Utilization of major fucosylated and sialylated human milk oligosaccharides by isolated human gut microbes

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Human milk oligosaccharides (HMOS) are not digested in the proximal intestine. In distal intestine, HMOS collectively modify the microbiota, but the response of individual bacteria to individual components of the HMOS is not well defined. Here, each of 25 major isolates of the human intestinal microbiota was fed individual major fucosylated and sialylated HMOS in anaerobic culture. This allowed for an assessment of the influence of specific HMOS on the growth and metabolic products of individual microbiota bacteria. Most Bifidobacterium spp. and Bacteroides spp. grew, induced α-L-fucosidase activity, and produced abundant lactate or short-chain fatty acids (SCFAs) when fed 2′-fucosyllactose (2′-FL), 3-FL, and lactodifucotetraose (LDFT). Lactobacillus delbrueckii ATCC7830, Enterococcus faecalis ATCC19433, and Streptococcus thermophilus ATCC19258 exhibited slight growth, pH reduction, and lactate production when supplemented with 2′-FL or 3-FL, but not LDFT. Supplementation with 3′-sialyllactose (3′-SL) and 6′-SL promoted moderate growth of Bifidobacterium longum JCM7007, 7009, 7010, 7011, 1272, 11347, ATCC15708, Bacteroides vulgatus ATCC8482, and B. thetaiotaomicron ATCC29148; accordingly, these bacteria exhibited greater neuraminidase activity and produced copious lactate, SCFA, or both. Lactobacillus delbrueckii ATCC7830 also consumed 6′-SL. In contrast, Clostridium spp., L. rhamnosus ATCC53103, E. faecalis ATCC29200, Staphylococcus spp., Enterobacter spp., and Escherichia coli K12 did not consume milk oligosaccharides nor produce appreciable acidic fermentation products. Specific Bifidobacteria and Bacteroides differentially digest specific individual HMOS, with the major fucosylated milk oligosaccharides most strongly stimulating key species of mutualist symbionts. This suggests strategies for treating dysbiosis of the microbiota and associated inflammatory disorders.

Keywords: commensal bacteria / glycosidase / human milk oligosaccharides / mutualist bacteria / organic acids

Introduction

Human milk oligosaccharides (HMOS) contain lactose at the reducing end, and most contain a polygalactosamine or lacto-N-biose core. The lactose and core structures are often decorated by fucose via α1-2, α1-3, and α1-4 linkages to galactose, and sialic acid via α2-3 and α2-6 linkages to N-acetylgalactosamine (Newburg 2000; Ninonuevo et al. 2006). In typical milk, ~70% of HMOS are fucosylated and ~30% are sialylated (Ninonuevo et al. 2006; Weiss and Hennet 2012). HMOS inhibit the pathogenesis of enteropathogens. Acting as structural analogs to cell surface receptors, HMOS can prevent the adhesion of pathogenic bacteria to the epithelial surface in the gastrointestinal tract. Several enteric pathogens such as stable toxin of Escherichia coli, Campylobacter, and noroviruses, are inhibited by fucosylated glycans (Ruiz-Palacios et al. 2003; Newburg, Ruiz-Palacios, Altaye, Chaturvedi, Guerrero, et al. 2004; Newburg, Ruiz-Palacios, Altaye, Chaturvedi, Meinzen-Derr, et al. 2004). Sialylated milk oligosaccharides can also inhibit the adhesion of bacteria to sialylated receptors of the intestinal epithelium (Sohanpal et al. 2004). α2,3-sialyllactose (3′-SL) binds Helicobacter pylori and inhibits its binding to human intestinal cells (Simon et al. 1997) and likewise reduces the adhesion of enteropathogenic E. coli (Angeloni et al. 2005).

Furthermore, because minimal HMOS are absorbed in the proximal human intestine, the bulk of ingested oligosaccharides pass into the distal intestine where mutualist symbiotic bacteria can use them as a source of energy (Gevers et al. 2012). HMOS are believed to be a specific growth factor that enriches gut bacteria. Breastfed infants have a microbiota uniquely rich in Bifidobacteria (Schack-Nielsen and Michaelsen 2007), and Bifidobacterium infantis and B. bifidum are major HMOS consumers found in breastfed infant feces. However, most gut bacteria do not grow well using HMOS as a sole carbon source (LoCascio et al. 2007, 2009; Ward et al. 2007; Marcobal et al. 2010; Sela et al. 2011).

