

# The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota

Zhuo-Teng Yu<sup>2,4</sup>, Ceng Chen<sup>2,4</sup>, David E Kling<sup>4</sup>, Bo Liu<sup>2,4</sup>, John M McCoy<sup>3</sup>, Massimo Merighi<sup>3</sup>, Matthew Heidtman<sup>3</sup>, and David S Newburg<sup>1,2,4</sup>

<sup>2</sup>Department of Pediatric Gastroenterology and Nutrition, Harvard Medical School, Massachusetts General Hospital, Boston, MA 02129, USA and  
<sup>3</sup>Glycosyn, LLC, Medford, MA 02155, USA

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Breast-fed infant microbiota is typically rich in bifidobacteria. Herein, major human milk oligosaccharides (HMOS) are assessed for their ability to promote the growth of bifidobacteria and to acidify their environment, key features of prebiotics. During *in vitro* anaerobic fermentation of infant microbiota, supplementation by HMOS significantly decreased the pH even greater than supplementation by fructooligosaccharide (FOS), a prebiotic positive control. HMOS elevated lactate concentrations, increased the proportion of *Bifidobacterium* spp. in culture, and through their fermentation into organic acids, decreased the proportion of *Escherichia* and *Clostridium perfringens*. Three principal components of HMOS, 2'-fucosyllactose, lactodifucotetraose and 3-fucosyllactose, were consumed in these cultures. These three principal oligosaccharides of human milk were then individually tested as supplements for *in vitro* growth of four individual representative strains of infant gut microbes. *Bifidobacterium longum* JCM7007 and *B. longum* ATCC15697 efficiently consumed oligosaccharides and produced abundant lactate and short-chain fatty acids, resulting in significant pH reduction. The specificity of fermentation differed by microbe species and strain and by oligosaccharide structure. *Escherichia coli* K12 and *C. perfringens* did not utilize appreciable fucosylated oligosaccharides, and a typical mixture of organic acid fermentation products inhibited their growth. In summary, 2'-fucosyllactose, lactodifucotetraose, and 3-fucosyllactose, when cultured with *B. longum* JCM7007 and *B. longum* ATCC15697, exhibit key characteristics of a prebiotic *in vitro*. If these bifidobacteria are

representative of pioneering or keystone species for human microbiota, fucosylated HMOS could strongly promote colonization and maintenance of a mutualist symbiotic microbiome. Thus, these simple glycans could mediate beneficial effects of human milk on infant health.

**Keywords:** bifidobacteria / fucosylated oligosaccharides / human milk / prebiotic

## Introduction

After birth, the vacant infant gut undergoes seeding by pioneering microbial species such as bifidobacteria followed by succession by commensals and mutualists, resulting in the establishment of a complex stable microbiota as the infant matures (Mackie et al. 1999). Human milk, ideally the principal food of infants during the first year of life, has been proposed to contain factors that stimulate growth by mutualist bacteria (Gyorgy et al. 1954). During the period of infancy, there is a lower rate of morbidity and mortality in breast-fed infants relative to artificially fed infants (Cunningham 1979).

Historically, the lower incidence of disease in infants while they are breast feeding has been attributed to many causes, including less exposure to contaminated water, better nutritional status and psychological factors (Goldman et al. 1996). More recently, bioactive molecules in human milk per se are recognized as directly reducing disease among breast-fed infants, including secretory IgA, enzymes (Goldman et al. 1996) and the high numbers and amounts of many other complex carbohydrates of human milk, particularly oligosaccharides (Newburg et al. 1986; Newburg 2000).

Oligosaccharides are present in the third highest concentration of all the solid components in human milk, but do not play an appreciable role in meeting the nutritional needs of the infant, as they are essentially indigestible by mammalian intestinal enzymes (Newburg et al. 1986). In the last six decades, more than 120 oligosaccharides have been isolated and identified (Newburg and Neubauer 1995). Human milk oligosaccharides (HMOS) can directly inhibit colonization by enteric pathogens (Yolken et al. 1992; Ruiz-Palacios et al. 2003; Newburg et al. 2005) during infancy. In human milk, many of the glycans are fucosylated, and the content of 2'-linked fucosyloligosaccharides in human milk is significantly associated with a lower risk of many types of diarrhea in breast-fed infants (Newburg et al. 2004). A prebiotic function of the complex array of HMOS could also contribute

<sup>1</sup>To whom correspondence should be addressed: Program in Glycobiology, Higgins Hall, Biology, 140 Commonwealth Avenue, Boston College, Chestnut Hill, MA 02467-3961, USA. Tel: +1-617-552-1529; Fax: +1-617-552-2011; e-mail: david.newburg@bc.edu

<sup>4</sup>Current address: Department of Biology, Boston College, Chestnut Hill, MA 02467, USA.

toward their association with the lower risk of disease in breast-fed infants (Newburg 2001b).

Typical prebiotics in use today, such as fructooligosaccharides (FOS) and galactooligosaccharides, enhance the growth of bifidobacteria and lactobacilli; bacteria digest the prebiotic to release sugars that can then be fermented to produce organic acids, lowering the pH (Gibson and Roberfroid 1995). Prebiotics are reported to suppress the numbers of potentially harmful bacteria in the microbiota and confer other health benefits to the host. Human milk glycans can promote colonization of the infant gut by bifidobacteria (Gyorgy et al. 1954), a prebiotic effect that may indirectly inhibit colonization by pathogens. These could work in concert with fucosylated glycans expressed on the surface of the intestinal mucosa toward establishing a normal, beneficial microbial community in the gut. Low expression of fucosylated glycans in the intestine of premature infants is associated with especially a high risk of negative outcomes (Morrow et al. 2011). Incongruent early microbiota are associated with the risk of necrotizing enterocolitis (Mshvildadze et al. 2010). Thus, if fucosylated glycans of human milk function as prebiotics to promote colonization by fucose-utilizing mutualist symbionts of the immature intestinal mucosa of infants, they could confer health benefits to the neonate.

