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Research Article

In vitro fermentability of human milk oligosaccharides by several strains of bifidobacteria

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This study was conducted to investigate the catabolism and fermentation of human milk oligosaccharides (HMO) by individual strains of bifidobacteria. Oligosaccharides were isolated from a pooled sample of human milk using solid-phase extraction, and then added to a growth medium as the sole source of fermentable carbohydrate. Of five strains of bifidobacteria tested (*Bifidobacterium longum* biovar *infantis, Bifidobacterium bifidum, Bifidobacterium longum* biovar *longum, Bifidobacterium breve, and Bifidobacterium adolescentis)*, *B. longum* bv. *infantis* grew better, achieving triple the cell density then the other strains. *B. bifidum* did not reach a high cell density, yet generated free sialic acid, fucose and N-acetylglucosamine in the media, suggesting some capacity for HMO degradation. Thin layer chromatography profiles of spent fermentation broth suggests substantial degradation of oligosaccharides by *B. longum* bv. *infantis*, moderate degradation by *B. bifidum* and little degradation by other strains. While all strains were able to individually ferment two monosaccharide constituents of HMO, glucose and galactose, only *B. longum* bv. *infantis* and *B. breve* were able to ferment glucosamine, fucose and sialic acid. These results suggest that as a potential prebiotic, HMO may selectively promote the growth of certain bifidobacteria strains, and their catabolism may result in free monosaccharides in the colonic lumen.

Keywords: Bifidobacteria / Fermentation / Milk / Oligosaccharide / Prebiotic

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1 Introduction

One characteristic that differentiates human milk from that of most other mammals is the high concentration and diversity of free oligosaccharides. These molecules are synthesized in the mammary gland by further elongation of lactose, and are composed of glucose, galactose, N-acetylglucosamine, fucose and sialic acid. They consist of linear and branched polymers, and from the structures provided by Kunz *et al*. [1], are linked together by at least 12 different types of glycosidic bonds. Human milk oligosaccharides (HMO) are heterogeneous among women and four basic phenotypic groups, fitting with the Lewis blood group system, have been recognized based on the expression and activity of two fucosyltransferases [2]. Literature values for the HMO concentration in mature milk are quite variable. For example, from three studies the concentration has been estimated to be 12–14 g/L [3], 5–8 g/L [1], and ~3 g/L [4]. The differences may represent regional diversity, different time points in lactation, differences in analytical methodology, or a combination of these factors. Nonetheless, HMO are a major constituent of human milk, and yet their functions are not well understood.

Based on two *in vitro* studies, HMO appear to be resistant to digestion by host hydrolases during transit through the small intestine and thus should arrive in the lower gastrointestinal tract relatively intact [5, 6]. To a small extent some HMO are absorbed intact and excreted in the urine [7], yet estimates of between 40–50% [8] and 97% [5] are recovered in the feces of infants consuming them. Beneficial physiological activities have been ascribed to HMO. For example, they antagonize pathogen binding to human epithelial cells [9], and the acidic fraction may be involved in immune modulation [1, 10, 11]. In addition, it has been speculated that the HMO may constitute a form of nutri-

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Abbreviations: HMO, human milk oligosaccharides

tional support for the rapid neural development human infants experience in the first 6 months of life by providing substrates for neural tissue such as galactose and sialic acid [1, 12]. However, such a nutritional role would require the intact oligomeric molecules were effectively degraded in the colon to liberate free monosaccharides.

Since the beginning of the last century breast-feeding has been associated with an infant fecal microbiota dominated by bifidobacteria, whereas the fecal microbiota of infants consuming alternative diets is described as mixed and adult-like [13, 14]. This led to the suggestion that breast milk contains specific growth factors for bifidobacteria. More recently, the bifidogenic effect of breast milk and the high HMO concentration has led to the hypothesis that HMO may function as a prebiotic [1, 15, 16], yet to date few studies have addressed this activity.

In the 1950's, Gyorgy and co-workers [17] conducted several studies that indicated a unique activity of HMO in providing a growth factor for a *Bifidobacterium* isolated from the feces of an infant. They isolated a bacterium that could not grow in a lactose-rich media unless human milk was added, and this strain was named *Lactobacillus bifidus* var *pennsylvannicus*. According to the ATCC (www.ATCC.org), this strain has been reclassified as *Bifidobacterium bifidum* ATCC 11863. Growth promotion of this strain by human milk fractions was attributed to substances containing N-acetylglucosamine, such as HMO molecules, and the amino sugar was subsequently shown to be incorporated into cell wall muramic acid [18]. Since all of the N-acetylglucosamine located in HMO is in the core of the polymer, according to published structures, it seems that *B. bifidum* ATCC 11863 must have the ability to degrade HMO to access the amino sugar. However, as the media contained lactose, this work did not address whether the strain could utilize HMO as the sole carbon source.

