JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

In Vitro Fermentation Characteristics of Select Nondigestible Oligosaccharides by Infant Fecal Inocula

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Supporting Information

ABSTRACT: This study sought to determine the fermentation potential of human milk oligosaccharides by mixed cultures of fecal microbiota from breast-fed (BF; $n = 4$) and formula-fed (FF; $n = 4$) infants. Infant fecal inocula were incubated with galactooligosaccharide (GOS), gum arabic (GA), HP inulin (HP), 2′-fucosyllactose (2′FL), 6′-sialyllactose (6′SL), and lacto-*N*-neotetraose (LNnt). GOS, 2′FL, and LNnT had a lower pH than other substrates after 3 h ($P < 0.05$). Total short chain fatty acids were greater in FF compared to BF infants at 6 h ($P = 0.03$) and 12 h ($P = 0.01$). GOS, 2′FL, and LNnT led to more lactate than 6′SL, HP, and GA ($P < 0.05$). Bifidobacteria populations were greater ($P = 0.02$) in FF at 6 and 12 h. Overall, GOS, 2′FL, and LNnT were rapidly fermented by infant fecal inocula, 6′SL and HP had intermediate fermentability, while GA had little fermentation. Inocula from FF infants fermented substrates more rapidly than inocula from BF infants, which should be accounted for when evaluating substrate fermentability. These data will aid in future infant formulas to promote optimal gut health in FF infants.

KEYWORDS: *human milk oligosaccharides, fermentation, in vitro, infant*

INTRODUCTION

Human milk is considered a near perfect food, providing nutrients in the correct ratios for young, growing infants. It is made up predominantly of lactose and lipids, but also includes functional compounds believed to play a major role in immunity and gut development. Oligosaccharides are the third largest component of human milk and are estimated to be present at 5−23 g/L[1,2] with much higher concentrations in colostrum than in mature milk.[1] Oligosaccharides are present in bovine milk, and thereby there are some of these oligosaccharides present in infant formula.[3] Bovine milk oligosaccharides, however, vary in composition compared to human milk oligosaccharides (HMO) and may vary in infant formula based on processing conditions.[3] Like other oligosaccharides, HMO resist hydrolytic digestion by infant enzymes, but are believed to be fermented by bacteria in the large bowel to a similar extent.[2] Estimates of the amount of ingested HMO that are completely undigested vary. Engfer et al.[4] noted that very few HMO are hydrolyzed by human enzymes in vitro, indicating the potential to reach the large bowel, while Coppa et al.[5] estimated 40−50% HMO in infant feces, indicating incomplete fermentation.

It is well documented that disease risk among formula-fed (FF) babies is greater than for breast-fed (BF) babies;[6] therefore, it is thought that compounds present only in human milk modulate the immune system. It is believed that HMO act to bind potentially pathogenic bacterial species, thereby preventing their attachment to the epithelial cells of the gut.[7,8] Human milk oligosaccharides are noted to be protective against a wide number of pathogens, which is likely due to the large variety of oligosaccharides present in human milk.[9]

Human milk oligosaccharides are also thought to function as prebiotics[10] in that they are believed to promote the growth of bifidobacteria in breast-fed infants, but there are limited data regarding the ability of bifidobacteria and other enteric species to utilize HMO. Recently, Marcobal et al.[11] noted that pure cultures of *Bifidobacterium longum* subsp. *infantis*, *Bacteroides fragilis*, and *Bacteroides vulgatus* were able to use pooled HMO as a growth substrate, while representative strains of *Enterococcus*, *Streptococcus*, *Lactococcus*, *Eubacterium*, *Clostridium*, *E. coli*, *Lactobacillus*, and *Veillonella* spp. only had minimal growth. Although this particular study demonstrated that *B. infantis* grew well on pooled HMO, the ability to utilize HMO may not be common to all *Bifidobacterium* strains.[12,13] Indeed, in vitro incubations of various strains of bifidobacteria with lacto-*N*-biose[14,15] or sialyllactose[16] suggest that few species are able to grow using individual HMO as the sole carbon source.

The objective of this study was to evaluate the fermentation of individual HMO in vitro using bacteria from BF and FF infant fecal inoculum, in a mixed culture system. These oligosaccharides also were compared to known prebiotics, galactooligosaccharides, and inulin, and to the slowly fermented carbohydrate, gum arabic.

MATERIALS AND METHODS

Donors and Collection Methods. Eight infants were enrolled for participation in this study between March and June of 2010 in the

Received: August 24, 2012
Revised: February 4, 2013
Accepted: February 5, 2013
Published: February 5, 2013

Champaign-Urbana area, and consumed their normal diets consisting exclusively of either breast milk (n = 4) or infant formula (n = 4). The formula-fed infants received Similac Advance (Abbott Laboratories Columbus, OH; n = 1), Member's Mark infant formula (Sam's Club, Bentonville, AR; n = 1), or Enfamil Premium (Mead Johnson, Glenview, IL; n = 2). Other inclusion/exclusion criteria included: the infant was full term at birth with a gestational age of 38 to 42 wk; the infant was at or above the fifth percentile for weight at birth; the infant had no maternal medical history of diabetes, tuberculosis, or perinatal infection with proven adverse effects on the fetus; were vaginal births; were at least 2 mo of age at study entry, but not older than 4 mo of age; had no known cardiac, respiratory, gastrointestinal, or other systemic disease such as urinary tract infection or otitis media; had no history of blood group incompatibility serious enough to result in hematological problems; and were not receiving any medications (except for supplemental vitamins) and had never received antibiotics. The experimental protocol was approved by the University of Illinois Institutional Review Board, and all legally acceptable representatives signed an informed consent prior to initiation of the experiment.

On the day of the in vitro experiments, a fecal sample was collected in the diaper and prepared within 15 min of defecation. The diaper with feces were double-sealed in plastic baggies and placed in a cooler containing tepid water to maintain temperature and transported immediately to the University of Illinois laboratory for analysis. Fecal samples were diluted 1:10 (wt/vol) in anaerobic dilution solution[17] by blending for 15 s in a Waring blender under a stream of CO_2. Blended, diluted feces were filtered through four layers of cheesecloth and sealed in 125-mL serum bottles under CO_2. Inocula were stored at 37 °C until inoculation of in vitro tubes.

