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Individual and Combined Effects of 2′-Fucosyllactose and *Bifidobacterium longum* subsp. *infantis* on the Gut Microbiota Composition of Piglets

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A B S T R A C T

Background: Human milk is a source of oligosaccharides that promote the growth of beneficial bacteria, including *Bifidobacterium longum* subsp. *infantis*, which can utilize human milk oligosaccharides.

Objectives: To evaluate the individual and combined effects of 2′-fucosyllactose (2′-FL), *B. infantis* Bi-26 (Bi-26) on piglet gut microbiota composition, and short-chain fatty acid (SCFA) concentrations.

Methods: Intact male pigs ($n = 63$) had ad libitum access to milk replacer without (control; CON) or with 1.0 g/L 2′-FL (FL) from postnatal day 2 to 34/35. Pigs were further stratified to receive either 12% glycerol or 10^9 CFU/d Bi-26 in glycerol (BI and FLBI). Gut microbiota and SCFA concentrations were determined in ascending colon contents (AC) and rectal contents (RC) by 16S ribosomal ribonucleic acid gene sequencing and gas chromatography, respectively. Microbiota composition and functional profiles were analyzed using QIIME 2 and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States-2 (PICRUSt2).

Results: Supplementation of 2′-FL increased valerate concentration in AC ($P = 0.03$) and tended to modulate the overall bacterial composition in RC ($P = 0.06$). Compared with CON, 2′-FL alone increased the acetate concentration in AC ($P < 0.05$). The addition of Bi-26 decreased Shannon indices and reduced propionate and butyrate concentrations in AC ($P < 0.05$). Bi-26 alone affected the relative abundances of several bacterial amplicon sequence variants (ASVs) in AC and RC, including the ASVs identified as *Phocaeicola* (*Bacteroides*) *vulgatus* and *Faecalibacterium prausnitzii*. Additionally, 2′-FL and Bi-26 individually increased the relative abundances of 9 PICRUSt2-predicted metabolic pathways related to fatty acid and lipid biosynthesis or carboxylate degradation/secondary metabolite degradation in the RC; however, these effects were negated, and the values were identical to the CON group when 2′-FL and Bi-26 were supplemented together.

Conclusions: 2′-FL and Bi-26 added to milk replacer exerted distinct influences on gut bacterial composition and metabolic function, and 2′-FL alone increased specific SCFA concentrations, demonstrating its prebiotic potential.

Keywords: gut microbiota, short-chain fatty acids, human milk oligosaccharides, 2′-fucosyllactose, *Bifidobacterium longum* subsp. *infantis*

Abbreviations: 2′-FL, 2′-fucosyllactose; AAP, American Academy of Pediatrics; AC, ascending colon contents; ASV, amplicon sequence variants; Bi-26, *Bifidobacterium longum* subs. *infantis*; BF, breastfed; FF, formula-fed; FOS, fructooligosaccharides; FUT2, fucosyltransferase 2; GOS, galactooligosaccharides; GRAS, generally recognized as safe; HMOs, human milk oligosaccharides; PICRUSt2, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States-2; PND, postnatal day; RC, rectal contents; RDP, Ribosomal Database Project; SCFA, short-chain fatty acid.

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Introduction

The development and maturation of infant gut microbiota constitutes a dynamic process that exerts a short- and long-term influence on human health [1]. Mutualistic relations between the host and gut microbiota are essential for host metabolic programming and gastrointestinal, immune, and neurological development in infancy. Disturbance in this process (dysbiosis) has been associated with childhood and later life disorders, such as eczema, asthma, inflammatory bowel diseases, obesity, diabetes, and autism [2]. The development of the gut microbiota occurs primarily during the first year of infant life and continues for the first 2–3 years of age, when it starts to resemble the adult microbiota [3]. The establishment of gut microbiota is influenced by host genetics and multiple environmental factors, of which feeding mode (breastmilk or infant formula) is one of the major drivers [2]. Many studies have shown that the fecal microbiota of breastfed (BF) infants is less diverse and has higher proportions of *Bifidobacterium* spp. and less *Firmicutes* compared with formula-fed (FF) infants [4].

The dissimilarity in microbiota composition and diversity between the BF and FF infants could be partially attributed to the presence of a high concentration of oligosaccharides in human milk. Human milk oligosaccharides (HMOs) are the third largest solid component of human milk following lactose (70 g/L) and lipids (41 g/L) [5]. HMO concentrations range from 20 to 25 g/L in colostrum and 5 to 15 g/L in mature milk with as many as 200 unique structures having been identified [5]. In contrast, bovine milk, the basis of most infant formula, contains far lower amounts of oligosaccharides (~1–2 g/L in colostrum and 100 mg/L in mature milk) with less structural diversity (30–50 structures identified) [5]. Compared with bovine milk, a larger proportion of HMO is fucosylated (50%–80% compared with ~1%) and a smaller proportion is sialylated (10%–20% compared with ~70%) [6]. Porcine milk was reported to contain 29 distinct oligosaccharides, including 6 fucosylated forms, making it more similar to human milk than bovine milk [7]. HMOs are minimally metabolized in the small intestine and the majority reach the colon, where they function as prebiotics to promote the growth of special bacteria, including certain species of *Bifidobacterium* and Bacteroides, and shape the composition and activity of gut microbiota [8–10]. Among the identified HMOs, 2′-fucosyllactose (2′-FL) is often the most abundant and has antimicrobial, immunomodulating, and prebiotic properties [11].

Bifidobacterium is a major constituent of the infant gut microbiota, and convincing evidence suggests that they confer various health benefits to the host by accelerating immune maturation of the response, balancing the immune system to suppress inflammation, and improving intestinal barrier function [12,13]. Furthermore, metabolites produced by *Bifidobacterium*, such as short-chain fatty acid (SCFA) and vitamins, also contribute positive effects on both host and gut microbes [12]. A lower concentration of *Bifidobacterium* in infancy has been linked to an increased risk of obesity, diabetes, and all-cause mortality later in life [14]. *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) is often the dominant inhabitant of the gastrointestinal tract of BF infants and displays anti-inflammatory properties and decreases intestinal permeability [15]. Genomic analysis revealed that strains of *B. infantis* contain an HMO utilization gene cluster that encodes oligosaccharide transport proteins and glycosyl hydrolases, enabling this subspecies to grow to a high density on a wide variety of HMOs, including 2′-FL [16,17].

Our previous publication assessed the safety of 2′-FL and/or *B. infantis* Bi-26 (Bi-26) administration, reporting that formula supplemented with 2′-FL and/or Bi-26 was well tolerated and supported the normal growth of piglets [18]. Herein, the effects of 2′-FL, Bi-26, and a combination of 2′-FL and Bi-26 on gut microbial community structure and function were evaluated in the piglet. Neonatal pigs have been extensively used as a preclinical model to study the effect of early life nutrition on the colonization of gut microbiota [19] as well as gastrointestinal, immune, and neural development because pigs and humans share striking similarities in nutrition requirements, anatomy, physiology, and gut microbiota development [20–22]. Also, both species are colon fermenters and share similar communities of colonic microbiota, such as *Firmicutes* and *Bacteroidetes phyla* [19], and ~96% of the functional pathways described in the human gut microbiome are common to the pig [23]. Piglets have been used for testing the safety and bioactivity of HMO [24]. We hypothesized that supplementation of 2′-FL and/or Bi-26 to formula would modulate the gut microbiota composition and their metabolic activity, and the abundance of *B. infantis* would increase by the synbiotic administration of 2′-FL and Bi-26.