We recently looked at the growth of two strains of fecal bacteria (*Bifidobacterium longum* biovar *infantis* ATCC 15697, and *Lactobacillus gasseri* ATCC 33323) using HMO as the sole fermentable carbohydrate [19]. *B. longum* bv. *infantis* grew to a high cell density and was active in the catabolism of the HMO, while *L. gasseri* did not grow measurably, yet seemed to degrade some of the HMO.

This study was conducted to investigate the ability of several strains of bifidobacteria to ferment HMO, or the constituent monosaccharides, in comparison to lactose as a positive control and inulin as a well established prebiotic of commercial use.

2 Materials and methods

Three liters of human milk from several donors was provided by the Mother's Milk Bank of San Jose, CA and three liters was provided by Dr. Jimi Francis, University of Nevada, Reno. Milk was stored at –80°C until use. Unless otherwise noted, all chemicals and materials utilized were purchased from Fisher (Fairlawn, NJ).

Oligosaccharides were extracted from human milk as described by Gnoth *et al*. [6], with minor modifications. Lipids were removed by centrifugation, re-extracted two times with deionized water, and aqueous phases combined. Protein was precipitated with two volumes of 95% ethanol v/v, and precipitate was re-extracted two times with 95% ethanol-water (2:1, v/v). Extracts were combined, and lactose was hydrolyzed to monosaccharides using β-galactosidase from *Kluyveromyces fragilis* (catalog number G3665, Sigma Aldrich, St. Louis, MO). The oligosaccharides were isolated from the resulting monosaccharides using solid phase extraction with graphitized non-porous carbon as a matrix (catalog number 57130, Sigma Aldrich) [19].

Bacteria strains used in this study are *B. longum* bv. *infantis* ATCC 15697, *B. longum* bv. *longum* ATCC 15707, *B. adolescentis* ATCC 15703, *B. breve* ATCC 27539 and *B. bifidum* ATCC 29521. Bacteria were grown in and maintained in MRS media supplemented with L-cysteine [20].

For the substrate fermentability studies, various carbohydrates were used at a concentration of 1% w/v in place of glucose in the MRS medium. Substrates used were lactose, inulin (Synergy 1®, Orafti, Tienen, Belgium), purified HMO, glucose, galactose, N-acetylglucosamine, fucose, and sialic acid. Synergy 1® is a mixture of fructans with a degree of polymerization (DP) >10 (inulin HP) and oligomers with a DP <10 (oligofructose) [21], and will be referred to as inulin-OF. Media was autoclaved separately from the carbohydrate, which was added to the cooled broth after sterile filtration. All strains were grown in triplicate at 37°C on each carbohydrate source. For negative controls, tubes containing media devoid of carbohydrate were inoculated with bacteria, and tubes containing carbohydrate were left uninoculated.

Bacterial growth was measured in a Klett-Summerson colorimeter (Klett Manufacturing, New York, NY) at specific time points using the no. 45 (green) filter. After growth, tubes were centrifuged at $2000 \times g$ for 30 min to pellet the bacteria and the supernatant was collected, and analyzed.

Effect of bacterial growth on the HMO profile was measured by HPTLC with specific carbohydrate detection [22]. In short, 2 μL of media was applied to a silica gel plate, and plates were run with butanol-ethanol-water-acetic acid-pyridine (5:50:15:1.5:5 v/v) solvent system. To detect carbohydrates, plates were sprayed with orcinol solution (20 mg in 10 mL 70% H_2SO_4) and placed in a 100°C oven for 15 min.