Substrates. Substrates used included: galactooligosaccharides (GOS) 95 (Inalco Pharmaceuticals, Italy), α-(2-6′)-*N*-Acetylneuraminyl-lactose sodium salt (6′-sialyllactose) (6′SL; Inalco Pharmaceuticals, Italy); 2′- α-L-Fucopyranosyl-D-Lactose (2′-fucosyllactose) (2′FL; Inalco Pharmaceuticals, Italy); Lacto-*N*-neotetraose (LNnT; Boehringer Mannheim, Germany); Orafti HP inulin (HP; BENEO-Orafti Belgium); and gum arabic (GA; Fisher Scientific, Pittsburgh, PA). All substrates were analyzed for dry matter (DM) and organic matter (OM) according to AOAC[18] and for free and hydrolyzed monosaccharide concentrations. Substrates were hydrolyzed according to Hoebler et al.[19] for hydrolyzed monosaccharide analysis. Free sugars and hydrolyzed monosaccharides were quantified using a Dionex CarboPac PA-1 column on a Dionex ICS-3000 high-performance liquid chromatography system with pulsed electrochemical detection (HPLC-PED) (Dionex Corp., Sunnyvale, CA). Human milk oligosaccharides were quantified using pure standards (V-Labs, Inc., Covington, LA) and analyzed according to Thurl et al.[20] using Dionex HPLC-PED as above (chromatograph, Supporting Information Figure S1). All other compounds were quantified using standards of sugars and monosaccharides. Free monosaccharides were injected at a volume of 25 μL. All assays were conducted using a CarboPac PA-1 (4 × 250 mm) column and guard column (4 × 50 mm) following methods cited by Smiricky et al.[21] Briefly, an initial degassed mobile phase was 120 mM NaOH increasing linearly to 150 mM NaOH by 10 min and 240 mM by 20 min. At that time, the NaOH concentration was increased to 300 mM for 10 min to clean the column. It was then re-equlibrated for 15 min with 120 mM of NaOH. Flow rate was constant at 1.0 mL/min.

In Vitro Fermentation Model. Approximately 80 mg of each substrate was weighed in triplicate for each sampling time into 16 mL Balch tubes that were used in a model that simulated large bowel fermentation.[22] An aliquot (7.2 mL) of medium (Table 1) was aseptically transferred into the Balch tubes, capped with butyl rubber stoppers, and sealed with aluminum caps. Tubes containing HP and GA were stored with medium at 4 °C for approximately 12 h to enable hydration of the substrates before initiating fermentation. These tubes were placed in a 37 °C water bath approximately 30 min before inoculation. Due to the limited supply of substrates and difficulty in obtaining samples from infants, tubes containing GOS, 6′SL, 2′FL, and LNnT were hydrated upon obtaining a fecal sample and placed in a 37 °C water bath until inoculation.

Table 1. Composition of Microbiological Medium Used in the in Vitro Experiment

component	concentration in medium
	mL/L
solution A[a]	330.0
solution B[b]	330.0
trace mineral solution[c]	10.0
water-soluble vitamin solution[d]	20.0
folate:biotin solution[e]	5.0
riboflavin solution[f]	5.0
hemin solution[g]	2.5
resazurin[h]	1.0
distilled H_2O	296.1
	g/L
Na_2CO_3	4.0
cysteine HCl-H_2O	0.5
trypticase	0.5
yeast extract	0.5

[a]Composition (g/L): NaCl, 5.4; KH_2PO_4, 2.7; $CaCl_2$-H_2O, 0.16; $MgCl_2$-$6H_2O$, 0.12; $MnCl_2$-$4H_2O$, 0.06; $CoCl_2$-$6H_2O$, 0.06; $(NH_4)_2SO_4$, 5.4. [b]Composition (g/L): K_2HPO_4, 2.7. [c]Composition (mg/L): ethylenediaminetetraacetic acid (disodium salt), 500; $FeSO_4$-$7H_2O$, 200; $ZnSO_4$-$7H_2O$, 10; $MnCl_2$-$4H_2O$, 3; H_3PO_4, 30; $CoCl_2$-$6H_2O$, 20; $CuCl_2$-$2H_2O$, 1; $NiCl_2$-$6H_2O$, 2; Na_2MoO_4-$2H_2O$, 3. [d]Composition (mg/L): thiamin-HCl, 100; d-pantothenic acid, 100; niacin, 100; pyridoxine, 100; p-aminobenzoic acid, 5; vitamin B_{12}, 0.25. [e]Composition (mg/L): folic acid, 10; d-biotin, 2; NH_4HCO_3, 100. [f]Composition: riboflavin, 10 mg/mL in 5 mmol/L of Hepes. [g]Composition: hemin, 500 mg/mL in 10 mmol/L of NaOH. [h]Composition: resazurin, 1 g/L in distilled H_2O.

Sample and blank tubes were aseptically inoculated with 0.8 mL of diluted feces. Tubes were incubated at 37 °C with periodic mixing every 2 h for up to 12 h. At 0, 3, 6, and 12 h after inoculation, tubes were removed from the 37 °C incubator and processed immediately for analyses. The pH of tube contents was measured with a standard pH meter (Denver Instrument Co., Arvada, CO). A 3 mL subsample of fluid was collected and used for short-chain fatty acid (SCFA), branched-chain fatty acids (BCFA), and lactate analyses.[23,24] A 2 mL subsample was taken and frozen at −80 °C for bacterial analyses.

Short-Chain Fatty Acid and Lactate Analyses. The 3 mL aliquot of fluid removed from the sample tubes for SCFA, BCFA, and lactate analyses was immediately added to 0.75 mL of 25% metaphosphoric acid. Concentrations of acetate, propionate, butyrate, isobutyrate, isovalerate, and valerate were determined using a Hewlett-Packard 5890A series II gas chromatograph (Palo Alto, CA) and a glass column (180 cm × 4 mm i.d.) packed with 10% SP-1200/1% H_3PO_4 on 80/100+ mesh Chromosorb WAW (Supelco Inc., Bellefonte, PA). Oven temperature, detector temperature, and injector temperature were 125, 180, and 175 °C, respectively. The supernatants were analyzed for lactate concentration by the spectrophotometric method described by Barker and Summerson.[23] Short-chain fatty acid, BCFA, and lactate concentration values were corrected for blank tube production and 0 h concentrations for each substrate. Blank tubes that contained only media and fecal inoculum but did not contain test substrate, allowed for estimation of fermentation not attributable to test substrates. Total SCFA were calculated as the total amount of acetate, propionate, and butyrate, and total BCFA were calculated as the total amount of valerate, isovalerate, and isobutyrate. All values were standardized to an OM basis prior to statistical analyses.

