronic Dis*. 2006;3:A129–32.
24. Dewey KG, Heinig MJ, Nommsen LA. Maternal weight-loss patterns during prolonged lactation. *Am J Clin Nutr*. 1993;58:162–6.
25. Ferris AM, Jensen RG. Lipids in human milk: a review. 1: sampling, determination, and content. *J Pediatr Gastroenterol Nutr*. 1984;3:108–22.
26. Slupsky CM, Rankin KN, Wagner J, Fu H, Chang D, Weljie AM, Saude EJ, Lix B, Adamko DJ, Shah S, et al. Investigations of the effects of gender, diurnal variation, and age in human urinary metabolomic profiles. *Anal Chem*. 2007;79:6995–7004.
27. Weljie AM, Newton J, Mercier P, Carlson E, Slupsky CM. Targeted profiling: quantitative analysis of <sup>1</sup>H NMR metabolomics data. *Anal Chem*. 2006;78:4430–42.
28. Reichard GA Jr, Haff A, Skutches C, Paul P, Holroyde C, Owen O. Plasma acetone metabolism in the fasting human. *J Clin Invest*. 1979;63:619–26.
29. Graham TE, Sgro V, Friars D, Gibala M. Glutamate ingestion: the plasma and muscle free amino acid pools of resting humans. *Am J Physiol Endocrinol Metab*. 2000;278:E83–9.
30. Viña JR, Puertes IR, Rodriguez A, Saez GT, Viña J. Effect of fasting on amino acid metabolism by lactating mammary gland: studies in women and rats. *J Nutr*. 1987;117:533–8.
31. Bales JR, Bell JD, Nicholson JK, Sadler PJ. <sup>1</sup>H NMR studies of urine during fasting: excretion of ketone bodies and acetylcarnitine. *Magn Reson Med*. 1986;3:849–56.
32. Chaiyabutr N, Faulkner A, Peaker M. Changes in the concentrations of the minor constituents of goat's milk during starvation and on refeeding of the lactating animal and their relationship to mammary gland metabolism. *Br J Nutr*. 1981;45:149–57.
33. Lenz EM, Bright J, Wilson ID, Morgan SR, Nash AFP. A <sup>1</sup>H NMR-based metabolomic study of urine and plasma samples obtained from healthy human subjects. *J Pharm Biomed Anal*. 2003;33:1103–15.
34. Sunehag A, Tigas S, Haymond MW. Contribution of plasma galactose and glucose to milk lactose synthesis during galactose ingestion. *J Clin Endocrinol Metab*. 2003;88:225–9.
35. Rasmussen LG, Winning H, Savorani F, Toft H, Larsen TM, Dragsted LO, Astrup A, Engelsen SB. Assessment of the effect of high or low protein diet on the human urine metabolome as measured by NMR. *Nutrients*. 2012;4:112–31.
36. Bertram HC, Hoppe C, Petersen BO, Duus J, Molgaard C, Michaelsen KF. An NMR-based metabolomic investigation on effects of milk and meat protein diets given to 8-year-old boys. *Br J Nutr*. 2007;97:758–63.
37. Stella C, Beckwith-Hall B, Cloarec O, Holmes E, Lindon JC, Powell J, van der Ouderaa F, Bingham S, Cross AJ, Nicholson JK. Susceptibility of human metabolic phenotypes to dietary modulation. *J Proteome Res*. 2006;5:2780–8.
38. Rasmussen LG, Winning H, Savorani F, Ritz C, Engelsen S, Astrup A, Larsen T, Dragsted L. Assessment of dietary exposure related to dietary GI and fibre intake in a nutritional metabolomic study of human urine. *Genes Nutr*. 2012;7:281–93.
39. Xu J, Yang S, Cai S, Dong J, Li X, Chen Z. Identification of biochemical changes in lactovegetarian urine using <sup>1</sup>H NMR spectroscopy and pattern recognition. *Anal Bioanal Chem*. 2010;396:1451–63.

40. Walsh MC, Brennan L, Pujos-Guillot E, Sébédio J-L, Scalbert A, Fagan A, Higgins DG, Gibney MJ. Influence of acute phytochemical intake on human urinary metabolomic profiles. *Am J Clin Nutr.* 2007;86:1687–93.
41. Chorell E, Svensson MB, Moritz T, Antti H. Physical fitness level is reflected by alterations in the human plasma metabolome. *Mol Biosyst.* 2012;8:1187–96.