Total and glycosidically bound sialic acid was measured using the method of Jourdian and Roseman [23] adapted to a microassay by Bhavanandan and Sheykhnazari [24]. In this method, samples (40 μL) containing less than 0.4 mM sialic acid are added to wells in 96-well plates. Ten microliters of 0.032 M periodic acid was added to each well, and mixed on a minishaker for 5 min. The plate was then placed

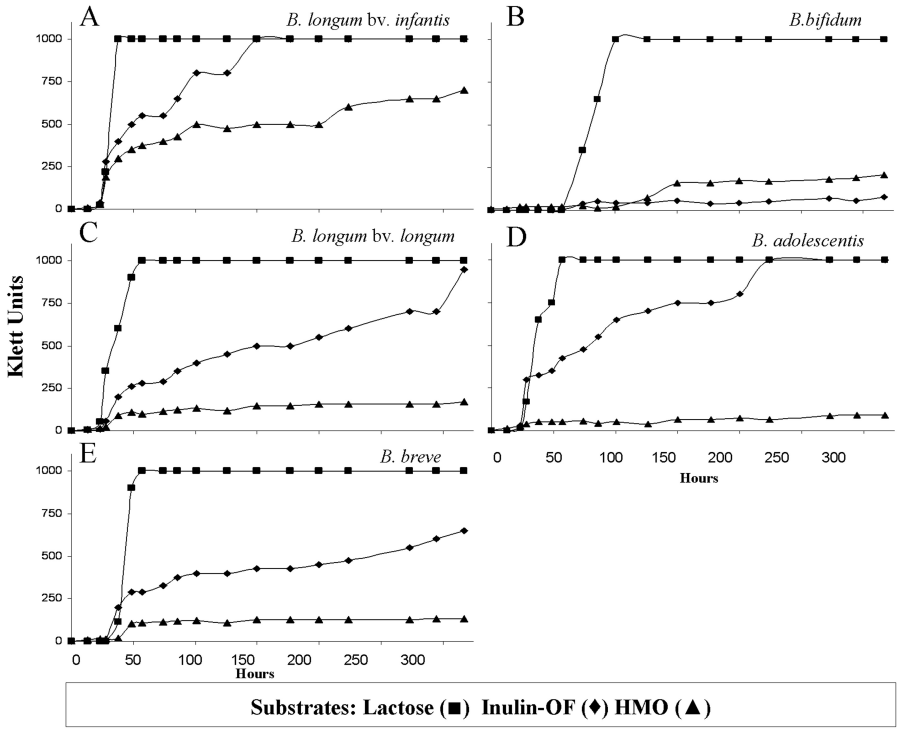

Figure 1. Growth curves for all five strains of bifidobacteria grown with lactose (■), inulin-OF (♦) and HMO substrates (▲). Fermentation assays were carried out in triplicate, and a representative curve for each condition is shown.

on ice for 35 min to oxidize the sialic acids. Resorcinol reagent (0.6 g resorcinol in solution of 60 mL 28% HCl, 40 mL water, and 25 μmoles $CuSO_4$) was added (100 μL), and plate was mixed as above. The cover was put on the plate and it was placed in an oven at 80°C for 60 min. The plate was removed from oven, cooled, and 100 μL of 95% tert-butyl alcohol v/v was added to stabilize the color. The plate was placed on a minishaker for 5 min, and absorbance was measured at 630 nm in a plate reader (Bio-Tek Instruments, Winooski, VT).

Monosaccharide constituents of the HMO, except sialic acid, were measured as alditol acetate derivatives by gas chromatography using the method of York *et al*. [25]. In the first step of this method, addition of TFA hydrolyzes carbohydrate polymers to monosaccharides, which are subsequently reduced and acetylated. This method was modified to measure free monosaccharides by the elimination of the hydrolysis step.

3 Results

Growth curves are shown in Fig. 1 for the five strains tested with the dimeric and polymeric substrates (lactose, inulin-OF and HMO). In general, all strains reached high cell densities with lactose as a substrate. With the two polymeric substrates, inulin-OF and HMO, growth was slower, and in some instances growth was still being recorded after 10 days. Using the inulin-OF substrate, *B. longum* bv. *infantis*, *B. longum* bv. *longum* and *B. adolescentis* all reached high cell densities, whereas *B. breve* reached an intermediate cell density (Fig. 1E), and *B. bifidum* (Fig. 1B) achieved little growth. With the HMO substrate, *B. longum* bv. *infantis* (Fig. 1A) achieved cell densities threefold higher than any of the other strains. Of the others, *B. bifidum* grew slightly better on the HMO than *B. longum* bv. *longum*, *B. adolescentis* and *B. breve*.

