Maternal and Pediatric Nutrition

Evaluation of Sialyllactose Supplementation of a Prebiotic-Containing Formula on Growth, Intestinal Development, and Bacterial Colonization in the Neonatal Piglet

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Abstract

Background: Sialyllactose (SL) is a highly abundant oligosaccharide in human milk that has been shown to influence intestinal maturation and cognitive development and exert bifidogenic effects on the gut microbiota. The SL content of infant formula is significantly less than that of human milk, therefore there is interest in determining the effect of supplementing SL to infant formula at the levels in human milk on neonatal outcomes.

Objective: The aim of this study was to investigate the effect of varying doses of dietary SL compared with a milk replacer formula on weight gain, gastrointestinal development, and microbiota composition in piglets.

Methods: Thirty-eight intact male piglets were randomly assigned to 1 of 4 experimental diets from 2 to 32–33 d of age. Diets were formulated to contain SL at 0 mg/L (CON), 130 mg/L (LOW), 380 mg/L (MOD), or 760 mg/L (HIGH). At 32–33 d of age, blood was collected for serum chemistry and blood cellular analyses, and coagulation time. Immediately after humane killing, the small intestine was excised and intestinal segments fixed for quantification of mucin-producing goblet cells and morphologic analysis. In addition, mucosal disaccharide activity was assessed. Colonic luminal contents and feces were collected for measurement of pH, dry matter, volatile fatty acids, and the microbiota.

Results: SL at ≤760 mg/L supported normal growth, intestinal development, and enzyme activity as well as serum chemistries and hematology ($P > 0.05$). In addition, SL supplementation did not affect overall microbiota structure and diversity in ascending colon contents and feces, but had minor effects on the relative abundances of specific microbes.

Conclusions: The findings in this study demonstrate that SL addition to a prebiotic-containing formula was well-tolerated by neonatal piglets, supported normal growth, and did not result in any adverse effects on serum chemistries or intestinal development. *Curr Dev Nutr* 2018;0:nzy067.

Keywords: infant formula, oligosaccharide, piglet, sialyllactose

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Supplemental Tables 1–5 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/cdn/.

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Abbreviations used: AC, ascending colon; aPTT, activated partial thromboplastin time; BW, body weight; CON, control; db_RDA, distance-based redundancy analysis; FDR, false discovery rate; GLDH, glutamate dehydrogenase; GOS, galacto-oligosaccharide; HIGH, high-SL concentration; HMO, human milk oligosaccharide; LNnT, lacto-*N*-neotetraose; LOW, low-SL concentration; MO, milk oligosaccharide; MOD, moderate SL concentration; OTU, operational taxonomic unit; PDX, polydextrose; PND, postnatal day; PT, partial prothrombin time; SA, sialic acid; SL, sialyllactose; VFA, volatile fatty acid; WBC, white blood cell; 2′FL, 2′fucosyllactose; 3′SL, 3′sialyllactose; 6′SL, 6′sialyllactose.

Introduction

Despite changes in infant formula composition over the past 2 decades, substantial compositional differences with human milk persist, a notable example being the absence of structurally complex milk oligosaccharides (MOs) due to the low concentrations in the bovine milk starting material (1). MOs are synthesized in the mammary gland as elongation products of lactose with combinations of galactose, *N*-acetylglucosamine, fucose, and/or sialic acid (2). MOs are known to modulate the infant microbiota and to influence immune and neurologic development

(3–5) and their low concentrations in formula may be a factor that contributes to the differences in respiratory tract and gastrointestinal infections and cognitive outcomes between breast- and formula-fed infants (5, 6).

MOs are present in human milk at concentrations ranging from 5 to 15 g/L (7) and are mostly found as neutral or fucosylated, whereas sialic acid (SA)-containing MOs comprise <20% (8). SA in mature human milk is present as oligosaccharides (69–76%), whereas 21–28% is bound to proteins, and ∼3% is present in the free form and bound to gangliosides (5). In stark contrast, SA in cow milk–based infant formula is mostly bound to protein, with 27.8% present as oligosaccharides and <1% is found in its free form (5). The most abundant SA-containing MOs in human milk are 6′sialyllactose (6′SL), 3′sialyllactose (3′SL), disialyllactose-*N*-tetraose, and sialyllacto-*N*-tetraose. A recent worldwide survey of human MO (HMO) concentrations showed that 6′SL and 3′SL were present in all milk samples and concentrations ranged from 0.13 to 0.56 and 0.26 to 0.39 g/L, respectively (9). Others have reported broader variation, with higher concentration of 6′SL in colostrum than in mature milk, but no differences between early and later milk concentrations of 3′SL (10).

Studies in human infants and neonatal piglets have demonstrated that MOs, including SA-bound oligosaccharides, survive digestion and reach the large intestine intact (11, 12). Once in the intestine, MOs shape the composition of microbial communities by stimulating the growth of specific bacteria (13). Of interest is the study reported by Wiese et al. (14), in which 3′SL, 6′SL, 3′-fucosyllactose, and fructo-oligosaccharide fermentability was investigated in an in vitro colon model. 3′SL produced the strongest bifidogenic effect and higher butyrate production. In addition, in vivo studies have also shown the ability of SA-containing oligosaccharides to manipulate the microbiota. Studies in mice indicated that exposure to 3′SL in milk not only affected the microbiota, but also the resistance to dextran sodium sulfate-induced colitis (15). Similarly, neonatal pigs fed 6′SL presented a microbiome profile that differed from those fed a control (CON) diet (16).