To date, most studies on HMOS utilization by microbes focus on the interaction of naturally occurring mixtures of HMOS with gut bacteria, but HMOS consist of more than 200 oligosaccharides (Newburg and Neubauer 1995). The present study was designed to measure the relative degree to which individual isolated human mutualist bacteria are able to utilize major individual fucosylated HMOS [2′-fucosyllactose (FL), 3-FL, and lactodifucotetraose (LDFT)] and major individual sialylated HMOS (3′-SL and 6′-SL) for their growth. The metabolism of each of the oligosaccharides into acidic fermentation products was also measured.
Results

Fucosylated HMOS affect the growth of gut microorganisms

Each of the major fucosylated HMOS, 2'-FL, 3-FL, or LDFT, was added to culture media as the sole sugar, and the physiological digestion in vitro by each of 25 individual microbial strains was measured. The individual oligosaccharides remaining in the supernatants after 48 h of culture were purified, reduced, and quantified by liquid chromatography–mass spectrometry (LC/MS; Table I).

All Bifidobacteria spp. and Bacteroides spp. consumed 40% or more of the 2'-FL, except for Bifidobacterium longum JCM15708, which consumed 10–40% of the 2'-FL. Bifidobacterium longum JCM7007, 7011, 1210, 11347, B. longum ATCC15697, and all Bacteroides spp. responded to supplementation with the induction of significantly greater activities of α-L-fucosidase (AFU; \( P < 0.05 \)). The Bifidobacteria spp. grew strikingly in response to 2'-FL supplementation, accompanied by a significant pH reduction (\( P < 0.05 \)) in the culture medium, but the response of B. longum ATCC15708 was much less pronounced (Figure 1). Bifidobacterium longum JCM7007, 7009, 7010, 7011, 1272, 11347, and B. longum ATCC15697 produced copious lactate. Bifidobacterium longum JCM7007, 7009, 7010, 1210, 1272, and B. longum ATCC15697 also produced ample SCFAs. Bacteroides spp. exhibited a significant growth increase and pH reduction when supplemented with 2'-FL (\( P < 0.05 \)), but they produced less lactate and SCFA than most of the Bifidobacteria spp. These features are consistent with a mutualistic relationship in which the human host supplies specific sugars which bacteria are able to adapt to and utilize, and the bacteria provide small organic acids that are beneficial to the human.

2'-FL induced only sparse activity of AFU in Lactobacillus delbrueckii ATCC7830, Enterococcus faecalis ATCC19433, and Streptococcus thermophilus ATCC19258, with these bacteria utilizing only 10–40% of the 2'-FL. Accordingly, L. delbrueckii ATCC7830, E. faecalis ATCC19433, and S. thermophilus ATCC19258 showed slight growth when supplemented with 2'-FL, displaying only a modest reduction in pH and sparse lactate production in the culture medium.

Typical commensal bacteria, Clostridium spp., L. rhamnosus ATCC53103, E. faecalis ATCC29200, Staphylococcus spp., Enterobacter spp., and E. coli K12, consumed less than 10% of the 2'-FL. They showed little induction of AFU, little increase in growth, little decrease in pH, and little production of lactate or SCFAs. These data are consistent with commensals differing from mutualists in that they are not as interdependent with the host.

Across all of the 25 bacteria tested, the relationships between changes in the primary variables, growth and pH, and the secondary variables, induction of fucosidase (2'-FL utilization) and production of acid, were significant: changes in fucosidase correlated significantly with changes in growth (\( r = 0.442, P = 0.027 \)) and pH (\( r = 0.514, P = 0.009 \)), and changes in organic acid production correlated with changes in pH (\( r = 0.739, P < 0.001 \)).

When supplemented with 3-FL, all Bifidobacteria spp. and Bacteroides spp. displayed appreciable induction of AFU activity and consumed 40% or greater of the 3-FL. The growth of Bifidobacteria spp. was accompanied by a significant reduction in pH (\( P < 0.05 \); Figure 2). Although all Bifidobacteria spp. converted 3-FL into SCFAs, 3-FL was metabolized into lactate only by B. longum JCM7007, 7010, 1210, 1272, 11347, and B. longum ATCC15708 and 15697. The three Bacteroides spp. tested exhibited growth in response to 3-FL and reduced pH in the culture medium. All produced elevated SCFAs (acetate, propionate, and butyrate) from 3-FL, but only Bacteroides

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<th>Species</th>
<th>2'-FL</th>
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<th>LDFT</th>
<th>3'-SL</th>
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+, consumption > 40%; ±, consumption between 10 and 40%; −, consumption < 10%. 

Table I. Consumption profiles (by LC/MS) of individual oligosaccharide by different bacteria species.
A TCC8482 produced lactate. These results suggest mutualism.

