DOI:10.1021/jf9044205

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Consumption of Human Milk Oligosaccharides by Gut-Related Microbes

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Human milk contains large amounts of complex oligosaccharides that putatively modulate the intestinal microbiota of breast-fed infants by acting as decoy binding sites for pathogens and as prebiotics for enrichment of beneficial bacteria. Several bifidobacterial species have been shown to grow well on human milk oligosaccharides. However, few data exist on other bacterial species. This work examined 16 bacterial strains belonging to 10 different genera for growth on human milk oligosaccharides. For this propose, a chemically defined medium, ZMB1, was used, which allows vigorous growth of a number of gut-related microorganisms in a fashion similar to complex media. Interestingly, *Bifidobacterium longum* subsp. *infantis*, *Bacteroides fragilis*, and *Bacteroides vulgatus* strains were able to metabolize milk oligosaccharides with high efficiency, whereas *Enterococcus, Streptococcus*, *Veillonella*, *Eubacterium*, *Clostridium*, and *Escherichia coli* strains grew less well or not at all. Mass spectrometry-based glycoprofiling of the oligosaccharide consumption behavior revealed a specific preference for fucosylated oligosaccharides by *Bi. longum* subsp. *infantis* and *Ba. vulgatus*. This work expands the current knowledge of human milk oligosaccharide consumption by gut microbes, revealing bacteroides as avid consumers of this substrate. These results provide insight on how human milk oligosaccharides shape the infant intestinal microbiota.

KEYWORDS: Human milk oligosaccharides; gut microbiota; chemically defined medium; ZMB1; MALDI-FTICR-MS; bacteroides

INTRODUCTION

Human milk, the sole nourishment for breast-fed infants, is an interesting model of a food shaped by evolution to promote the healthy development of newborns. Of the various components in human milk, oligosaccharides constitute a significant fraction, being the third most abundant molecular species in terms of concentration after lactose and lipids (*1*). Up to 200 different structures have been defined for human milk oligosaccharides (HMOs) (*2*). All HMO structures follow the same basic configuration: a lactose core at the reducing end, elongated by *N*-acetyllactosamine units with at least 12 different types of glycosidic bonds, wherein fucose and sialic acid residues are added to terminal positions (*3*). The linear and branched HMOs vary in size, from 3 to 32 sugars, being mostly fucosylated neutral oligosaccharides (*2*). Infants digest a minor portion of HMOs present in the breast milk, whereas a fraction passes undigested through the intestine (*4*). Different researchers have confirmed that HMOs are resistant to enzymatic hydrolysis from intestinal brush border membrane and pancreatic juices (*5*, *6*). We hypothesize that, given the high concentration of oligosaccharides in human milk, these polymers must have an important role in the well-being of the baby, particularly when considering the evolutionary forces that have shaped the contents of human milk to ensure the least energy burden on the mother and the greatest survival benefit for the infant (*7*). One of the functions attributed to HMOs is a role in the development of neonatal intestine. HMOs modulate intestinal cell proliferation and maturation in vitro, suggesting that the mucosal barrier of the gut can be affected by these milk components (*8*). In addition, HMOs are considered to be a mechanism to protect the newborn against exogenous infections (*3*). Study of the response of epithelial cells to the presence of 3′-sialyllactose, an acidic HMO, has suggested that the expression of various glycosyltransferases is diminished. Thus, this particular HMO seems able to modify the glycan content of the epithelial cell surface and the receptor sites for some pathogens (*9*). Moreover, different in vitro studies have demonstrated that HMOs bind and block the infection of pathogenic bacteria to animal cells by acting as receptor analogues to the intestinal cell glycans (*10*−*12*). A recent study shows how HMOs can inhibit transfer of HIV-1 to CD4+ lymphocytes (*13*).

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 Published on Web 04/15/2010