Oligosaccharides are also known to modulate gut maturation as demonstrated in in vitro studies (17–19). Holscher et al. (19) used intestinal cell lines to build a model of the crypt-villus axis and were able to show that the presence of HMO [6′SL, 2′fucosyllactose (2′FL), lacto-*N*-neotetraose (LNnT)] reduced cell proliferation and barrier function, and increased gut maturation, but had no impact on alkaline phosphatase, an enzyme used as a marker of crypt-villus differentiation (20). Lactase and sucrase are among the digestive enzymes present at the villus tip and are often used as markers of cell differentiation. Berding et al. (21) demonstrated that the incorporation of galacto-oligosaccharides (GOSs) and polydextrose (PDX) in neonatal piglets increased jejunal lactase activity. Furthermore, GOSs have also been shown to influence goblet cell function by increasing the expression of mucin (*MUC*) genes (22). However, information on the in vivo effects of sialyllactose (SL) on digestive enzymes and goblet cells is lacking.

The most commonly added prebiotics to infant formula are fructo-oligosaccharide, GOS, and PDX. Compared to a standard infant formula, infants fed formula supplemented with GOS and PDX at 4 g/L (1:1 ratio) had stool consistency that more closely resembled that of breastfed infants; the supplemented formula also promoted an increase in *Bifidobacterium infantis* and *Bifidobacterium longum* in feces of healthy infants during a 60-d feeding period, while having no impact on weight gain (23, 24). More recently, neutral and fucosylated MOs have been added to infant formula and have shown health benefits compared with unsupplemented formula (6, 25, 26). To date, no commercial infant formula has been supplemented with SA-containing oligosaccharides, although the effects of SL have been tested in preclinical animal models. In neonatal pigs, adding 2 or 4 g/L of 3′SL or 6′SL to a formula devoid of other prebiotics did not affect weight gain, but increased brain ganglioside–bound SA and changed the gut microbiota profile (16). Pure 3′SL and 6′SL have not been tested in human infant clinical trials; however, a standard formula supplemented with 10 g/L bovine milk-oligosaccharides, a mixture containing 3′SL, 6′SL, and GOS, fed to infants for 4 mo led to microbiota changes (26).

These preliminary findings suggest a potential benefit of supplementing infant formula with SL; however, the bioactivity of SL as part of an infant formula containing other prebiotics was unknown. In our initial findings, prebiotic-enriched formula supplemented with SL in piglets influenced brain development with changes in levels of bound SA in the prefrontal cortex, the ratio of free SA to bound SA in the hippocampus, and axial and radial diffusivity in the corpus callosum (27). In addition to the effects of SL on brain development, our lab set out to investigate the impact of adding SL to a formula containing GOS and PDX on weight gain, serum chemistries, intestinal morphology, disaccharidase activity, and the composition of the microbiota in newborn piglets. The piglet is a commonly used preclinical model for formula ingredient testing owing to anatomic, developmental, and brain growth trajectory similarities to the human infant (28, 29). We hypothesized that dietary SL would be well-tolerated and would modify the microbiota compared with milk replacer without supplemental SL.

Methods

Animal study design

All animal care and experimental procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals (30). Beginning at postnatal day 2 (PND 2), 38 naturally farrowed intact male piglets were randomly assigned to 1 of 4 milk replacer diets until 32 or 33 d of age. The trial was completed in 4 replicates (5–12 piglets/replicate), with 8–11 piglets/diet, selected from 14 litters, to control for genetics and initial body weight (BW). A total of 12 pigs/dietary treatment were initially started on the study; however, several pigs were removed from the study for reasons not associated with the dietary treatments (27). All pigs were housed in custom piglet-rearing units and were maintained on a 12-h light/dark cycle. Researchers were blinded to treatment groups throughout the study and sample analyses.

Dietary treatment

All diets were produced by Mead Johnson Nutrition with the use of a proprietary blend of nutrients formulated to meet the nutritional needs of growing piglets. The bovine milk-based CON and experimental diets are shown in **Supplemental Table 1**. The CON diet contained DHA

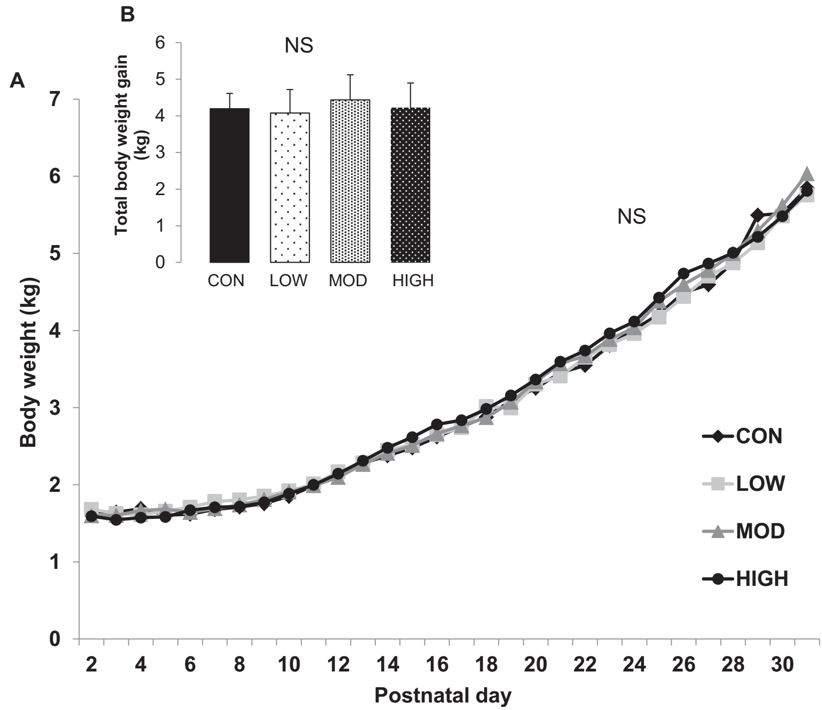

FIGURE 1 Body weights (A) of piglets fed formula containing various concentrations of sialyllactose from postnatal days 2 to 31; total weight gain (kilograms) (B) calculated as the difference in weights on postnatal days 2 and 31. Values are means (A) or means ± SEMs (B). NS, not significant at $P < 0.05$. CON, control; HIGH, high-SL diet; LOW, low-SL diet; MOD, moderate-SL diet.