Quantitative Polymerase Chain Reaction (qPCR). A 2 mL subsample of the in vitro material from each tube at each time point was collected for determination of microbial species. Two tubes from each substrate at each time point were processed. Genomic DNA was extracted and isolated using the repeated bead beating plus column (RBB+C) method described by Yu and Morrison.[25] *Escherichia coli*, *Bifidobacterium* spp., *Lactobacillus* spp., and *Clostridium perfringens*

were quantified via qPCR using specific primers.[24] DNA from each standard curve serial dilution was amplified along with in vitro DNA samples using a Taqman ABI PRISM 7900HT Sequence Detection System (Applied BioSystems), and colony forming units (CFU), based on the standard curves, were determined as described by Hernot et al.[24] Briefly, standard curves were obtained from pure cultures of the bacterium of interest in the logarithmic growth phase in triplicate. DNA was extracted using the above procedures followed by serial dilution and used to quantify amount of each bacterium present in test samples. In a deviation to Hernot et al.,[24] due to the small quantity of DNA extracted in time 0 and 3 h samples, only 2 ng of DNA was amplified during qPCR. Bacterial population values were corrected for blank tube production and 0 h values for each substrate.

Statistical Analysis. Data for SCFA production were fitted to a logistic model equation to determine the rate of production and the time to attain maximal rate of production for each substrate. This is frequently used to model biological growth,[26] and is a sigmoidal curve that describes accelerating and, after passing through an inflection point, decelerating phases of growth.[27] The time at which maximal rate of SCFA production occurred was calculated according to the following equation:

$$Y = A/(1 + e^{-(t-C)/B})$$

where Y = SCFA production, A = asymptote, or maximal production, t = incubation time in h, C = time in h at which the rate of SCFA production is maximum (the inflection point), and B = a measure of the duration of SCFA production expressed in μg per gram of DM. Variables (A, B, and C) were estimated for each substrate using nonlinear regression (NLREG).[28] The model explained 90% or more of the variation in SCFA production in all cases.

Additionally, maximal rates of SCFA production were estimated using the derivative of the logistic function according to the following equation:

$$(A \times e^{(C+t)/B})/(B \times [e^{(C/B)} + e^{(t/B)}]^2) = dY/dt$$

where A = asymptote, or maximal SCFA production; t = time in hours; C = time in hours at which maximal rate of SCFA production occurs; and B = measure of the duration of SCFA production expressed as μg per gram of DM.

Data were analyzed as a split−split-plot in a completely randomized block design using the Mixed procedure of SAS (SAS Inst., Inc., Cary, NC). Block was defined as the diet of the infant (breast milk or formula). Fixed effects tested included diet, substrate, and time, and the interactions were investigated if significant. Infant, and the interaction of infant and substrate, were included as random effects in the model to ensure the use of the proper error term. Means for SCFA, BCFA, lactate, and microbial populations were separated using the Holm−Bonferroni method to control for experiment-wise error. Means for SCFA production and time to attain maximal rates were separated using a protected LSD with a Tukey adjustment. Least square means were reported along with the pooled standard error of the mean (SEM) for all response criteria. A probability of $P < 0.05$ was accepted as statistically significant.

RESULTS

Chemical composition of the test carbohydrates is provided in Supporting Information Table S1. Dry matter was similar among substrates, except GOS, which was a syrup. Organic matter was similar among substrates. Of the free sugars present in these substrates, glucose and galactose often were the predominant components. 2′FL was the only substrate that contained no free sugars. The 2′FL substrate contained only 2′-fucosyllactose, while 6′SL contained traces of sialic acid, siayllaco-*N*-tetraose, as well as larger amounts of 6′- and 3′-sialyllactose. LNnT contained traces of sialic acid and lacto-*N*-fucopentaose.

Change in pH had diet by time ($P = 0.0045$) and time by substrate ($P < 0.0001$) interactions. Culture pH from BF and FF infants decreased by approximately one pH unit over the course of 12 h with pH declining more rapidly ($P < 0.05$) for FF compared with BF infants (Figure 1(A)). No differences

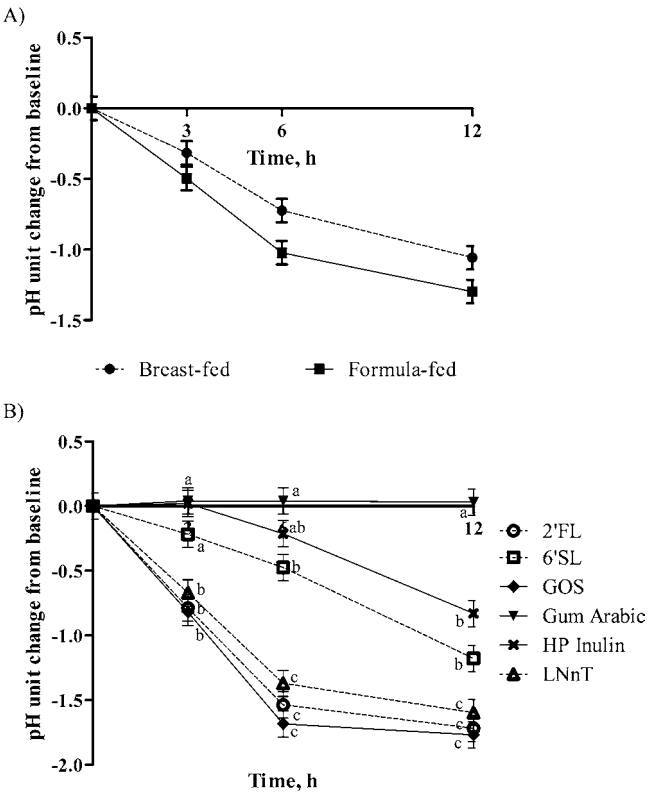

Figure 1. Change in pH over time during in vitro incubation of infant fecal microbiota with human milk oligosaccharides and other substrates. (A) Effect of infant diet over all substrates. (B) Effect of substrate regardless of infant diet. Superscripts a, b, and c refer to means lacking a common superscript differ at each time point.

were observed between BF and FF infants at any single time point. With regard to time by substrate effects, pH decreased over time for all substrates except GA (Figure 1 (B)). Three hours after inoculation, pH was lower ($P < 0.05$) for GOS, 2′FL, and LNnT compared with 6′SL, HP, and GA. No differences were noted in substrates by diets over time ($P = 0.07$; Supporting Information Figure S2(A,B)).

Acetate production had a diet by time by substrate interaction ($P = 0.04$). FF inocula produced more acetate overall than BF inocula ($P < 0.001$) over time, but no statistically significant differences were found at any individual time point (Figure 2(A)). 6′SL had a greater ($P < 0.05$) acetate production after 12 h of fermentation by FF infant inocula compared to BF infant inocula (Figure 3(A),(B)). Acetate production differed over time among substrates ($P < 0.0001$) (Figure 3). Fermentation of GA and HP produced minimal acetate over 12 h, while GOS generated large amounts, regardless of diet. BF infant inocula produced intermediate amounts of acetate from LNnT, 2′FL, and 6′SL, but these substrates generated more acetate than either HP or GA, especially at 6 ($P < 0.05$) and 12 h ($P < 0.05$), when incubated with inocula from FF versus BF infants.