42. Psihogios NG, Gazi IF, Elisaf MS, Seferiadis KI, Bairaktari ET. Gender-related and age-related urinalysis of healthy subjects by NMR-based metabolomics. *NMR Biomed.* 2008;21:195–207.
43. Kochhar S, Jacobs DM, Ramadan Z, Berruex F, Fuerholz A, Fay LB. Probing gender-specific metabolism differences in humans by nuclear magnetic resonance-based metabolomics. *Anal Biochem.* 2006;352:274–81.
44. Holmes E, Loo RL, Stamler J, Bictash M, Yap IKS, Chan Q, Ebbels T, De Iorio M, Brown IJ, Veselkov KA, et al. Human metabolic phenotype diversity and its association with diet and blood pressure. *Nature.* 2008;453:396–400.
45. Krupp D, Doberstein N, Shi L, Remer T. Hippuric acid in 24-hour urine collections is a potential biomarker for fruit and vegetable consumption in healthy children and adolescents. *J Nutr.* 2012;142:1314–20.
46. O'Sullivan A, Gibney MJ, Brennan L. Dietary intake patterns are reflected in metabolomic profiles: potential role in dietary assessment studies. *Am J Clin Nutr.* 2011;93:314–21.
47. Rana SK, Sanders T. Taurine concentrations in the diet, plasma, urine and breast milk of vegans compared with omnivores. *Br J Nutr.* 1986;56:17–27.
48. Agostoni C, Carratù B, Boniglia C, Lammardo AM, Riva E, Sanzini E. Free glutamine and glutamic acid increase in human milk through a three-month lactation period. *J Pediatr Gastroenterol Nutr.* 2000;31:508–12.
49. Harzer G, Franzke V, Bindels JG. Human milk nonprotein nitrogen components: changing patterns of free amino acids and urea in the course of early lactation. *Am J Clin Nutr.* 1984;40:303–9.
50. Hoppe B, Roth B, Bauerfeld C, Langman CB. Oxalate, citrate, and sulfate concentration in human milk compared with formula preparations: influence on urinary anion excretion. *J Pediatr Gastroenterol Nutr.* 1998;27:383–6.
51. Coppa GV, Gabrielli O, Pierani P, Catassi C, Carlucci A, Giorgi PL. Changes in carbohydrate composition in human milk over 4 months of lactation. *Pediatrics.* 1993;91:637–41.
52. Hülsemann J, Manz F, Wember T, Schöch G. Administration of creatine and creatinine with breast milk and infant milk preparations. *Klin Padiatr.* 1987;199:292–5.
53. Ogasa K, Kuboyama M, Kiyosawa I, Suzuki T, Itoh M. The content of free and bound inositol in human and cow's milk. *J Nutr Sci Vitaminol (Tokyo).* 1975;21:129–35.
54. Neville MC, Keller RP, Seacat J, Casey CE, Allen JC, Archer P. Studies on human lactation. I. Within-feed and between-breast variation in selected components of human milk. *Am J Clin Nutr.* 1984;40:635–46.
55. Mahadevan S, Shah SL, Marrie TJ, Slupsky CM. Analysis of metabolomic data using support vector machines. *Anal Chem.* 2008;80:7562–70.
56. McNiven EMS, German JB, Slupsky CM. Analytical metabolomics: nutritional opportunities for personalized health. *J Nutr Biochem.* 2011;22:995–1002.
57. Marincola FC, Noto A, Caboni P, Reali A, Barberini L, Lussu M, Murgia F, Santoru ML, Atzori L, Fanos V. A metabolomic study of preterm human and formula milk by high resolution NMR and GC/MS analysis: preliminary results. *J Matern Fetal Neonatal Med.* 2012;25:62–7.
58. O'Sullivan A, He X, McNiven EMS, Hinde K, Haggarty NW, Lönnerdal B, Slupsky CM. Metabolomic phenotyping validates the infant rhesus monkey as a model of human infant metabolism. *J Pediatr Gastroenterol Nutr.* 2013;56:355–63.
59. Allen JC, Keller RP, Archer P, Neville MC. Studies in human lactation: milk composition and daily secretion rates of macronutrients in the first year of lactation. *Am J Clin Nutr.* 1991;54:69–80.
