Influence of sulfonated and diet-derived human milk oligosaccharides on the infant microbiome and immune markers

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ABSTRACT

That human milk oligosaccharides (HMOs) promote the development of the neonatal intestinal, immune, and nervous systems has recently received considerable attention. Here, we investigated how the maternal diet affects HMO biosynthesis and how any diet-induced HMO alterations influence the infant gut microbiome and immunity. Using capillary electrophoresis (CE) and MS-based analyses, we extracted and measured HMOs from breast milk samples and then correlated their levels with results from validated 24 h diet-recall surveys and breast milk fatty acids. We found that fruit intake and unsaturated fatty acids in breast milk were positively correlated with an increased absolute abundance of numerous HMOs, including 16 sulfonated HMOs we identified here in humans for the first time. The diet-derived monosaccharide 5-N-glycolyl-neuraminic acid (Neu5Gc) was unambiguously detected in all samples. To gain insights into the potential impact of Neu5Gc on the infant microbiome, we used a constrained-ordination approach and identified correlations between Neu5Gc levels and Bacteroides spp. in infant stool. However, Neu5Gc was not associated with marked changes to infant immune markers, in contrast with the sulfonated HMOs, whose expression correlated with the suppression of two major Th2 cytokines, interleukin-10 (IL-10) and IL-13. The findings of our work highlight the importance of maternal diet for HMO biosynthesis and provide as yet unexplored targets for future studies investigating interactions between HMOs and the intestinal microbiome and immunity in infants.

The most abundant solid component of human milk is lactose, followed by lipids, free human milk oligosaccharides (HMOs), and glycoproteins. To date, over 200 HMOs have been identified in human milk (1) and a typical infant will consume several grams per day. HMOs, in contrast with lactose, are indigestible by infants (2–4), but they are often readily digested by bacteria present in the large intestine and colon, provided they express the requisite glycolytic enzymes (5, 6). By providing these microbes with a nutrient source HMOs thus actively promote the colonization of the neonatal gastrointestinal (GI) tract (7–11). Since HMOs are often chemically similar to the protective mucus layer lining the GI tract, they may also serve as decoys for pathogenic bacteria, mimicking the glyco-epitopes to which pathogens prefer to bind (12). Additionally, some HMOs directly impact immune signalling events (13, 14), which, in light of the fact that they have been shown to enter the blood stream(15), is suggestive of systemic immune functions (16). Identifying the principle bio-active HMOs and
defining their health-promoting effects is imperative given the clear links between these compounds and the proper establishment of the infant microbiome, alterations in which are associated with numerous immunological disorders or infectious diseases (17, 18).

HMOs vary significantly among women (19) with genetic factors affecting the expression of the glycosyltransferases (GTs) required for HMO biosynthesis being an important source of this variance. HMOs are biosynthesized by a non-template-directed process in which the numerous GTs expressed within the cells of the mammary gland elongate lactose with other monosaccharides including galactose (Gal), fucose (Fuc), N-acetylgalactosamine (GlcNAc) and 5-N-acetylenuraminic acid (Neu5Ac). The best known genetic sources of HMO variance is a woman’s Lewis blood group (Le) and secretor (Se) status which are dictated by polymorphisms α1,4 and α1,2-fucosyltransferase genes, respectively (19–21). This variation is correlated with pediatric infectious disease susceptibility (22), and gut microbiome diversity (23).

A recent international study observed that HMOs from ethnically similar mothers varied geographically, suggesting an environmental and/or dietary influence on HMO biosynthesis (24). Obesity (21), malnourishment (7) and hyperglycemia (21) impact both HMO concentrations and structures; it is possible that these conditions affect the levels of nucleotide-activated GlcNAc and Neu5Ac available for HMO biosynthesis as obesity and hyperglycemia are both linked to altered metabolic flux through the hexosamine biosynthetic pathway (25). The levels of Gal and Fuc in the maternal diet may likewise influence the levels of these monosaccharides incorporated into HMOs as there is evidence that both Gal (26) and Fuc (27) may be directly recycled by specific monosaccharide-salvage pathways present in mammalian cells. The incorporation of diet-derived Neu5Ac into new glycoconjugates has been well-established. Dietary Neu5Ac is primarily observed glycosidically-bound to other biomolecules but it must be released into its free form by neuraminidases expressed by the GI mucosa or microbes before incorporation into infant tissue (28). Among the clearest evidence for the salvaging of dietary Neu5Ac in vivo comes from studies on the structurally-related analogue 5-N-glycolyl-neuraminic acid (Neu5Gc). Humans are unable to biosynthesize Neu5Gc, instead obtaining it from the diet, red meats and dairy products being particularly rich sources; however, human metabolic enzymes do not differentiate between Neu5Gc or Neu5Ac in vitro (29) or in vivo (30). Experiments with mice unable to biosynthesize Neu5Gc have demonstrated that diet-derived Neu5Gc that is glycosidically-bound is more bioavailable than free Neu5Gc (31).

To date Neu5Gc incorporation into HMOs has not been demonstrated, although it has been detected in a single sample of whole, pooled human milk (32). Establishing the capacity for diet-derived Neu5Gc to be incorporated into newly biosynthesized HMOs is relevant to infant health since the human immune system is able to distinguish between Neu5Ac and Neu5Gc-containing glycans, specifically recognizing the latter as foreign (33). Experiments in rodents have led to the proposal that the anti-Neu5Gc antibodies, appearing in human neonates around six months after birth (34), are linked to cancer (35), atherosclerosis (36), and autoimmune disease (37).

Recently, we tested the potential influence of diet on milk oligosaccharide biosynthesis in dairy cattle (38). Cow milk shares some oligosaccharides in common with human milk, although the concentration and variety of those observed in cow milk are lower than that of human milk. This research has led to the identification of nine previously undescribed sulfonated milk oligosaccharides in bovine milk; these sulfonated glyco-epitopes chemically resemble both the O-glycans lining the infant GI tract (39) and the ligands for cell adhesion molecules borne by immune cells (40). Accordingly, the research presented herein sought to address three questions. Does the human diet impact the biosynthesis of HMOs? Are sulfonated HMOs present in human milk? If so, do these have an observable prebiotic effect in exclusively breast-fed infants? Our observations indicate that all three questions are answered in the affirmative.