Although HMOS have been proposed to function as a prebiotic for bifidobacteria (Ward et al. 2006; LoCascio et al. 2010; Marcobal et al. 2010; Asakuma et al. 2011), the relative activity of the most common isolated fucosylated HMOS had not been determined. This study investigates in vitro the physiology of consumption of the natural mixture of HMOS and of its principal individual fucosylated oligosaccharides by the entire infant fecal microbiota community and selected major individual bacteria from this community. The organic acids produced by bacteria as they ferment individual oligosaccharides were measured, as well as the effects of these acids on growth and function of non-fermenting bacteria. FOS, a well recognized prebiotic, is used as a reference positive control.

## Results

### *Effect of HMOS supplementation on bacterial growth, pH, lactate and oligosaccharide consumption*

To determine how HMOS affect the infant intestinal microbiome, fecal slurries representing the comprehensive microbial communities from each of nine infants were inoculated into basal medium; the medium was supplemented with HMOS or FOS as the sole sugar source to test the interactions between fecal microbiota and different substrates. Several parameters of the microbiome were analyzed, including bacterial growth, pH changes, lactate production and human milk oligosaccharide consumption. HMOS supplementation significantly increased the number of bifidobacteria, as determined by real-time polymerase chain reaction (PCR), while the numbers of *Escherichia* spp. and *Clostridium perfringens* declined (Fig. 1).

Lactate concentrations were significantly higher in both HMOS- and FOS-supplemented microbiota than in controls ( $P < 0.05$ ). Consistent with these results, fecal microbiota cultures grown in the medium containing HMOS had

significantly lower pH after 48 h of incubation than control cultures grown in the basal medium. HMOS decreased the pH even more than FOS-supplemented positive controls ( $P < 0.05$ , Fig. 2), which is noteworthy because FOS is an archetypal prebiotic.

After 48 h of fermentation, the remaining HMOS in the fecal culture supernatants was extracted, reduced and quantified by liquid chromatography–mass spectrometry (LC–MS; Newburg 2001a). Over 90% of 2'-fucosyllactose (2'-FL) and lactodifucotetraose (LDFT) and 53% of 3-fucosyllactose (3-FL) from the HMOS supplement were consumed by cultured fecal microbiota (Fig. 2). These data indicate that 2'-FL and LDFT are readily metabolized by fecal bacteria. Although 3-FL is also consumed, it appears to be more resistant to catabolism by the microbiota of infants.

These results suggested the hypothesis that some oligosaccharides may have stronger prebiotic effects than others and that these differences may be driven more by some component bacteria of the microbiome than others. This hypothesis was tested: First, representative major individual bacteria of the infant microbiota were fermented with total milk oligosaccharide supplementation. Then, the responses of these individual bacteria to supplementation by the major individual oligosaccharides of typical human milk were compared with their responses to total HMOS supplementation.

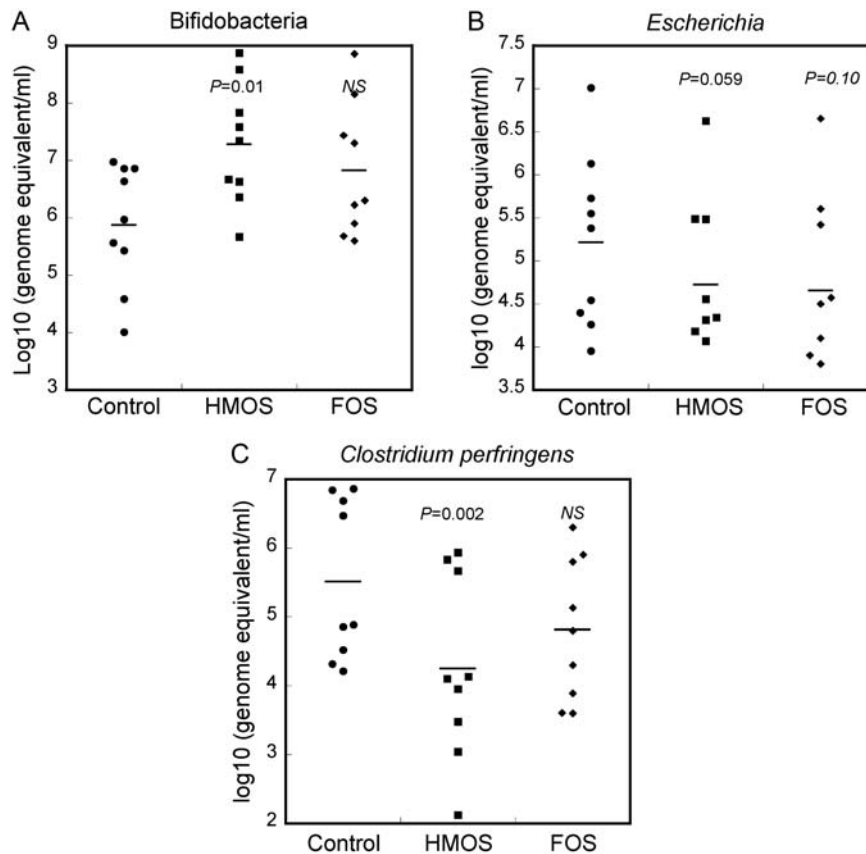
### *Growth of isolated bacteria fed HMOS or individual fucosylated oligosaccharides*

Four representatives from human microbiota were selected to represent two opposite responses of fecal microbiota to HMOS: Two of the bifidobacteria, *B. longum* (designated as *B. infantis*) ATCC15697 and *B. longum* JCM7007, exhibited strong growth stimulation in response to HMOS. In contrast, *C. perfringens* and *E. coli* K12 exhibited growth suppression when fecal slurry was supplemented with HMOS.