60. Mitoulas LR, Kent JC, Cox DB, Owens RA, Sherriff JL, Hartmann PE. Variation in fat, lactose and protein in human milk over 24h and throughout the first year of lactation. *Br J Nutr.* 2002;88:29–37.
61. Bauer J, Gerss J. Longitudinal analysis of macronutrients and minerals in human milk produced by mothers of preterm infants. *Clin Nutr.* 2011;30:215–20.
62. Kent JC, Mitoulas LR, Cregan MD, Ramsay DT, Doherty DA, Hartmann PE. Volume and frequency of breastfeedings and fat content of breast milk throughout the day. *Pediatrics.* 2006;117:e387–95.
63. Khan S, Prime DK, Hepworth AR, Lai CT, Trengove NJ, Hartmann PE. Investigation of short-term variations in term breast milk composition during repeated breast expression sessions. *J Hum Lact.* 2013;29:196–204.
64. Hinde K, German J. Food in an evolutionary context: insights from mother's milk. *J Sci Food Agric.* 2012;92:2219–23.
65. Neville MC. The physiological basis of milk secretion. *Ann N Y Acad Sci.* 1990;586:1–11.
66. Beck FX, Schmolke M, Guder WG. Osmolytes. *Curr Opin Nephrol Hypertens.* 1992;1:43–52.
67. Holub BJ. Metabolism and function of *myo*-inositol and inositol phospholipids. *Annu Rev Nutr.* 1986;6:563–97.
68. Hallman M, Bry K, Hoppu K, Lappi M, Pohjavuori M. Inositol supplementation in premature infants with respiratory distress syndrome. *N Engl J Med.* 1992;326:1233–9.
69. Mephram TB, Linzell J. Urea formation by the lactating goat mammary gland. *Nature.* 1967;214:507–8.
70. Metges CC. Contribution of microbial amino acids to amino acid homeostasis of the host. *J Nutr.* 2000;130 Suppl:1857S–64S.
71. Metges CC, Petzke KJ, El-Khoury AE, Henneman L, Grant I, Bedri S, Regan MM, Fuller MF, Young VR. Incorporation of urea and ammonia nitrogen into ileal and fecal microbial proteins and plasma free amino acids in normal men and ileostomates. *Am J Clin Nutr.* 1999;70:1046–58.
72. Li X, Min X, Xiao J, Kawasaki K, Ohta N, Sakaguchi E. Utilization of dietary urea nitrogen is stimulated by D-mannitol feeding in rabbits. *Anim Sci J.* 2012;83:605–9.
73. De Preter V, Vanhoutte T, Huys G, Swings J, Rutgeerts P, Verbeke K. Effect of lactulose and *Saccharomyces boulardii* administration on the colonic urea-nitrogen metabolism and the bifidobacteria concentration in healthy human subjects. *Aliment Pharmacol Ther.* 2006;23:963–74.
74. Sela DA, Chapman J, Adeuya A, Kim JH, Chen F, Whitehead TR, Lapidus A, Rokhsar DS, Lebrilla CB, German JB, et al. The genome sequence of *Bifidobacterium longum subsp. infantis* reveals adaptations for milk utilization within the infant microbiome. *Proc Natl Acad Sci USA.* 2008;105:18964–9.
75. Rassin DK, Gaull GE, Heinonen K, Rähä NC. Milk protein quantity and quality in low-birth-weight infants: II. Effects on selected aliphatic amino acids in plasma and urine. *Pediatrics.* 1977;59:407–22.
76. Burrin DG, Stoll B. Metabolic fate and function of dietary glutamate in the gut. *Am J Clin Nutr.* 2009;90 Suppl:850S–6S.
77. Brosnan JT, Brosnan ME. Branched-chain amino acids: enzyme and substrate regulation. *J Nutr.* 2006;136 Suppl:207S–11S.
78. Wiederschain GY, Newburg DS. Human milk fucosyltransferase and  $\alpha$ -L-fucosidase activities change during the course of lactation. *J Nutr Biochem.* 1995;6:582–7.
79. Wiederschain GY, Newburg DS. Glycoconjugate stability in human milk: glycosidase activities and sugar release. *J Nutr Biochem.* 2001;12:559–64.
80. Adibi SA, Mercer DW. Protein digestion in human intestine as reflected in luminal, mucosal, and plasma amino acid concentrations after meals. *J Clin Invest.* 1973;52:1586–94.