RESULTS

Non-human monosaccharides are present in HMOs but are not associated
specifically with dairy intake - Red meat, (bovine) milk and dairy products represent the most prevalent dietary sources of the non-human sialic acid Neu5Gc in the human diet (30, 41). It was hypothesized that in women, milk and dairy products would be the major dietary source of Neu5Gc and that this monosaccharide would consequently be observed at elevated levels in the HMOs biosynthesized by women consuming cow’s milk rather than alternative, almond-based beverages. Accordingly, breastmilk samples from two groups of women (n=8 for each)—classified as milk- or almond beverage-consumers as self-assessed on pre-study questionnaires—were selected from a larger cohort of samples obtained from donors who had completed a detailed diet-recall survey for the 24 hour period preceding milk collection. Close analysis of participants’ diets post-group selection revealed that although not all the women in the almond group refrained entirely from dairy products, these groups were different in their mean intake of milk (0.2 ± 0.06 vs. 1.2 ± 0.45 food group equivalents (FTE); p = 0.028, one-tailed Student’s t-test), and total dairy products (0.8 ± 0.32 vs. 2.3 ± 0.39 FTE; p = 0.005). Based on the diet recalls, estimated levels of ingested Neu5Ac and Neu5Gc were predicted for each participant (Fig. 1a and Sup. Table S1) by multiplying the FTE (i.e. mass) of each Neu5Ac or Neu5Gc-source by their putative abundance in common sources as previously evaluated (30, 41). Overall, the estimated amounts of ingested Neu5Ac and Neu5Gc were similar in the milk and almond-consuming groups, however milk-consumers obtained a greater fraction of Neu5Gc from dairy products than almond-beverage consumers (Fig. 1a). Across both groups the ratio of ingested Neu5Gc-to-Neu5Ac remained essentially constant. The actual levels of Neu5Ac and Neu5Gc glycosidically-bound to HMOs were quantitated (42) in all samples (Fig. 1a). Intriguingly, low amounts of Neu5Gc were detected in all (n=16) samples tested, representing, to our knowledge, the first conclusive report of the presence of this diet-derived monosaccharide on HMOs. However, despite estimated amounts of Neu5Gc being higher in dairy-consumers, their levels in HMOs were unchanged relative to the almond-group and thus not associated specifically with dairy intake.

Differences in the levels of HMO-bound Neu5Gc or Neu5Ac between women in the milk- and almond beverage-consuming groups may have been obscured by genetic variability with respect to the Se status of individuals. Accordingly, monosaccharide levels, including Neu5Ac and Neu5Gc, were compared (Fig. 1b) between Se+ or Se- individuals, groups that were defined on the basis of relative HMO levels as described below. However, Neu5Gc and Neu5Ac levels in HMOs did not significantly vary with secretor type. When considering a subset of Se+ samples only, it was observed that HMOs from women in the milk-consuming group (n = 5) contained essentially equivalent amounts of Neu5Gc as was observed for almond-beverage consumers (n = 3), although the former did contain higher average levels of Neu5Ac (p = 0.055; two-tailed, Student’s t-test). Due to the human inability to biosynthesize Neu5Gc, its presence in human milk is clear evidence of a direct dietary influence on HMO biosynthesis (Sup. Fig. S1). Spearman rank correlations were therefore determined in order to uncover potential associations between estimated levels of ingested Neu5Gc (from all possible sources) and the concentration of HMO-bound Neu5Gc in human milk (Fig. 1c). A positive, but not significant, association was observed between ingested and observed (on HMO) Neu5Gc levels. Interestingly, the opposite trend emerged for Neu5Ac where a negative correlation was observed between the estimated levels of ingested Neu5Ac and the abundance Gal in HMOs. Overall, these results reveal that while Neu5Gc in HMOs is dependent on dietary intake, a clear correlation between estimated dietary intake within the previous 24 h and HMO-prevalence was not apparent.

Elucidation of secretor status and discovery of sulfonated HMOs by CE – We (38), and others (43), have previously used capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection to rapidly profile milk oligosaccharides. CE-LIF analysis of HMOs extracted from our 16 samples permitted up to 93 (median = 87) different oligosaccharides to be distinguished within ten min (Figure 2), 21 of which could be identified on the basis of their co-migration with HMO standards (Sup. Fig. S2). Both the absolute and relative peak areas for 70
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HMOs present in the majority of samples were recorded (Sup. Table S2). Traces of lactose (Fig. 2, HMO17) remained in all samples, accounting for, on average, 13 ± 6% of the total integrated peak area; this peak was excluded in the determination of relative HMO peak areas.

The relative abundance of several HMOs, including 2′-fucosyllactose (2′FL), lactodifucotetraose (LDFT), and lacto-N-fucopentaose I (LNFP1), has been demonstrated to accurately establish an individual’s Se status (44). Determination, by CE, of the median levels of 2′FL, and LDFT, in the 16 samples in the present study permitted them to be clearly differentiated into Se+ (n = 12) and Se− (n = 4) groups (Fig. 3a). Semi-quantitative analysis of the same HMOs by HPLC-MS also classified samples into the same secretor groups as predicted by CE (Sup. Fig. 3). Note that by CE, 2′FL and 3FL could not be clearly resolved, however, HPLC-MS could distinguish between these HMOs, demonstrating that among samples identified as Se+ by CE, the median levels of 2′FL were nearly 300-fold higher than 3FL; in contrast, Se− samples contained median levels of 3FL over four times above those observed for 2′FL. Mean absolute concentrations for 11 selected HMOs were determined by CE-LIF, yielding values that were of comparable magnitude and precision to those previously reported by other groups (Sup. Table 3).