Inulin is a linear polymer of β-D-fructofuranoses and degradation of inulin-OF by bifidobacteria will lead almost exclusively to free fructose for fermentation. Degradation of HMO, on the other hand, will result in a mixture of five monosaccharides, which a given strain may or may not be able to utilize. Therefore, each strain investigated in this study was grown individually on monosaccharide constituents of HMO to determine whether they could be used as a carbon source, and the result are presented in Table 1. Glucose was well fermented by all species tested, and all but *B. breve* grew to a high density with galactose as the substrate. Only two strains, *B. longum* bv. *infantis* and *B. breve*, grew to any extent on N-acetylglucosamine, fucose and sialic acid.

Carbohydrates from the spent fermentation broth originally containing HMO or Inulin-OF were separated using HPTLC (Figs. 2A and B). It is readily apparent from these profiles that the Bifidobacterium strains differ in catabolism of oligosaccharides. Comparing the HMO (Fig. 2A, lane 2) and inulin-OF (Fig. 2B, lane 2) profiles of *B. longum* bv. *infantis* to controls (Figs. 2A and B, lane 1), it is appa-

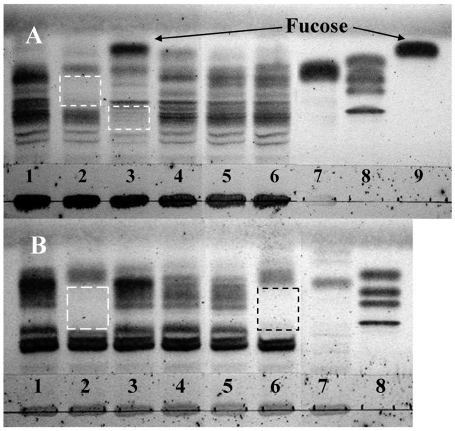

Figure 2. HPTLC of carbohydrates in bacterial media after fermentation for (A) HMO and (B) inulin-OF. Lane designations: 1) control 2) *B. longum* bv. *infantis* 3) *B. bifidum* 4) *B. longum* bv. *longum* 5) *B. breve* 6) *B. adolescentis* 7a) galactose 7b) MRS with no added carbohydrate 8) glucose, lactose, raffinose, stachyose (from top) 9a) fucose. Superimposed boxes highlight changes referred to in text.

rent a similar change has occurred, which is outlined in the superimposed box. In each case, a gap was created in the carbohydrate profile that corresponds to species of equal or greater mobility than stachyose (Figs. 2A and B, lane 8). Incubation of *B. adolescentis* on inulin-OF caused the same effect, which is also highlighted by superimposition of a box (Fig. 2B, lane 6). However, *B. adolescentis* growth did not have the same effect on the HMO profile (Fig. 2A, lane 6) which is consistent with its low cell density resulting after growth on this substrate (Fig. 1D).

B. bifidum achieved the second highest cell density on the HMO substrate, and the effects of this growth on the oligosaccharide profile is visible (Fig. 2A, lane 3). Whereas growth of most of the *Bifidobacterium* strains did not alter the profile of the more slowly migrating HMO bands, growth of *B. bifidum* did. Another observation is that the growth of *B. bifidum* on the HMO substrate resulted in a new band at the top of the lane, which was not present in the control (Fig. 2A, lane 3). Comparison to the fucose standard (Fig. 2A, lane 9) indicates this band is most likely fucose, which must have been cleaved off larger, slower migrating HMO species. This result agrees with the observation that *B. bifidum* is unable to ferment free fucose (Table 1).

B. longum bv. *longum* reached the third highest cell density on the HMO, and the third highest cell density on the inulin-OF, and the effects of this growth are shown in Figs. 2A and B, lane 4. Although *B. longum* bv. *longum* grew well on the inulin-OF, it is not possible to determine with these methods which fraction of the inulin-OF was fermented.

Lanes 5–6 in each Figs. 2A and B show the carbohydrate profiles for *B. breve,* and *B. adolescentis*, respectively. Neither of these three strains grew well on the HMO, and consequently their carbohydrate profiles are similar to that of the control. While the same is true for B. breve on the inulin-OF substrate, *B. adolescentis* grew to a high cell density on this substrate, and the effects are visible in Fig. 2B, lane 6. One striking observation is the similarity of this lane with that of *B. longum* bv. *infantis* (Fig. 2B, lane 2). In both cases, several bands have disappeared after growth of these strains, and at least one slow migrating band remained unchanged.