Methods

Experimental design

All animal care and experimental procedures were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the University of Illinois Institutional Animal Care and Use Committee (protocol #17286). Study design, experimental treatment, and animal care were previously described by Daniels et al. [18]. Briefly, naturally farrowed, intact male piglets ($n = 63$) were sow-reared until postnatal day (PND) 2 in a commercial swine farm and then transferred to the Piglet Nutrition and Cognition Laboratory on the campus of the University of Illinois. The piglets were assigned to 2 dietary treatments by equalizing initial body weight and genetics (that is, litter of origin): control (CON) or FL (CON + 1.0 g/L 2′-FL). The 2′-FL (CARE4U) is a high purity (≥94%) infant-grade 2′-FL produced by microbial fermentation and the final product containing ≤ 300 EU endotoxins/g product (International Flavors & Fragrances) [25]. Within each dietary treatment, piglets were further stratified to receive orally either 10^9 CFU/d *B. infantis* Bi-26 (Bi-26; Danisco Global Culture collection DGCC11473 Niebüll, Germany) in 12% glycerol (BI and FLBI) or 12% glycerol (CON and FL). Bi-26 was selected because this strain was isolated from infant stool, tolerates exposure to acidic pH and bile salts in vitro, and is specifically adapted for fast and efficient utilization of fucosylated HMOs [26]. Two milk replacer diets (CON and FL) were identical to ProNurse specialty milk replacer (Land O′Lakes), except for lactose being added to CON to match the 2′-FL content in the FL diet. The nutritional composition of the milk replacers was reported previously [18]. Milk replacer powder was reconstituted fresh each day at 200 g of dry powder per 800 g of water. Piglets were fed 1 of the 2 reconstituted milk replacers ad libitum from PND 2-34/35. Milk was dispensed into clean reservoirs and automatically delivered to piglets using a pump [18]. Bi-26 was resuspended in 12% glycerol (10^9 CFU/mL) and aliquoted before

the start of animal experiment and stored at –80°C. The viability of frozen Bi-26 stock was confirmed by culturing on Reinforced Clostridial Agar. Bi-26 (at a dose of 10^9 CFU/pig/d) or 12% glycerol was administrated orally to the piglets once daily [18]. Piglets were housed in custom pig-rearing units in the same room with a 12-h light/dark cycle [27]. The experiment was performed in 5 replicates. Ten piglets were removed from the study due to failure to thrive unrelated to experimental treatments. The number of piglets completed the study per treatment group was CON ($n = 12$), BI ($n = 14$), FL ($n = 15$), and FLBI ($n = 12$).

Sample collection

On PND 34/35, piglets were sedated with an intramuscular injection of Telazol (Tiletamine HCl and Zolazepam HCl, 3.5 mg/kg BW each, Pfizer Animal Health, Fort Dodge, IA) and then killed by an intracardiac injection of Euthasol (sodium pentobarbital, 72 mg/kg BW; Virbac). The colon was excised, and ascending colon contents (AC) and rectal contents (RC) were collected into sterile cryogenic tubes, snap-frozen in liquid nitrogen, and stored at –80°C. For volatile fatty acid (VFA) analysis, additional AC and RC rectal samples were mixed with 2N HCl in Eppendorf tubes and stored at –20°C. AC and RC were chosen for microbiome analysis, as previous studies have shown that HMOs are minimally digested by intestinal enzymes in the small intestine with >90% reaching an infant's large intestine, where they serve as prebiotics to stimulate the growth of specific bacteria, such as certain species of *Bifidobacterium* and Bacteroides [8,10].

Microbiota analysis

DNA extraction, PCR amplification, and sequencing of 16S rRNA genes

DNA was extracted from AC and RC by combining bead beating on the FastPrep-24 System (MP Biomedicals) with the QIAamp Fast DNA Stool Mini Kit (Qiagen), as described previously [28]. DNA concentration was measured with a Qubit 3.0 Fluorometer using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). PCR amplification and sequencing of 16S rRNA genes were performed at the Roy J. Carver Biotechnology Center, University of Illinois. The V3-V4 regions of bacterial 16S rRNA genes were amplified using primers V3_F357_N (5′-CCTAC GGGNGGCWGCAG -3′) and V4_R805 (5′- GACTACHVGGGTA TCTAATCC-3′) [29]. PCR was performed on a Fluidigm June (Fluidigm Corporation), as described previously [30] with the temperature profiles: 50°C for 2 min (1 cycle), 70°C for 20 min (1 cycle), 95°C for 10 min (1 cycle), followed by 10 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min, 2 cycles at 95°C for 15 s, 80°C for 30 s, 60°C for 30 s, and 72°C for 1 min, 8 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min, 2 cycles at 95°C for 15 s, 80°C for 30 s, 60°C for 30 s, and 72°C for 1 min, 8 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min, and 5 cycles at 95°C for 15 s, 80°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The amplicons from 48 separate reactions were pooled for each sample, and DNA concentration was measured. The amplicons were mixed in equimolar concentrations and analyzed on a 2% agarose E-gel (Thermo Fisher Scientific). The band of expected size (~460 bp) was excised from the gel, and DNA was extracted from the band with a Qiagen Gel Extraction Kit. The extracted DNA was analyzed on an Agilent 2100 bioanalyzer (Agilent Technologies) to confirm an appropriate profile and determine the average size, as described previously [31]. Sequencing was performed on an Illumina MiSeq flowcell for 251 cycles from each end of the fragments (2 × 250 nt paired-end reads) using a MiSeq Reagent Kits v2 (Illumina).

Sequence processing

Sequences were processed using the QIIME 2 pipeline (Version 2020.6) [32]. Paired-end reads obtained from the sequencing facility were imported into QIIME 2 and demultiplexed using the emp-paired method of the plugin demux. Demultiplexed reads were denoised, primer trimmed, dereplicated, and chimera removed using command DADA2 denoise paired [33]. A feature table representing the counts of amplicon sequence variants (ASVs) for each sample was generated, and representative sequences were picked for each feature. The representative sequences were aligned, and a rooted phylogenetic tree was constructed from filtered alignment, as described previously [34]. Alpha diversity (observed features, Shannon and evenness indices, Faith phylogenetic diversity [Faith PD]) and UniFrac distance metrics were computed through the plugin diversity with the core metrics-phylogenetic method on the feature table and phylogenetic tree. When α diversity and UniFrac distance metrics were calculated, all samples were rarefied to 9986 reads to control for differences in sequencing depth.

Taxonomy assignment of ASVs and identification of Phocaeicola vulgatus

Taxonomy was assigned to the ASVs in QIIME 2 using the q2-feature-classifier plugin (classify-sklearn), which was trained on the V3–V4 region of the 16S rRNA reference sequences contained the Ribosomal Database Project training set No. 18 (v. 2.13; July 2020 release) [35–37]. Species designations for ASVs within the genus *Phocaeicola* were manually verified to be correctly assigned based on 100% sequence similarity to the type strains contained in the EzBioCloud database [38]. The taxonomic compositions are reported as relative abundance (% of total sequences).

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States-2 metabolic pathway prediction

Microbiota functional profiles were predicted from the ASV features using the QIIME 2 plugin q2-picrust2, and the relative abundance of MetaCyc metabolic pathway was summarized for the RC samples [39]. The predicted functional profiles were visualized using the q2-feature-table heatmap plugin, where data were normalized using a pseudocount and $\log^{10}$ transformation followed by a 2-way unsupervised clustering by pathway features and treatment groups using the Euclidian distance metric [40]. Metabolic pathway prediction focused on RC as a proxy for stool, the only sample that could be collected from human infants for gut microbiome analysis.

VFA concentrations

Branch chain fatty acid (isobutyrate, valerate, isovalerate) and SCFA (acetate, propionate, butyrate) concentrations were measured in AC and RC via a Hewlett-Packard 5890A Series II gas chromatograph, as described previously [41]. A subset of animals (9–11 piglets/group) was analyzed for VFA due to limited sample availability.