The growth of each of the four bacteria supplemented with HMOS as the sole sugar source is shown in Supplementary data, Figure S1. The total HMOS stimulated significant growth of the two bifidobacteria strains, and the growth increase was even greater than that of the positive prebiotic control, FOS, when fed at equal concentrations of 5 g/L. Supplementation with 2'-FL or 3-FL at 2 g/L, or LDFT at 1 g/L, also promoted the growth of ATCC15697 and JCM7007. However, the degree of growth increase was lower with LDFT than 2'-FL or 3-FL. In contrast, *E. coli* and *C. perfringens* grew only somewhat more in HMOS supplemented media beyond that of the unsupplemented controls (Fig. 3), and this low level of growth stimulation may be attributed to the residual lactose in the HMOS preparations.

### *Anaerobic fermentation products by isolated bacteria fed HMOS or individual fucosylated oligosaccharides*

When bacteria of the microbiota metabolize sugars anaerobically, much of the fermentation product is typically lactate and short-chain fatty acids (SCFA). In vitro this results in pH reduction in the culture medium, and in vivo results in pH reduction in the gut lumen. Therefore, the production of lactate and SCFA due to oligosaccharide fermentation by the individual bacteria was determined (Fig. 3).



**Fig. 1.** Differences in microbial composition between fecal microbiota incubated in the presence of HMOS or FOS and their unsupplemented media controls. The microbes monitored included genus *Bifidobacteria* (A), genus *Escherichia* (B) and species *C. perfringens* (C) as a function of HMOS or FOS supplementation for 48 h. Quantitative PCR determined relative gene frequencies. Each data point represents the microbiome of one individual.  $P \leq 0.05$  was considered statistically different from controls.

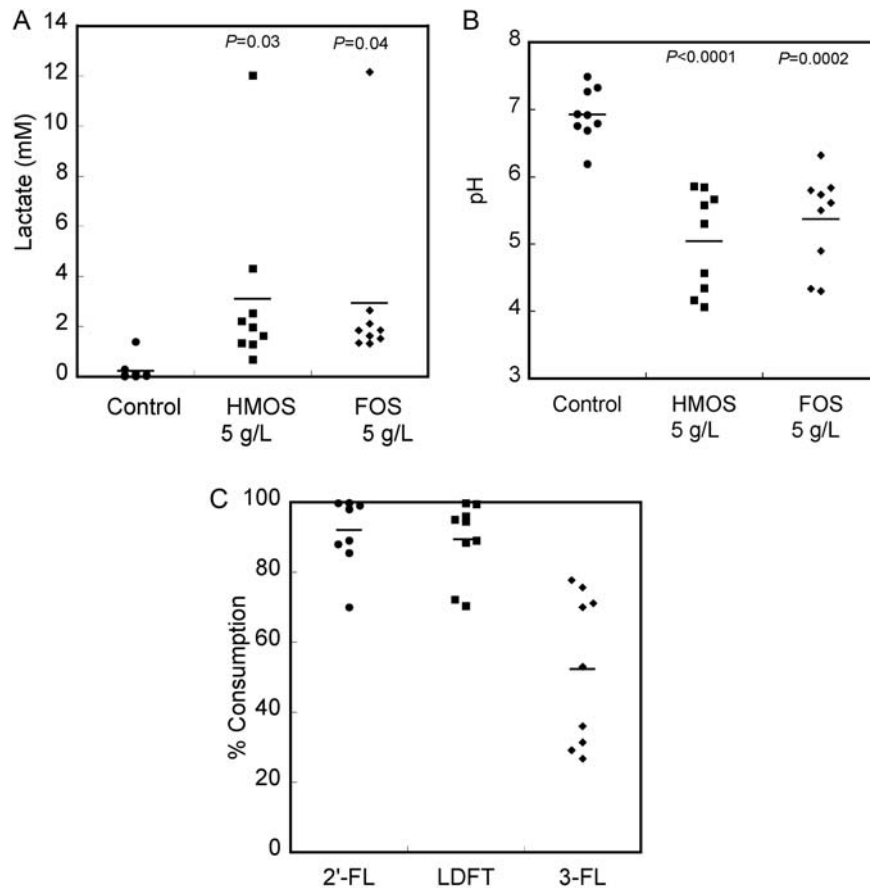
When supplemented with HMOS, *B. longum* ATCC15697 and JCM7007 produced copious lactate and SCFA, accompanied by a significant pH reduction in the culture medium. When supplemented with only 2'-FL, ATCC15697 and JCM7007 also produced ample lactate and SCFA, with ATCC15697 producing more lactate and less SCFA than JCM7007. ATCC15697 and JCM7007 also metabolized 3-FL and LDFT into lactate and SCFA, with JCM7007 displaying greater ability to transform 3-FL into lactate and SCFA than ATCC15697. Thus, even in closely related bifidobacteria strains, fermentation of the same oligosaccharides can result in differences in the relative amounts of fermentation products. The pH reduction induced by the total HMOS at 5 g/L was greater than that of 2 g/L supplementation by individual 2'-FL or 3-FL, or by 1 g/L of LDFT, their approximate physiologic levels in typical human milk. Therefore, no single oligosaccharide accounts for all of the prebiotic activity of the natural HMOS mixture. The pH reduction by the total HMOS at 5 g/L also exceeded that of FOS at 5 g/L in the *Bifidobacterium* spp. (Fig. 3). In stark contrast, very little of these individual fucosylated oligosaccharides were converted by *E. coli* K12 into lactate or SCFA, and *C. perfringens* produced significantly less of these metabolic products than either of the bifidobacteria. In contradistinction, supplementation with these acids at the concentrations produced by the