The carbohydrate stain used on the HPTLC plates in Fig. 2 does not detect N-acetylglucosamine or sialic acid. Therefore, the concentration and linkage of sialic acid was investigated separately using a spectrophotometric assay. Figure 3 shows the concentration of both total and bound sialic acid in the spent broth. As free sialic acid is unstable to periodate oxidation, whereas bound sialic acid is not, manipulation of the conditions of the assay allowed for a measurement of both total and bound sialic acid. Therefore, the difference between the two is an indication of free sialic acid, which is presumably generated by neuraminidases present in the bifidobacterial strains. As indicated in Fig. 3, there was 0.6 mg/mL total sialic acid in the control media, which is about 6% of the carbohydrate. *B. longum* bv. *infantis* reduced the total sialic acid concentration 68% and the remaining 32% appears to mostly be free monosaccharide (80%). Of the other strains, only *B. bifidum* had a measurable effect on the sialic acid profile. The difference between total and bound sialic acid was at least 45% of the total, suggesting that this strain also has neuraminidase activity.

To better characterize the catabolism of HMO by the bacteria, the monosaccharides constituents of the HMO were analyzed as alditol acetate derivatives using gas chromatog-

Table 1. Maximum growth for bifidobacteria with free monosaccharide constituents of HMO as fermentable substrates[a]. NG=no growth detected

Strain	Glucose	Galactose	Glucosamine	Fucose	Sialic acid
B. longum bv. *infantis* ATCC 15697	1000 (0)	1000 (0)	237 (0)	150 (23)	367 (14)
B. longum bv. *longum* ATCC 15707	1000 (0)	1000 (0)	NG	NG	NG
B. breve ATCC 15700	1000 (0)	200 (10)	303 (40)	57 (8)	550 (0)
B. adolescentis ATCC 15703	1000 (0)	1000 (0)	NG	NG	NG
B. bifidum ATCC 15696	1000 (0)	1000 (0)	NG	NG	NG

a) Cell densities measured in Klett units with SD in parentheses.

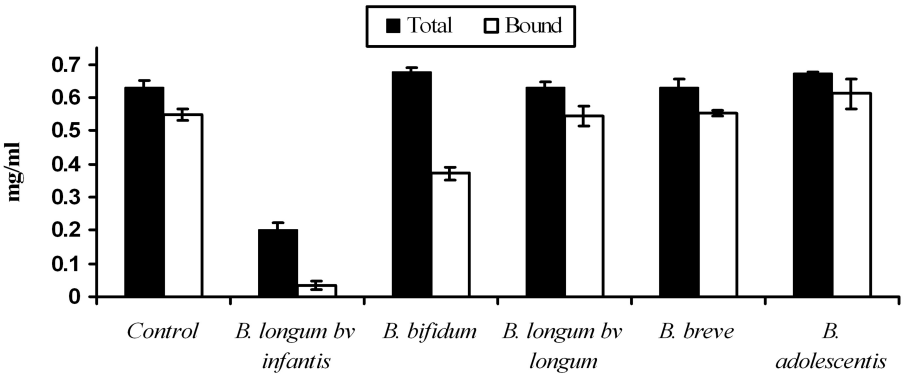

Figure 3. Total and bound sialic acid in HMO media after fermentation.

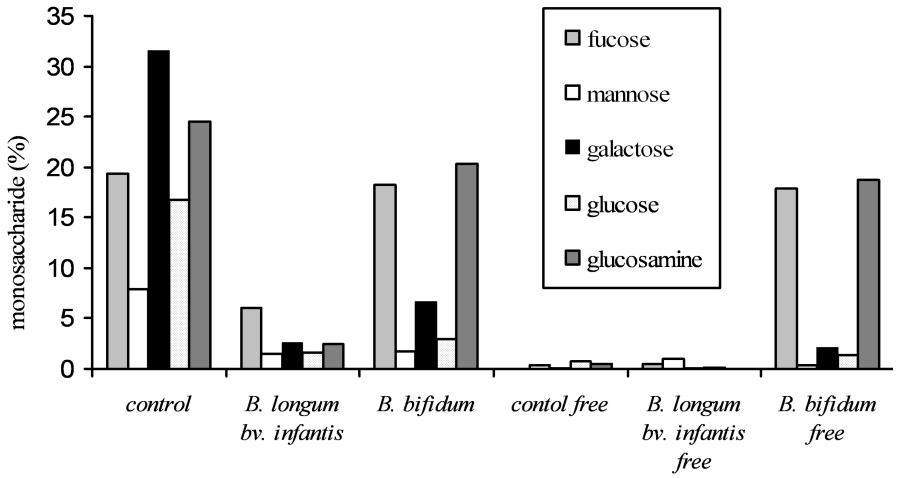

Figure 4. Total and free monosaccharides in HMO media after growth of *B. longum bv. infantis* and *B. bifidum*.