Another hypothesis regarding the bioactive function of HMOs is a role as "prebiotic" (*14*). Prebiotics are defined as those substances that allow specific changes, in both the composition and activity in the gastrointestinal microbiota, conferring benefits upon host health (*15*). Prebiotics stimulate the growth of the "beneficial" bacteria including bifidobacteria, the genus often predominant in the intestinal microbiota of breast-fed infants (*16*). We recently demonstrated that *Bifidobacterium longum* subsp. *infantis* ATCC15697 (termed *Bifidobacterium infantis*) grows on HMOs as a sole sugar source, whereas *Lactobacillus gasseri*, an adult gut isolate, does not (*17*). To examine this further, we developed a method for quantifying consumption of individual HMOs using matrix-assisted laser desorption/ionization−Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FTICR MS) (*2*, *18*). With this method, we can detect individual neutral oligosaccharides, which represent 90−95% of total HMOs (*2*). This approach allowed us the determination of the HMO consumption profile of different species belonging to the bifidobacterial group (*Bifidobacterium breve*, *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium infantis*, *Bifidobacterium bifidum*, and *Bifidobacterium adolescentis*) (*19*, *20*). Comparisons among the selected species and strains revealed significant differences in their consumption profiles. Some of the examined microorganisms show preferences for consuming specific nonfucosylated or nonsialylated oligosaccharide structures, whereas other strains reveal a broader glycoside consumption profile including fucosylated glycans. Subsequent genomic analysis of a "high consumer" of HMOs, *Bi. infantis* ATCC15697, revealed the existence of a 43 kb gene cluster dedicated to HMO import and processing (*7*).

After birth, the infant gut undergoes a complex process of microbial colonization. Aerobic microbes initially colonize the intestine of the newborn; however, as oxygen is consumed, the microbiota switches to anaerobic species, composed mainly by bifidobacteria and, to a lesser extent, bacteroides and clostridia (*21*). Among the components of human milk, oligosaccharides are believed to directly influence the final microbial composition of the infant gut. As described, we have recently demonstrated that specific strains of bifidobacteria can grow vigorously on HMOs. However, the influence of these oligosaccharides on the growth of other bacterial genera present in the gut during the first months of life remained unexplored. In this work, we used a scalable method previously developed by our group (*19*), to examine 16 strains of bacteria belonging to 10 different genera related to gut microbiota. We add the advantage of using a chemically defined medium, termed ZMB1, initially designed for the growth of lactococci, enterococci, and streptococci (*22*). We demonstrate that ZMB1, which does not contain any complex components, is a useful defined medium for growth of a fairly wide array of intestinal microbes in order to test the specificity of HMOs *in vitro*. Growth curves and detailed glycoprofile analysis of HMOs consumption using MALDI-FTICR MS, indicate that bifidobacteria and bacteroides strains are able to metabolize this substrate, whereas other species, such as clostridia, enterococci, or *Escherichia coli*, are not. The results obtained in this work provide an insight into the selectivity of HMOs, showing for the first time bacteroides as a species able to consume these sugars.

MATERIALS AND METHODS

HMOs Purification. The oligosaccharide purification was performed according to the method described by Ward et al. (*17*). Milk samples were provided by the Milk Bank of San Jose, CA, and Austin, TX.

Bacterial Strains and Growth Conditions. Bacteria listed in **Table 1** were tested for growth in the presence of HMOs. On the basis of the results obtained by Ward et al. (*17*), *Lactobacillus gasseri* UCD235 was included as a control for low growth on HMOs. Seed cultures of all bacteria were prepared as following. *Enterococcus faecalis*, *Streptococcus thermophilus*, *Escherichia coli*, *Lactobacillus acidophilus*, *Lactobacillus gasseri*, and *Lactococcus lactis* strains were incubated overnight, whereas 2 days of incubation was necessary for *Clostridum perfringens*, *Clostridium difficile*, *Eubacterium rectale*, *Bifidobacterium infantis*, *Bacteroides fragilis*, *Bacteroides vulgatus*, and *Veillonela parvula* strains. All bacteria were grown in anaerobic conditions at 37 °C, using an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). Reinforced clostridial medium (RCM) was used for growing clostridia, bacteroides, and *Eu. rectale*, M17 supplemented with 2% glucose for enterococci, streptococci and lactococci growth, MRS for *Lactobacillus* and *Bi. infantis* strains, BHI for *Veillonela*, and LB for *Es. coli* strains. All complex media were purchased from Becton Dickinson (Franklin Lakes, NJ). Two microliters of each resulting overnight culture was used to inoculate 200 μL of various media distributed in the wells of a microplate. Bacterial growths were tested in the complex media LB, MRS, M17, and RCM, as well as in the chemically defined medium ZMB1, prepared according to the description of Zhang et al. (*22*). ZMB1 contains 2% glucose as a sole carbon source. After verifying that ZMB1 allowed growth of the various gut bacteria on glucose, each strain was grown overnight in the same medium. Two microliters of the overnight cultures was added to 200 μL of modified ZMB1 in which the glucose was replaced with 2% HMO, and another 2 μL was inoculated into ZMB1 without added sugar. In all cases the cultures in the wells of the microtiter plates were covered with 40 μL of sterile mineral oil to avoid evaporation. Cell growth was monitored in real time by assessing optical density at 600 nm using a BioTek PowerWave 340 plate reader (BioTek, Winooski, VT) reading every 30 min preceded by 15 s of shaking at variable speed. Two biological replicates (three technical replicates each) were performed for every studied strain and medium. Maximum OD and growth rates were calculated and expressed as the mean of all replicates with the respective standard deviation. These calculations were done with the Bacterial Growth Kinetics Software (F. Breidt, personal communication). The maximum OD observed for each strain grown on HMOs was compared with the maximum OD obtained in the absence of sugar source. This difference in OD (ΔOD) was used as a criterion to evaluate the strain's ability for growing on milk oligosaccharides.