Only 10–40% of the initial 3-FL was utilized by \textit{L. delbrueckii} A TCC7830, \textit{E. faecalis} A TCC19433, and \textit{S. thermophilus} A TCC19258, and their induction of AFU activities was modest relative to bifidobacteria and bacteroides. 3-FL promoted slight growth of \textit{L. delbrueckii} A TCC7830, \textit{E. faecalis} A TCC19433, and \textit{S. thermophilus} A TCC19258, with modest reductions in pH and little lactate production in the culture medium.

In contrast, \textit{Clostridium} spp., \textit{Lactobacillus} ATCC53103, \textit{Enterococcus} ATCC29200, \textit{Staphylococcus} spp., \textit{Enterobacter} spp., and \textit{E. coli} K12 consumed less than 10% of the 3-FL. They exhibited negligible AFU induction, growth, pH reduction, and organic acid production in response to 3-FL. Thus, the strains that seem to be strictly commensal did not respond to 3-FL, similar to their lack of response to 2'-FL.

Across all of the 25 bacteria tested, the relationships between changes in the primary variables, growth and pH, and the

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**Fig. 1.** Ability of 25 individual species of gut microbiota to utilize 2'-FL. (A) Difference in growth of 25 species after 48 h incubation with purified 2'-FL. Growth was the difference in OD600 between the media control and the supplemented group for each species (mean ± SD). Changes in (B) pH, (C) lactate production (mmol/L), (D) SCFA production (mM) and (E) AFU activity (U/L) were the differences in these parameters between these 25 bacteria species after 48 h incubation with 2'-FL and their unsupplemented media controls (mean ± SD).

\textit{Vulgatus} ATCC8482 produced lactate. These results suggest mutualism.

Only 10–40% of the initial 3-FL was utilized by \textit{L. delbrueckii} ATCC7830, \textit{E. faecalis} ATCC19433, and \textit{S. thermophilus} ATCC19258, and their induction of AFU activities was modest relative to bifidobacteria and bacteroides. 3-FL promoted slight growth of \textit{L. delbrueckii} ATCC7830, \textit{E. faecalis} ATCC19433, and \textit{S. thermophilus} ATCC19258, with modest reductions in pH and little lactate production in the culture medium.
secondary variables, induction of fucosidase (3-FL utilization) and production of acid, were significant: changes in fucosidase correlated significantly with changes in growth ($r = 0.544$, $P = 0.005$) and pH ($r = 0.747$, $P < 0.001$) and changes in organic acid production correlated with changes in pH ($r = 0.548$, $P = 0.005$). When supplemented with LDFT, all Bifidobacteria spp. (except B. longum ATCC15708), B. vulgatus ATCC8482, and B. fragilis ATCC25285 consumed greater than 40% of the LDFT. Consistent with these data, most of the bacteria exhibited significant induction of AFU activity ($P < 0.05$) and grew more in response to LDFT, with a reduction in pH in the culture media.
With the exception of *B. longum* JCM7009 and 1260, they produced significant lactate and ample SCFAs. *Bifidobacteria longum* JCM7009 produced only SCFAs, while strain JCM1260 did not produce either of these families of acidic fermentation products after consuming LDFT. When supplemented with LDFT, *B. vulgatus* ATCC8482 and *B. fragilis* ATCC25285 also displayed increased growth and pH reduction, but metabolized LDFT only into SCFAs. Thus, most, but not all, of the bacteria that strongly utilized 2′-FL and 3-FL were also able to utilize LDFT.

*Bifidobacteria longum* ATCC15708 utilized between 10 and 40% of the available LDFT. It showed modest induction of AFU, slight growth, displayed a modest pH reduction, and little organic acid production when supplemented with LDFT.

The group of bacteria unable to use LDFT included those that did not utilize 2′-FL and 3-FL (*Clostridium* spp., *Lactobacillus* spp., *Enterococcus* spp., *Streptococcus* spp., etc.).
Bacteroides vulgatus, grew modestly, and exhibited a moderate pH reduction. Production of acid, were significant changes in fucosidase correlated significantly with changes in growth \((r = 0.642, P = 0.001)\) and pH \((r = 0.592, P = 0.002)\), and changes in organic acid production correlated with changes in pH \((r = 0.749, P < 0.001)\).

Across all of the 25 bacteria tested, the relationships between changes in the primary variables, growth and pH, and the secondary variables, induction of fucosidase (LDFT utilization) and production of acid, were significant: changes in fucosidase correlated significantly with changes in growth \((r = 0.642, P = 0.001)\) and pH \((r = 0.592, P = 0.002)\), and changes in organic acid production correlated with changes in pH \((r = 0.749, P < 0.001)\).