(87 mg/100 g milk replacer powder; DSM) and arachidonic acid (174 mg/100 g milk replacer powder; DSM). In addition, diets contained GOS (1.0 g/100 g milk replacer powder; FrieslandCampina) and PDX (1.0 g/100 g milk replacer powder; Danisco). Experimental diets were formulated with the CON diet as the base and supplemented with bovine-derived modified whey enriched with SL (Lacprodan SAL-10; Arla Foods Ingredients Group P/S) to provide final SL concentrations of: low SL (65 mg SL/100 g milk replacer powder; LOW), moderate SL (190 mg SL/100 g milk replacer powder; MOD), and high SL (380 mg SL/100 g milk replacer powder; HIGH). The milk replacer powder was reconstituted fresh daily at 200 g of dry powder/800 g of water, and piglets were fed at 285 and 325 mL of reconstituted diet/kg of BW starting on 3 and 8 d of age, respectively. At this reconstitution rate, all diets contained arachidonic acid (348 mg/L), DHA (174 mg/L), and PDX/GOS (2 g/L each). The reconstituted experimental milk replacers were formulated to contain 130 mg SL/L (LOW), 380 mg SL/L (MOD), or 760 mg SL/L (HIGH). Analytic assessment conducted after study completion showed the levels of SL in the diets were: CON (55 mg SL/L), LOW (159 mg SL/L), MOD (429 mg SL/L), and HIGH (779 mg SL/L), owing to inherent SL.

Sample collection

At the end of the study, piglets were deprived of food for 8 h before being killed. Piglets were sedated with an intramuscular injection of Telazol [tiletamine hydrogen chloride (HCl) and zolazepam HCl, 3.5 mg/kg BW each; Zoetis] and blood samples were collected by cardiac puncture into heparin-laced and 3.2% sodium citrate–containing vacutainer tubes (BD Biosciences) for coagulation time and cell blood count with differential, respectively. In addition, blood samples were also collected in heparin-free tubes for the isolation of serum for chemistry analysis. Piglets were then killed by an intracardiac injection of sodium pentobarbital (72 mg/kg BW Fatal Plus; Vortech Pharmaceuticals). The small intestine was excised between the pyloric sphincter and ileocecal valve for measurement of total small intestinal weight and length. Thereafter, the small intestine was cut at 10% and 85% from the

TABLE 1 BW, and small intestinal length and weight presented as absolute value and normalized by BW, of piglets fed formula containing various concentrations of sialyllactose[1]

		Intestinal length		Intestinal weight	
Diet and SL concentration	Body weight, kg	cm	cm/kg BW	g	g/kg BW
CON, 0 mg/L	5.93 ± 0.44	858 ± 40.1	148 ± 6.9	248 ± 19.2	42 ± 1.8
LOW, 130 mg/L	5.82 ± 0.68	826 ± 41.9	145 ± 9.8	238 ± 26.4	41 ± 1.9
MOD, 380 mg/L	6.14 ± 0.68	861 ± 34.3	149 ± 9.7	240 ± 22.9	40 ± 1.9
HIGH, 760 mg/L	5.81 ± 0.72	823 ± 35.1	155 ± 16.1	210 ± 19.5	37 ± 1.5

[1]Values are means ± SEMs. No significant differences were detected at $P < 0.05$ (ANOVA). BW, body weight; CON, control; HIGH, high SL concentration; LOW, low SL concentration; MOD, moderate SL concentration.

TABLE 2 Complete blood count and differential analysis measured in the serum of piglets fed formula containing various concentrations of sialyllactose[1]

Cell type	Unit	Reference ranges[2]	Concentration of sialyllactose in formula			
			CON (0 mg/L)	LOW (130 mg/L)	MOD (380 mg/L)	HIGH (760 mg/L)
RBCs	$\times 10^6/\mu L$	4.1–8.2	7.2 ± 0.32	7.4 ± 0.24	7.0 ± 0.23	7.7 ± 0.16
Hemoglobin	g/dL	4.3–13.3	13.2 ± 0.45	13.7 ± 0.43	13.1 ± 0.44	14.3 ± 0.30
Hematocrit	%	16–41	41.5 ± 1.5	43.0 ± 1.4	41.1 ± 1.3	45.0 ± 0.86
MCV	Fl	34.2–61.3	58.3 ± 0.90	57.8 ± 0.83	59.0 ± 0.52	58.5 ± 0.56
MCH	Pg	9.4–19.8	18.5 ± 0.29	18.4 ± 0.22	18.8 ± 0.14	18.6 ± 0.14
MCHC	g/dL	26.5–33.6	31.8 ± 0.19	31.8 ± 0.16	31.8 ± 0.22	31.7 ± 0.26
Platelet	$\times 10^3/\mu L$	192–832	523 ± 57.7	606 ± 56.6	601 ± 36.3	572 ± 33.9
MPV	Fl	6.5–12.7	10.3 ± 0.54	9.2 ± 0.35	8.7 ± 0.28	8.9 ± 0.37
WBCs	$\times 10^3/\mu L$	5.6–18.5	8.6 ± 0.35	9.8 ± 0.66	8.6 ± 0.82	9.5 ± 0.43
Neutrophils	%	10.8–70.6	30.3 ± 5.0[ab]	21.2 ± 6.8[b]	41.9 ± 5.1[a]	21.8 ± 5.3[b]
Lymphocytes	%	26.2–82.9	61.5 ± 4.1[b]	62.2 ± 4.5[b]	47.8 ± 2.4[a]	64.5 ± 2.5[b]
Band	%	0–4	0	0.3 ± 0.24	0	0
Monocytes	%	1.4–8.3	4.3 ± 0.68	5.2 ± 0.68	5.0 ± 0.57	5.8 ± 1.1
Eosinophils	%	0–1.9	1.16 ± 0.27	0.79 ± 0.31	0.7 ± 0.26	0.7 ± 0.31
Basophils	%	0–0.9	0.2 ± 0.14	0.81 ± 0.23	0.4 ± 0.20	0.3 ± 0.17
Neutrophil count	$\times 10^3/\mu L$	0.8–9.7	2.5 ± 0.38	2.2 ± 0.69	3.5 ± 0.53	2.2 ± 0.53
Lymphocyte count	$\times 10^3/\mu L$	2.7–12.8	5.4 ± 0.46[ab]	6.1 ± 0.61[b]	4.1 ± 0.47[a]	6.1 ± 0.33[b]
Monocyte count	$\times 10^3/\mu L$	0.1–1.1	0.36 ± 0.06	0.5 ± 0.06	0.4 ± 0.08	0.6 ± 0.1
Eosinophil count	$\times 10^3/\mu L$	0–0.2	0.1 ± 0.02	0.09 ± 0.04	0.04 ± 0.02	0.07 ± 0.03
Basophil count	$\times 10^3/\mu L$	0–0.13	0.02 ± 0.01	0.08 ± 0.02	0.13 ± 0.1	0.02 ± 0.02