HMOS-supplemented bifidobacteria cultures significantly inhibited the growth of *E. coli* K12 and *C. perfringens* (Fig. 4). These data demonstrate and confirm that bifidobacteria ferment milk oligosaccharides into acids more efficiently than representatives of other bacterial genera; in mixed microbiota cultures, the inhibition of *E. coli* and *C. perfringens* growth by human milk oligosaccharide supplementation seems to be mediated not by the HMOS per se, but by their organic acid fermentation products.

After 48 h, for each bacterial culture condition, oligosaccharide consumption was determined by LC-MS (Fig. 5). *B. longum* ATCC15697 and JCM7007 consumed  $\geq 40\%$  of the 2'-FL, 3-FL or LDFT in the medium. ATCC15697 utilized 2'-FL better than JCM7007. In contrast, *E. coli* K12 and *C. perfringens* did not utilize 2'-FL, 3-FL or LDFT appreciably. Even between the two closely related dominant bifidobacteria that consume HMOS and produce organic acids, each bacterium differs in the magnitude of specific oligosaccharide consumed and of specific organic acids produced.

## Discussion

Human milk is unique in its amount and variety of complex carbohydrate structures, particularly oligosaccharides. Human



**Fig. 2.** Fermentation products of fecal microbiota. (A) L-Lactate levels of fecal microbiota after 48 h incubation with HMOS (5 g/L) or FOS (5 g/L) relative to media controls. (B) Corresponding pH changes. (C) Human milk oligosaccharide consumption by fecal microbiota after 48 h of incubation. Consumption is the difference for each oligosaccharide between initial concentration and that after 48 h of incubation. Each data point represents the microbiome of one individual. Differences between supplemented microbiota and their media controls were considered significant at  $P \leq 0.05$ .

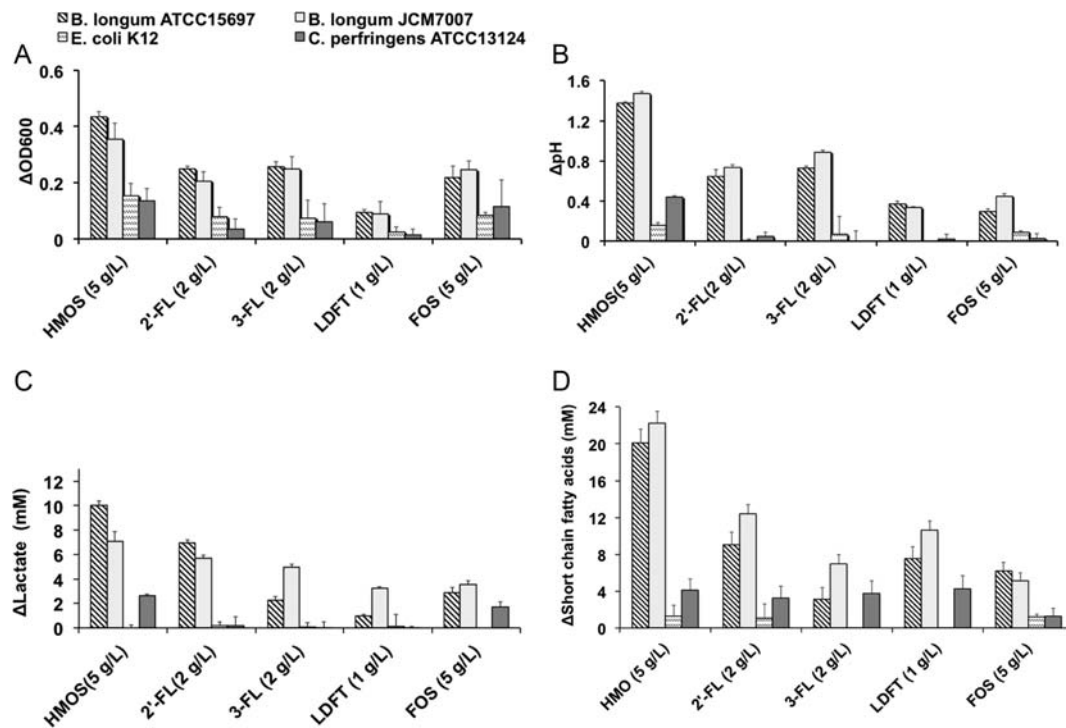
milk glycans are essentially indigestible by the mammalian gut and, therefore, pass into the distal gut where they influence the composition of the intestinal microbiota (Zivkovic et al. 2011). Learning details of human milk oligosaccharide interaction with microbiota was the objective of this study.