raphy (Fig. 4). As *B. longum* bv. *infantis* and *B. bifidum* were the only strains that caused marked changes to the HMO HPTLC profiles, only these samples and the control were analyzed. In the control HMO broth, galactose was the most prominent constituent, followed by glucosamine and fucose, which is in agreement with the published HMO structures of Kunz [1]. In addition, little of the carbohydrate in the control sample was in the form of free monosaccharides. There was little free monosaccharide in the *B. longum* bv. *infantis* sample, as well. After the growth of *B. longum* bv. *infantis* on the HMO substrate, all monosaccharide species were present in lower concentration, and the free monosaccharide concentration was low. This is in accordance with the HPTLC, in which no new bands were present, and with the growth studies on monosaccharide constituents of HMO, indicating *B. longum* bv. *infantis* could use each monosaccharide as a fermentable carbon source.

In the *B. bifidum* media there was a similar concentration of fucose and glucosamine as in the control, yet the concentration of galactose, glucose and mannose was much lower. These two monosaccharides are present primarily as free monosaccharides, presumably generated by the catabolism of HMO, and are in agreement with the findings of the sialic acid assay. Furthermore, the change in the HMO HPTLC profile after *B. bifidum* growth indicates catabolism of the large, slowly migrating HMO species. In sum, these data indicate *B. bifidum* contains the necessary enzymes to catabolize many of the glycosidic covalent linkages of HMO, yet only utilized the resulting free glucose, mannose and galactose for growth.

4 Discussion

Various studies have demonstrated the predominance of bifidobacterial species in the gastrointestinal tracts of nursing infant with respect to those fed synthetic diets [26]. Several species have been associated with this niche including, *B. longum* bv. *longum*, *B. longum* bv. *infantis*, *B. breve*, and *B. bifidum* among others [27]. From the few studies concerning the presence of particular strains of bifidobacteria in breast-fed infants and formula-fed infants using culturing techniques no clear pattern emerges (reviewed by Mackie *et al* [28]). In one study, *B. breve* was the dominant species in infants regardless of diet, and *B. adolescentis*, *B. longum* and *B. bifidum* were present to a

lesser extent [29]. In another, *B. infantis* was dominant in breast-fed infants, and *B. longum* and *B. bifidum* were the next most common [30].

In recent years, the distribution of bifidobacteria in breast-fed and formula-fed infants has been addressed using molecular techniques, and the results are in general agreement with culture-based studies, in that there does not seem to be a particular strain promoted by breast-feeding. Matsuki *et al.* [31] used nine bifidobacteria specific primers to identify all strains of this bacteria known to colonize humans in 27 breast fed infants, and found *B. breve* in 70%, *B. infantis* in 41%, *B. longum* 37%, *B. bifidum* in 22%, *B. catenulatum* in 19%, and *B. dentium* in 11%. The rare isolates in infants were *B. adolescentis* and *B. angulatum.* Favier *et al.* [32] followed the microflora of 4 breast-fed infants over 4 months, and *B. infantis* was detected in all four, *B. psedocatenulatum* was detected in two, and *B. bifidus*, *B. longum* and *B. adolescentis* were each detected only in one. Sakata *et al.* [27] analyzed fecal matter of one-month-old breast-fed, mixed-fed, and bottle-fed infants for bifidobacteria using species-specific primers. Bifidobacteria distribution among fecal samples was not affected by diet. *B. longum* was the most commonly detected. It was found in 43/68 specimens, while *B. infantis* was detected in 23/68 and *B. breve* in 16/68. *B. adolescentis*, *B. bifidum* and *B. catenulatem* were all detected in 10 specimens or less.