Propionate production differed over time within diet ($P < 0.0001$) (Figure 2(B)). Overall, inocula from FF infants

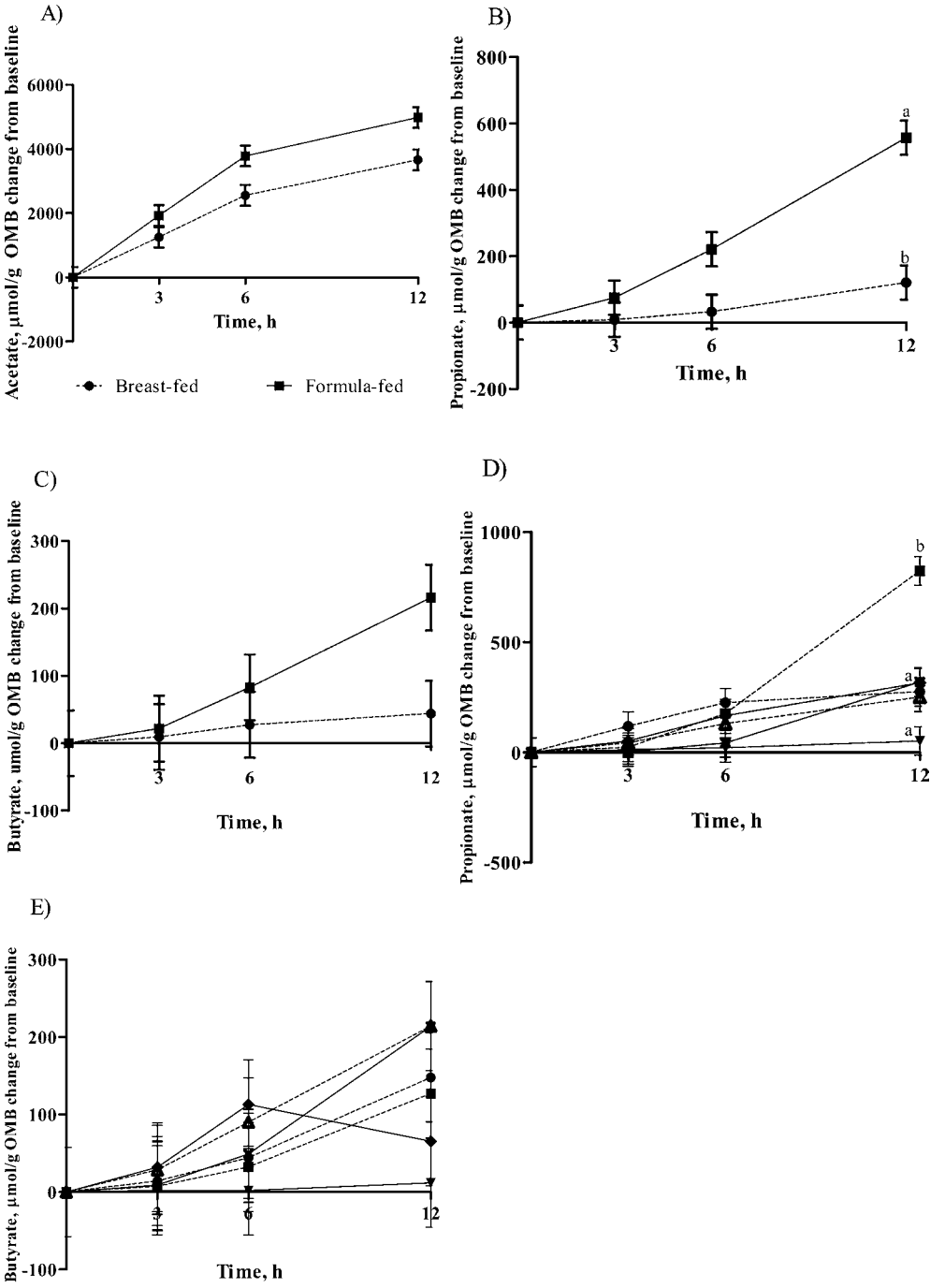

Figure 2. Changes in acetate (A), propionate (B), and butyrate (C) during in vitro incubation of infant fecal microbiota with human milk oligosaccharides and other substrates as affected according to infant diet, regardless of substrate. Changes in propionate (D) and butyrate (E) over time by substrate in both BF and FF infants. OMB, organic matter basis.

generated more ($P = 0.03$) propionate than inocula from BF infants due to the large difference ($P < 0.05$) after 12 h of incubation. The change in propionate over time was also affected by substrate ($P < 0.0001$) due to a disproportionate increase ($P < 0.0001$) in propionate at 12 h as a result of fermentation of 6′SL (Figure 2(D)). There was no difference in diet by substrate by time ($P = 0.44$; Figure 3(C,D)).

FF infant inocula generated more ($P = 0.01$) butyrate overall than BF inocula, but no differences were detected at any individual time point (Figure 2(C)). Butyrate concentrations also tended to increase ($P < 0.10$) over time for most substrates (Figure 2(E)), but neither time by substrate interaction ($P = 0.74$) or diet by substrate by time interaction ($P = 0.65$) were detected (Figure 3(E,F)).

Total SCFA results (Supporting Information Figure 3(A,B)) were nearly identical to those for acetate, and were affected by the interaction of diet, time, and substrate ($P = 0.01$). FF inocula produced greater ($P = 0.03$) overall total SCFA than BF infant inocula, particularly for 6′SL ($P < 0.05$) and LNnT ($P < 0.05$). Total SCFA production was greater ($P = 0.04$) for FF compared to BF inocula at 6 h ($P = 0.03$) and 12 h ($P = 0.01$) postinoculation (Figure 2(A)). Lactate was not affected by diet ($P = 0.73$) or time ($P = 0.19$) (Figure 4(A)), but differed ($P < 0.0001$) over time among substrates (Figure 4(B)). GOS