The first consideration was defining the ability of the infant fecal microbial community to utilize HMOS, a precondition of a prebiotic effect, in our anaerobic culture system. When infant fecal microbiota was cultured in the presence of HMOS, the growth of *Bifidobacterium* spp. was stimulated, accompanied by a decrease in pH level and an increase in lactate concentration. In aggregate, these results are consistent with the earlier reports of a prebiotic effect of HMOS (Coppa et al. 2004). Moreover, the principal prebiotic activities, stimulation of bifidobacteria growth and production of organic acids, are more strongly manifest by HMOS supplementation than by an equivalent amount of FOS. This is remarkable due to the wide acceptance of FOS as the most established and efficacious prebiotic. These results imply that HMOS are powerfully prebiotic for the infant microbiome.

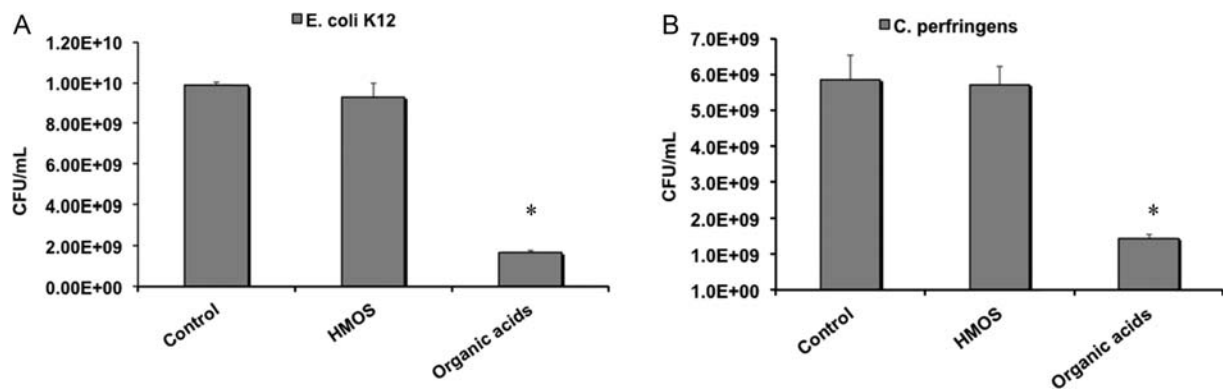
During anaerobic fermentation of HMOS, the fecal microbiota consumed >90% of 2'-FL and LDFT, identifying these two principal fucosylated oligosaccharides in human milk as

major components consumed by the infant microbiome. The lesser consumption (53%) of 3-FL raised the possibility that this HMOS could be catabolized less efficiently by the same major bacteria (structure specific) or could be a primary source of energy for a minor member of the infant microbiota (microbe specific). These possibilities were tested in cultures of two isolated *B. longum* strains, as well as *E. coli* K12 and *C. perfringens*, to compare the specificity of oligosaccharide utilization by bacteria individually and collectively, their utilization of individual isolated major milk oligosaccharides and their fermentation products.

Among closely related *B. longum* strains, the most robust growth on HMOS had been observed in *B. longum* subsp. *infantis* (LoCascio et al. 2009; LoCascio et al. 2010). Accordingly, our HMOS preparation enhanced the growth of *B. longum*. *B. longum* ATCC15697 has been described as an archetype HMOS-utilizing strain containing several gene regions that allow milk glycan utilization (LoCascio et al. 2010). Comparative genomic hybridizations indicate that *B. longum* ATCC15697 milk utilization gene regions contain glycosyl hydrolases such as fucosidase and sialidase and contain ATP-binding cassette transporter genes likely to support the efficient metabolism of glycans such as the



**Fig. 3.** Ability of four individual species of infant microbiota to utilize milk oligosaccharides. (A) Differences in growth of *B. longum* ATCC15697, *B. longum* JCM7007, *E. coli* and *C. perfringens* after 48 h incubation with purified 2'-FL, 3-FL, LDFT, FOS or a complete HMOS mixture. Growth was the difference in OD<sub>600</sub> between the media control and supplemented group for each species (mean ± SD). (B) Changes in pH, (C) lactate production (mmol/L) and (D) SCFA production (mM) were the differences in these parameters between these four bacteria after 48 h incubation with HMOS, 2'-FL, 3-FL, LDFT or FOS and their unsupplemented media controls (mean ± SD).



**Fig. 4.** Growth of *E. coli* and *C. perfringens* (CFU/mL) supplemented with either typical organic acid fermentation products of HMOS (100 mM) or HMOS per se. Growth was the final plate count after 48 h incubation of *E. coli* (A) and *C. perfringens* (B), supplemented with HMOS (5 mg/mL), a mixture of organic acids typically produced by fermentation of HMOS (100 mM) and unsupplemented media controls. \* $P \leq 0.05$  relative to control.

HMOS (LoCascio et al. 2010). *B. longum* JCM 7007 display similar phenotypic characteristics as *B. longum* ATCC15697, exhibiting the same strong bifidogenic response to supplementation by each of the three principal HMOS. This shared phenotype suggests conserved genes that provide metabolic capacity to utilize carbon of milk glycans, and such conserved genes have been proposed as genotypic biomarkers that might indicate shared adaptation to a unique nutrient ecological niche (LoCascio et al. 2010). The metabolic efficiency for

HMOS of strains such as *B. longum* ATCC15697 and *B. longum* JCM7007 would be consistent with these bacteria having a central role in the microbiome of the breast-fed infant gut, such as being founder colonizers or keystone species.