Oligosaccharide Quantitation Using Deuterium-Labeled Internal Standard Method. Bacteria cultures in modified ZMB1 (2% HMO) were collected and centrifuged at 2000*g* for 30 min. Supernatants were boiled for 5 min and filtered using a MultiScreen 96-well filtration plate (Millipore, Billerica, MA). Remaining oligosaccharides recovered in the supernatants (25 μL) were reduced using 25 μL of 2.0 M sodium borohydride and incubated at 65 °C for 1 h. For quantitative analysis deuterated HMOs (50 μL) were added as internal standard. The oligosaccharides were desalted and purified by solid phase extraction, following the method described by LoCascio et al. (*20*).

MALDI-FTICR MS Analysis. The mass spectra analyses were performed on a HiRes Matrix-assisted laser desorption/ionization−Fourier transform ion cyclotron resonance mass spectrometry instrument with an external MADI source, a 355 nm pulsed Nd:YAG laser, a quadrupole ion guide, and a 7.0 T superconducting magnet (IonSpec Corp., Irvine, CA). 2,5-Dihydroxybenzoic acid (DHB) was used as matrix, and samples were prepared following the fast evaporation technique. One microliter of analyte (glycans) was spotted onto a 100-well stainless steel sample plate (Applied Biosystems, Foster City, CA) followed by the addition of 0.2 μL of 0.1 mM NaCl as a dopant and 1 μL of 0.4 M DHB. The spots were allowed to dry under a stream of air before analysis. Fifteen acquisitions were recorded for each replicate. MALDI-FTICR MS analysis was performed in the positive ion mode in the m/z scan range of 220−4500 with 1024K data points and 1 MHz ADC rate acquired. The ratio of deuterated species to undeuterated species (D/H) and percent of consumption were calculated according to Ninonuevo et al. (*20*) for the 14 most abundant HMO signals present in the spectra. Standard deviations for the percent consumption values were calculated from the measured mass spectral intensities via error propagation. Student's t tests ($p = 0.05$) were performed to determine the significance of the difference in consumption values between strains.

RESULTS

Application of ZMB1 as Chemically Defined Medium for Growth of Intestinal Bacteria. Our previous work to characterize

Table 1. Kinetic Analysis of Bacterial Growth[a]