We have previously documented the presence of nine sulfonated and two phosphorylated milk oligosaccharides in bovine milk, the availability of a 3′-sulfolactose (3′SO4L) standard permitting a level one identification (45) of this oligosaccharide by both CE-LIF and HPLC-MS. CE-LIF also permitted the detection and relative quantitation of 3′SO4L (Fig. 2, HMO1) in every human milk sample tested (Sup. Table S2); relative levels of 3′SO4L did not significantly differ between Se+ and Se− samples, although they tended to be higher in the latter (Fig. 3a). As previously observed with bovine milk samples, the CE analysis of human milk revealed the presence of 13 HMOs of higher electrophoretic mobility than the well-known anionic HMOs 3′ or 6′-sialyllactose (3′/6′-SL; Fig. 2, HMO15 and 14, respectively). These HMOs have been putatively identified as sulfonated or phosphorylated milk oligosaccharides on the basis of their sensitivity to chemical or enzyme-catalyzed hydrolys; seven of these HMOs (HMO1-6, and 10) possessed identical electrophoretic mobilities to oligosaccharides previously observed in bovine milk, while one (HMO9) appeared to be unique to human milk (Sup. Fig. S4). Among these anionic HMOs median levels of only two differed significantly between the Se+ and Se− groups as assessed by CE (Fig. 3b): HMO4 was elevated in Se+ samples while, in contrast, HMO8 was non-detectable. HMO 3, 9-11 all tended to have higher median levels in Se− individuals; indeed, when considered as a group (i.e. HMOs 1-13), these anionic HMOs comprised a significantly greater fraction of the total HMO pool than in Se+ individuals (median levels 15.7 and 7.1%, respectively). None of these HMOs (1-13) differed significantly between the almond- or milk-consuming groups.

HMOs were also analyzed by HPLC-MS to further confirm the presence of a class of previously uncharacterized sulfonated and/or phosphorylated HMOs. Monoisotopic masses of 16 unique HMOs were detected in our set of human milk samples (Sup. Fig. S5 and S6); for each secretor type, median levels were determined relative to a common internal standard (ISTD; Fig. 3c)). To our knowledge, there has been only one previous report of the existence of sulfonated HMOs in which structures consistent with monoisotopic m/z values for xii – xv (Fig. 3c) were identified in a sample extracted from 20 liters of pooled human milk (46). We have observed equivalent oligosaccharides to HMOs i – vi, and S′SO4L, in bovine milk (Table S4), i.e. structures matching both HPLC retention times and monoisotopic mass (38).

To further establish the identities of putative sulfonated HMOs in the absence of authentic standards, several neutral HMOs were tested as substrates for recombinant human galactose-3-O-sulfotransferase 2 (GAL3ST2) and carbohydate sulfotransferase 1 (CHST1). GAL3ST2 and CHST1 have been previously demonstrated to transfer sulfate residues to lactose (47) and sialyllactosamine-containing oligosaccharides (48), respectively. Both CE and HPLC-MS analyses reveal that these enzymes were capable of producing a range of sulfonated
HMO analogues from lactose and 2'-FL (Fig. 4). More specifically, both enzymes produced at least four products from lactose with CE mobilities identical to HMO1, 3, 4 and 5; similarly, 2'-FL yielded products of identical mobilities to HMOs 8–10. HPLC-MS analyses of these sulfotransferase mixtures similarly indicated that lactose and 2'-FL were converted into four and six distinct sulfonated products, respectively; for each substrate, only two products were detectible in human milk samples by HPLC-MS (Fig. 3c). Tandem MS analyses (Sup. Fig. S7) demonstrated that in vitro, sulfotransferases were able to transfer a sulfonate moiety to both the reducing and non-reducing ends of lactose and 2’FL. These data support the existence of a large, previously unrecognized, class of at least 16 (Fig. 3c) sulfonated HMOs in human milk, at least 13 of which (Fig. 2, HMOs1-13) were quantifiable by CE.

**Correlative analysis between HMO levels, maternal diet and breast milk fatty acids** - Spearman’s rank correlation coefficients were determined in order to assess correlations between the maternal diet and the absolute CE detector responses of HMOs, of particular interest being the anionic, putative sulfonated HMOs (1–13) with electrophoretic mobilities exceeding that of 6’S’L (Fig. 5 and Sup. Fig. S8). Only Se+ individuals were assessed in order to eliminate the major source of genetic HMO variability. Several correlations were observed between this group of HMOs and breast milk fatty acids which are known to directly reflect their levels in the maternal diet (49). First, significant negative correlations were observed between HMO1, 2, 5, 10 and 13 and the total saturated fats quantitated in breast milk. HMO1 and HMO12 negatively correlated with breast milk docosanoic acid (C12:0), while HMO2 negatively correlated with tridecyl acid (C13:0). HMO8 negatively correlated with pentadecyl acid (C15:0), HMO10 negatively correlated with stearic acid (C18:0), and HMO13 negatively correlated with docosanoic acid, myristic acid (C14:0), and stearic acid. No notable correlations were observed between HMOs1-13 and capric (C10:0), undecylic (C11:0) and palmitic (C16:0) acid. Interestingly, unsaturated fats showed opposite correlations. Total monounsaturated fats in breast milk positively correlated with HMOs1, 6 and 7. Oleic acid (C18:1) was positively associated with HMO1, and HMO4 whereas gondoic acid (C20:1) correlated with HMO2, HMO7 and HMO8. Total polyunsaturated fatty acids and linoleic acid (C18:2 n-6) positively correlated with HMO13, whereas alpha linolenic acid (C18:3 n3) correlated with Fuc, Gal, HMO2, 7 and 11. Arachidonic acid positively correlated with Neu5Ga. Docosahexanoic acid (C22:6 n-3) was the only unsaturated fat which negatively correlated with HMO11. Dietary cholesterol levels, as assessed from 24 hour recalls, appeared to negatively influence the levels of all 13 sulfonated/phosphorylated HMOs and, when the 70 quantitated HMOs are considered, significant inverse correlations between cholesterol and 18 different HMOs were observed (Sup. Fig. S8).