### Sialylated HMOS affect the growth of gut-related microorganisms

Supplementation with acidic milk oligosaccharides also elicited specific responses from individual bifidobacteria species. The major sialylated human milk trisaccharides, 3′SL and 6′SL, were fed to the 25 different strains (Figure 4). When fed physiologically relevant concentrations of 3′SL, *B. longum* JCM7007, 7009, 7010, 7111, 11347, ATCC15697, and *B. thetaiotaomicron* ATCC29148 induced appreciable neuraminidase activity, digested over 40% of the 3′SL supplemented in the media, strongly promoting the growth of these bacteria, accompanied with a significant reduction in pH \((P < 0.05)\). *Bifidobacteria longum* strains JCM7007, 7009, 7010, 7111, 11347, and ATCC15697 metabolized 3′SL into significant amounts of both lactate and SCFAs. *Bifidobacteria longum* JCM1272 and *B. thetaiotaomicron* ATCC29148 only produced SCFAs. Thus, only some of the bacteria that efficiently utilize 2′-FL and 3′-FL also are able to utilize 3′SL.

*Bifidobacteria longum* ATCC15708 and *B. vulgatus* ATCC8482 consumed 10–40% of 3′SL, induced modest neuraminidase activity, grew modestly, and exhibited a moderate pH reduction. *Bifidobacteria longum* ATCC15708 only produced SCFAs. *Bacteroides vulgatus* ATCC8482 metabolized 3′SL into lactate and SCFAs.

However, more of the bacteria were unable to utilize 3′SL supplementation. *Bifidobacteria longum* JCM1210, *B. fragilis* ATCC25285, *Lactobacillus* spp., *Clostridium* spp., *Enterococcus* spp., *S. thermophilus* ATCC19258, *Staphylococcus* spp., *Enterobacter* spp., and *E. coli* K12 induced little or no expression of neuraminidase; these bacteria utilized less than 10% of 3′SL and exhibited negligible growth or production of fermentation products.

Across all of the 25 bacteria tested, the relationships between changes in the primary variables, growth and pH, and the secondary variables, induction of neuraminidase (3′SL utilization) and production of acid, were significant: changes in neuraminidase correlated significantly with changes in growth \((r = 0.592, P = 0.002)\) and pH \((r = 0.747, P < 0.001)\) and changes in organic acid production showed a trend toward correlating with changes in pH \((r = 0.357, P = 0.08)\). The lower correlation of the relationship between organic acids and pH may reflect the influence of other organic acids that were not measured in this study.

6′SL supplementation of *B. longum* JCM7007, 7009, 7010, 7011, 1260, 1272, 11347, *B. vulgatus* ATCC8482, *B. thetaiotaomicron* ATCC29148, and *L. delbrueckii* ATCC7830 induced elevated levels of neuraminidase. Greater than 40% of the 6′SL supplement was consumed and resulted in a reduction in pH \((P < 0.05)\; \text{Figure 5)} (*Bifidobacteria longum* JCM7009, 7010, 7011, and 11347 produced copious lactate and SCFAs. *Bifidobacteria longum* JCM7007 only produced lactate. *Bifidobacteria longum* JCM1260, 1272, and ATCC15708 and *L. delbrueckii* ATCC7830 produced SCFAs but no lactate. These bacteria were among those that could efficiently utilize 2′-FL and 3′-FL, but not all of the fucosyltriose utilizers could metabolize 6′SL.

*Bifidobacteria longum* ATCC15697 and *B. fragilis* ATCC25285 utilized only 10–40% of 6′SL. Each showed modest induction of neuraminidase, slight growth, a modest reduction in pH, and little lactate production in the culture medium.

Supplementation of 6′SL to *B. longum* JCM1210, *Clostridium* spp., *L. rhamnosus* ATCC53103, *Enterococcus* spp., *S. thermophilus* ATCC19258, *Staphylococcus* spp., *Enterobacter* spp., and *E. coli* K12 did not induce neuraminidase. They consumed less than 10% of the 6′SL, which resulted in little change in pH or production of lactate or SCFAs. All of those bacteria that could not utilize the other HMOS were in this group, whereas some that were able to utilize some or all of the other HMOS could not utilize 6′SL.

Across all 25 bacteria tested, the relationships between changes in the primary variables, growth and pH, and the secondary variables, induction of neuraminidase (6′SL utilization) and production of acid, were significant. Changes in neuraminidase correlated significantly with changes in growth \((r = 0.524, P = 0.007)\) and pH \((r = 0.698, P < 0.001)\) and changes in organic acid production showed a trend toward correlating with changes in pH \((r = 0.387, P = 0.056)\). The lower correlation of the relationship between organic acids and pH may reflect the influence of other organic acids that were not measured in this study.

In summary, bacteria characteristic of the feces of breastfed infants are able to adapt to the presence of major individual oligosaccharides of human milk and utilize them for growth, fermenting them into small organic acids that acidify the gut.