Statistical analyses

All data were analyzed as a 2 × 2 factorial design. The effect of 2′-FL and Bi-26 on overall bacterial communities (beta diversity) was evaluated with principal coordinate analysis (PcoA) and permutational multivariate analysis of variance (adonis). PcoA and adonis were performed on weighted UniFra distances using the QIIME 2 plugins diversity pcoa and diversity adonis, respectively [32]. A $P \leq 0.05$ was considered significant and $0.05 > P \leq 0.1$ was reported as a trend of significance. Alpha diversity, relative abundances of ASVs and predicted metabolic pathways, and SCFA concentrations were analyzed by 2-way analysis of variance (ANOVA) using the MIXED procedure of SAS (version 9.4, SAS Institute) with 2′-FL, Bi-26, and the interaction between 2′-FL and Bi-26 as fixed effects and replicate nested within sow as random effect. Dunnett's test was applied to compare CON with other treatment groups. For α diversity and SCFA concentrations, data were reported as means ± SEMs and a P value of ≤ 0.05 was set as significant. For relative abundances of bacterial ASVs and metabolic pathways, the ASVs were filtered at a minimum 0.1% total abundance and presence in ≥50% of samples for each intestinal section. An arcsine-square root transformation was applied to normalize the residual distribution, and data were presented as Least Squares Mean (LSM) estimate and 95% confidence interval (CI) in original relative abundance scale. The Benjamini–Hochberg procedure was applied to control the false discovery rate (FDR), and a P value ≤0.05 and q-value (FDR-adjusted P) ≤ 0.2 were considered statistically significant. ASVs or metabolic pathways with $P \leq 0.05$ and $q > 0.2$ were reported as a trend of significance.

Statistical analyses for clustering and pathway abundance

The MetaCyc functional pathways are generated from the microbiota for the RC and represented as relative abundances (sum equal to 100%) in each sample. Only the pathways with mean relative abundances >0.1% and presented in >50% of samples were included in the analyses. A MIXED model is fitted to the data, with a fixed model structure according to the 4 diet levels and random structure aligned to the study design (split-plot design with whole-plots equal to sow and nested effect of replicate; split-plots equal to pig and split-plot treatment equal to diet). The statistical scale for all inferential statistics uses the arcsine-square root transformation. The data are reported as the diet LSM estimate (95% CI) in the (back-transformed) original scale. The overall ANOVA significance (ANOVA P) is reported by the main effect of 2′-FL, the main effect of Bi-26, and the interaction between 2′-FL and Bi-26. The treatment-control ANOVA significance (Dunnett's test P) is reported by 2′-FL compared with CON, Bi-26 compared with CON, 2′-FL + Bi-26 compared with CON. The Benjamini–Hochberg procedure was applied to control the FDR, and a P value ≤0.05 and q-value (FDR-adjusted P) ≤ 0.2 were considered statistically significant. Pathways with $P \leq 0.05$ and $q > 0.2$ were reported as a trend of significance. All statistical analyses are performed using the statistical software SAS (version 9.4, SAS Institute).

Results

Microbiota analysis

Illumina sequencing of 16S rRNA amplicons yielded 8 million paired-end reads across 100 samples. After denoising, dereplicating, chimera removal, and joining of paired-end reads with DADA2, 3,477,338 sequences (mean ± SD: 34,773 ± 8495 per sample) were utilized for further analysis. PcoA of weighted UniFrac distances generated from AC and RC samples is shown in Figures 1A and B, respectively. Adonis indicated no main effect of 2′-FL on overall microbiota composition in the AC ($P = 0.216$; Figure 1A); however, there was a tendency for 2′-FL to influence the microbial community structure in RC ($P = 0.06$; Figure 1B). Neither Bi-26 nor the interaction between the 2′-FL and Bi-26

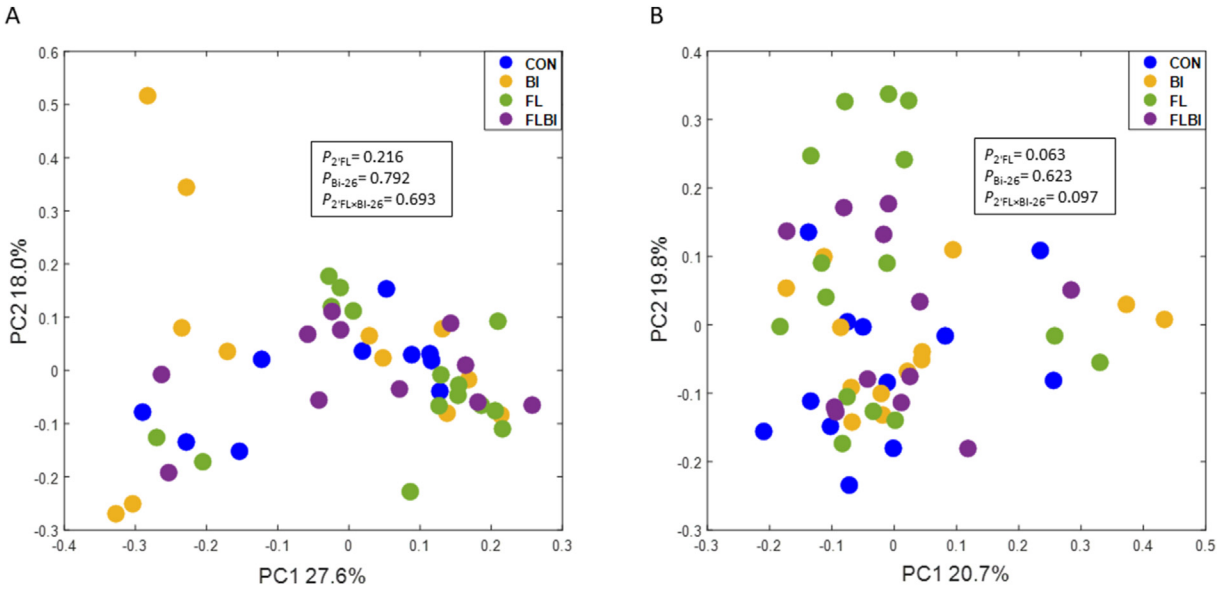

FIGURE 1. Principal coordinate analysis based on weighted UniFrac distances generated from ascending colon and rectal (B) contents of piglets fed different diets. (A) Ascending colon content. (B) Rectum content. $n = 10$–15; P values were obtained by adonis. The statistical model included 2′-FL, Bi-26 and the interaction of 2′-FL and Bi-26 (2′-FL × Bi-26). Bi-26 did not affect overall microbiota communities in AC and RC ($P > 0.05$). 2′-FL tended to influence the microbial community structure in RC ($P = 0.063$). AC, ascending colon content; RC, rectal content.

affected the overall microbiota composition in AC and RC ($P > 0.05$; Figures 1A and B).

The effects of treatment on α diversity measures are presented in Table 1. Shannon indices were lower in AC of piglets administered Bi-26 alone (BI) or with 2′-FL (FLBI); $P = 0.05$) relative to piglets not receiving Bi-26 (CON and FL). Observed features, evenness indices, and Faith PD in AC were similar among the treatment groups ($P > 0.05$). Neither 2′-FL nor Bi-26 influenced α diversity in RC ($P > 0.05$); however, 2′-FL and Bi-26 interactively affected the observed features in RC ($P = 0.017$; Table 1).

Main and interaction effects of 2′-FL and Bi-26 on the relative abundance of ASVs in AC and RC are shown in Supplemental Tables 1 and 2, respectively. Neither the main effects (2′-FL or Bi-26) nor the interaction between the 2 significantly affected the relative abundances of ASVs (ANOVA $q > 0.2$). However, supplementation of 2′-FL or Bi-26 tended to impact the relative abundance of some ASVs in AC and RC (ANOVA $P < 0.05$, $q > 0.2$, Supplemental Figures 1 and 2). The relative abundance of ASVs classified as Holdemanella biformis (ASV39) and Lactobacillus johnsonii (ASV63) were lower and ASVs identified as Faecalibacterium prausnitzii (ASV78), Blautia luti (ASV74), Flintibacter butyricus (ASV35), Prevotella stercorea (ASV53, ASV82), and unclassified Bacteroidales (ASV71) were higher in the AC of piglets either fed diets containing 2′-FL (FL and FLBI groups) than piglets fed diets without 2′-FL (CON and BI) (Figure 2A). In RC, 8 bacterial ASVs showed a trend of being affected by 2′-FL, including enrichment of ASV21 *Catenibacterium mitsuokai*, ASV48 *Mediterraneibacter faecis*, ASV18 *Blautia obeum* and ASV88 *Negativibacillus massiliensis*, and reduction of ASV79 *Blautia glucerasea*, ASV43 *Anaerotignum lactatifermentans*, ASV6 unclassified *Ruminococcaceae*, and ASV81 unclassified *Eggerthellaceae* (Figure 2B).