strain		ZMB1 without sugar	ZMB1 (2% glucose)	modified ZMB1 (2% HMO)	ΔOD
Enterococcus faecalis OG1RF	max OD	0.370 (0.070)	1.388 (0.054)	0.245 (0.001)	
	growth rate	0.053 (0.001)	0.335 (0.001)	0.039 (0.001)	
Enterococcus faecalis KA117	max OD	0.403 (0.075)	1.515 (0.017)	0.309 (0.001)	
	growth rate	0.079 (0.001)	0.230 (0.001)	0.027 (0.001)	
Streptococcus thermophilus MTC330	max OD	0.388 (0.012)	1.525 (0.188)	0.469 (0.002)	0.165
	growth rate	0.028 (0.001)	0.404 (0.001)	0.026 (0.001)	
Streptococcus thermophilus MTC360	max OD	0.242 (0.082)	1.329 (0.073)	0.551 (0.001)	0.243
	growth rate	0.496 (0.026)	0.330 (0.002)	0.059 (0.001)	
Bifidobacterium infantis ATCC15697	max OD	**0.175** (**0.156**)	**1.069** (**0.062**)	**0.698** (**0.295**)	**0.523**
	growth rate	0.015 (0.001)	0.191 (0.001)	0.023 (0.001)	
Lactococcus lactis IL1403	max OD	0.322 (0.008)	1.353 (0.009)	0.417 (0.001)	0.124
	growth rate	0.014 (0.001)	0.253 (0.009)	0.054 (0.001)	
Eubacterium rectale ATCC35183	max OD	0.305 (0.028)	1.103 (0.028)	0.454 (0.001)	0.231
	growth rate	0.047 (0.001)	0.275 (0.002)	0.052 (0.001)	
Clostridium perfringens ATCC13124	max OD	0.112 (0.007)	0.937 (0.017)	0.378 (0.001)	0.177
	growth rate	0.096 (0.031)	0.147 (0.003)	0.025 (0.001)	
Clostridium perfringens AB1	max OD	0.369 (0.055)	1.471 (0.089)	0.434 (0.001)	0.171
	growth rate	0.031 (0.001)	0.117 (0.001)	0.029 (0.001)	
Clostridium difficile AB2[a,b]	max OD	0.411 (0.078)	1.108 (0.012)		
	growth rate	0.011 (0.001)	0.184 (0.001)		
Escherichia coli OP50	max OD	0.288 (0.066)	1.460 (0.220)	0.386 (0.001)	0.152
	growth rate	0.073 (0.001)	0.231 (0.001)	0.056 (0.001)	
Escherichia coli EC100	max OD	0.319 (0.061)	1.095 (0.149)	0.383 (0.001)	0.028
	growth rate	0.053 (0.001)	0.138 (0.001)	0.042 (0.001)	
Lactobacillus acidophilus NCFM	max OD	0.222 (0.029)	0.815 (0.067)	0.517 (0.001)	0.290
	growth rate	0.070 (0.001)	0.010 (0.001)	0.078 (0.001)	
Bacteroides fragilis ATCC25285	max OD	**0.297** (**0.001**)	**1.352** (**0.001**)	**1.081** (**0.001**)	**0.940**
	growth rate	0.023 (0.001)	0.068 (0.001)	0.052 (0.001)	
*Bacteroides vulgatus*ATCC8482	max OD	**0.085** (**0.002**)	**1.049** (**0.001**)	**0.744** (**0.001**)	**0.660**
	growth rate	0.002 (0.001)	0.629 (0.001)	0.028 (0.001)	
Veillonella parvula ATCC10790	max OD	0.199 (0.001)	1.220 (0.001)	0.214 (0.001)	0.015
	growth rate	0.033 (0.001)	0.081 (0.001)	0.047 (0.001)	

[a] Optical densities were measured at a wavelength of 600 nm. Growth rates (h^{-1}) were calculated using the Bacterial Growth Kinetic Software Package (F. Breidt, personal communication). Values are reported as a mean (± SD). Bold numbers correspond to obtained values from high HMO consumers. [b] During growth the strain precipitated, forming filaments that did not allow OD measurements.

HMO consumption by bifidobacteria employed a rich medium, MRS (*19*, *20*). However, when tested, other bacteria showed significant background growth in MRS medium lacking any added sugar, thus making testing for HMO growth problematic because it would be impossible to discern weak, or even moderate, growth on HMO from background growth (see Supporting Information, Figure 1). To circumvent this problem, we employed the newly developed chemically defined medium ZMB1 (*22*), which allowed robust growth of all species examined here. To our knowledge, no other chemically-defined medium has been described for use among this many genera. The maximum OD values obtained using glucose as a sugar source ranged from 0.815, in the case of *Lb. acidophilus* NCFM, to 1.5 for *E. faecalis* 117 (**Table 1**). By substitution of glucose with other sugar source, ZMB1 can be used to evaluate growth of a wide range of bacteria on select sugars. An additional advantage that we found in the use of ZMB1 is a significant reduction in background growth in the absence of sugar. When a complex rich medium was used, and sugar was eliminated from its composition, many strains generally still grew on other components in the rich media, such as yeast or beef extract (see Supplementary Figure 1 as an example). As it is shown in Supplementary Figure 2 we have found ZMB1 without glucose particularly useful in lowering this background growth. Thus, this broadly-applicable medium ZMB1 was ideal for the evaluation of HMOs consumption by the 16 studied strains belonged to 10 different genera.

Growth of Gut-Related Microorganisms on HMOs. The various strains were grown in modified ZMB1 with HMOs as sole sugar source. In addition, all of the selected strains were grown in ZMB1 media without sugar. Maximum optical densities (ODs), ΔOD (maximum OD in modified ZMB1 minus maximum OD in ZMB1 without sugar), and growth rates are shown in **Table 1**.