### Discussion

HMOS is the third most abundant fraction of human milk (Newburg et al. 1986; Kunz et al. 2000), accounting for ~10% of maternal energy input into milk. Although the inability of HMOS to be digested by the intestinal mucosa of the infant precludes a direct role in infant nourishment (Newburg 1996), its bioactive functions were discovered only gradually. In 1890, Moro observed that the composition of intestinal bacteria of breastfed infants differed from bacteria of adults or of weaned infants (Moro 1900), in that it contained more *L. rhamnosus*. Now, HMOSs are considered prebiotic because they stimulate growth by several bifidobacteria of the infant gut microbiota (Coppi et al. 2006; Chichlowski et al. 2011; Yu et al. 2013).

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Prebiotics are indigestible dietary glycans that confer health benefits via modification of the intestinal microbiota. The defining features of prebiotics include their carbohydrate nature, oral ingestion, resistance to digestion by mucosal enzymes, minimal absorption in the proximal gastrointestinal tract, and selective fermentation by beneficial bacteria of the distal gut (Gibson et al. 2004). Generally, molecules that cause an increase in bifidobacteria and lactobacilli of the gut microbial community, and are fermented by microbiota to produce organic acids that reduce the pH, are considered prebiotic. After ingestion, HMOS pass mainly unabsorbed through the small intestine into the colon. HMOS stimulate the growth of specific bacteria.
(Coppa et al. 2006), and are fermented to SCFAs and lactic acids, creating an acidic environment (Ogawa et al. 1992). Among the *Bifidobacteria* spp., *B. longum* (originally typed as *B. infantis*) and *B. bifidum*, the major bacteria found in breastfed infant feces, can grow using HMOS as a sole carbon source, whereas *B. breve* and *B. adolescentis*, often associated with the adult gastrointestinal tract, do not utilize HMOS as efficiently for growth (Ward et al. 2006, 2007; LoCascio et al. 2007). The extent to which individual oligosaccharides induce the growth of individual bacteria is less well defined. The major individual HMOS tested herein are all, to various degrees, prebiotic to *B. longum* and *B. bifidum*.

Humans exhibit extensive diversity in their expression of glycosyltransferase genes (Wiederschain and Newburg 1996), and relatively common null alleles, especially of *FUT2* and *FUT3*, result in high individual variation among mothers in their

Fig. 5. Ability of 25 individual species of gut microbiota to utilize 6′-SL. (A) Difference in growth of 25 species after 48 h incubation with purified 6′-SL. Growth was the difference in OD600 between the media control and the supplemented group for each species (mean ± SD). Changes in (B) pH, (C) lactate production (mmol/L), (D) SCFA production (mM) and (E) neuraminidase activity (U/L) were the differences in these parameters between these 25 bacteria species after 48 h incubation with 6′-SL and their unsupplemented media controls (mean ± SD).
milk oligosaccharide expression (Newburg, Ruiz-Palacios, Altaye, Chaturvedi, Meinzen-Derr, et al. 2004). Factors driving the changes in HMOS amounts and patterns across lactation are not well defined, but include changes in glycosyltransferase expression driven by hormonal factors (Nanthakumar et al. 2013). Different patterns of expression, and individual HMOS structures, are associated with diverse functions (Newburg et al. 2005b). The data presented above now define the specificity of growth stimulation and metabolism in individual mutualist symbionts in populations of infants. For example, more bifidobacteria tested herein could utilize the fucosylated HMOS for growth and metabolism than those that could utilize the sialylated HMOS.

Among the 25 different strains tested, Bifidobacteria spp. generally displayed the greatest ability to utilize the fucosylated and sialylated HMOS. This study confirmed that most B. longum are capable of consuming major individual HMOS, including not only the fucosylated milk oligosaccharides, 2'-FL, 3-FL, or LDFT, but also the sialylated milk oligosaccharides, 3'-SL or 6'-SL. Bifidobacteria longum JCM7007, 7009, 7010, 7011, 1272, and 11347 exhibited the most robust induction of fucosidase or sialidase activity, growth responses, and were able to produce the greatest amount of total acidic fermentation products (lactate and SCFAs) from these individual HMOS. Other bifids also responded to the presence of these oligosaccharides, but with distinct differences: B. longum ATCC15708, when supplemented with 2'-FL, LDFT, and 3'-SL, exhibited little induction of fucosidase activity, poor utilization of the sugar, little extra growth, and quite low production of acidic fermentation products. Bifidobacterium longum JCM1210 and 1260 had no ability to utilize 3'-SL and little ability to consume 6'-SL. Thus, individual infant-borne bifidobacteria display different catabolic strategies for digestion of individual components of HMOS.