On the other hand, administration of Bi-26 showed a tendency to decrease colonic proportions of 4 ASVs related to *Catenibacterium mitsuokai* (ASV21), *Prevotella stercorea* (ASV92), *Prevotellamassilia timonensis* (ASV77), and *Escherichia/Shigella coli* (ASV96) and to increase ASV62 *P. vulgatus* (Figure 3A). In RC, Bi-26 supplementation tended to lower an ASV identified as *F. prausnitzii* (ASV78) and an ASV (ASV65) identified as unclassified Ruminococcus2, whereas Bi-26 enriched 5 ASVs classified as *Ruminococcus bromii* (ASV42), *Flintibacter butyricus* (ASV35), unclassified *Ruminococcaceae* (ASV1), *Fournierella massiliensis* (ASV49), and unclassified *Bacteroidales* (ASV86) (Figure 3B).

A trend for an interaction effect was observed in AC for the relative abundances of ASV14 (Unclassified Muribaculaceae), ASV50 (unclassified Ruminococcus2), ASV16 (unclassified Bacteria), ASV8 and ASV95 (both identified as *Desulfovibrio piger*) (Supplemental Table 1 and Supplemental Figure 1; $P < 0.05$, $q > 0.2$). In RC, there was a trend for interaction of 2′-FL and Bi-26 to impact the proportions of ASV62 (*P. vulgatus*), ASV22 (unclassified Butyricimonas), ASV41 (*F. prausnitzii*), ASV 75 (unclassified Ruminococcaceae) and ASV 72 (*Fusobacterium mortiferum*) (Supplemental Table 2 and Supplemental Figure 2; $P < 0.05$, $q > 0.2$).

When Dunnett's test was applied to compare CON with other treatment groups (FL, BI or FLBI), significant differences ($P < 0.05$, $q < 0.2$) in the relative abundances of ASVs were observed between CON and BI groups (1 in AC and 4 in RC), but not between CON and FL or FLBI groups. The proportion of ASV50 (unclassified Ruminococcus2) was lower in AC of BI compared with CON (Figure 4A; $P = 0.003$, $q = 0.068$). In RC, 2 ASVs (ASV62 *P. vulgatus*, ASV1 unclassified Ruminococcaceae) were higher and 2 ASVs (ASV41 *F. prausnitzii*, ASV72 *Fusobacterium mortiferum*) were lower in BI than in CON (Figures 4B-4E; $P < 0.05$, $q < 0.2$).

Four ASVs showed a tendency to differ ($P < 0.05$, $q > 0.2$) between CON and other treatment groups (Supplemental Tables 1 and 2). In AC, ASV50 (unclassified Ruminococcus2) was lower in AC of FL (0.05%) and FLBI (0.03%) compared with CON piglets (0.35%; $P < 0.05$, $q > 0.2$). The proportions of ASV65 unclassified Ruminococcus2 were lower in BI than CON piglets in both AC (0.01% compared with 0.14%; $P = 0.044$, $q > 0.2$) and RC (0.04% compared with 0.24%; $P = 0.043$, $q > 0.2$). Compared with CON, RC ASV48 *Mediterraneibacter faecis* and ASV35 *Flintibacter butyricus* were higher in FL and FLBI, respectively (1.24% compared with 0.24% and 0.48% compared with 0.15%; $P < 0.05$, $q > 0.2$).

Metabolic pathway prediction by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States-2

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States-2 (PICRUSt2) was used to predict the

TABLE 1
α diversity obtained from microbiota of ascending colon and rectal contents of piglets fed different diets.

	CON	BI	FL	FLBI	ANOVA *P*		
					2′-FL	Bi-26	Interaction
Ascending colon contents							
Observed features	240 ± 7.9	216 ± 10	219 ± 6.1	206 ± 12.5	0.107	0.053	0.554
Shannon	5.8 ± 0.1	5.4 ± 0.2	5.6 ± 0.1	5.5 ± 0.2	0.710	0.048[1]	0.513
Evenness	0.73 ± 0.01	0.70 ± 0.02	0.72 ± 0.01	0.71 ± 0.02	0.775	0.091	0.579
Faith PD	24.7 ± 1.00	22.8 ± 1.02	23.6 ± 0.80	22.3 ± 1.10	0.368	0.091	0.757
Rectal contents							
Observed features	221 ± 7.3	242 ± 6.6	238 ± 8.7	220 ± 11.4	0.922	0.778	0.017
Shannon	5.5 ± 0.1	5.6 ± 0.1	5.6 ± 0.1	5.6 ± 0.1	0.511	0.984	0.892
Evenness	0.71 ± 0.01	0.70 ± 0.02	0.71 ± 0.01	0.72 ± 0.01	0.440	0.949	0.518
Faith PD	24.7 ± 0.71	25.1 ± 0.70	25.3 ± 0.80	24.3 ± 1.5	0.803	0.768	0.425

Abbreviations: BI, control diet + 10^9 CFU Bi-26/d; CON, control diet; FL, control formula + 1.0 g/L 2′-FL; FLBI, control formula + 1.0 g/L 2′-FL + 10^9 CFU Bi-26/d; PD, phylogenetic diversity.
Values are expressed as means ± SEMs; $n = 10$–15/group.
Data were analyzed by ANOVA using MIXED procedure of SAS.
[1] Value indicates significant difference (ANOVA $P < 0.05$).

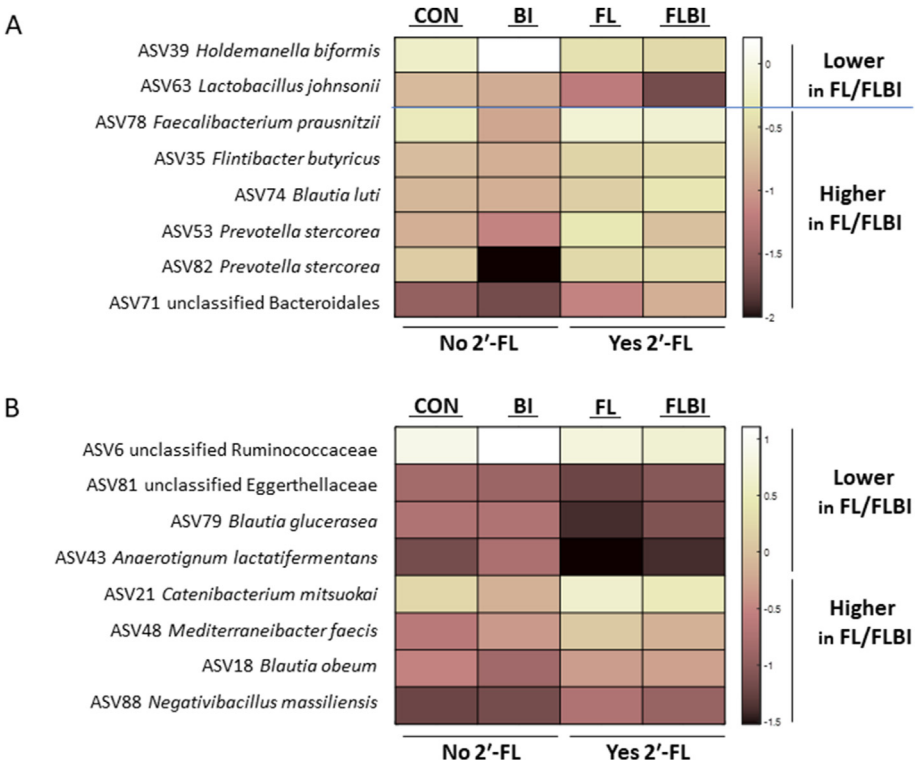

FIGURE 2. Relative abundances of ASVs that tended to be affected by 2′-FL in the ascending colon (A) and rectal (B) contents of piglets ($P < 0.05$, $q > 0.2$). LSM estimates for the heat maps were log10 transformed; $n = 10–15$. 2′-FL, 2-fucosyllactose; ASV, amplicon sequence variant.