Ingested carbohydrates, including both simple sugars and dietary fiber, were observed to significantly correlate positively with relative levels of both Gal and Fuc present in HMOs to the decrement of Neu5Ac (total carbohydrates) and Neu5Ga (total sugars; Fig. 5). Similar correlations were observed for the total amount of ingested fruits, a major dietary source of both sugars and fiber, the latter of which is a known source of Fuc (Sup. Fig. S8); specifically, significant positive correlations were observed for Gal and Fuc while Neu5Ac levels were significantly lower in HMOs biosynthesized by women consuming high amounts of fruit. Total fruit intake was positively correlated with the absolute levels of 15 different HMOs (including putative sulfonated HMOs 1, 4, 5 and 10). Similar correlations were not observed for any other food group, with the exception of cured meats which was negatively correlated with the abundance of 22 HMOs. Finally, it was hypothesized that Neu5Ga levels would be highest in milk samples from women who had consumed the largest amounts of red meats and dairy products. In contrast the data indicate that only cheese intake positively correlated with Neu5Ga levels. Overall, these results suggest that in Se+ women unsaturated fat- and fruit-derived dietary fiber are correlated with increases in multiple HMOs, while the opposite trend was observed for saturated fat and cholesterol. With the exception of cheese intake, no positive associations were observed between known Neu5Ga sources and its levels in HMOs, suggesting that class of
biomolecule (*i.e.* glycolipid vs. glycoprotein) might influence Neu5Gc bio-availability.

**Secretor status and HMOs explain overall bacterial community composition in infants** - The redundancy analysis (RDA) ordination with a Monte Carlo permutation test indicated that Se status as defined by levels of 2′FL, LDFT and LNFP1(44) (Fig. 3a), and levels of Fuc, Gal and Neu5Gc were marginally (MCPP \( p = 0.05 \)) related to infant gut microbial composition (Fig. 6). Stringent adjustments for multiple testing revealed *Escherichia* to be the only significant genera associated with maternal HMOs among the 43 genera included. Without adjusting for multiple testing, several taxa exhibited a positive association. While acknowledging the increased potential for type-1 errors, these taxa included: *Bacteroides*, *Escherichia*, *Lactobacillus*, *Enterobacteriaceae*, *Erysipelotrichaceae*, and *Veillonella* spp. Overall, ~20 percent of the total variation in the microbial community could be explained by the selected HMOs.

Our results indicate that the abundances of *Enterobacteriaceae* were associated with maternal Se status whereas *Bacteroides* spp. correlated with HMOs bearing more Neu5Gc and Fuc. Fuc likewise positively correlated with *Escherichia* abundances, while Gal positively correlated with *Lactobacillus* spp along RDA2. Other genera that aligned with RDA2 but were not correlated with the selected predictors included *Viellonella* and *Erysipelotrichaceae* spp. These results suggest that HMO type in breast milk can predict the type of microbes harboring in the infant gut.

**HMOs correlate with distinct immune-markers** - The absolute levels of HMOs correlated with distinct immune markers expressed in breast milk and infant stool. The Spearman rank correlation heatmap of breast milk (Fig. 7) depicts a positive association \( (P <0.05) \) between HMO9 with the pro-inflammatory cytokine, tumor necrosis factor alpha (TNF-\( \alpha \)) and the chemokine, interleukin (IL)-8. 6′SL associated with pro-inflammatory cytokine IL-6 whereas HMO2 was negatively associated with IL-13, a major anti-inflammatory cytokine. In contrast, Neu5Ac negatively correlated with the pleotropic cytokine, IL-6, and the chemokines, myocyte chemoattractant protein-1 (MCP-1) and IL-8. Neu5Gc negatively correlated with the granulocyte-macrophage colony stimulating factor (GM-CSF). Finally, Gal levels exhibited a negative association with pro-inflammatory cytokines IL-2 and IL-12.

In infant stool, there was a negative association between absolute abundances of HMO1, 3, 5, 6′SL and galactose with the expression of anti-inflammatory cytokine IL-10. Similarly, HMO1, 3, 6′SL and galactose were all negatively associated with anti-inflammatory IL-13 in infant stool. Uniquely, there was a positive association observed between galactose and stool secretory immunoglobulin-A (sIgA). Following a similar trend to the other sulfonated/phosphorylated HMOs, HMO6 negatively correlated with IL-6 whereas HMO9 negatively correlated with the pro-inflammatory cytokine IFN-\( \gamma \). In stark contrast, 2′FL, sialyllacto-N-tetraose c (LSTc) and LNFP1 were associated with higher expression of pro-inflammatory cytokines in infant stool. 2′FL, LSTc and LNFP1 all positively correlated with GM-CSF expression. Similarly, 2′FL, LSTc, LNFP1 and also fucose positively associated with pro-inflammatory IL-12. Likewise, 2′FL, LSTc, LNFP1, and fucose were associated with IL-17a expression. These results suggest that HMO type in breast milk is associated with the overall immune status of the infant.

**DISCUSSION**

The neonatal period represents a critical time in mammalian life, particularly with respect to nutrition and development of the intestinal microbiome. The intestinal microbiome is a diverse microbial community composed of 500-1,000 different species that lie at the interface of the intestinal and the external environment, forming a relationship between the host epithelial cells and dietary antigens. Collectively, these microbes provide a multitude of benefits to their mammalian host including nutrient absorption, immune system maturation, and pathogen defense. However recent evidence suggests that any disturbance in the host-microbiota relationship, termed dysbiosis, is associated with diseases such as inflammatory bowel disease (IBD), metabolic syndrome, obesity and diabetes. Consequently, there has been an increase in research aimed at manipulating the intestinal microbiota in an attempt to promote health and
prevent dysbiosis-associated diseases. Of all the exogenous factors implicated in structuring the phylogenetic makeup of the gut microbiota, diet is the most extensively studied. Yet, this area of research remains to be fully elucidated.

Breast milk is considered the ‘gold standard’ of infant nutrition and is known to provide bioactive components which help infants develop their immune system and microbiome. Of these bioactive components, HMOs are thought to play a vital role in nourishing the developing infant gut microbiome. Despite the high abundance and diversity of HMOs, the structure and function of individual HMOs remain unclear as well as factors which influence their biosynthesis.