The Bacteroides are another major component of infant microbiota. Bacteroides fragilis and B. thetaiotaomicron utilize the mixture of HMOS found in milk, can metabolize stachyose acid (Chang et al. 2004; Almagro-Moreno and Boyd 2009a, b) and the HMOS induce the expression of specific genes, suggesting mutualism (Marcobal et al. 2010, 2011). In contrast, B. ovatus and B. stercoris do not utilize HMOS.

The data herein also identify Bacteroides that are strong mutualists. Bacteroides vulgatus responds to the three principal fucosylated HMOS and the two sialylated HMOS with robust growth and fermentation. Bacteroides fragilis efficiently utilize the three fucosylated milk oligosaccharides and 6'-SL for robust growth and metabolism, but not 3'-SL. Bacteroides thetaiotaomicron consume 2'-FL, 3-FL, 3'-SL, and 6'-SL and display robust growth and generation of acidic fermentation products, but have less ability to utilize LDFT. All three Bacteroides spp. metabolized the HMOS into SCFAs, and they also produced lactic acid when fermenting most, but not all, of the oligosaccharides. In all cases, the induction of fucosidase and neuraminidase activity accompanied supplementation by the corresponding utilizable HMOS molecule. The oligosaccharide utilization patterns and induction of the two glycosidase activities measured in this study suggest that these three mutualist Bacteroides species express glycoside hydrolases capable of accommodating most of the structural diversity of the non-reducing termini in milk oligosaccharides.

The most popular prebiotic in infant formula is a 9:1 ratio of galactosyloligosaccharides and fructosyloligosaccharides. When fed to animals and humans, it strongly increases the content of both bifidobacteria and lactobacilli in the microbiota. However, this prebiotic activity is distinct from that exhibited by HMOS. The individual HMOS that we tested also stimulate bifids, but their stimulation of lactobacilli is weak, at best. Lactobacillus delbrueckii ATCC7830 showed only slight ability to consume 2'-FL, 3-FL, and 6'-SL. This weak response is similar to the responses of E. faecalis ATCC19433 and S. thermophilus ATCC19258, two probiotics commonly added to foods such as yoghurt. Enterococcus faecalis and S. thermophilus utilized small amounts of 2'-FL or 3-FL, which resulted in slight growth, slightly reduced pH, and little lactate production. These three representative probiotic dietary bacteria of the microbiota are only weakly responsive to the three major fucosyloligosaccharides and two major sialyloligosaccharides of human milk. These findings are consistent with L. acidophilus and S. thermophilus exhibiting little growth on total HMOS (MARCObAL et al. 2010). Therefore, in the context of the intestinal microbiota of the infant, these bacteria should be considered weakly mutualistic, as best.

The response of L. rhamnosus ATCC53103 more closely resembles typical non-mutualists, which did not demonstrate appreciable signs of utilizing any of the HMOS tested. These non-mutualist (commensal) bacteria in this study include Clostridium spp., E. faecalis ATCC29200, Staphylococcus spp., Enterobacter spp., and E. coli K12, which exhibited only faint growth with either fucosylated or sialylated HMOS, and did not produce appreciable acidic fermentation products. In these Enterococcus, Streptococcus, Clostridium, and E. coli strains, growth was stimulated less well or not at all when supplemented with individual milk oligosaccharides.

In this study, the oligosaccharides were tested in concentrations resembling those found in human milk to assess which species of human microbiota had the genetic ability to utilize the individual oligosaccharides as presented by breast feeding. The results indicate that individual infant-borne bifidobacteria display different catabolic strategies for digesting individual components of the HMOS. This suggests evolutionary adaptations by each to gain a distinct metabolic niche that can provide a growth advantage over other symbionts of the microbiota that compete for the same complex mixture of HMOS (Sela and Mills 2010). These data are also consistent with the idea of spatial specialization by microbes as the mixture of HMOS is gradually depleted of individual oligosaccharides during its traverse through the distal gut.

The protection afforded to the breastfed infant by HMOS includes the prebiotic effect, whereby individual HMOS provide complementary prebiotic effects for the growth of major mutualists of the infant microbiota. These mutualists would occupy mucosal receptors that might otherwise be available to pathogens for their colonization, thereby inhibiting the first step in pathogenesis. The prebiotic HMOS also serve as substrates for fermentation into SCFAs and lactate, among others. Fermentation products per se inhibit pathogens (Yu et al. 2013), but they are also strong modulators of the maturation of the intestinal mucosa (Augenlicht et al. 1999). Oligosaccharides may also be anti-inflammatory (Boehm and Stahl 2007). These activities can now be considered in the context of the ability of HMOS to inhibit binding of enteropathogens to their receptors in the intestinal mucosa, with both
fucosylated and sialylated HMOS playing an important role in protection from pathogen (Newburg et al. 2005a). We conclude that HMOS are an essential part of an innate immune system of human milk whereby the mother protects her infant by several complementary mechanisms.