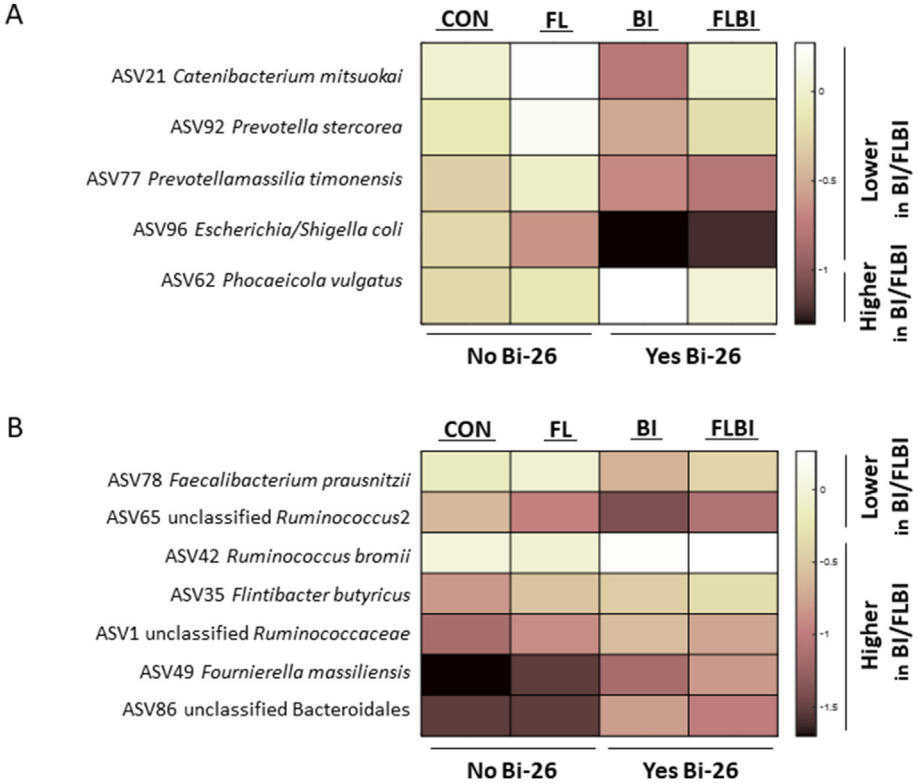

FIGURE 3. Relative abundances of ASVs that tended to be affected by Bi-26 in the ascending colon (A) and rectal (B) contents of piglets ($P < 0.05$, $q > 0.2$). LSM estimates for the heat maps were log10 transformed; $n = 10–15$. ASV, amplicon sequence variant; Bi-26, *Bifidobacterium longum* subsp. *infantis* Bi-26.

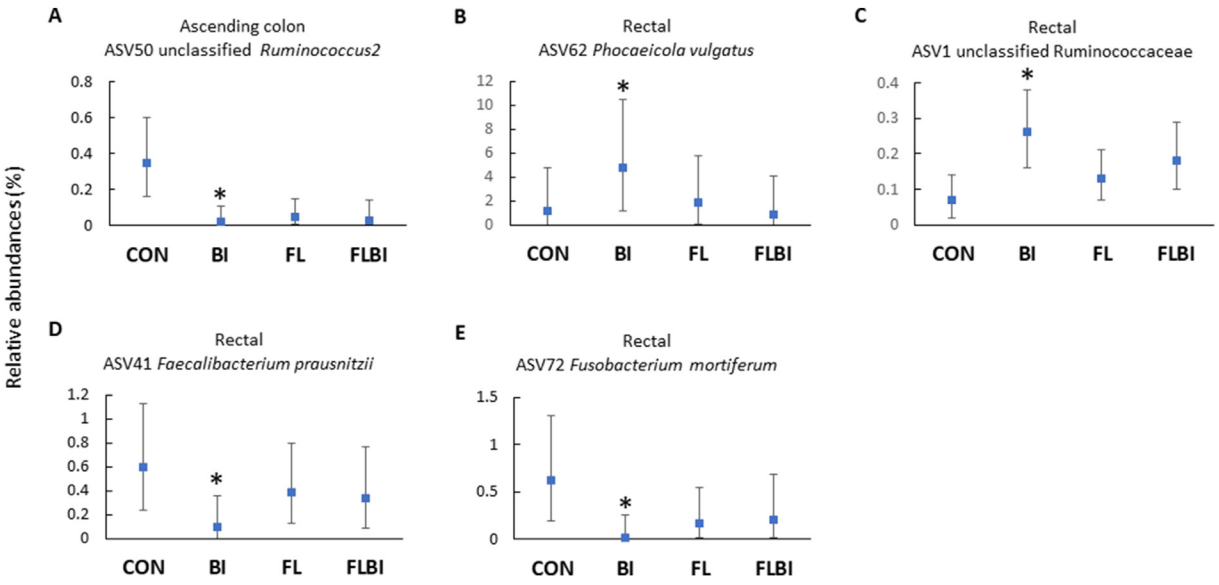

FIGURE 4. Relative abundances of ASVs differing between CON and BI in ascending colon (A) and rectal (B–E) contents of piglets. Data are LSM estimates with 95% CIs; $n = 10$–15; * indicated differed from CON by Dunnett's test ($P < 0.05$, $q < 0.2$). ASV, amplicon sequence variant; CI, confidence interval.

metabolic pathway profiles of RC bacterial communities from the 16S rRNA sequences. A limitation of this method is that it infers function compared with direct metagenomic sequencing; however, it does provide preliminary data for hypothesis generation and future research. A total of 359 MetaCyc pathways were predicted in RC. Cluster analysis indicated no clear clustering of pathway abundances among treatment groups (data not shown). A filtered set of 189 pathways having mean relative abundances >0.1% and presenting in >50% of samples were further compared among the treatment groups.

Statistical analyses of individual pathways demonstrated that neither 2′-FL nor Bi-26 significantly affected the relative abundances of any metabolic pathways ($q > 0.2$). Significant interaction effects were observed for 9 pathways (Table 2 and Figure 5; $P < 0.05$, $q < 0.2$), including 2 pathways belonging to the superclass of degradation/utilization/assimilation and 7 to biosynthesis. In general, supplementation with 2′-FL and Bi-26 independently increased the relative abundances of the pathways, but these effects were negated when 2′-FL and Bi-26 were administered together (Figure 5).

A total of 13 metabolic pathways showed a trend ($P < 0.05$, $q > 0.2$) to be influenced by 2′-FL or Bi-26 administration (Supplemental Table 3). Among these, 2′-FL affected 12 pathways, including 9 pathways belonging to the superclass of biosynthesis and 3 to degradation/utilization/assimilation. Compared with the piglets fed diets without 2′-FL (CON and BI groups), the rectal microbiome of piglets consuming diets with 2′-FL (FL and FLBI groups) appeared to have higher abundances of pathways related to L-methionine biosynthesis III (HSERMETANA-PWY), L-tryptophan biosynthesis (TRPSYN-PWY), super pathway of L-methionine biosynthesis (trans-sulfuration) (PWY-5347), L-methionine biosynthesis I (HOMOSER-METSYN-PWY), thiamine diphosphate salvage II (PWY-6897), super pathway of S-adenosyl-L-methionine biosynthesis (MET-SAM-PWY), sucrose degradation III (sucrose invertase) (PWY-621), and guanosine nucleotides degradation III (PWY-6608). In contrast, piglets fed diet without 2′-FL had higher proportions of folate transformations III (*E. coli*) (1CMET2-PWY), biotin biosynthesis I (BIOTIN-BIOSYNTHESIS-PWY), pyrimidine deoxyribonucleotides de novo biosynthesis III (PWY-6545), and super pathway of glucose and xylose degradation (PWY-6901). Two microbiome pathways, aspartate super pathway (PWY0-781) and L-methionine biosynthesis III (HSERMETANA-PWY), were impacted by Bi-26 at a trend level ($P < 0.05$, $q < 0.2$) with their abundances being lower in Bi-26 supplemented (BI and FLBI) compared with unsupplemented groups (CON and FL). A trend of interaction effect ($P < 0.05$, $q > 0.2$) was observed for 9 pathways (Supplemental Table 3 and Supplemental Figure 3). Of these pathways, 3 were associated with the superclass of degradation/utilization/assimilation, 4 were related to the generation of precursor metabolites and energy, and 2 belonged to biosynthesis.