When the bifidobacteria isolates were supplemented with the individual fucosylated oligosaccharides, ATCC15697 and JCM7007 consumed >40% of the supplement over 48 h, which is lower than fecal microbiota collectively (consumption >90%

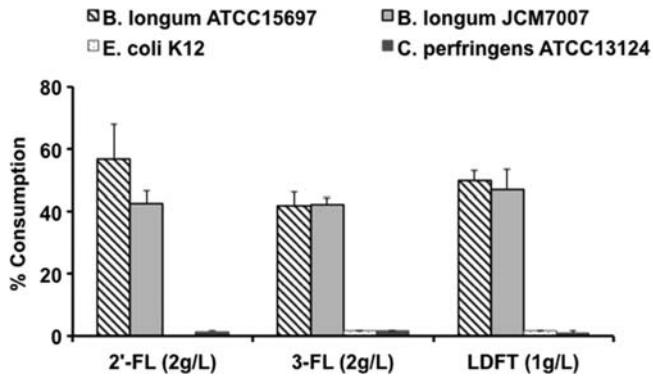


Fig. 5. Ability of the four bacteria to consume purified 2'-FL, 3-FL and LDFT. The levels of oligosaccharide (LC-MS) were compared before and after 48 h incubation with each bacterial isolate (mean  $\pm$  SD).

of 2'-FL and LDFT). In a fecal microbiota community, cooperation between different species allows the community to more efficiently utilize substrates than is possible by a single bacterium (Faust et al. 2012). The more complete metabolism of HMOS by whole infant microbiota than by their isolated bacterial components provides a concrete example of cooperative communal behavior enhancing the metabolism over that possible by even the major isolates of the community supplemented with the major oligosaccharides of human milk. This has ramifications for human health. Within the two bifidobacteria, ATCC15697 showed greater ability to consume 2'-FL and produced more lactate, but less SCFA, than JCM7007, while JCM7007 transform 3-FL into lactate and SCFA more efficiently than ATCC15697. These findings confirm that different strains and species within a human gut microbiome each have overlapping but unique specificities with regard to their ability to metabolize the individual milk oligosaccharides from the mixture found in human milk. In aggregate, human infant microbiota is able to utilize HMOS to an extent that equals, and often exceeds, their ability to metabolize the most common and active plant-derived prebiotic.

In the feces of breast-fed infants, the human milk dependent decrease in pH was thought to contribute to the lower numbers of potential pathogens and other non-mutualists (Newburg et al. 1990). Similarly, in our in vitro fermentation model, feeding HMOS to an infant fecal microbiota community did not support, but rather suppressed, the growth of *E. coli* and *C. perfringens* relative to unsupplemented controls. Lactate and SCFA are the major metabolic end-products of milk oligosaccharide fermentation by the human microbiota, lowering the pH. To assess the degree to which the acid fermentation products of oligosaccharides contribute to this suppression of non-mutualists, a mixture of organic acids mimicking the fermentation end products of HMOS-supplemented *B. longum*, both qualitatively and quantitatively (including pH), were added to the media of *E. coli* and *C. perfringens*. The growth inhibition by the acids per se was comparable with that observed in HMOS-supplemented fecal cultures, pointing to their mediating most of the oligosaccharide effect.

*E. coli* cultured in isolation grew on ZMB1 medium, consistent with their ability to utilize the amino acids of this

relatively rich medium; in the process they produce some organic acid. However, this *E. coli* basal growth did not change significantly upon supplementation with total HMOS or individual fucosylated oligosaccharides, nor was there any appreciable change in levels of lactate or pH, confirming that *E. coli* is a non-HMOS consumer (Marcobal et al. 2010; Shen et al. 2011). Overall, these results support the longstanding notion that the inhibition of gut pathogens by HMOS is mediated to a major extent through the change in organic acid concentrations and pH by bifidobacteria.

Because the oligosaccharide pattern in milk varies among individual mothers (Newburg et al. 2004), it would follow that individual mothers may have milk that varies in its prebiotic potential, both qualitatively and quantitatively. Likewise, the glycan content of milk of an individual varies over the course of lactation (Newburg 1996), and the data in this current report imply that human milk may vary in prebiotic potential or specificity over the course of lactation. This is consistent with recent findings that the infant microbiota varies among individuals and changes as the infant matures (personal communication). Perhaps individual variation in HMOS directs colonization by specific bacteria, leading to systematic differences in microbiota. If so, this phenomenon could contribute toward the differential risk of disease that has been observed in breast-fed infants of genetically distinct mothers (Newburg et al. 2004).

The conclusions reported herein are supported by the changes in mean values of each parameter from all nine of our fecal samples. The changes in each parameter were also evaluated within each individual; each significant change in mean values was reflecting either eight of the nine or nine of the nine fecal samples exhibiting a concordant change. Thus, notwithstanding individual differences in microbiota from individual infants (four first generation Chinese and five North Americans of European ancestry), the phenomena reported herein were common to our entire sample set.

Putting these results into a broad perspective, total HMOS and their specific fucosylated oligosaccharide components confer benefits to breast-fed infants through multiple complementary mechanisms. Some HMOS can directly inhibit the pathogen binding to intestinal cell surface receptors, an innate immune function (Newburg et al. 2004). Other human milk glycans, like classic prebiotics, stimulate the growth of specific bifidobacteria, allow them to ferment the glycans into organic acids and drastically reduce the pH of the chyme in the lumen of the gut. The strength of these HMOS prebiotic effects equals or exceeds that of our model prebiotic. Acidification of the chyme suppresses the growth of non-mutualists, while oligosaccharides concurrently select desired bacteria as early colonizers, stimulating the development of the complex normal community of interdependent microbiota important to human health and development. Future studies may define putative contributions of HMOS in promoting colonization by pioneering species, microbes essential to succession and keystone species of the mature microbiome. These functions could also help with subsequent preservation and renewal of the human gut microbiota. Failure of these functions may underlie chronic diseases of microbial dysbiosis. This strong prebiotic effect of specific HMOS adds to the

known human milk functions in protecting the infant from microbial challenges and supporting proper colonization of the gut during development. These data support the promotion of breast feeding and the provision of HMOS upon weaning.