dx.doi.org/10.1021/jf305056f | J. Agric. Food Chem. 2013, 61, 2109−2119

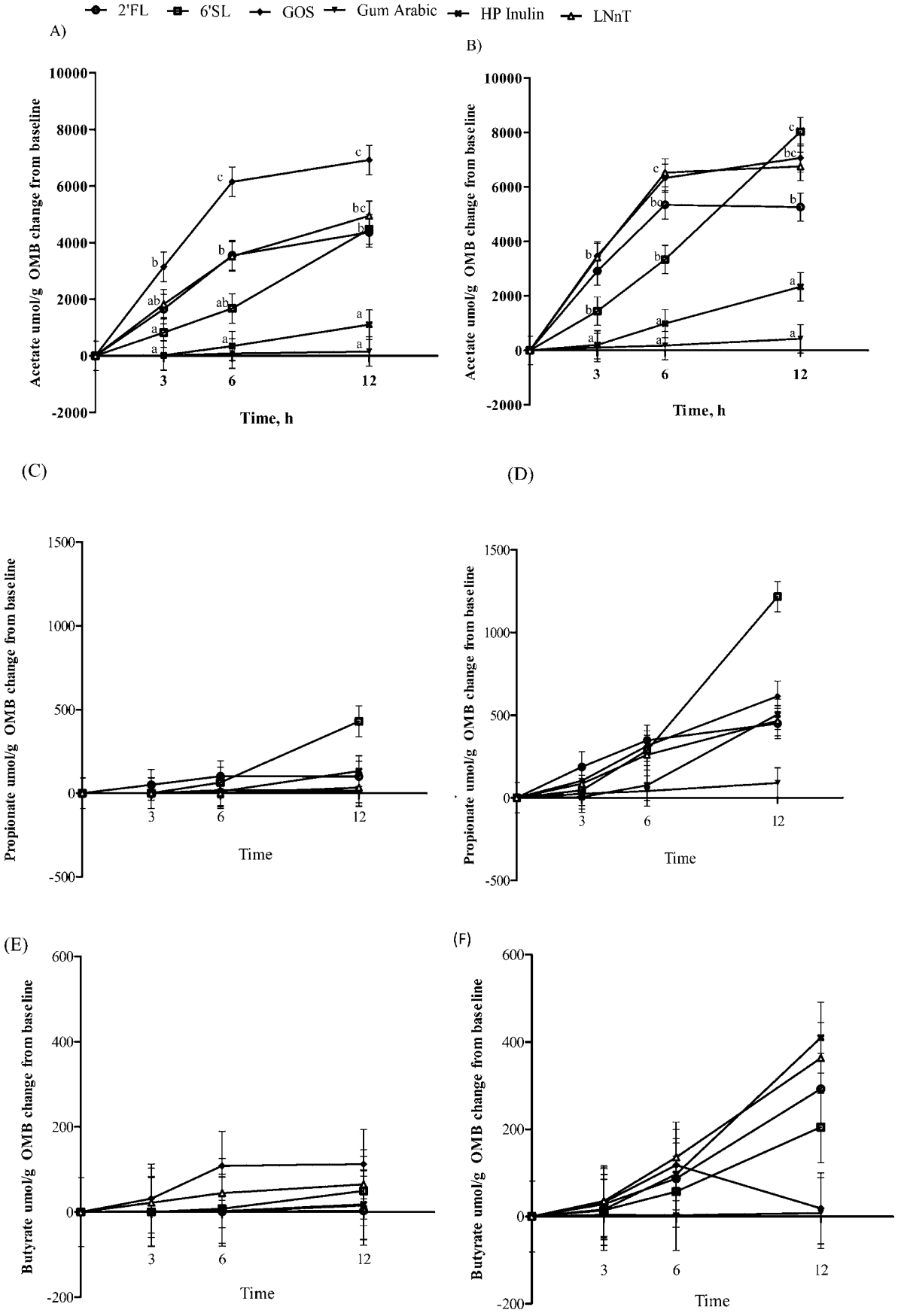

Figure 3. Changes from baseline in acetate (A, B), propionate (C, D), and butyrate (E, F) during in vitro incubation of infant fecal microbiota with human milk oligosaccharides and other substrates for BF and FF infants, respectively. OMB, organic matter basis. Superscripts a, b, and c refer to means lacking a common superscript at each time point.