The major oligosaccharides of human milk strongly stimulate bacteria that are commonly found in the feces of breastfed infants. In aggregate, this stimulation is confined to bacteria that are mutualist symbionts, and most of the major oligosaccharides stimulate most of the mutualists. However, there is pronounced oligosaccharide specificity among the different species and strains of the microbiota, especially with regard to the types of fermentation products produced. Collectively, the mutualist components of the human microbiota are able to utilize all of these major oligosaccharides of human milk for growth, to induce the glycosidases needed for utilization, and to produce biologically active acidic fermentation products. When taken together as an interdependent community, mutualists thrive on HMOS and protect the infant through several independent but overlapping mechanisms.

The significant maternal investment in producing HMOS as protective components is consistent with the central role of milk in supporting the human reproductive strategy. Human parents invest large amounts of effort and resources to bring each of their offspring into a successful adulthood through a vulnerable period of immune development. The prolonged developmental period allows for sufficient information transfer to maintain a complex culture. There is emerging appreciation for an important role of successful transfer of gut microbiota in the vulnerable infant. Thus, HMOS play multiple concerted roles in protecting the neonate: prebiotic activity directs proper colonization, untoward inflammatory responses to newly colonizing microbes are attenuated, and inhibition of pathogen binding directly protects the infant from infection. These cooperative mechanisms of protection are entirely distinct from the protection conferred by the current families of antibiotics in use. Thus, the protective human milk glycans may prove useful as a basis for the development of novel prophylactic and therapeutic agents for diseases whose etiology includes dysbiosis of the microbiota.

**Materials and methods**

**Substrates**

Isolated HMOS were tested at physiologically relevant concentrations, including 2'-FL (2 g/L), 3-FL (2 g/L), LDFT (1 g/L; Glycosyn, LLC, Medford, MA), 3'SL (0.5 g/L), and 6'SL (1 g/L; Carbosynth Limited, UK).

**In vitro fermentation with bacteria species**

Bacteria strains (Supplementary data, Table S1) were obtained from the Japanese Collection of Microorganisms (RIKEN BioResource Center, Japan) and the American Type Culture Collection (Manassas, VA). *Bifidobacteria* spp., *Bacteroides* spp., and *Clostridium* spp. were propagated in reinforced clostridial medium, *Lactobacillus* spp. in de Man, Rogosa, Sharpe medium, *Enterococcus* spp. and *Streptococcus* spp. in brain–heart infusion broth, *Staphylococcus* spp. and *Enterobacter* spp. in nutrient broth, and *E. coli* in Luria broth. Seed cultures were incubated overnight until the optical density at 600 nm reached 0.5. Bacteria were grown in anaerobic conditions at 37°C in an anaerobic workstation (DG250 Anaerobic Workstation, Don Whitley Scientific Limited, West Yorkshire, UK).

To study the growth of individual bacteria strains in the presence of individual oligosaccharides, sugar-free basal medium ZMB1 was prepared according to Zhang et al. (2009). All glycans were dissolved for an hour in ZMB1 medium before inoculation with a 10% (v/v) bacteria slurry in phosphate-buffered saline (PBS). Baseline controls were ZMB1 plus inoculum in PBS with no added carbon sources. Preliminary experiments indicated that 48 h of incubation was well beyond the transition into the final stationary phase of fermentation under all conditions used herein; thus, all comparisons were of bacteria after 48 h of culture, when in the final stationary phase of metabolism. Anaerobic fermentation was at 37°C. Culture fluid taken at 48 h was used to measure growth as optical density at 600 nm in a microtiter plate. Each data point from an experiment was the average of three wells, and all experiments were carried out in triplicate (*n* = 3).

**pH and lactate levels**

Culture medium pH was measured after 48 h of bacterial fermentation using a pH meter (Corning, pH meter 240). Lactate concentration in the medium was determined using lactate assay kit K607-100, from BioVision Inc. (CA).

**SCFA analysis by LC/MS**

The SCFA fermentation products were quantified by LC/MS. After 48 h incubation, the culture sample was brought to 4°C, a 1 mL aliquot was centrifuged at 4°C 10,000 × g for 10 min, the supernatant was passed through 0.2 μm syringe filter, sealed, and stored at 4°C briefly until analysis. All reagents were analytical grade from Sigma-Aldrich (Pennsylvania). Ultra-pure water was generated through a Super-Q water purification system (Millipore, Billerica). Chromatographic resolution was on an Agilent ZORBAX Eclipse XDB-C8 (4.6 × 150 mm, 5 μm) column in an Agilent 1100 LC/MS, using water as the mobile phase. The predominant SCFAs were acetic, propionic, and butyric acid. Subsequently, selected ion monitoring included the ions of acetic acid, *m/z* = 84 (M + Na)+, propionic acid *m/z* = 98 (M + Na)+, and butyric acid *m/z* = 112 (M + Na)+.