[1]Values are means ± SEMs. Different letter superscripts indicate significant differences at $P < 0.05$ (ANOVA). CON, control; HIGH, high SL concentration; LOW, low SL concentration; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MOD, moderate SL concentration; MPV, mean platelet volume; WBC, white blood cell.
[2]From Ventrella et al. (45).

proximal end resulting in 3 segments: duodenum, jejunum, and ileum, respectively (31). Intestinal segments were flushed with PBS and 1- to 2-cm pieces were fixed in Bouin's solution for 24 h at room temperature for goblet cell analyses and intestinal morphology. In addition, a section of each segment was cut longitudinally, and mucosal scrapings were obtained and frozen in liquid nitrogen. Ascending and descending colon samples were obtained immediately following (distal to) the cecum or proximal to the rectum, respectively. Ascending content and feces were sampled for volatile fatty acids (VFA) analyses, 16S rRNA sequencing, and qPCR for quantification of bacteria species.

Hematologic analyses

Serum chemistries and coagulation time [partial prothrombin time (PT) and activated partial thromboplastin time (aPTT)] were determined with the use of an Olympus AU680 chemistry analyzer (Beckman Coulter) and an STA-Compact coagulation analyzer (Diagnostica STAGO), respectively, at the Clinical Pathology lab within the University of Illinois College of Veterinary Medicine. Serum was obtained after blood vials were centrifuged at 2200 × g for 20 min at 4°C in a benchtop centrifuge (CS-6R Centrifuge, Beckman Coulter Life Sciences). The present serum chemistry panel included minerals (calcium, phosphorus, magnesium), electrolytes (sodium, potassium, chloride), protein (total protein, albumin, and globulin), metabolites (glucose, total cholesterol, TGs, creatinine, urea, total bilirubin, and bicarbonate), and the enzymes alkaline phosphatase, aspartate transaminase, γ-glutamyltransferase, creatine phosphokinase, and glutamate dehydrogenase (GLDH). In addition, complete blood count was performed on CELL-DYN 3700 (Abbott), and trained technicians at the College of Veterinary Medicine performed differential analysis. The variables evaluated in our study were RBC count, hemoglobin concentration, hematocrit value, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular and hemoglobin concentration. Total white blood cell (WBC) count and differential WBC analyses (neutrophils and lymphocytes) were also performed. Platelet indexes were analyzed and included platelet count and mean platelet volume.

Intestinal analyses

Histomorphologic analyses. Bouin's-fixed sections of duodenum, jejunum, ileum, and ascending colon were processed into paraffin blocks, sectioned into 5-μm slices, and placed on glass slides. For the histomorphologic analyses, digital images (10× magnification) of hematoxylin-eosin–stained tissues were captured with the use of the NanoZoomer Slider Scanner (Hamamatsu Corporation; Institute for Genomic Biology Microscopy and Imaging Facility, University of Illinois). Small intestinal villus height (micrometers), villus area (millimeters squared), crypt depth (micrometers), crypt area (millimeters squared), and colon cuff depth (micrometers) and cuff area (millimeters squared) were measured from 10–15 well-oriented crypts-villi with the use of AxioVision 4.8 Digital Image Processing Software (Carl Zeiss MicroImaging, Inc.).

Goblet cell quantification. Slides with ileum and ascending colon sections were stained with High Iron Diamine/Alcian Blue to detect sulfated and carboxylated mucin-producing cells as described by Croix et al. (32). Digital images were obtained as described earlier. Goblet cell numbers were determined by enumerating the number of stained cells within an area delimited by a colon cuff, ileal villus, and ileal crypt.

TABLE 3 Partial PT and aPTT measured in the serum of piglets fed formula containing various concentrations of sialyllactose[1]

		Concentration of sialyllactose in formula			
Variable	Reference ranges[2]	CON (0 mg/L)	LOW (130 mg/L)	MOD (380 mg/L)	HIGH (760 mg/L)
PT, s	9.3–13.3	13.7 ± 0.2	13.8 ± 0.2	13.9 ± 0.1	13.4 ± 0.4
aPTT, s	12.3–17.8	14.1 ± 0.2[a]	13.8 ± 0.4[a]	13.6 ± 0.2[a]	12.6 ± 0.5[b]

[1]Values are means ± SEMs. Different letter superscripts indicate significant differences at $P < 0.05$ (ANOVA). aPTT, activated partial thromboplastin time; CON, control; HIGH, high SL concentration; LOW, low SL concentration; MOD, moderate SL concentration; PT, prothrombin time.
[2]From Rispat et al. (46).