Dunnett's test demonstrated that 10 RC microbiome pathways showed a trend of significant difference ($P < 0.05$, $q > 0.2$) between CON and other treatment groups (Supplemental Table 4). Of the 10 pathways, 9 were associated with the superclass of biosynthesis and 1 was related to degradation/utilization/assimilation. One pathway (PWY-6897: thiamine diphosphate salvage II) belonged to the subclass of cofactor, carrier, and vitamin biosynthesis was higher in FL than CON. The remaining 9 pathways differed between CON and BI. Among these, 1 pathway related to subclass of carbohydrate degradation (RHAMCAT-PWY: L-rhamnose degradation I), and 7 belonged to fatty acid and lipid biosynthesis, including palmitate biosynthesis II (PWY-5971), mycolate biosynthesis (PWYG-321), oleate biosynthesis IV (PWY-7664), palmitoleate biosynthesis I (PWY-6282), stearate biosynthesis II (PWY-5989), (5Z)-dodecanoate biosynthesis I (PWY0-862), and super pathway of fatty acid biosynthesis initiation (FASYN-INITIAL-PWY), were higher in BI compared with CON. On the contrary, relative abundance of L-methionine biosynthesis III (HSERMETANA-PWY) was lower in BI than CON.

TABLE 2
Relative abundances of microbiome pathways in rectal contents that were significantly impacted by the interaction between 2′-FL and Bi-26.

BioCyc ID: pathway name	Superclass → subclass	CON	BI	FL	FLBI	ANOVA *P*		
						2′-FL	Bi-26	interaction
PWY-7242: D-fructuronate degradation	Degradation/utilization/assimilation → carboxylate degradation/ secondary metabolite degradation	0.29 (0.20, 0.39)	0.38 (0.28, 0.5)	0.34 (0.25, 0.45)	0.25 (0.17, 0.35)	0.158	0.884	0.001[1]
GALACTUROCAT-PWY: D-galacturonate degradation I	Degradation/utilization/assimilation → carboxylate degradation/ secondary metabolite degradation	0.25 (0.20, 0.30)	0.29 (0.23, 0.35)	0.30 (0.24, 0.36)	0.24 (0.19, 0.30)	0.885	0.602	0.006[1]
PWY-5971: palmitate biosynthesis II (type II fatty acid synthase)	Biosynthesis → fatty acid and lipid biosynthesis	0.30 (0.19, 0.45)	0.51 (0.36, 0.69)	0.38 (0.25, 0.53)	0.30 (0.18, 0.45)	0.239	0.241	0.007[1]
PWYG-321: mycolate biosynthesis	Biosynthesis → fatty acid and lipid biosynthesis	0.28 (0.15, 0.45)	0.48 (0.31, 0.70)	0.35 (0.21, 0.53)	0.26 (0.14, 0.43)	0.185	0.324	0.008[1]
PWY-7664: oleate biosynthesis IV (anaerobic)	Biosynthesis → fatty acid and lipid biosynthesis	0.28 (0.15, 0.45)	0.48 (0.31, 0.69)	0.35 (0.21, 0.53)	0.26 (0.14, 0.43)	0.192	0.339	0.007[1]
PWY-6282: palmitoleate biosynthesis I (from (5Z)-dodec-5-enoate)	Biosynthesis → fatty acid and lipid biosynthesis	0.25 (0.13, 0.42)	0.45 (0.28, 0.66)	0.33 (0.19, 0.50)	0.24 (0.12, 0.40)	0.183	0.336	0.006[1]
PWY-5989: stearate biosynthesis II (bacteria and plants)	Biosynthesis → fatty acid and lipid biosynthesis	0.25 (0.13, 0.42)	0.45 (0.27, 0.66)	0.32 (0.18, 0.51)	0.23 (0.11, 0.40)	0.189	0.340	0.006[1]
PWY0-862: (5Z)-dodecenoate biosynthesis I	Biosynthesis → fatty acid and lipid biosynthesis	0.25 (0.13, 0.41)	0.44 (0.28, 0.65)	0.32 (0.19, 0.49)	0.23 (0.12, 0.39)	0.182	0.342	0.006[1]
FASYN-INITIAL-PWY: superpathway of fatty acid biosynthesis initiation	Biosynthesis → fatty acid and lipid biosynthesis	0.22 (0.11, 0.36)	0.38 (0.24, 0.56)	0.28 (0.17, 0.43)	0.21 (0.11, 0.35)	0.243	0.351	0.008[1]

Abbreviations: 2′-FL, 2-fucosyllactose; BI, control diet + 109 CFU Bi-26/d; Bi-26, *Bifidobacterium longum* subsp. *infantis* Bi-26; CON, control diet; FL, control formula + 1.0 g/L 2′-FL; FLBI, control formula + 1.0 g/L 2'-FL + 10^9 CFU Bi-26/d.
Values are expressed as LSM estimate (95% CI) in original scale; $n = 10–15$.
Data were analyzed by ANOVA using MIXED procedure of SAS. Only the pathways with mean relative abundances >0.1% and presented in >50% of samples were included in the analyses.
[1] Indicates that the effect was significant after false discovery rate correction (ANOVA $P < 0.05$, $q < 0.2$).

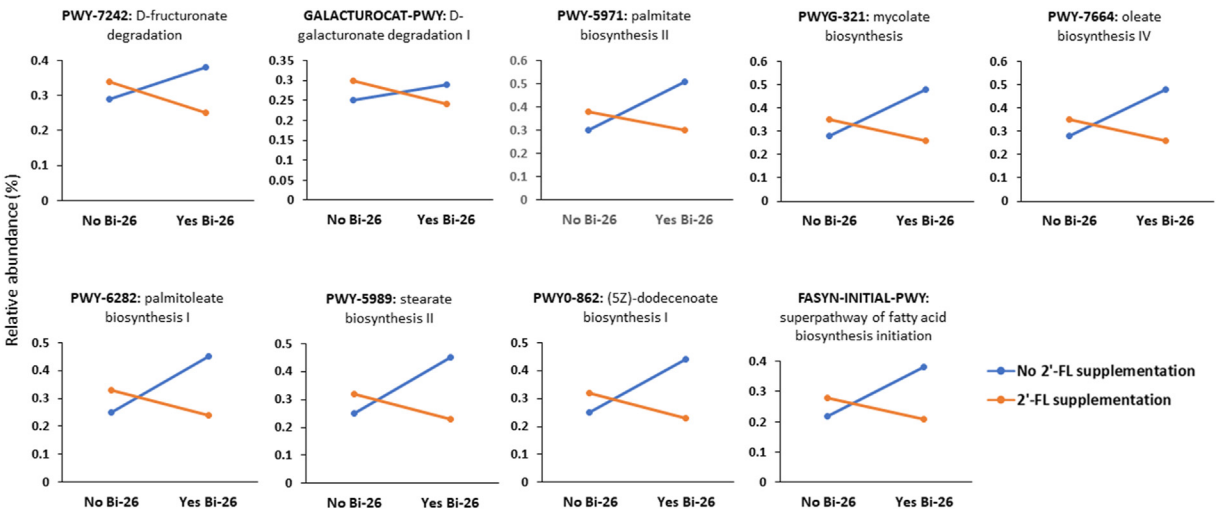

FIGURE 5. Interaction results of metabolic pathways in rectal contents. A similar trend was observed for 9 pathways ($P < 0.05$ and $q < 0.2$). For these pathways, supplementation with 2′-FL and Bi-26 separately increased values, but these effects were negated when 2′-FL and Bi-26 were supplemented together. 2′-FL, 2-fucosyllactose; Bi-26, *Bifidobacterium longum* subsp. *infantis* Bi-26™.