dx.doi.org/10.1021/jf305056f | J. Agric. Food Chem. 2013, 61, 2109−2119

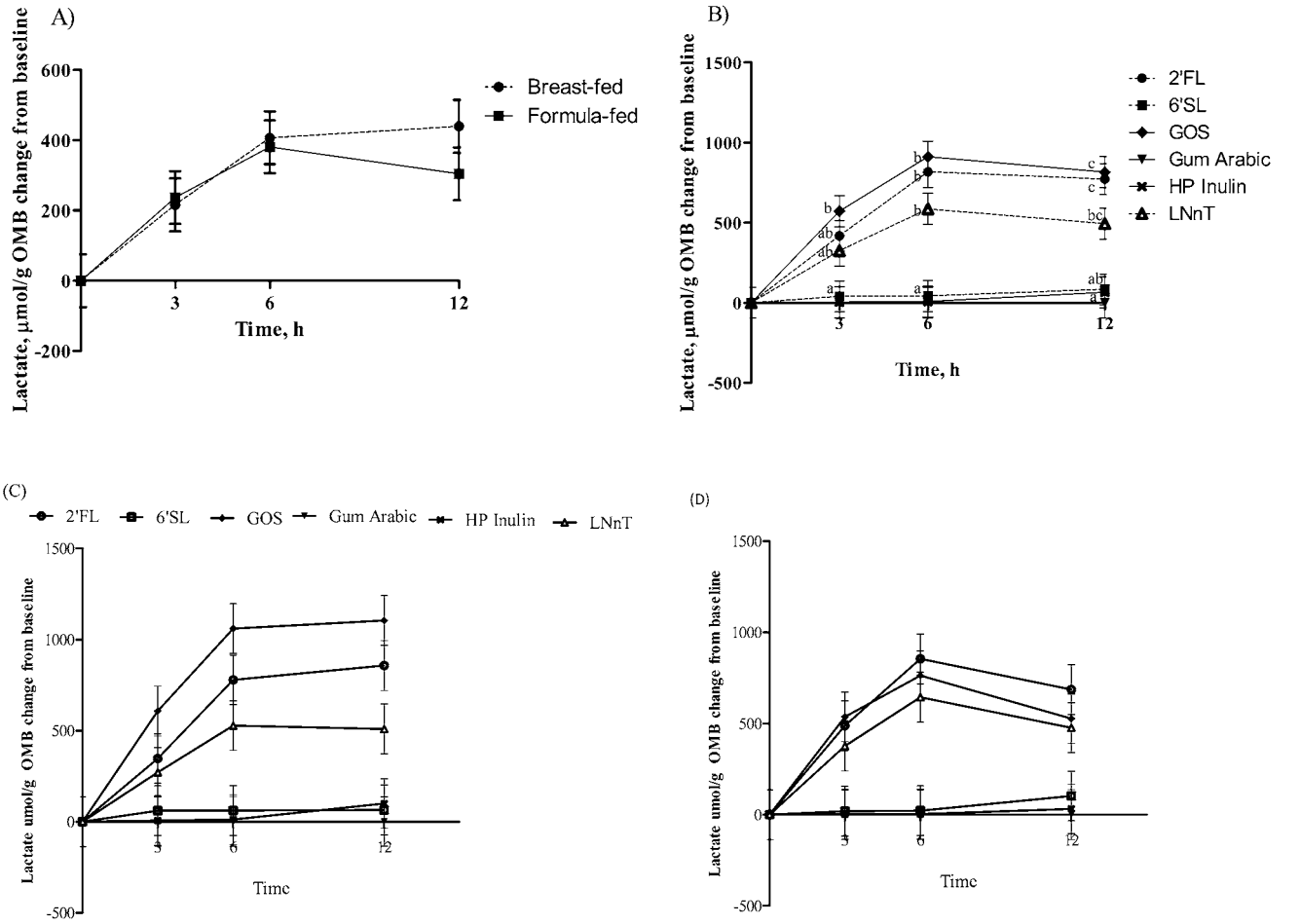

Figure 4. Changes in lactate during in vitro incubation of infant fecal microbiota with human milk oligosaccharides and other substrates. (A) Effect of infant diet, regardless of substrate. (B) Effect of substrate, regardless of infant diet. (C and D) Effects of substrate over time in BF and FF infants, respectively. Superscripts a, b, and c refer to means lacking a common superscript at each time point.

Table 2. Rate of Short-Chain Fatty Acid (SCFA) Production and Time to Attain Maximal Rate of Production for Human Milk Oligosaccharides and Other Fermentable Substrates Using Infant Fecal Inoculum[a,b]

	substrate[c]						
SCFA	2′FL	6′SL	LNnT	GOS	gum arabic	HP inulin	SEM[d]
rate of production, µg/g DM/h							
acetate	4747.0^{AB}	818.7^{A}	4275.9^{AB}	9106.2^{B}	784.9^{A}	1034.7^{AB}	2154.84
propionate	61.1*	943.4†	143.3*,†	99.3*,†	NE[e]	400.4*,†	247.74
butyrate	64.1	47.1	68.6	98.6	NE	NE	47.69
time to attain maximal rate of production, h							
acetate	4.2^{A}	8.1^{B}	4.7^{AB}	3.6^{A}	6.7^{AB}	6.7^{AB}	0.89
propionate	3.4^{A}	7.3^{C}	5.6^{BC}	4.5^{AB}	NE	6.8^{C}	0.56
butyrate	5.7^{AB}	8.0^{B}	5.4^{A}	4.7^{A}	NE	NE	0.96

[a]Means not sharing a common symbol (*, †) tend to differ ($P < 0.10$). [b]Means in the same row not sharing a common superscript letter (A, B, C) differ ($P < 0.05$). [c]Substrates: GOS, galactooligosaccharide; 6′SL, 6′-sialyllactose; 2′FL, 2′-fucosyllactose; LNnT, lacto-*N*-neotetraose; HP inulin Orafti HP inulin; gum, gum arabic. [d]SEM, standard error of the mean. [e]NE, not estimable.

produced more ($P < 0.0001$) lactate compared to HP, 6′SL, or GA at 3 h. At 6 h, lactate production was greater ($P < 0.0001$) for GOS, 2′FL, and LNnT compared to 6′SL, HP, or GA. This pattern continued after 12 h of fermentation, as GOS, 2′FL, and LNnT lactate production was greater ($P < 0.0001$) compared to GA. Overall, lactate production was greater ($P < 0.05$) for GOS, 2′FL, and LNnT compared to HP, 6′SL, and GA. Lactate did not differ in diet by substrate over time ($P = 0.75$; Figure 4(C,D)).

The rate of SCFA production and time to attain maximal rate of production are presented in Table 2. Rate of acetate production was faster ($P = 0.02$) for GOS compared to GA or 6′SL, with all other values being intermediate. Rates of propionate and butyrate production did not differ among substrates. Time to attain maximal rate of acetate production was affected by substrate and infant diet (data not presented). Time to attain maximal rate of acetate production was longer ($P = 0.001$) for 6′SL compared to 2′FL and GOS. Time to

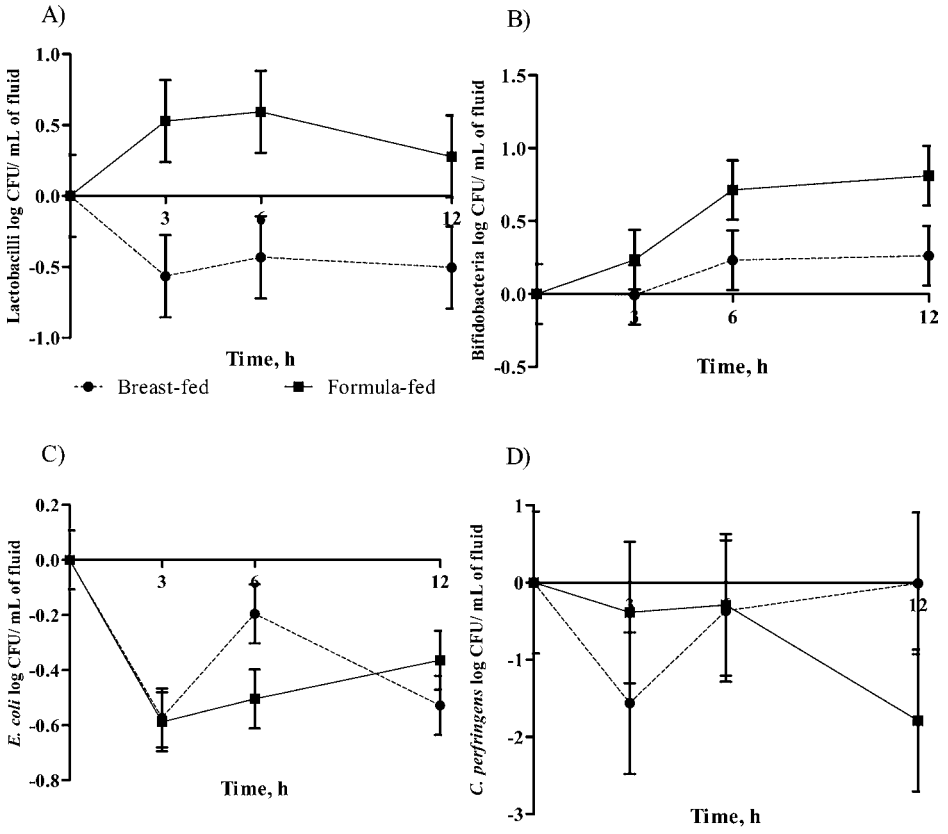

Figure 5. Changes in *Lactobacillus* spp.(A), *Bifidobacterium* spp. (B), *E. coli* (C), and *Clostridium perfringens* (D) populations during in vitro incubation of infant fecal microbiota with human milk oligosaccharides and other substrates as affected by infant diet regardless of substrate. CFU, colony forming units.

attain maximal rate of propionate production was shorter ($P < 0.001$) for 2′FL compared to all other substrates except GOS, and longest for 6′SL and HP. Time to attain maximal rate of butyrate production was greater ($P = 0.01$) for 6′SL compared to LNnT and GOS.