The association of breast-feeding with gut microbiota enriched in bifidobacteria, and the high concentration of HMO in breast milk suggests the possibility that fermentation of these indigestible carbohydrates may drive amplification of these bifidobacterial populations in the infant gut. However, to date there has been little direct evidence of fermentation of HMO by bifidobacteria. In order to explore the hypothesis we characterized the growth of several bifidobacterial species on HMO as a sole carbon source. Of all the strains tested, *B. longum* bv. *infantis* reached cell density threefold higher than the other bifidobacterial species when HMO were provided as a substrate. The HPTLC profile indicates that *B. longum* bv. *infantis* was able to remove many of the smaller bands, and did not produce new monosaccharide bands. Growth of this strain on free HMO monosaccharides indicated that this strain could utilize all five constituents as energy sources. As HMO are linked by at least 12 different glycosidic bonds, complete depolymerization and fermentation of the resulting monosaccharides requires an extensive armory of enzyme activities. While *B. longum* bv. *infantis* contains at least some of these activities, some intact HMO were present in the media after growth (Fig. 2). As the final pH was not prohibitive of further growth (data not shown), it appears some HMO species are resistant to catabolism by *B. bifidum* bv. *infantis*.

In contrast to the growth of *B. longum* bv. *infantis* on HMO, other bifidobacterial strains did not grow well. Interestingly, *B. breve* possesses the ability to ferment each of the HMO monosaccharide constituents individually, yet was presumably unable to access these monosaccharides from the HMO polymers. *B. bifidum*, on the other hand, did not achieve a high cell density on HMO substrates, yet was able to markedly affect the HMO TLC profile (Fig. 2A, Lane 3). However, when grown on pure monosaccharide constituents of HMO, this strain was only able to grow on glucose, mannose and galactose even though it liberated fucose (Fig. 2) and sialic acid (Fig. 3) from the HMO.

Several studies have previously demonstrated fucosidase activity in *B. bifidum.* Screening fecal isolates for the ability to degrade blood group antigens, Hoskins *et al.* [33] isolated strains of *B. bifidum* and *B. infantis* which could degrade the H antigen, but not the A nor the B antigen. The H antigen contains a terminal Fucα1→2Gal bond, and the A and B antigens are formed by the further addition of either a GalNacα1→3Gal or a Galα1→3Gal to the H antigen. Each strain also displayed neuraminidase activity. Katayama *et al.* [34] screened several bacteria for an α1→2 fucosidase, and detected the activity in the same *B. bifidum* strain as used in this study (ATCC# 29521), as well as in a *B. infantis* strain. They subsequently cloned the enzyme from *B. bifidum*, and determined it had the highest activity with 2′sialyllactose, followed by the H antigen and the HMO constituent, lacto-*N*-fucopentaose I. The enzyme was inactive with α1–3 fucose linkages.

We propose that *B. bifidum* is depolymerizing the HMO as a means to access glucose and galactose for energy, while leaving fucose, glucosamine and sialic acid in the media. The different sets of activities observed – the depolymerizing activity of *B. bifidum* with the monosaccharide fermentation capacity of *B. breve*–provides a conceptual basis for commensal activities between bifidobacterial strains whereby those capable of degrading the large HMO polymers provide monosaccharide substrates for other strains.

Higher physiological concentration of sialic acid in breast-fed infants [35] suggests they are somehow able to access the bound sialic acid in breast milk, yet little is known about any such mechanism (reviewed in [12]). Sialyted oligosaccharides resist digestion by host glycosyl hydrolases [5, 6], and a small percentage may be absorbed intact [1]. According to current understanding, the majority of both neutral and sialyated oligosaccharides consumed by breast-fed infants should arrive in the colon of the infant relatively intact. Coppa *et al.* [8] compared the profiles of 20 HMO species in breast milk, and in the feces of infants consuming it, and found that between 39–49% of oligosaccharides were excreted. Four of the HMO monitored in the milk and feces were sialyated, and from the chromatograms provided, the profiles seemed to change very little. Speculating as to a potential mechanism by which sialic acid may be made accessible from breast milk oliogosaccharides and glycoconjugates, Wang and Brand-Miller [12] suggested that free sialic acid may be produced by sialidases from the microflora, and added that colonic mucosal absorption of sialic acids has not been demonstrated. Thus, the free sialic

acid in the media generated by the neuraminidases of *B. longum* bv. *infantis* and *B. bifidum* provides evidence for the first part of this mechanism, and subsequent studies will be needed to confirm the latter.

Breast-feeding has long been associated with a fecal flora dominated by bifidobacteria. According to the results of this study, only *B. longum* bv. *infantis* was able to grow substantially using HMO as a substrate. Fermentation of the HMO by *B. longum* bv. *infantis* clearly indicates that this significant fraction of human milk may act as a prebiotic *in vivo*, and suggests *B. longum* bv. *infantis* is a good candidate for understanding the specifics of this activity.

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