**Glycosidase assays**

Activity of AFU in the culture medium was measured by an AFU assay kit (Diazyme, CA). The fucosidase activity was measured as the cleavage of the fucosylated substrate. This enzyme cleaves α-1-fucosyl residues bound to Gal through α1-2, α1-3, α1-4, and α1-6 linkages at the non-reducing termini of HMOS. One unit of AFU is the amount that cleaves 1 μmol of substrate per min at 37°C.

Neuraminidase activity catalyzes the hydrolysis of terminal sialic (neuraminic) acid. The combined α2,3 and α2,6 neuraminidase activity was measured using an Abcam assay kit (ab138888, Abcam, MA). One unit of neuraminidase is the amount that cleaves 1 μmol of substrate per hour at 37°C.
Oligosaccharide utilization by gut microbes

Oligosaccharide consumption

Fermentation samples (48 h) were thawed, mixed and centrifuged at 4000 x g for 15 min at 4°C. To 0.5 mL of clear supernatant was added 0.25 mL of a fresh aqueous solution of 0.5 M sodium borohydride with vigorous mixing. Reduction proceeded overnight at 4°C whereupon the reaction was terminated by addition of 0.25 mL of 0.5 M acetic acid. In 5 mL of serological pipettes, 5 x 0.5 cm ion exchange beds were built over glass wool, sand, and celite with 0.6 g (3 meq) AG50W-X8 cation-exchange resin (pyridinium form) and another with 0.9 g (3 meq) AG1-X8 anion-exchange resin (acetate form, BioRad, Hercules, CA). AG50W-X8 cation-exchange resin (pyridinium form) was converted to the pyridinium form by soaking for 1 h in a solution of 1 M pyridine in an Erlemeyer flask thrice with gentle swirling, settling and decanting, followed by rinsing in water three times; the resulting pyridinium form of AG50W-X8 cation-exchange resin was added to the column. The column was rinsed with 5 mL of water, followed by application of the reduced samples in 1 mL of water. Residue from the sample tube was rinsed into the column with 0.5 mL of water, and the resin column washed with an additional 18 mL of water. The eluates of the cation exchange column were applied to the AG1-X8 anion exchange column, whose eluate was the neutral oligosaccharides. The acidic oligosaccharides were eluted from the anion exchange column by 20 mL of ammonium acetate (200 mM), and the eluates frozen and lyophilized. Acidic oligosaccharides were analyzed by an Agilent high-performance liquid chromatography (1200 series) with a triple-quadrupole mass spectrometer (6460) equipped with a porous graphitic column (3 µm, 100 x 2.1 mm, Hypercarb, Thermo Scientific, Waltham, MA) set for 25°C. Methods were validated by authentic oligosaccharides from GlycoSeparations (Moscow, Russia; Newburg 2001).

Statistical analysis

Data are expressed as the mean ± SD. The primary outcome variables are growth and pH, which define prebiotic activity. Secondary outcome variables are the induction of glycosidases and organic acid production. Induction of the appropriate glycosidase, fucosidase for fucosyloligosaccharides, and staldidase for sialyoligosaccharides, allows the utilization of the respective oligosaccharide for growth and fermentation. Organic acid production reflects utilization for fermentation and should drive the change in pH. The statistical significance of differences among groups was determined by one-way ANOVA. When differences were found, Student’s t-test was used for pairwise comparisons between groups; P ≤ 0.05 was considered significant. The relationship between primary and secondary outcomes was calculated as the Pearson correlation coefficient.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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This investigation was supported by NIH AI075563, HD013021 and HD059140 and by Abbott Nutrition. 2'-FL, 3-FL and LDF were a kind gift of John M. McCoy, Massimo Merighi, Matthew Heidtman, Scott Rose and Debatosh Majumdar of Glyscon, LLC.

Conflict of interest

DSN is a shareholder in Glyscon, LLC, which synthesizes individual HMOS. This potential conflict is managed by Boston College.

Abbreviations

AFU, α-L-fucosidase; 2'-FL, 2'-fucosyllactose; 3'-FL, 3'-fucosyllactose; HMOS, human milk oligosaccharide(s); LC/ MS, liquid chromatography–mass spectrometry; LDF, lactodi-fucotetraose; SCFA, short-chain fatty acid; 3'-SL, 3'-sialyllactose; 6'-SL, 6'-sialyllactose.

References

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