Here, CE-LIF analysis was used to detect a median of 87 distinct HMOs (n = 16 samples) within ten minutes (Fig. 2). The presence of 13 HMOs of electrophoretic mobility in excess of that of 6'SL (HMO14) suggested that human milk, like cow milk (38), contains small, highly charged HMOs consistent with the presence of either phosphate- or sulfate-bearing oligosaccharides. Co-migration of a CE-LIF peak with a 3'-sulfo-lactose standard in all 16 samples, and HPLC-MS evidence for 16 unique oligosaccharides bearing sulfate and/or phosphate groups (Fig. 3c and 4b) provides further evidence for a large and, with a sole exception (46), previously undescribed class of sulfate-bearing HMOs. The abundance of sulfonated HMOs in human milk appears to be due to the ability of human sulfo-transferases to produce multiple products from lactose and/or other neutral HMOs (Fig. 4). The sulfonation of mucus-linked oligosaccharides in the colon has been shown to be correlated with a decrease in susceptibility to parasitic infections (50) and the inflammation associated with human inflammatory bowel disease or colon cancer (51).

Since the sulfonated HMOs identified herein in many instances (Sup. Fig. S7) bear the sulfate moiety on a non-reducing hexose, i.e. galactose, residue—the predominant site of oligosaccharide sulfonation in the human colon (52)—we hypothesize that these unique HMOs fill a similar protective role in maintaining colon homeostasis. The non-sulfonated HMOs such as 2'FL, LSTc, LNP1 associated with higher Th1 and Th17 immune mediators in infant stool, whereas the sulfonated HMOs correlated with the suppression of two major Th2 cytokines, IL-10 and IL-13. Considering this in addition to the correlation of HMOs to Enterobacteriaceae, a microbe that drives Th1 immune responses in the gut, this study highly suggests that both sulfonated and non-sulfonated HMOs are stimulating Th1 immunity in the infant gut, but through unique immune mediators. Curiously this was in the absence of strong immune stimulation in the breast milk suggesting this is a mild or specific effect in the naive infant gut. Given that the infant immune system is under development during the post-natal period, we propose the HMOs are important modulators in training of the infant immune system from moving away from a Th2 dominance through Th1 stimulation. HMO ingestion by the infant thus has wide reaching consequences for protection against infectious disease susceptibility as well as chronic immune conditions including allergies. As discussed by Xu and Knight (53), a challenge in understanding the dietary effects on the gut microbiota is the confounding variations in host genotypes. Thus, here we used the relative abundances of 2'FL, LDFT, and LNFP1, to determine and account for maternal Se status. In this study, maternal genetics (as assessed by HMO analysis) had modest effects on the infant fecal microbiome. For example, Se status was associated with Enterobacteriaceae in infants, suggesting a defining role for genetics in the establishment of early colonizers in infants. While this manuscript focusses on HMOs, it should be noted that other components in breastmilk may also directly or indirectly influence the microbiome. For instance, Hill et al. (54) have shown that low molecular weight hyaluronan reported in human milk is able to alter expression of beta-defensins in the gastrointestinal tract, which could conceivably alter gut microbial communities. Thus, future studies should seek to isolate the HMOs identified here for mechanistic studies.

In addition to genetic factors, maternal dietary intake during lactation appears to influence the community composition of the infant microbiome. Long-term dietary intake of animal protein and saturated fats are associated with high abundances of Bacteroides taxa (55). Red meats, such as beef, pork and lamb contain considerable amounts of Neu5Gc which has
Recently been shown to incorporate into tissues. We show for the first time that dietary-derived Neu5Gc is also incorporated into HMOs. To facilitate insights into the potential impact of Neu5Gc on the infant microbiome, an RDA was used to understand assemblage composition differences in relation to predictors of interest. The RDA indicated that Neu5Gc correlates with Bacteroides spp. abundances in infant stool. However, Bacteroides are known for their ability to metabolize a variety of HMOs (56) and therefore this observation should be tested in a controlled setting. Alongside Neu5Gc, our study shows that fucose levels in breast milk are associated with Bacteroides spp. and Escherichia spp. in infant stool. These findings align with previous literature which show that some bacteria of the Bacteroides family, such as B. thetaiotaomicron, produce multiple fucosidases that cleave fucose from host glycans, resulting in free fucose available in the intestinal lumen (57). Certain species of Escherichia carry a fucose-sensing signal transduction system which can sense the monosaccharide and use it to their competitive advantage (58), suggesting a rationale for the association observed between Bacteroides and Escherichia spp. and dietary fucose. Finally, our results show that Lactobacillus spp. in infant stool correlated with the total galactose concentration in human milk. This is unexpected given most Lactobacillus spp. do not grow well on HMOs and do not typically utilize them as a carbon source (59). Rather, Lactobacillus spp. tend to metabolize the glucose moiety of lactose and release galactose and lactic acid into their environment.

Given that the gut microbiome and immune responses are tightly interrelated, this study also sought to generate hypotheses with respect to HMO-immune associations. We observed a distinct separation between the sulfonated/phosphorylated HMOs from 2’FL, LSTc, LnNT and LNFP1 in infant stool which, similar to the bacterial associations, should be investigated further.

Overall, the findings from this exploratory study suggest that maternal diet affects the biosynthesis of HMOs and in turn, may influence their infants’ bacteriome and immune development. However, interactions between host genetics, diet and the gut microbiota are convoluted and-multifaceted (53), and the effect size in this analysis is small compared with the huge variability intrinsic in human data. As such, we acknowledge that large-scale studies with greater statistical power are required to reveal definite contributions of maternal diet on their infant’s gut microbiome. Future studies may benefit in using the HMO-microbe-immune associations presented here as a possible a priori interest.

**EXPERIMENTAL PROCEDURES**

**Study design**

This study was conducted according to the Declaration of Helsinki guidelines and all procedures were approved by the UBC Clinical Research Ethics Board and BC Interior Health Ethics Board. Informed, signed consent was obtained from each participant at enrolment.