All substrates, except 2′FL, produced little BCFA, with final accumulation amounting to less than 10 μmol/mL (OMB; data not presented). Total BCFA production was greater ($P < 0.0001$) for 2′FL compared to all other substrates after 12 h, due to an increase ($P < 0.0001$) in isobutyrate. Valerate production was greater ($P = 0.004$) for FF infants compared to BF infants after 12 h. Valerate production did not differ among substrates at 0, 3, or 6 h after fermentation, but was greater ($P < 0.01$) with HP compared to all other substrates at 12 h.

Lactobacilli populations were affected by diet over time ($P < 0.0001$), but there were no differences found between diets at the individual time points (Figure 5(A)). Bifidobacteria populations were greater ($P = 0.02$) in FF inocula at 6 and 12 h compared to BF infant inocula (Figure 5(B)). *E. coli* populations were affected by diet over time with inocula from BF and FF infants showing similar decreases ($P = 0.01$) over time (Figure 5(C)). *C. perfringens* populations decreased ($P = 0.04$) over time (Figure.5(D)), regardless of diet or substrate. No bacterial species tested was affected by diet by substrate over time ($P > 0.05$).

DISCUSSION

It is well established that feeding breast milk, especially within the first 6 months of life, confers multiple health benefits to infants.[29] Many mothers are unable to provide breast milk and, therefore, finding ways to improve infant formula is of great importance. Human milk is highly digestible and leads to little stool production, but it is apparent that some material must reach the large bowel to support the rapidly developing colonic microbiota. Oligosaccharides represent a major component of human milk and likely serve as an important energy source for the gastrointestinal microbiota in young infants. Shen et al.[30] recently reported the effects of incubating fecal inocula from 7-month-old infants during the weaning process with mixed HMO from a single donor, pooled HMO from multiple donors, and a 1:1 (w/w) mixture of hydrolyzed inulin plus galactooligosaccharides. In contrast, our study sought to determine the rate and extent to which fecal microbiota from younger preweaning infants were able to ferment individual HMO in an in vitro model of colonic fermentation.

The test substrates used in our study contained detectable amounts of free sugars, but the concentrations were very low. This finding is important because quantifying both the free sugar and hydrolyzed monosaccharide contents of a novel carbohydrate is important in assessing its potential role in the gastrointestinal tract. Free sugars represent those that would likely be absorbed by the small intestine, while hydrolyzed monosaccharides represent the components of carbohydrate polymers that would be potentially fermented in the large bowel. It is also important to note that free sugars can affect in vitro fermentation by artificially elevating end-product production via the metabolism of substrates that would otherwise be digested and absorbed in the small bowel.

With the exception of GOS, our data indicate that fecal inocula from FF infants fermented the selected substrates more

rapidly than those from BF infants. This result was not entirely surprising given that: (i) feces from FF infants contain a more diverse microbiota population with a greater proportion of *Bacteroides* than exclusively BF infants,[31,32] (ii) members of the *Bacteroides* genus are known for their ability to utilize a wide range of polysaccharides,[33] and (iii) all of the FF infants who participated in our study were being fed commercial formulas that contained GOS. Moreover, the greater in vitro fermentation capacity of microbiota from FF infants, as reflected by total SCFA production, agrees with results from previously published studies showing higher total SCFA concentration in feces from infants fed formula compared to human milk.[34,35] In contrast, Edwards et al.[36] showed no difference in the in vitro fermentation capacity of FF and BF infants, but the infants were younger (2 to 13 wk of age) and may have had a less developed microbiota population than those sampled in the current study.

Of the HMO tested, 2′FL and LNnT were fermented rapidly in vitro. In vitro evaluations using formula and sow-reared piglet inocula (9 and 17 d of age) resulted in similar rapid fermentation of an HMO mixture and LNnT.[36] Similar findings were noted in the current study, with greater fermentation by FF versus BF inocula with acetate comprising the largest proportion of SCFA, and a greater production of propionate after 12 h of fermentation of HMO. Li et al.,[37] however, used a pooled mixture of extracted HMO, not individual substrates as in the current study. Interestingly, increased propionate only was noted for the 6′SL substrate in the current study, which may be the HMO within the mixture that led to the increase noted by Li et al.[37] Rate of production and time to attain maximal rate of production were not able to be determined for all substrates. This may have been due to either (i) no change above concentrations in the blank tubes, leading to an inappropriate model equation, or (ii) an inflection point not being able to be determined through selection of collection times, or (iii) because accelerated fermentation was still occurring.

While previous researchers reported an estimated 97% of milk oligosaccharides pass intact through the infant gastrointestinal tract into the feces,[38] results from the current study suggest that microbiota from breast-fed infants are able to ferment these substrates. These seemingly contradictory observations may be due to changes in HMO metabolism within the GI tract during infancy.[39] HMO metabolism progresses through three stages as defined by fecal oligosaccharide profiles: (i) dominance of either neutral or acidic oligosaccharides between birth and approximately two months postpartum; (ii) reduction of HMO in infant feces and an increase in HMO metabolites between 2 and 4 mo postpartum; and (iii) absence of fecal HMO and metabolites at 4 months postpartum and coincident with introduction of solid food.[39] Because BF infants participating in the current study were between 2 and 4 mo of age and had not yet received solid food, they likely fit the criteria for "Stage 2." During stage 2, colonic microbiota have colonized and adapted to a diet of human milk and have developed the ability to degrade most HMO.[39] Likewise, FF infants in our study were solely fed formula and would likely have fit into either "Stage 2" or "Stage 3." Hence, if younger infants were used in this study, we may have observed slower or more variable fermentation rates.