## Materials and methods

### Substrates

Materials that support bacterial growth when provided as the sole sugar source included HMOS (5 g/L) isolated and purified from pooled human milk (Stahl et al. 1994), 2'-FL (2 g/L), 3-FL (2 g/L), LDFT (1 g/L; Glycosyn, Inc., MA). FOS from chicory was from Sigma (Sigma-Aldrich Chemical Co., St. Louis, MO, F8052).

### *In vitro* fermentation with infant fecal bacteria community

Fresh fecal samples were collected from nine healthy babies who had not received antibiotics or pre/probiotics since birth and had no recent history of gastrointestinal disorders. The study protocol and consent forms were approved by the human studies Institutional Review Board of Boston College. Experiments were initiated within 2 h of initial stool collection to ensure that the fecal microbiota were representative of fresh stools and, therefore, of infant colon microbiota. An aliquot of each sample was prepared as a 10% (w/v) slurry of feces in pre-reduced phosphate-buffered saline (PBS; pH 7.2; Oxoid Ltd., Basingstoke, UK); an aliquot of the slurry (1 mL) served as the inoculum. Fermentation was performed *in vitro* in an anaerobic workstation (DG250 Anaerobic Workstation, Don Whitley Scientific Ltd., West Yorkshire, UK). Each substrate, HMOS and FOS, (5 g/L final concentration) was loaded into tubes containing 9 mL of autoclaved medium plus a 1 mL inoculum of slurry. Medium contained per liter: 2 g peptone, 2 g yeast extract, 0.1 g NaCl, 0.04 g K<sub>2</sub>HPO<sub>4</sub>, 0.01 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g CaCl<sub>2</sub>·6H<sub>2</sub>O, 2 g NaHCO<sub>3</sub>, 0.005 g hemin (Sigma-Aldrich), 0.5 g L-cysteine HCl, 0.5 g bile salts, 2 mL Tween 80, 10 µL vitamin K and 4 mL of 0.025% (w/v) resazurin solution (Oxoid Ltd.). Incubation was at 37°C. The baseline control was medium and inoculum without any added sugar source. Culture fluid was taken for analysis after 48 h, a time that we had determined to be several hours into the maximum stationary phase for HMOS-treated and negative control populations. All experiments were carried out in triplicate, and each tube was stored at -20°C until analysis.

### *In vitro* fermentation with bacteria species

Four bacteria strains were selected as representative species to test their ability to utilize HMOS and individual fucosylated milk oligosaccharides, *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (originally typed as *B. infantis* subsp. *infantis*; Sakata et al. 2002), *Bifidobacterium longum* subsp. *infantis* JCM 7007, *Escherichia coli* K12 and *C. perfringens* ATCC13124. *Bifidobacterium* spp. and *C. perfringens* were propagated in reinforced clostridial medium and *E. coli* in Luria broth. Seed cultures were incubated overnight and the optical density at 600 nm reached 0.5. Bacteria were grown in an anaerobic chamber at 37°C.

The growth of individual bacteria strains in the presence of fucosylated oligosaccharides was in sugar-free basal medium

ZMB1, a chemically defined medium containing 57 chemical components (including various essential amino acids, vitamins and minerals) that supports the growth of various bacteria (Zhang and Block 2009). All glycan test materials were dissolved for an hour in the ZMB1 medium before inoculation with the 10% (v/v) bacteria slurry. Bacteria were harvested by centrifugation at 4000 × *g* for 10 min at 4°C, and the pellets were suspended with PBS at approximately 10<sup>8</sup> bacteria per mL. Baseline controls were ZMB1 plus inoculum containing no additional sugar source substrate. Positive controls were ZMB1 containing FOS (5 g/L) plus inoculum. Preliminary experiments indicated that 48 h of incubation was well beyond the formation of the final stationary phase of fermentation for all conditions used herein, and the final stationary phase was used for all comparisons. Anaerobic fermentation was at 37°C. The culture medium taken at 48 h was used to measure growth as optical density at 600 nm in a microtitre plate using a BioTek synergy H4 plate reader (BioTek, Winooski, VT). Two biological replicates of three technical replicates each were performed for every distinct experimental condition. The optical density (OD) and growth rates at 48 h were expressed as the mean and standard deviation of all replicates. The OD observed for each strain and substrate was compared with the OD in the absence of any sugar source. Difference in OD ( $\Delta$ OD) was one criterion for evaluating the ability of a strain to utilize the different substrates.

### *Non-mutualist incubation with typical organic acid fermentation products*

*E. coli* and *C. perfringens* were grown in the presence of a mixture of organic acids typical of bifidobacterial oligosaccharide fermentation products. To seed each culture tube, bacteria were harvested by centrifugation at 4000 × *g* for 10 min at 4°C, and the pellets re-suspended with PBS to approximately 10<sup>8</sup> bacteria per mL. Test materials were HMOS (5 g/L) or organic acid mixture (mixture of 25 mM each of lactate, acetic acid, propionic acid and butyric acid). ZMB1 plus inoculum containing no additional sugar source was the baseline control. Test materials were dissolved for an hour in the ZMB1 medium before inoculation. Media was incubated at 37°C. The experiment was carried out in triplicate and total bacteria at 48 h were enumerated by plating.