A prospective cohort clinical study was conducted in the Okanagan Valley, Canada. The full study design has been previously published elsewhere (60). Prior to enrolment, participants were screened to include healthy exclusively breast-feeding mother-infant pairs (self-reported). Participants were excluded if the mother or infant had a gastrointestinal disorder or if the infant was born prematurely. In total, 109 women-infant pairs were recruited. Of these, women who exclusively drank dairy milk were selected for this study (n=8) and matched with women who did not drink dairy (n=8) to encompass a range in maternal dietary patterns, while limiting other covariates such as maternal age, education and socioeconomic status.

**Stool and breast milk sample collection**

The method for infant stool collection, storing and processing has been described previously (61). For this study, only samples collected at five months of age were utilized. For collecting breast milk samples, mothers were instructed to clean the nipple and surrounding area with warm water and soap, and to manually express a few drops of milk before collecting 10 mL of foremilk in the morning of infant stool collection. Participants were given a sterile collection kit and instructed to store the breast milk and stool samples in their home freezer for up to a maximum of three days. Samples were transported to the research facility on dry ice and stored in -80 °C until further analysis.

**HMO extraction and analyses**
HMOs were extracted from milk samples essentially as previously described (38). In brief, milk samples were thawed at 4 °C and a 500 μL aliquot was fortified with 40 μg maltoheptaose (Sigma) as an internal standard (ISTD). HMOs were subsequently removed from the proteins and lipids by liquid-liquid extraction and dried before salts and the majority of lactose were removed by solid-phase extraction (SPE) using graphitized carbon cartridges (ENVI-Carb; Sigma). HMO-containing fractions were re-dissolved in 18 MΩcm⁻¹ water, and divided into five equal portions for: (a) quantitation of total reducing sugars (62), (b) quantitation of Neu5Ac and Neu5Ac (42), (c) neutral monosaccharide analysis (38), (d) HMO profiling by CE-LIF, and (e) targeted HMO analysis by HPLC-MS (38). All monosaccharide standards were purchased from Carbosynth, while HMO standards were purchased from Carbosynth or Dextra Laboratories, Inc. Where possible, HMO identifications were made based on their co-migration (CE-LIF), or co-elution and mass-to-charge ratio (HPLC-MS), to these commercially-available standards.

CE-LIF data were acquired under normal (monosaccharides) and reverse (HMOs) polarity, exactly as previously described, using a ProteomeLab PA800 (Beckman-Coulter) (38). Peak areas were determined by manual integration using 32 Karat software (version 7.0); in instances where peaks could not be detected, the relevant region of baseline was integrated and the background signal was recorded. All CE peaks areas were divided by their migration times and subsequently normalized to the area of the ISTD (maltoheptaose) to permit comparison of absolute HMO levels; similarly, the extracted ion chromatogram peak areas for HMOs detected by HPLC-MS were normalized to the area of the ISTD present in each sample. Relative HMO levels, as assessed by CE-LIF, were calculated by expressing each peak area as a fraction of the total detectable area of all HMOs with the exception of residual lactose. HPLC-MS analysis of HMOs (38) and Neu5Ac/Neu5Gc (42) were performed as previously described. HPLC was conducted on an Agilent 1290 Infinity system (Agilent Technologies) with a 1290 Infinity binary pump, 1290 Infinity autosampler, and a 1290 Infinity column compartment. HMOs were separated on a Thermo Scientific Hypercarb 100 mm x 2.1 mm column (3 μm particle size) at a temperature of 50 °C. Samples were analyzed using an injection volume of 5 μL at a flow rate of 0.250 mL/min. Mobile phases A and B were H₂O and ACN, respectively, each with 0.1% formic acid. Gradient elution was programmed with a total runtime of 30 min as follows: 0-5 min, 0-5% B; 5-15 min, 5-20% B; 15-20 min, 20-40% B; 20-25 min, 40-80% B; 25-27 min, 80% B; 27-27.1 min, 80-5% B; 27.1-29 min, 5% B; 29-30 min, 5-0% B. Mass spectrometry was conducted in negative ion mode on an Agilent 6530 QToF mass spectrometer with an Agilent Jet Stream electrospray ionization source. Source parameters were as follows: drying gas (N₂) temperature 300 °C with flow rate 10 L/min, sheath gas (N₂) temperature 350 °C with flow rate 10 L/min, nebulizer pressure 35 psig, capillary voltage 3500 V, nozzle voltage 1000 V, and fragmentor voltage 175 V. Reference ion solution containing 10 μM purine and 2.0 μM HP (m/z 121.0509 and 922.0098, respectively) in 95:5 ACN:H₂O was added post-column at 6 μL/min by an Agilent 1260 Infinity II isocratic pump.

The QToF was tuned and calibrated in the 2 GHz extended dynamic range mode for the 100-3200 m/z range immediately prior to sample analysis. MS/MS spectra were acquired using the data-dependent Auto MS/MS mode with a preferred precursor ion list containing calculated m/z for each HMO and sulfonated HMO. Precursor ions were selected for MS/MS product ion scans with absolute and relative thresholds set to 1,000 counts and 0.01%, respectively. Active exclusion was disabled for HMO standards and enabled for human samples, with precursors excluded after 10 spectra and released after 0.75 minutes. Abundance dependent accumulation was enabled for all samples; scan speed was varied based on precursor abundance with a target of 30,000 counts per spectrum for HMO standards and 20,000 counts per spectrum for human samples. Full-scan MS spectra were collected at a rate of Hz with a mass range of 100-1100 m/z for HMO standards and 100-3200 m/z for human samples. All MS/MS spectra were collected at a rate of Hz with the isolation width set to ~1.3 m/z. The mass range for MS/MS spectra was adjusted depending on the sample to maximize the number of transients per spectrum,
with the minimum set to 100 m/z for all samples and the maximum set to that of the highest-m/z sulfonated HMO precursor ion, rounded up in increments of 50 m/z units.