Among all of the tested microorganisms, *Ba. fragilis* ATCC25285 and *Ba. vulgatus* ATCC8482 reached the highest cell density on HMOs, with $\Delta OD > 0.6$. We have previously demonstrated that *B. infantis* ATCC15967 is able to consume milk oligosaccharides (*19*, *20*). Thus, this strain was used as a positive control for consumption. However, the maximum OD reached using modified ZMB1 with 2% HMO (0.698; **Table 1**) was lower than that observed previously in rich media (modified MRS with 1.6% HMO) (*17*). Interestingly, the species with higher OD (*Ba. fragilis*, *Ba. vulgatus*, and *Bi. infantis*) also show the longest lag times among the strains tested (data not shown). Some strains show weak, but noticeable, growth on HMOs including *Lb. acidophilus, Cl. perfringens, Es. coli* OP50, *Eu. rectale*, and *St. thermophilus*, with ΔODs between 0.1 and 0.29. *Cl. difficile*, *En. faecalis*, *Ve. parvula*, and *Es. coli* EC100 did not grow at all in this substrate. It should be noted that all of these species were capable of vigorous growth on ZMB1 medium containing glucose (to ODs of 0.8−1.5).

Determination of HMO Consumption Profile Using MALDI-FTICR MS. To determine the specific HMO structures consumed by the species that grew well on HMOs, supernatants from *Ba. fragilis*, *Ba. vulgatus*, and *Bi. infantis* cultures were recovered after fermentation, and remaining HMOs were purified, reduced, and profiled by MALDI-FTICR MS as previously described by LoCascio et al. (*19*). Consumption of 14 neutral oligosaccharides with a degree of polymerization from 4 to 12 was monitored, representing up to 95% of milk oligosaccharides (*2*). **Table 2** shows the mass to charge ratio (*m*/*z*) of the types of sugars analyzed, their composition in hexose, *N*-acetylhexosamine and fucose residues, degree of polymerization (DP), and relative concentration of each oligosaccharide in the total HMO pool extracted from multiple samples of human milk, according to the data described by Ninonuevo et al. (*2*).

Table 2. Masses and Compositions of Human Milk Oligosaccharides Analyzed[a]

HMO (*m*/*z*)	hexose	*N*-acetylhexosamine	fucose	DP	relative abundance[b] (%)
732.25	3	1		4	20
878.31	3	1	1	5	1
1024.36	3	1	2	6	<1
1097.38	4	2		6	10
1243.44	4	2	1	7	25
1389.50	4	2	2	8	15
1462.51	5	3		8	3
1535.55	4	2	3	9	<1
1608.57	5	3	1	9	7
1754.63	5	3	2	10	8
1827.64	6	4		10	2
1900.69	5	3	3	11	5
1973.70	6	4	1	11	2.5
2119.76	6	4	2	12	3

[a] *m*/*z*, mass to charge ratio; DP, degree of polymerization. [b] As measured in Ninonuevo et al. (*2*).

Glycoprofiling of *Bi. infantis* ATCC15697 confirmed the high metabolic capacity for HMOs of this strain. The consumption profile appeared different from that described in a previous work where modified MRS (1.6% HMO) was used as growth medium (*20*). When grown in modified ZMB1 with 2% HMO, *Bi. infantis* ATCC15697 showed significant consumption of highly fucosylated structures with a high DP, a phenotype not witnessed previously in the MRS-grown cells (*20*). Interestingly, the relative consumption of oligosaccharides with two (*m*/*z* 1024.36, 1389.50, and 1754.63) and three (*m*/*z* 1535.55 and 1900.69) fucose residues was higher than the corresponding monofucosylated (*m*/*z* 878.31, 1243.44, 1608.57, and 1973.70) or nonfucosylated oligosaccharides (*m*/*z* 732.25, 1097.97, and 1462.52) (**Figure 1**). In addition, *Bi. infantis* did not completely consume glycans with DP ≤ 7 (*m*/*z* 732.25, 878.31, 1097.39, 1243.44, and 1389.50), which constitute almost 70% of the total HMO pool. Thus, in this culture medium, the total consumption of HMOs from the initial pool ranges from 45 to 65%. The data contrast with the complete utilization of these small polymers when *Bi. infantis* is grown in modified MRS (*20*). Moreover, *Bi. infantis* manifested a strong ability to catabolize specific oligosaccharides with DP > 7. These results reinforce the concept that *Bi. infantis* ATCC15697 has the ability to consume the wide range of structures present in the HMO pool.