### *pH and lactate levels*

Culture medium pH was recorded after 48 h of bacterial fermentation using a Corning pH meter (Corning, New York, pH meter 240). Lactate concentration in the medium was determined using a lactate assay kit (kit no. K607-100; BioVision Inc., CA). Two biological replicates (three technical replicates each) were performed for every studied strain and substrate. The difference in pH for each strain grown on a different carbohydrate minus the pH obtained in the absence of the sugar ( $\Delta$ pH) reflected the ability of a strain to produce acids on different substrates.

### *SCFA analysis by LC-MS*

SCFA were quantified by LC-MS. The culture sample was brought to 4°C, 1 mL aliquot was centrifuged at 10 000 × *g*

for 10 min at 4°C, the supernatant passed through 0.22 µm syringe filter and the filtrate was sealed and stored at 4°C briefly until analysis. All reagents were of analytical grade from Sigma-Aldrich (PA). Ultra-pure water was generated through a Super-Q water purification system (Millipore, Billerica, MA). The chromatographic separation was on an Agilent ZORBAX Eclipse XDB-C8 (4.6 × 150 mm, 5 µm) column in an Agilent 1100 LC/MSD, using water as the mobile phase. The predominant SCFA were acetic, propionic and butyric acid. Hence, selected ion monitoring included the ions of acetic acid,  $m/z = 84$  (M+Na)<sup>+</sup>, propionic acid  $m/z = 98$  (M+Na)<sup>+</sup> and butyric acid  $m/z = 112$  (M+Na)<sup>+</sup>.

#### Analysis of microbial populations by real-time PCR

At the end of fermentation, 2 mL of each sample were centrifuged at 12,000 × *g* for 30 min. DNA was extracted from the cultures by the method of Zhu et al. (2003). Bacterial DNA was amplified by real-time PCR (Bio-Rad Laboratories, Hercules, CA) according to Collado et al. (2009) using a series of genus-specific or species-specific primer pairs (Frahm and Obst 2003; Matsuki et al. 2002; Rinttilä et al. 2004) described in Supplementary data, Table S1. Each reaction mixture (25 µL) was composed of iQ<sup>TM</sup> SYBR<sup>®</sup> Green Supermix (Bio-Rad Laboratories), 1 µL of each of the specific primers at a concentration of 0.25 µM and 1 µL of template DNA. After amplification, melting curves were calculated to distinguish the targeted PCR product from the non-targeted product. Standard curves were eight 10-fold dilutions of bacterial DNA extracted from pure cultures of between 2 and 9 log<sub>10</sub> colony forming units (CFUs) of each of the following selected representative species: *B. longum* ATCC 15697, *C. perfringens* ATCC13124, *E. coli* H10407 ATCC 35401.

#### Consumption of each oligosaccharide

Fermentation samples (48 h) were thawed, mixed and centrifuged at 4000 × *g* for 15 min at 4°C. The clear supernatant (0.5 mL) was treated with 0.25 mL fresh aqueous solution of sodium borohydride (0.5 M). After vigorous mixing, the mixture was reduced overnight at 4°C and the reaction terminated by the addition of 0.25 mL acetic acid (0.5 M). In 5 mL serological pipettes, 5 × 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, Bio-Rad). AG50W-X8 cation-exchange resin (hydrogen form, Bio-Rad) was converted to the pyridinium form with 1 M pyridine in an Erlenmeyer flask, 1 h × 3 times with gentle swirling, followed by three water rinses, and the resulting pyridinium form was added to the column. The column was rinsed with 5 mL water, followed by the application of the reduced samples in 1 mL water. The sample tube was rinsed into the column with water (0.5 mL), 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, which were frozen and lyophilized. The neutral oligosaccharides were analyzed by an Agilent HPLC (1200 series) with a triple-quadrupole mass spectrometer (6460), equipped with a porous graphite column (3 µm,

100 × 2.1 mm, Hypercarb, Thermo Scientific, Waltham, MA) set for 25°C. Methods were validated by authentic oligosaccharides from GlycoSeparations (Moscow, Russia; Newburg 2001a). The percent consumption of each fucosylated HMOS was calculated using the ratio of the intensities of internal standard maltopentaose to the sample oligosaccharides (M/O). Standard curves of given fucosylated oligosaccharides were constructed from the M/O ratio. Consumption was calculated by subtracting the values of each oligosaccharide at 48 h from that at 0 h.

#### Statistical Analysis

Data are expressed as mean ± standard deviation (SD). The statistical significance of differences between groups was determined by one-way analysis of variance. When differences were found, Student's *t*-test was used for pairwise comparisons;  $P \leq 0.05$  was considered significant.

#### Supplementary data

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

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#### Conflict of interest

J.M.M., M.H. and M.M. are employees of Glycosyn LLC, which is a for-profit company engaged in the manufacture of human milk oligosaccharides.

#### Abbreviations

CFUs, colony forming units; 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; FOS, fructooligosaccharide; HMOS, human milk oligosaccharides; HPLC, high-performance liquid chromatography; LDFT, lactodifucotetraose; LC-MS, liquid chromatography-mass spectrometry; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; OD, optical density; SCFA, short-chain fatty acid.

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