HPLC-MS data acquisition and analysis were performed using MassHunter Workstation software (Agilent Technologies): Data Acquisition Workstation (v B.06.01, SP1), and Qualitative Analysis (v B.07.00, SP2). The Findby-Formula algorithm in Qualitative Analysis was used to generate extracted ion chromatograms (EICs) for analyte m/z values with a ±15.00 ppm mass accuracy limit and ±0.300 min retention time window, as established by analyzing HMO standards and enzyme-treated HMO standards without retention time filtering. Peak areas and retention times were processed further in Microsoft Excel. In silico fragmentation of HMOs and sulfonated HMOs was performed in GlycoWorkbench (v 2.1, build 146). MS/MS data were processed, and spectra were generated and annotated in R (v 3.6.1; R Core Team) with major B/Y and C/Z fragments from GlycoWorkbench.

**Dietary intake and breast milk analysis**

To understand the relationship between dietary intake and HMOs, a validated 24-hour dietary recall was given to all participants to track food consumption. Participants were asked to fill out the recall on sample collection days; all dietary data were assessed and entered into Self-Administered 24 hour (ASA24) Dietary Assessment Tool, a web-based tool for dietary analysis (63). The data output from the ASA24 includes a summary of dietary nutrients consumed by the participant. Since dietary recalls are not always good indicators of breast milk fatty acids, the medium and long-chain fatty acids in breast milk were quantitated using gas chromatography (GC) exactly as previously described (64).

**Synthesis of sulfonated HMOs**

3.8 mg of each sulfotransferase (CHST1 and GAL3ST2; R&D Systems) were mixed with 10 nmol of either lactose or 2’FL, 49.25 nmol 3’-phosphoadenosine-5’-phosphosulfate (PAPS; Sigma) and reacted at 37 °C. Reactions were conducted in 50 mM Tris, 500 mM NaCl 15 mM MgCl₂, pH 7.5 (CHST1) or 25 mM 2-(N-morpholino)ethanesulfonic acid, 15 mM MgCl₂, pH 5.6 (GAL3ST2) in total reaction volumes of 50 μL. Following overnight incubations, samples were applied directly to conditioned ENVICarb SPE cartridges and subsequently prepared for CE-LIF and HPLC-MS/MS analysis exactly as described above.

**Immune function analysis**

The expression of sIgA, IgE, and cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12 (p70), MCP-1, TNF-α, IL-13 and IL-5) were measured in infant stool and breast milk by Eve Technologies, Calgary, Canada.

**High throughput sequencing**

**Sequencing methods:** The QIAamp DNA stool mini kit (Qiagen; Cat No 51504) was used to extract total DNA fecal samples following manufacturer’s specifications as described previously (65). Briefly, 5 ng of extracted DNA was used in a PCR reaction to amplify the V3-V4 region of the 16S ribosomal DNA using 341F and 805R primers (66) attached to the Illumina adapter overhang. A Nextera XT dual index kit was used to attach unique identifiers to both 5’ and 3’ ends using a second PCR reaction. PCR products were cleaned using Agencourt Ampure XP beads (Beckman Coulter) and pooled amplicons were checked for quality and quantity on the Experion Automated Electrophoresis System (Bio-Rad). The resulting 2 x 300 bp, paired end reads were sequenced on a MiSeq system at The Applied Genomics Core (TAGC) in Edmonton, AB.

**Bioinformatics and microbial analysis:** All bioinformatics processing was performed within QIIME2 (67). Forward sequences underwent quality-filtering, dereplication, chimera removal, denoising, and merging using the Deblur plugin with default settings. Using the most recent version of Greengenes available (13_8), a naïve Bayes classifier was trained on a specific region targeted by our primer sets. MAFFT-aligned (68) sequences were used to produce a phylogeny tree using FastTree2 (69) with default settings for all microbiome analyses requiring phylogenetic information. This resulted in ten samples being included for microbial comparisons. All used software packages, versions, and parameters are available under the “provenance” section of the QIIME2 feature-table artifact available at: https://osf.io/dnsg4/. The file can be viewed by
dragging and dropping onto https://view.qiime2.org/.

**Statistics**

Median HMO levels between different secretor groups were deemed statistically significant if the two-tailed Mann-Whitney U values were lower than $U_{crit}$ at $P < 0.05$ or 0.01. Associations between absolute HMO levels (assessed by CE-LIF) and estimated dietary metrics were determined by calculating the two-tailed Spearman rank correlations for each comparison. Following a previous approach (65), an RDA, performed using R version 3.4.1, was used to simultaneously evaluate the relationship between dietary components, HMOs and the infant microbiome. This constrained ordination looks at composition differences in the infant microbiome in relation to potential predictors. Given our low sample size (n=10), we limited our predictor variables (n= 70) to include: maternal secretor status (44), Neu5Ac, Neu5Gc, fucose and galactose. Microbial data was first Hellinger-transformed (70) to accommodate the high occurrences of zero values in the count data using the “Vegan” package. The `ordistep` function was used for variable selection using default settings. To identify genera that contributed to the variance in microbial data, a Spearman correlation was used between the genus abundance and the first two RDA axes. Any significant correlation evaluated at an alpha level=0.05, was displayed on the RDA plot with type II scaling. For comparisons between immune markers and HMOs, a Spearman rank correlation was conducted using the `rcorr` function in the “Hmisc” package in R and presented as a heatmap. Holm’s method was applied to correct for multiple inference.
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Data availability
Microbiome, HPLC-MS, and tandem MS data have been deposited to the publicly-accessible repository Open Science Framework (https://osf.io/2xygh/).

Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

REFERENCES
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ABBREVIATIONS

The abbreviations used are: APTS, 8-aminopyrene-1,3,6-trisulfonate; CE, capillary electrophoresis; Fuc, L-fucose; Gal, D-galactose; Glc, D-glucose; GlcNAc, N-acetyl-D-glucosamine; HMO, human milk oligosaccharide; HPLC, high-performance liquid chromatography; LDFT, lactodifucotetraose; LIF, laser-induced fluorescence; LNFP, lacto-N-fucopentaose; LSTc, sialyllacto-N-tetraose c; MS, mass spectrometry; Neu5Ac, 5-N-acetyl-D-neuraminic acid; Neu5Gc, 5-N-glycolyl-D-neuraminic acid; Se, Secretor; SL, sialyllactose; TOF, time-of-flight.
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Figure 1. Diet-derived Neu5Gc was present as a constituent of HMOs in all \( n = 16 \) samples tested. a, Milk (i.e. bovine milk) was hypothesized to be the most significant source dietary Neu5Gc. It was determined that milk-consuming women did not ingest significantly more Neu5Gc than those consuming an almond-milk beverage (top), nor did these groups significantly differ in median levels of HMO-borne Neu5Ac or Neu5Gc (bottom). b, Maternal secretor status did not appear to affect levels of Neu5Gc in HMOs. (* and ** indicate \( p < 0.05 \) and \( 0.01 \), respectively, by the Mann-Whitney U test). For all box-and-whisker plots, centre lines depict medians while box limits indicate 25\(^{th}\) and 75\(^{th}\) percentiles. Whiskers extend to 1.5 times the interquartile range. c, Spearman rank correlation heatmap depicting correlations
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between selected HMO monosaccharides and calculated Neu5Ac and Neu5Gc dietary intake levels over the 24 h period prior to sample collection. Colour indicates directionality (red = positive; blue = negative); values for \( \rho_{\text{crit}} \) are provided for each association and are indicated in bold font when exceeding the critical value \( (p < 0.01; n=16) \) for a two-tailed test.

**Figure 2.** Representative capillary electrophoresis (CE) electropherogram of fluorescently-labeled HMOs. Peaks attributable to HMOs were sequentially numbered (in bold font); # indicates the migration times of HMOs observed in only one to three (of 16) samples. An original, unscaled electropherogram is depicted with the bold line, while the boxed regions, and the region from 8.4 to 10 min, are individually scaled to more clearly illustrate the HMO peaks. Peak areas were recorded for all numbered HMOs; those above 71 could not be reliably aligned between different samples and were recorded collectively as the sum of all HMO peaks in this region. ISTD = internal standard (maltoheptaose).
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Figure 3. Relative quantitation of sulfonated and phosphorylated HMOs by CE and HPLC-MS. a, CE analysis of HMOs permitted the identification of maternal Secretor (Se) status based on significant differences in the relative levels of 2'FL, LDFT, and 6'SL (44). Numbers in bold font refer to CE peaks as numbered in Fig. 2. 3'SL and 3'SO₃L, a sulfonated HMO previously identified in dairy milk samples, did not significantly vary with maternal secretor status. For all box-and-whisker plots, centre lines depict medians while box limits indicate 25th and 75th percentiles. Whiskers extend to 1.5 times the interquartile range. Open circles are used rather than box-and-whisker plots when < 4 samples contained a specific HMO. b, With a single exception, all human milk samples contained a class of sulfate- or phosphate-bearing HMOs (HMO1–6, and 10) with identical electrophoretic mobilities to those previously identified for the first time in bovine milk samples (38). The occurrence of several other peaks in this region (e.g. 7-9, 11-13) suggests the presence of additional sulfonated HMOs. c, HPLC-MS analysis indicates the presence of HMOs with accurate masses (m/z) consistent with phosphorylated and sulfonated HMOs; HMOs i–vi and 3'SO₃L have been previously identified in bovine milk; HMOs with m/z consistent with xiii–xv have been previously documented in pooled human milk (46). In all cases, the reducing end residue was a hexose (i.e. glucose). Statistical differences between groups were assessed using a Mann-Whitney U test (*p < 0.05; **p < 0.01). n.d. = not detected.
Figure 4. Human sulfo-transferases accept lactose and 2′-fucosyllactose as substrates creating multiple sulfonated HMOs. a. Capillary electrophoresis (CE) analysis of sulfo-transferase reaction mixtures reveals at least four products (HMO1, 3, 4, and 5) that also observed in human milk samples; five products (HMO6 – 10) are detectible by CE when 2′FL is used as a substrate. Additional products are attributed to traces of lactose in the 2′FL. b. Consistent with CE, HPLC-MS analysis demonstrates that human sulfo-transferases convert lactose and 2′-FL into four and six unique sulfonated products, respectively.
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Figure 5. The total dietary intake of sugars and saturated fats significantly impacted the absolute levels of HMOs and monosaccharide HMO constituents observed in human milk. The Spearman rank correlation heatmap, for only Se+ samples (n=12), depicting the associations between a. estimated dietary intake of selected macronutrients and b. GC quantified breast milk fatty acids, indicates that the relative levels of Fuc and Gal in HMOs were positively correlated with both the total sugars ($p < 0.01$) and total dietary fiber ($p < 0.05$) ingested within the 24 h period prior to milk collection. Similarly, several of the putative sulfonated/phosphorylated HMOs were observed to be positively correlated with breast milk monounsaturated fats and polyunsaturated fats, and negatively correlated with levels of saturated fats. A complete heatmap depicting the associations between all HMOs and an extensive list of nutrients is included in the supplemental information (Figure 8). Note that the intensity of each colour indicates the directionality of the associations; all associations exceeding $\rho_{crit}$ ($p = < 0.05$) are highlighted in black. Numbers in bold font refer to CE peaks as labeled in Fig. 2.
Figure 6. RDA ordination of microbial community constrained by selected explanatory variables. Arrow length indicates the importance of each infant microbial community and their relationship with HMOs. Only the predictor variables that significantly explained variability in microbial community structures are displayed. The HMOs (left; blue arrows) and bacterial genera (right; red arrows) are presented separately for clarity; however, they are derived from the same RDA model. In total, RDA1 and RDA2 explain over 20% of the total variation in beta-diversity. The global model’s $P$ value was significant ($p = 0.05$) as calculated by a Monte Carlo Permutation Procedure (MCPP). ($n = 10$)
Figure 7. The Spearman rank correlation heatmap, for both Se+ and Se- samples ($n=16$), depicting the associations between the levels of HMOs and oligosaccharides in human milk with immune markers in breast milk (top) and infant stool (bottom). Note that the intensity of each colour indicates the directionality of the associations; all associations with $p < 0.05$ are highlighted in black. HMOs are numbed as depicted in Fig. 2.
Influence of sulfonated and diet-derived human milk oligosaccharides on the infant microbiome and immune markers
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