A total of 8 ascending colon cuffs, ileum villi, and crypts were used for the quantification. Data are presented as total number of cells or cell number normalized by crypt, cuff, and villus area (micrometers squared).

Disaccharidase activity. Intestinal lactase and sucrase activities were measured as previously described (33, 34). Briefly, mucosal homogenates were prepared and incubated with 0.6 M lactose (Thermo Fisher Scientific) or 0.3 M sucrose (Thermo Fisher Scientific) buffer (dissolved in 0.0625 M maleate buffer) for 60 min at 37°C. The reaction was stopped by the addition of 2.0% zinc sulfate (Thermo Fisher Scientific) and 1.8% barium hydroxide (Thermo Fisher Scientific), and the amount of glucose released was detected via a glucose oxidase reagent (Thermo Scientific). Enzyme activity was in μmoles glucose per minute per gram of protein. Protein was measured in intestinal homogenates via the Bradford method (Bio-Rad).

VFA concentrations. SCFA (acetate, butyrate, propionate) and branched-chain fatty acid (isobutyrate, valerate, and isovalerate) concentrations were determined in ascending colon (AC) contents and feces by GC as described previously (35), with data expressed as μmol/g of dry matter.

Microbiota analysis.

DNA extraction. DNA was extracted from AC contents and feces by the QIAamp DNA Stool Mini Kit (Qiagen) in combination with the FastPrep-24 System (MP Biomedicals) as previously described (35). DNA concentration was determined with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies).

Real-time qPCR. Bacterial DNA was analyzed for total bacteria, Enterobacteriaceae, *Bifidobacterium* spp., *Lactobacillus* spp., *Prevotella* spp., *Roseburia* spp./*Eubacterium rectale*, *Clostridium perfringens*, *Clostridium difficile*, *Faecalibacterium prausnitzii*, and *Bacteroides fragilis* with the use of primer/probe sequences indicated in **Supplemental Table 2**. Real-time qPCR was performed in the 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific) and used TaqMan (for *B. fragilis*) or SYBR Green (for other bacterial groups/species) assays (21). After amplification, a dissociation step was included to analyze the melting profile of the amplified products. Standard curves (4–10^8 16S rRNA gene copies per reaction) were generated with the use of purified pCR 4 TOPO-TA plasmids (Thermo Fisher Scientific) containing the 16S rRNA genes of *Eubacterium hallii* 27751 (for total bacteria), *Escherichia coli* 11775 (Enterobacteriaceae), *B. longum* 15707 (*Bifidobacterium* spp.), *Lactobacillus rhamnosus* 53103 (*Lactobacillus* spp.), *Prevotella ruminicola* 19189 (*Prevotella* spp.), *Eubacterium rectale* 33656 (*Roseburia* spp./*E. rectale*), *B. fragilis* 25285 (*B. fragilis*), *C. perfringens* 13124 (*C. perfringens*), *C. difficile* 9689 (*C. difficile*), and *F. prausnitzii* 27766 (*F. prausnitzii*). Data analysis was processed with SDS version 2.3 software supplied by Thermo Fisher Scientific and data are presented as number of 16S rRNA gene copies per gram of AC contents or feces.

PCR amplification and sequencing of 16S rRNA genes. PCR amplification and sequencing of 16S rRNA genes were performed at the High-Throughput Sequencing and Genotyping Unit, University of Illinois. The V3-V4 regions of bacterial 16S rRNA genes were amplified through the use of primers V3f (5′-CCTACGGGAGGCAGCAG-3′) and V4r (5′-GGACTACHVGGGTWTCTAAT-3′) (36) with appropriate linkers and sample barcodes. PCR was performed on a Fluidigm Biomark HD platform (Fluidigm Corporation) as previously described (37) with the temperature profiles: 50°C for 2 min (1 cycle), 70°C for 20 min (1 cycle), 9°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. Amplicons from 48 separate reactions were pooled for each sample and DNA concentration was measured on a Qubit 3.0 Fluorometer (Thermo Fisher Scientific). The size of amplicons was confirmed by running the samples on a Fragment Analyzer (Advanced Analytical Technologies). The amplicons were then mixed in equimolar concentrations and run on a 2% agarose E-gel (Thermo Fisher Scientific). The band of expected size (*c*.460 bp) was excised from the gel and the DNA was extracted with a Qiagen Gel Extraction kit (Qiagen). The extracted DNA was run on an Agilent 2100 Bioanalyzer (Agilent Technologies) to confirm an appropriate profile and determine the average size. Sequencing was performed on an Illumina MiSeq flow cell for 251 cycles from each end of the fragments with a MiSeq Reagent Nano Kit version 2 (2 × 250 nt paired end reads; Illumina) (38).