VFA concentrations

Administration of Bi-26 decreased the concentrations of propionate and butyrate in AC of piglets regardless of the presence of 2′-FL ($P = 0.039$ and $P = 0.043$, respectively; Table 3). Valerate concentrations were higher ($P = 0.033$) in AC of piglets consuming diets with 2′-FL (FL and FLBI groups) than without 2′-FL (CON and BI groups). Concentrations of acetate were higher in AC of FL piglets compared with CON ($P < 0.05$). The SCFA concentrations in RC were similar among all treatment groups ($P > 0.05$).

Discussion

Human milk provides bioactive components, including HMOs that serve functional roles to protect infants against infections and allergic and autoimmune diseases in childhood and is associated with a reduced risk of inflammatory bowel diseases, cardiovascular diseases, diabetes, and obesity later in life [42,43]. Exclusive breastfeeding is recommended by the American Academy of Pediatrics for the first 6 mo of life [44]. However, only 24.9% of American babies meet this recommendation, and 75.1% of U.S. infants receive exclusively formula- or mixed-feeding by 6 mo of age [45]. Feeding mode shapes the composition and function of infant gut microbiota and differences in microbial composition and diversity between BF and FF infants [2]. The presence of high quantities of HMOs and microbes in human milk has promoted efforts to mimic the intestinal microbiota of BF infants by supplementing infant formula with prebiotics (such as galactooligosaccharides [GOS],

TABLE 3
Short-chain fatty acid concentrations in ascending colon and rectal contents of piglets fed different diets.

	CON	BI	FL	FLBI	ANOVA *P*		
					2′-FL	Bi-26	Interaction
μmol/g dry matter of contents							
Ascending colon contents							
Acetate	353 ± 51.2	373 ± 74.8	580 ± 71.1[1]	366 ± 53.0	0.191	0.174	0.046[2]
Propionate	115 ± 19.3	83.6 ± 10.5	151 ± 17.6	109 ± 13.7	0.084	0.039[2]	0.588
Butyrate	47.5 ± 4.4	43.8 ± 9.6	75.2 ± 13.5	42.5 ± 3.7	0.165	0.043[2]	0.470
Isobutyrate	11.3 ± 0.8	11.0 ± 1.2	11.3 ± 1.6	11.3 ± 1.4	0.346	0.949	0.809
Isovalerate	13.4 ± 1.0	13.6 ± 1.8	14.6 ± 1.8	14.0 ± 1.7	0.358	0.721	0.765
Valerate	15.7 ± 2.2	12.6 ± 2.1	20.8 ± 3.0	18.9 ± 2.8	0.033[2]	0.357	0.870
Rectal contents							
Acetate	94.5 ± 8.9	117 ± 17.1	169 ± 27.9	117 ± 31.0	0.218	0.362	0.063
Propionate	44.8 ± 5.4	41.3 ± 7.2	56.1 ± 7.7	44.1 ± 11.7	0.427	0.224	0.386
Butyrate	22.0 ± 3.2	21.9 ± 3.4	27.9 ± 4.4	17.7 ± 2.0	0.843	0.175	0.162
Isobutyrate	8.7 ± 0.9	8.6 ± 1.1	11.0 ± 1.5	7.8 ± 1.8	0.615	0.210	0.216
Isovalerate	10.5 ± 0.8	10.5 ± 1.2	13.0 ± 1.7	9.7 ± 2.0	0.583	0.280	0.266
Valerate	7.5 ± 0.7	8.3 ± 1.1	10.1 ± 1.9	10.9 ± 3.3	0.165	0.681	0.997

Abbreviations: BI, control diet + 10^9 CFU Bi-26/d; CON, control diet; FL, control formula + 1.0 g/L 2′-FL; FLBI, control formula + 1.0 g/L 2′-FL + 10^9 CFU Bi-26/d.
Values are expressed as means ± SEMs; $n = 9$–11.
Data were analyzed by ANOVA using MIXED procedure of SAS. Dunnett's test with Tukey adjustment was applied to compare CON with other treatment groups.
[1] Indicates treatment group differed from CON (Dunnett's test $P < 0.05$).
[2] Values indicate significant differences (ANOVA $P < 0.05$)

fructooligosaccharides [FOS], and milk oligosaccharides) and/or probiotics [2,46–48]. In this study, 2′-FL, Bi-26, and a combination of 2′-FL and Bi-26 were supplemented to formula. Bi-26 was isolated from infant stool and has the genes required for efficient utilization of fucosylated HMOs [18]. In a semi-continuous colon simulator containing donor human infant feces, the combination of 2′-FL and *B. infantis* Bi-26 decreased α diversity, increased the relative abundance and number of *Bifidobacterium* species, and increased total SCFA and acetate, concentrations supporting the prebiotic potential of 2′-FL for *B. infantis* [49].

2′-FL is one of the most highly abundant HMOs, but its concentration varies depending on secretor status of the mother, stage of lactation, and geographic region [50,51]. In the milk of secretor mothers (women with active alleles of the fucosyltransferase 2 [FUT2] gene), 2′-FL represents 20%–30% of total HMOs with a mean concentration of 2–3 g/L [52–54]. However, in the milk of nonsecretor mothers (women with inactive gene for FUT2), who on average represent ∼20% of the mothers (0%–37% depending on the geographical region), 2′-FL was low or undetectable [51]. Compared with infants consuming milk from nonsecretors, infants fed with breastmilk of secretors had a higher *Bifidobacterium* population in feces [55]. Synthesized 2′-FL are generally recognized as safe and have been added to some commercial infant formula [11]. Infant formulas supplemented with 2′-FL (0.2 or 1 g/L) alone or in combination with other prebiotics (such as lacto-N-neotetraose, GOS, or short-chain FOS) were safe and well tolerated and supported the normal growth of healthy term infants [47,48,56]. Moreover, infants fed formula with 2′-FL (0.2 g/L) had fewer respiratory infections than infants fed unsupplemented formula [48]. In a recent double-blind randomized controlled trial, Alliet et al. examined the effects of supplementation of 2′-FL (1.0 g/L) to a formula containing *Lactobacillus reuteri* DSM 17938 (1×10^7 CFU/g) on the fecal microbiota of infants from ≤14 d to 6 mo of age [56]. In that study, α diversity (Faith PD) was not affected by 2′-FL supplementation; however, the overall microbiota composition (beta diversity) of infants consuming 2′-FL differed from that of infants fed control formula containing only *L. reuteri* at age 2 and 3 mo and was closer to that of BF infants. Like the results of Alliet et al. [56], in our study, a tendency ($P = 0.062$) for an overall bacterial community structure was observed in the RC between the piglets fed diets with 2′-FL (FL and FLBI) and without 2′-FL (CON and BI). Additionally, α diversity measures (observed features, Shannon and evenness indices, and Faith PD) were comparable between piglets fed diets with 2′-FL and without 2′-FL in both AC and RC.