Ba. fragilis ATCC2585 shows the highest HMO consumption rates from all bacteria analyzed, ranging from 25 to 90% (**Figure 1**), nearly depleting all structures from the HMO pool with a high DP. Oligosaccharides with lower DP (*m*/*z* 732.25, 878.32, and 1024), corresponding to tetra-, penta-, and hexasaccharides, were partially metabolized from 40 to 70%. Unlike *Bi, infantis*, *Ba. fragilis* exhibited a glycan utilization pattern in which

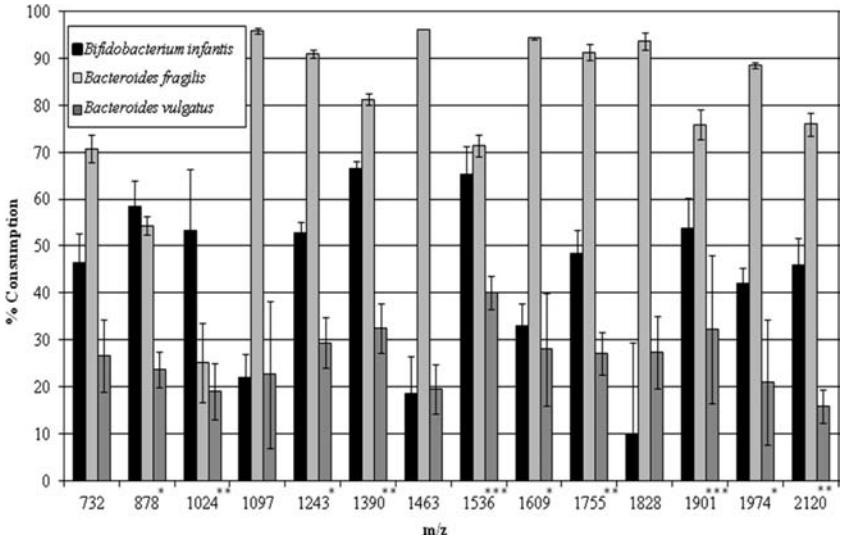

Figure 1. Human milk oligosaccharide consumption profiles of *Bifidobacterium infantis*, *Bacteroides fragilis*, and *Bacteroides vulgatus* in modified ZMB1 (2% HMO), as determined using MALDI-FTICR MS. Asterisks represent number of fucose residues on the specific oligosaccharide depicted by the mass/charge ratio (*m*/*z*).

the relative utilization of nonfucosylated glycans (m/z values 735.21, 1097.38, 1462.51, and 1827.64) was higher than the consumption of fucosylated oligosaccharides. Indeed, the relative consumption of fucosylated glycans by *Ba. fragilis* decreased as the number of fucose residues increased on the HMO structure.

Ba. vulgatus ATCC8482 exhibited a moderate growth phenotype on HMOs (ΔOD of 0.66), and the consumption of individual glycans ranged from 16 to 40%. For this strain, no selectivity was observed regarding the DP, and the higher consumption rates were those found for highly fucosylated species (m/z values of 1535 and 1901). *Ba. vulgatus* can partially consume all available HMO species, however, with noticeably lower efficiency than *Ba. fragilis* (**Figure 1**). Differences between these two *Bacteroides* strains were also witnessed in the growth kinetics. *Ba. fragilis* exhibited a growth rate of 0.052 h^{-1} in HMOs, whereas *Ba. vulgatus* had a rate of 0.028 h^{-1}. Oligosaccharide utilization by *Ba. vulgatus* is quite similar to the level of consumption observed by LoCascio et al. (*19*) for bifidobacteria classified as low HMO consumers.

DISCUSSION

Although breast milk constitutes the dominant nutritional source for newborns and infants during the first months of life, the bioactive effects of various milk components are poorly understood. HMOs constitute one of the most abundant molecules provided by the mother to the newborn through lactation. The high concentration of bifidobacteria in feces from breast-fed infants and the high concentration of HMOs in mothers' breast milk have suggested a "prebiotic" role of these components, which are thought to promote a predominantly bifidobacterial microbiota in the infant gut (*16*). In vitro analyses have shown that several bifidobacterial species can grow on HMOs, and the genome of *Bi. infantis* revealed clusters of glycosidases and oligosaccharide transporters, likely linked to HMO utilization by this phylotype (*7*, *19*, *20*). However, there has been little examination of the consumption of HMOs by other bacterial species, including those commonly found in the infant gut.