Sequence processing. Demultiplexed forward (read 1) and reverse reads (read 2) obtained from the sequencing facility were processed with the use of the QIIME software package (39). The paired-end reads were merged with the use of SeqPrep with a minimum overlap of 30 bp. Merged reads were filtered and split into libraries at Phred quality score ≥25. Sequences were assigned to operational taxonomic units (OTUs) via UCLUST with a 97% pairwise identity threshold. The representative sequences from each OTU were picked and the chimera sequences were identified via Chimera Slayer. After removal of chimeras, the remaining sequences were aligned to the greengenes reference alignment (gg_13_8_otus/rep_set_aligned/85_otus.pynast.fasta) with the use of PyNAST (40). The alignment was then filtered to remove highly variable regions and columns comprised of only gaps with the use of a lane mask. The phylogenetic tree was constructed from filtered alignment via FastTree (41). The representative sequence of each OTU was assigned to different taxonomic levels with the use of the Ribosomal Database

TABLE 4 Serum minerals, electrolytes, metabolites, and proteins measured in the serum of piglets fed formula containing various concentrations of sialyllactose[1]

Variable	Units	Reference ranges[2]	Concentration of sialyllactose in formula			
			CON (0 mg/L)	LOW (130 mg/L)	MOD (380 mg/L)	HIGH (760 mg/L)
Minerals						
Calcium	mg/dL	7.1–11.6	9.6 ± 0.2	9.8 ± 0.3	10.0 ± 0.2	9.9 ± 0.3
Phosphorous	mg/dL	5.3–9.6	10.7 ± 0.3	11.2 ± 0.9	10.7 ± 0.5	11.2 ± 0.7
Magnesium	mg/dL	2.7–3.7	2.3 ± 0.1	2.5 ± 0.2	2.5 ± 0.1	2.6 ± 0.2
Electrolytes						
Sodium	mmol/L	135–150	136 ± 0.7	135 ± 0.8	136 ± 0.6	136 ± 0.6
Potassium	mmol/L	4.4–6.7	6.3 ± 0.6	6.3 ± 1.9	6.0 ± 0.5	5.6 ± 0.4
Sodium:potassium			23 ± 1.7	23 ± 5.8	24 ± 1.7	25 ± 1.6
Chloride	mmol/L	94–106	102 ± 1.0	102 ± 1.1	102 ± 0.9	107 ± 1.2
Metabolites						
Glucose	mg/dL	85–150	137 ± 14.8	131 ± 15.6	141 ± 14.5	177 ± 31.5
Cholesterol total	mg/dL	78–116	77 ± 5.9	78 ± 5.6	80 ± 4.1	88 ± 4.4
TGs	mg/dL	29–80	51 ± 7.4	56 ± 6.1	54 ± 7.6	52 ± 6.7
Protein						
Total protein	g/dL	3.7–4.8	3.8 ± 0.08	3.9 ± 0.07	3.9 ± 0.1	4.0 ± 0.1
Albumin	g/dL	1.9–3.9	2.8 ± 0.07	2.7 ± 0.05	2.8 ± 0.09	2.7 ± 0.08
Globulin	g/dL	1.9–3.9	1.0 ± 0.06	1.2 ± 0.06	1.2 ± 0.07	1.3 ± 0.09
Albumin:globulin			2.8 ± 0.2	2.3 ± 0.1	2.5 ± 0.2	2.2 ± 0.2

[1]Values are means ± SEMs. No significant differences were detected at $P < 0.05$ (ANOVA). CON, control; HIGH, high SL concentration; LOW, low SL concentration; MOD, moderate SL concentration.
[2]From Ventrella et al. (45) and Merck Veterinary Manual (47).

Project naïve Bayesian rRNA Classifier at 80% confidence level (42) and an OTU table was created. The OTU table was further filtered to remove nonaligned and chimeric OTUs and singletons. α-Diversity (observed OTUs, Chao1, Shannon, and Simpson reciprocal indexes) and β-diversity analysis was performed from the filtered OTU table after rarefying to an equal number of reads.

Microbiome statistical analyses. Differences in bacterial communities among diet groups were evaluated with principal coordinate analysis and distance-based redundancy analysis (db_RDA) through the use of UniFrac distance matrices. Principal coordinate analysis and db_RDA were performed on both unweighted and weighted UniFrac distances with the use of QIIME and the function capscale of the vegan package of R (43, 44), respectively. Univariate statistical analysis (qPCR, α-diversity, relative abundances of bacterial taxa) was performed via the PROC MIXED procedure of SAS version 9.4 with diet as a main effect and replicate as a random effect (SAS Institute Inc.). Tukey's post hoc tests were applied for multiple comparisons. When the relative abundance data were not normally distributed, arcsine-square root transformation was applied. Real-time qPCR data were

TABLE 5 Enzymes, indicators of renal and liver function, and acid:base balance measured in the serum of piglets fed formula containing various concentrations of sialyllactose[1]

Variable	Units	Ref ranges[2]	Concentration of sialyllactose in formula			
			CON (0 mg/L)	LOW (130 mg/L)	MOD (380 mg/L)	HIGH (760 mg/L)
Enzymes						
ALP	U/L	110–1292	396 ± 28.2	380 ± 55.8	351 ± 21.1	332 ± 23.9
AST	U/L	13–65	43 ± 6.7	40 ± 7.2	43 ± 3.6	33 ± 5.0
GGT	U/L	10–60	36 ± 5.8	30 ± 4.1	29 ± 1.6	34 ± 4.8
CPK	U/L	153–5427	1474 ± 407	802 ± 212	1194 ± 332	699 ± 126
GLDH	U/L		0.9 ± 0.1[b]	1.3 ± 0.1[a]	1.5 ± 0.2[a]	1.2 ± 0.08[ab]
Kidney function						
Creatinine	mg/dL	0.51–1.39	0.8 ± 0.06	0.7 ± 0.03	0.7 ± 0.03	0.8 ± 0.06
Blood urea nitrogen	mg/dL	4–39	9.6 ± 1.3	9.7 ± 1.0	8.7 ± 0.8	8.6 ± 0.7
Liver function						
Total bilirubin	mg/dL	0–10	0.5 ± 0.08	0.3 ± 0.04	0.5 ± 0.1	0.3 ± 0.03
Acid:base status						
Bicarbonate	mmol/L		26 ± 1.1	27 ± 1.1	27 ± 1.1	26 ± 0.7
Anion gap[3]			13.8 ± 0.8	12.9 ± 1.0	12.9 ± 0.5	13.6 ± 0.8

[1]Values are means ± SEMs. Different superscript letters indicate significant differences at $P < 0.05$ (ANOVA). ALP, alkaline phosphatase; AST, aspartate transaminase; CON, control; CPK, creatine phospholinase; GGT, γ-glutamyltransferase; GLDH, glutamate dehydrogenase; HIGH, high SL concentration; LOW, low SL concentration; MOD, moderate SL concentration.
[2]From Ventrella et al. (45); Merck Veterinary Manual (47); Cooper et al. (48).
[3](Sodium + potassium) – (chloride + CO_2) = anion gap.