6′SL was fermented more slowly than either 2′FL or LNnT, regardless of infant diet, but the reason for the difference in fermentation rate is unclear. Measurements of fecal glycosidase activity of healthy adults[40−42] and children[40] uncovered considerable intraindividual variation in total neuraminidase and α-fucosidase activity. Differences in the activity of these enzymes, however, would only affect fermentation rates if the glycolytic cleavage of sialic acid, lacto-*N*-biose, and fucose moieties were rate-limiting during the catabolism of these oligosaccharides. In addition, although neuraminidases are believed to be widely distributed among both pathogenic and commensal bacterial species,[43] resistance of sialyl moieties to glycolytic cleavage makes intuitive sense. More specifically, many infectious diseases are caused by organisms that exhibit high levels of neuraminidase activity,[43] with the neuraminidase facilitating invasion of host tissues, acting directly as a toxin and/or hindering host defense mechanisms.[43] Therefore, slow degradation of sialyl-containing glycans could potentially be advantageous to the host.

Fermentation potential by BF versus FF microbiota differed in the current study compared to previous literature. In the current study, FF infants had consistently greater SCFA production compared to BF infants. This is in contrast to previous reports indicating that BF infants were able to produce greater amounts of SCFA in vitro.[44,45] The differences in SCFA production in vitro often are attributed to the known differences in microbiota of BF and FF infants. As mentioned above, FF infants are reported to have greater microbial diversity,[46,47] which may lead to more adult-like molar ratios for the SCFA, specifically propionate. Others have reported greater fecal propionate and butyrate for FF infants compared to BF infants.[36,48] Ogawa et al.[49] noted results similar to those of the current study, as fecal SCFA concentrations were greater in FF infants, which was driven mostly by fecal propionate and butyrate concentrations. Additionally, we collected samples from infants between 2 and 4 mo of age. FF infants in that age range have greater fecal acetate, propionate, and butyrate concentrations compared to BF infants.[49]

BCFA arise solely from the fermentation of peptides and amino acids, and their accumulation is considered an indicator of protein degradation.[50,51] In the present study, excess carbohydrate was added to each tube, so little BCFA accumulation was expected. Accordingly, the fermentation of GOS, LNnT, 6′SL, HP, and GA resulted in very little BCFA accumulation. Curiously, however, the fermentation of 2′FL resulted in a rapid rise in BCFA with the increase almost entirely due to isobutyrate, a product of valine fermentation.[52] Closer examination of the data indicated marked interindividual differences in isobutyrate production among the infants with three individuals producing copious amounts of this compound and three producing virtually none. Because infant diet did not appear to be related to isobutyrate production, these results suggest that some infants may be populated with higher numbers of amino acid-fermenting bacteria such as clostridia than others, a hypothesis that is consistent with the notion that microbial composition varies widely among infants during the first year of life.[53]

Human milk oligosaccharides were among the first prebiotics recognized for their potential ability to promote growth of bifidobacteria,[54] but in vitro fermentation of the individual HMO, 2′FL, LNnT, and 6′SL in the current study led to only modest increases in bifidobacteria concentrations in our mixed culture system. One possible explanation for this discrepancy is that these particular HMO were not as efficient as other HMO in promoting the growth of bifidobacteria. This hypothesis is supported by the observation of LoCasio et al.[55] that various

strains within the *B. longum* subsp. *infantis*, *B. bifidum*, and *B. breve* species differed in their ability to utilize smaller HMO molecules (DP $\leq$ 7) so that the fermentation of HMO might change the distribution of the bifidobacteria species without significantly altering the total concentration of bifidobacteria. It is reported that few gastrointestinal bacteria are capable of growing on HMO as a sole carbon source and, among bifidobacteria, only *B. longum* subsp. *infantis*, *B. bifidum*, and *B. breve* possess this ability.[56]

In summary, this study demonstrated that individual HMO substrates, particularly LNnT and 2′FL, were rapidly fermented in mixed culture systems by microbiota from BF and FF infants. Acetate was the most abundant fermentation metabolite produced, and none of the HMO appeared to have any butyrogenic or bifidogenic effect in these cultures. Differences in the fermentation rates between the two neutral HMO, LNnT, and 2′FL, and the acidic HMO, 6′SL, raise additional questions regarding the relative fermentability of individual HMO. For instance, are all sialylated HMO fermented more slowly than the neutral HMO? Does the degree of sialylation affect susceptibility to bacterial degradation, and if so, are sialylated HMO more likely to reach the distal colon? And, finally, are mixtures of these HMO utilized more rapidly than individual compounds? Clearly, further studies are needed to address these questions.

Our study results highlight the importance of fecal donor selection for in vitro mixed culture studies and raise several additional questions about the metabolism of HMO. Infant fecal inocula donor diet influences the rate of fermentation of HMO. Hence, future in vitro fermentation studies that aim to draw conclusions about the effects of HMO in infant formulas should use fecal inocula collected from FF infants. Likewise, studies that aim to make inferences regarding the bacterial metabolism of HMO in BF infants should use inocula from BF infants. In this study, GOS, 2′FL, and LNnT were rapidly fermented by mixed cultures of infant fecal inocula, regardless of donor source. Overall, inocula from FF infants fermented substrates more rapidly than inocula from BF infants, which may be due to the greater complexity of their microbiota populations. These data will aid in future infant formulas to promote optimal gut health in FF infants.

■ ASSOCIATED CONTENT

Supporting Information

Chromatograph of human milk oligosaccharide analysis using HPLC-PED (Figure S1); changes from baseline in pH over time during in vitro incubation of infant fecal microbiota with human milk oligosaccharides using breast-fed or formula-fed infant fecal inocula (Figure S2); changes from baseline in total short-chain fatty acids during in vitro incubation of breast-fed or formula-fed infant fecal microbiota with human milk oligosaccharides and other substrates (Figure S3); and dry matter, organic matter, free sugars, milk oligosaccharides, and monosaccharides of in vitro substrates evaluated using infant fecal inoculum (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): This research was supported by Abbott Nutrition. A.J.C. is an employee of Abbott Nutrition.

■ ACKNOWLEDGMENTS

The authors thank Geraldine Baggs of Abbott Nutrition for assistance with the statistical analyses.

■ ABBREVIATIONS USED

GOS, galactooligosaccharide; GA, gum arabic; 2′FL, 2′-fucosyllactose; 6′SL, 6′-sialyllactose; LNnT, lacto-*N*-neotetraose; SCFA, short-chain fatty acids; BCFA, branched-chain fatty acids; BF, breast-fed; FF, formula fed; HMO, human milk oligosaccharides; DM, dry matter; OM, organic matter; HPLC, high-performance liquid chromatography; qPCR, quantitative polymerase chain reaction; CFU, colony forming units; SEM, standard error of the mean

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