When one is comparing sugar consumption by many different species, an optimum situation is to use a common medium in which one can manipulate the sugar source. Unfortunately, the requirements of intestinal microbes are diverse and, as a consequence, rich (complex) medium components such as beef or yeast extract often are added to the "common" growth medium. However, as shown in Supporting Information, Figure 1, complex components can bias the results by allowing significant background growth even in the absence of sugar. For that reason, we suggest that a chemically defined medium that supports good cell growth is a better choice for the study of HMO consumption by the selected microbes. ZMB1 medium, originally designed for *La. lactis*, consists of known quantities of trace elements and vitamins, as well as defined nitrogen and carbon sources. The data presented in **Table 1** show that ZMB1 generates high cell density in all of the strains evaluated, with minor background growth in the same medium lacking a sugar source.

This work demonstrates that HMO consumption is not an exclusive property of specific strains of bifidobacteria. Two species of *Bacteroides* are able to metabolize free glycans from breast milk, growing to moderate or high cell densities. *Bacteroides* is known for its incredible ability to ferment an extended assortment of plant polysaccharides (*23*, *24*), and some species, such as *Ba. fragilis*, are known for their capability of consuming host-derived glycoconjugates found in the mucus gel layer from the gastrointestinal cells (*25*). Intestinal glycans are similar to the structures of HMOs, containing *N*-acetylglucosamine, galactose, fucose, sialic acid, and *N*-acetylgalactosamine (*25*). Thus, as most

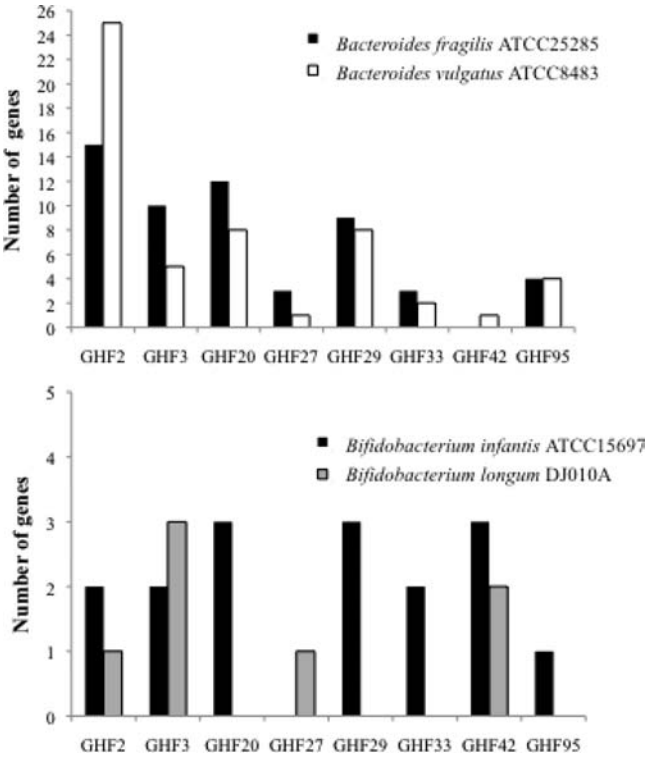

Figure 2. Annotated genes coding for some carbohydrate active enzymes in the genomes of *Bacteroides fragilis* ATCC25285, *Bacteroides vulgatus* ATCC8483, *Bifidobacterium infantis* ATCC15697, and *Bifidobacterium longum* DJO10A, as described at the CAZy database (http://www.cazy.org) (*27*). The glycoside hydrolase families (GHF) shown are potentially related to the degradation of HMOs, and they include the following activities: GH2, α-galactosidase; GH3, β-*N*-acetylgalactosaminidase; GH20, β-hexosaminidase; GH27, α-*N*-acetylgalactosaminidase; GH29, α-L-fucosidase; GH33, sialidase; GH42, β-galactosidase; GH95, α-1,2-L-fucosidase.