TABLE 6 Lactase and sucrase activity in the small intestine of piglets fed formula containing various concentrations of sialyllactose[1]

Disaccharidase	Concentration of sialyllactose in formula CON (0 mg/L)	LOW (130 mg/L)	MOD (380 mg/L)	HIGH (760 mg/L)
Lactase, μmol · min^{-1} · g protein^{-1}				
Duodenum	107 ± 16	115 ± 29	125 ± 27	115 ± 18
Jejunum	444 ± 88	291 ± 55	505 ± 102	568 ± 140
Ileum	25 ± 5.8	27 ± 6.3	32 ± 9.4	21 ± 5.2
Sucrase, μmol · min^{-1} · g protein^{-1}				
Duodenum	4.55 ± 0.74	5.15 ± 3.4	8.44 ± 2.0	9.25 ± 2.4
Jejunum	117 ± 20	91 ± 10	113 ± 16	109 ± 8.3
Ileum	21 ± 3.7	15 ± 2.0	14 ± 1.9	19 ± 3.1

[1]Values are means ± SEMs. No significant differences were detected at $P < 0.05$ (ANOVA). CON, control; HIGH, high SL concentration; LOW, low SL concentration; MOD, moderate SL concentration.

log10 transformed to achieve normal distribution. False discovery rate (FDR) was computed with the use of PROC MULTTEST of SAS. The original P value was considered significant when the calculated FDR was ≤0.2.

Statistical analyses

All data, with the exception of the microbiota data, were subjected to an ANOVA via the MIXED procedure of SAS version 9.4. Most outcomes were analyzed by a simple ANOVA, except for daily piglet BWs, which were analyzed with repeated-measures ANOVA. All statistical models included replicate as a random effect and the level of significance was set at $P < 0.05$, with trends accepted at $0.05 < P < 0.10$. Data are reported as means ± SEMs.

TABLE 7 SCFA and branched-chain fatty acid concentrations (μmole per gram of dry matter) in ascending colon contents and feces of piglets fed formula containing various concentrations of sialyllactose[1]

	Concentration of sialyllactose in formula CON (0 mg/L)	LOW (130 mg/L)	MOD (380 mg/L)	HIGH (760 mg/L)
Ascending colon				
Acetate	200 ± 53	201 ± 53	170 ± 39	151 ± 37
Propionate	64 ± 19	58 ± 17	49 ± 11	42 ± 12
Butyrate	20 ± 4.7	20 ± 5.9	16 ± 4.1	17 ± 5.1
Isobutyrate	3.6 ± 1.0	2.9 ± 0.5	2.3 ± 0.4	2.2 ± 0.3
Isovalerate	4.7 ± 1.5	3.7 ± 0.8	2.7 ± 0.4	3.0 ± 0.5
Valerate	3.8 ± 0.9	3.9 ± 0.9	2.9 ± 0.6	3.0 ± 0.8
Feces				
Acetate	55 ± 5.6	67 ± 10	53 ± 8.2	69 ± 17
Propionate	17 ± 2.3	24 ± 4.2	18 ± 3.7	22 ± 6.1
Butyrate	6.8 ± 1.0	8.4 ± 1.6	5.3 ± 0.7	8.2 ± 2.5
Isobutyrate	1.6 ± 0.3	2.2 ± 0.5	1.6 ± 0.3	2.0 ± 0.5
Isovalerate	2.2 ± 0.4	2.8 ± 0.6	2.3 ± 0.4	2.5 ± 0.6
Valerate	1.6 ± 0.3	1.8 ± 0.4	1.4 ± 0.3	1.7 ± 0.5

[1]Values are means ± SEMs. No significant differences were detected at $P < 0.05$ (ANOVA). CON, control; HIGH, high SL concentration; LOW, low SL concentration; MOD, moderate SL concentration.

Results

BW and intestinal length and weight

Average daily and total weight gain, calculated as the difference in weight between PND 32 and PND 1, were similar across all dietary treatments (**Figure 1**A, B). The average formula volume offered to the piglets in periods of 5–8 d did not differ among the treatment diets (**Supplemental Table 3**), and, as expected, increased significantly as BW increased. Dietary SL supplementation did not significantly affect intestinal length or weight (**Table 1**).

Hematologic analyses

Complete blood count and cell differential results are presented in **Table 2**. All values were within the reference ranges (45–48) and measures of RBCs and platelets did not differ among the dietary treatments. Differences were observed in the distribution of WBC types. The total number of lymphocytes ($\times 10^3/\mu$L) was lower in MOD than in LOW and HIGH ($P < 0.05$), but did not differ significantly from CON. Piglets in MOD had a higher percentage of neutrophils than did piglets in CON, LOW, and HIGH ($P < 0.05$). Conversely, the percentage of WBCs as lymphocytes was significantly lower in MOD than in the other 3 treatment groups.