VFAs are produced through gut microbial fermentation of nondigestible carbohydrates and amino acids, and their profiles represent the collective metabolic activity of the gut microbiota [57]. Several double-blind placebo-controlled trials have investigated the effects of traditional prebiotics (GOS or short-chain GOS/long-chain FOS) on infant fecal SCFA profiles and reported that the proportions of acetate were higher in infants fed formula with added prebiotics than unsupplemented controls [57,58]. In a recent study, supplementation of bovine milk-derived oligosaccharides to formula increased the acetate concentration in AC of piglets [59]. Consistent with the results of these previous studies, we found that acetate concentrations were significantly higher in AC of piglets supplemented with 2′-FL alone in comparison with CON. Acetate is the most abundant SCFA in the human colon, accounting for ∼60% of total SCFAs [60].

The influence of SCFAs on host physiology in early life is an active area of research; however, in vitro studies indicate that acetate contributes to the decreased intestinal pH milieu and inhibited the growth of many common pathogens [61]. Additionally, acetate can stimulate the growth of butyrate-producing bacteria and increase the production of butyrate, a preferred energy source for colonocytes, which has anti-inflammatory, antioxidant, and anticarcinogenic properties as well as improves intestinal barrier function [62]. Furthermore, ≤70% acetate is taken up by the liver, where it is used as a substrate for the biosynthesis of cholesterol, long-chain fatty acids, and the amino acids glutamine and glutamate [63]. Moreover, acetate has been shown to promote G protein-coupled receptor 43 mediated intestinal IgA response and modulate microglial functions in a mouse model, suggesting its important role in host immune response and the development of central nervous system [64,65].

Several lines of evidence support the use of *B. infantis* in infant formula. Human milk contains a variety of potential probiotic bacteria, including *Bifidobacterium,* and recent evidence has shown that breast milk microbiota can directly seed the infant gut microbiota [66]. Additionally, *B. infantis* dominates the gut microbiota of BF infants, and administration of *B. infantis* improves infant health, including preventing the development of atopic dermatitis, improving symptoms of allergic rhinitis and asthma, decreasing the risk of common infections, and reducing the incidence of necrotizing enterocolitis in preterm infants [13]. Although how *B. infantis* exerts its positive impact on infant health remains to be fully understood, it could be related to their direct interaction with the host immune system or indirect by their influence on the composition and/or metabolic activity of the gut microbiota [13].

In this study, the effect of Bi-26 supplementation on microbiota in AC and RC of piglets was assessed. We observed higher abundance of *B. infantis* in the AC and RC of piglets fed diets with Bi-26 (BI and FLBI) than without Bi-26 (CON, FL) [18]; however, the overall bacterial community structure (beta diversity) of the AC and RC was not affected by Bi-26 supplementation, which is in line with previous findings when *B. infantis* ATCC15697 was administrated to FF infants with gastroschisis [67]. Similar results were reported when other probiotic strains were supplemented to human adults [68].

Previous studies have shown that the fecal microbiota of BF infants is less diverse and has higher levels of *Bifidobacterium* spp. compared with FF infants [69]. Fecal SCFAs differ between BF and FF infants, with higher concentrations of propionate and butyrate in FF than in BF infants [69,70]. In this study, supplementation of Bi-26 reduced α diversity and decreased the concentrations of propionate and butyrate, shifting the patterns to be more like BF infants; however, the *Bifidobacterium* population were low in both AC and RC of the piglets (mean relative abundances of 0.002% in this study), and supplementation of BI-26 did not change the total abundance of *Bifidobacterium* [18]. Given that reduced microbial diversity in early life has been linked with childhood disorders such as atopic eczema and asthma [71,72] and the important roles of propionate and butyrate on antitumor and anti-inflammation [73], as well as the

positive effects of *Bifidobacterium* on the immune system as discussed above, whether the use of Bi-26 is beneficial on host health needs to be further investigated.

Because *B. infantis* contains a suite of HMO utilization genes and preferentially metabolizes small-mass HMO in vitro [74], we hypothesized that the combination of 2′-FL and Bi-26 would promote the growth of total *Bifidobacterium* or *B. infantis*. However, in this study, no significant differences in the overall *Bifidobacterium* population were detected among treatment groups [18]. Furthermore, *B. infantis* abundances were similar between BI and FLBI piglets [18]. Our results are in contrast with the findings of previous human infant studies, where a higher concentration of *Bifidobacterium* and *B. infantis* were detected in the stools of BF infants consuming a different strain of *B. infantis* (EVC001) compared with unsupplemented controls [75,76].

A possible explanation for these contradictory observations may be due in part to host specificity for microbes. The ability of microbes to colonize a host depends on their physiological interaction with the host and environmental requirements [77]. Indigenous bacteria are well adapted to thrive in their natural environment, whereas nonindigenous bacteria are relatively intolerant to the harsh conditions of GIT, such as gastric acidity and bile acid [78]. *B. infantis* Bi-26 is not a member of the indigenous microbiota of pig.

Even though Bi-26 survived through the gastrointestinal tract and reached the colon of piglets, niche availability within the resident gut microbiota and relative fitness of the probiotic strain may also influence the colonization success of administered probiotics. A recent review has demonstrated that host response to probiotics is unlikely when the available niche is low, and probiotics cannot outcompete with resident taxa for resource utilization [79]. In this study, Bi-26 was administered to piglets who were sow-reared for 2 d, which might provide a less available niche for Bi-26 to colonize as previous studies on human infants have reported increased effectiveness of colonization when the first dose of *Bifidobacterium* breve was administered a few hours after birth compared with when administrated >24 h after birth [80]. Furthermore, Bacteroides is a predominant bacterial genus in both AC and feces of piglets [81]. Similar to *Bifidobacterium*, some members of Bacteroides are equipped with specialized machinery dedicated to importing and metabolizing HMO [9]. It is possible that Bi-26, isolated from infant stool, might not outcompete the resident Bacteroides in the piglet for 2′-FL utilization.

Some interactive effects between 2′-FL and Bi-26 were observed in this study. For example, 2′-FL and Bi-26 each increased the relative abundances of 9 PICRUSt2-predicted metabolic pathways related to fatty acid and lipid biosynthesis or carboxylate degradation/secondary metabolite degradation in the RC; however, these effects were negated, and the values were identical to the CON group when 2′-FL and Bi-26 were supplemented together. Similar trends were also observed for the relative abundance of several bacterial taxa, including *Desulfovibrio piger* (ASV8) and unclassified Muribaculaceae (ASV14) in AC, as well as unclassified Butyricimonas (ASV22) in RC, suggesting that a synergistic effect was not achieved when 2′-FL and Bi-26 were coadministered. How the combination of 2′-FL and Bi-26 diminished the individual effects of 2′-FL and Bi-26 is not known, warranting further studies.

In summary, using the piglet model, we assessed the effects of 2′-FL and B-26 individually and synergistically on gut microbiota composition and their metabolic activity. We observed that supplementation of 2′-FL increased valerate and acetate concentrations in the AC and tended to modulate the overall bacterial composition in RC. Supplementation of Bi-26 alone reduced the α diversity and propionate and butyrate concentrations in the AC. Bi-26 alone affected the relative abundances of several bacterial ASVs in AC and RC. Higher Bi-26 was detected in the AC and RC, but an increase in total *Bifidobacterium* was not observed when 2′-FL and Bi-26 were added together to the formula. Taken together, our results demonstrate that 2′-FL and Bi-26 exert distinct actions on gut bacterial composition and metabolic function and that 2′-FL alone increased specific SCFA concentration demonstrating its prebiotic potential.

Author contributions

The authors' responsibilities were as follows – SMD, RND, JH, HMJ, ACO, MJL, RM: designed the study; VCD, MHM, MW: conducted the research and performed sample analyses; MW, AH: conducted microbiome bioinformatics; MW, NC: performed statistical analyses; MW: prepared the manuscript; and all authors: read and approved the final manuscript.

Conflict of interest

VCD, MHM, and MW declare no conflict of interest. SMD and RND received grant funding from IFF. JH, HMJ, NC, ACO, MJL, and AH are employees of IFF. RM was employed by IFF at the time the study was conducted. SMD has received funding from IFF for presentations at scientific conferences and service on advisory boards.

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Data availability

Data described in the manuscript and analytic code will be made available upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2024.12.022.

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