Bacteroides can consume host glycans, it is perhaps not surprising that they can also consume HMOs. Overall, *Ba. fragilis* ATCC25285 was most efficient in metabolism of HMOs, and its glycan consumption pattern exhibits a trend for preferential degradation of nonfucosylated HMO species and longer oligosaccharides. *Ba. vulgatus* ATCC8482 exhibited a lower consumption of these sugars than *Ba. fragilis*. It is interesting to note that *Ba. vulgatus* ATCC8482 has been revealed as a moderate consumer of host glycans and polysaccharides, which could be extended to its ability to consume HMOs (*26*). The potential ability to consume HMOs by *Bacteroides* was suggested by Bjursell et al. (*23*), after a transcriptomic-based in silico reconstruction of *Ba. thetaiotaomicron* carbohydrate metabolism, using data obtained in the study of gnotobiotic suckling mice.

As we have previously demonstrated (*19*, *20*), our results indicate that *Bi. infantis* metabolizes HMOs. However, the use of the chemically defined media ZMB1 reveals that *Bifidobacterium* can consume the fucosylated glycans, an ability not observed when *B. infantis* was grown on rich media (MRS). In addition, *Bi. infantis* growth on modified ZMB1 indicated consumption of glycans of all sizes, similar to what was witnessed previously on MRS using limiting levels of HMOs (*19*).

We hypothesize that the consumption differences between the studied strains are related to the specificity of the enzymes involved in cleaving and importing the complex oligosaccharides. Genomic comparisons of *Bi. infantis* ATCC15697, *Ba. fragilis* ATCC25285, *Ba. vulgatus* ATCC8482, and the low HMO

consumer *Bi. longum* DJO10A (*20*) show differences in the number of genes related to glycoside hydrolase families (GHFs) (**Figure 2**). A closer examination of the genomes using the CAZy database (*27*) reveals that *Ba. fragilis* ATCC25285 and *Ba. vulgatus* ATCC8482 have differences in the number of genes that encode glycoside hydrolase genes, with 126 and 159, respectively. The fact that the *Ba. vulgatus* genome encodes larger number of cleaving enzymes suggests that differences found in the growth curves and HMO consumption analysis are derived from cleavage specificities. Differences between these two *Bacteroides* strains were previously found in the analysis of their consumption of host glycans (*26*). The genome of *Bi. infantis* ATCC332 encodes 42 glycosidehydrolases, which is far lower than the number of carbohydrate-cleaving enzymes found in the genomes of the *Bacteroides* species used in this work. However, the genome of *Bi. infantis* encloses specific loci seemingly designed for HMO import and consumption (*7*), thus explaining the HMO growth phenotype of this strain. Conversely, the low HMO consumer *Bi. longum* DJO10A (*20*) does not have any fucosidases (GHF29 and GHF95) or sialidases (GHF33), which are directly related to the consumption of specific types of HMOs.

Studies of the influence of breast-feeding in the intestinal microbiota of newborns and infants reveal that breast-fed subjects develop a microbiota rich in bifidobacteria (*28*, *29*). Other anaerobes such as clostridia and *Bacteroides* are present in lower concentration, and facultative anaerobes such as enterococci or *Es. coli* are even less numerous (*30*). In contrast to breast-fed infants, formula-fed infants are often colonized by a more diverse microbiota (*29*,*31*). Among all of the components in human milk, such as proteins, lactose, or nucleotides, HMOs are the only component that has been demonstrated to play an important role in the stimulation of the growth of specific bacterial species (*32*). In this work, the clostridia, eubacteria, enterococci, and *Es. coli* strains were revealed as non-HMO consumers, reinforcing the concept that HMOs could enhance the growth of specific groups of bacteria in the gut. Such selectivity also helps to explain why enterococci, often the initial colonizers of the newborn gut, are commonly replaced by bifidobacteria within the first few weeks of breast-feeding (*16*). Even though a set of 18 isolates represents a small subset of the gut microbiome, in vitro analysis of the growth of different intestinal species in HMOs, combined with an in-depth analysis of oligosaccharide consumption profiles, constitutes the first and necessary step toward understanding the role of this breast milk component. The results obtained provide a basis for future in vivo studies, in order to understand the shifts in infant intestinal microbiota and a possible competition between *Bacteroides* and bifidobacterial populations during lactation.

ACKNOWLEDGMENT

We are grateful to Dr. Fred Breidt at the USDA-ARS for the Bacterial Growth Kinetics Software package.

Supporting Information Available: Growth curves as well as kinetic parameters of the species in complex media. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review December 14, 2009. Revised manuscript received March 12, 2010. Accepted March 26, 2010. This publication was made possible in part by grant support from the University of California Discovery Grant Program, the California Dairy Research Foundation, USDA NRI-CSREES Award 2008-35200-18776, and NIH (HD059127).