PT values did not differ among the treatment groups (**Table 3**). aPTT values of piglets in the HIGH group were 8–10% lower ($P < 0.05$) than in CON, LOW, and MOD. Serum minerals, electrolytes, proteins, metabolites, enzymes, biomarkers of liver and kidney function, and acid:base status were similar among the treatment groups (**Tables 4** and **5**), with the exception of glutamate dehydrogenase (GLDH, Table 5). Compared with piglets in the MOD group, GLDH values were 40% and 70% higher ($P < 0.05$) in CON and LOW piglets, respectively, whereas GLDH values in the HIGH group were not significantly different from the other dietary groups.

Assessment of intestinal development

Intestinal morphology and the number of goblet cells staining positive for sulfated-mucin did not differ among the dietary groups (**Supplemental Tables 4** and **5**). The pattern of enzyme activity in the small intestine was similar to previously published findings (49), with lactase activity being highest in the jejunum and lowest in the ileum and sucrase activity being highest in the jejunum and lowest in the duodenum. However, disaccharidase activity did not differ among the dietary treatment groups (**Table 6**).

VFAs and analysis of intestinal contents. The concentrations of VFAs in AC contents and fecal samples did not differ between the CON and SL-supplemented groups (**Table 7**). Similarly, pH and dry matter of the AC contents and feces were not significantly affected by dietary SL supplementation (**Figure 2**).

Real-time qPCR. The densities of total bacteria, Enterobacteriaceae, *Bifidobacterium* spp., *Lactobacillus* spp., *Prevotella* spp., *Roseburia* spp., *E. rectale*, *C. difficile*, *F. prausnitzii*, and *B. fragilis*, as determined by qPCR, were similar among the treatment groups in both AC contents and feces (**Table 8**). In the AC contents, *C. perfringens* density in MOD was significantly higher than in CON and HIGH, with no differences among CON, LOW, and HIGH. However, differences among the groups

TABLE 8 Bacterial densities (Log_{10} 16S rRNA gene copies per gram) in the ascending colon contents and feces of piglets fed formula containing various concentrations of sialyllactose[1]

Bacteria	Concentration of sialyllactose in formula: CON (0 mg/L)	LOW (130 mg/L)	MOD (380 mg/L)	HIGH (780 mg/L)
Ascending colon				
Total bacteria	12.3 ± 0.15	12.4 ± 0.09	12.2 ± 0.06	12.3 ± 0.14
Enterobacteriaceae	10.4 ± 0.22	10.8 ± 0.14	10.5 ± 0.11	10.4 ± 0.19
Bifidobacterium spp.	6.70 ± 0.42	6.66 ± 0.43	6.87 ± 0.45	6.02 ± 0.09
Lactobacillus spp.	10.6 ± 0.17	10.7 ± 0.22	10.6 ± 0.15	10.6 ± 0.25
Prevotella spp.	11.6 ± 0.25	11.7 ± 0.12	11.7 ± 0.10	11.7 ± 0.19
Roseburia spp./*E. rectale*	6.88 ± 0.32	7.08 ± 0.44	6.42 ± 0.45	6.65 ± 0.18
B. fragilis	9.02 ± 0.55	9.46 ± 0.23	9.63 ± 0.25	9.58 ± 0.33
C. perfringens	6.55 ± 0.43[ab]	6.14 ± 0.46[a]	7.33 ± 0.54[b]	6.18 ± 0.39[ab]
C. difficile	5.23 ± 0.22	5.14 ± 0.26	4.79 ± 0.18	5.23 ± 0.28
F. prausnitzii	9.30 ± 0.16	9.15 ± 0.29	9.00 ± 0.10	9.11 ± 0.15
Feces				
Total bacteria	12.0 ± 0.16	12.1 ± 0.14	11.9 ± 0.19	11.9 ± 0.26
Enterobacteriaceae	10.2 ± 0.24	10.4 ± 0.17	10.0 ± 0.25	9.85 ± 0.43
Bifidobacterium spp.	6.48 ± 0.37	6.45 ± 0.39	6.58 ± 0.51	5.93 ± 0.23
Lactobacillus spp.	10.3 ± 0.24	10.5 ± 0.17	10.1 ± 0.33	9.96 ± 0.29
Prevotella spp.	11.2 ± 0.23	11.4 ± 0.19	10.9 ± 0.28	11.1 ± 0.34
Roseburia spp./*E. rectale*	7.18 ± 0.30	7.16 ± 0.52	6.72 ± 0.27	6.61 ± 0.23
B. fragilis	8.67 ± 0.56	9.14 ± 0.30	8.97 ± 0.47	8.96 ± 0.43
C. perfringens	6.43 ± 0.33[b]	6.49 ± 0.45[ab]	7.54 ± 0.58[a]	5.83 ± 0.21[b]
C. difficile	5.26 ± 0.18	4.90 ± 0.19	4.78 ± 0.15	4.97 ± 0.22
F. prausnitzii	9.21 ± 0.15	9.05 ± 0.20	8.96 ± 0.19	8.88 ± 0.20

[1]Values are means ± SEMs. Labeled means in a row without a common superscript letter differ at $P \leq 0.05$ (ANOVA).

disappeared after correction for false discovery (FDR = 0.286). In feces, *C. perfringens* density was greatest in MOD, intermediate in CON and HIGH, and lowest in LOW, and differences were not affected by FDR correction.

16S rRNA gene sequencing

Effect of diet on the microbiota structure and relative abundances. After the assembling of paired-end reads and performing the quality control depletions as described, 7,221,345 sequences with a mean of 91,409 sequences/sample were used for further analysis. db_RDA of both unweighted and weighted UniFrac distance matrices revealed that the overall bacterial communities were similar among treatment groups in both AC contents and feces ($P > 0.05$; Figure 3). Similar results were also obtained by permutational multivariate ANOVA (data not shown) with the use of the vegan function Adonis of R. Statistical analysis of the relative abundances of bacterial phyla and genera showed that

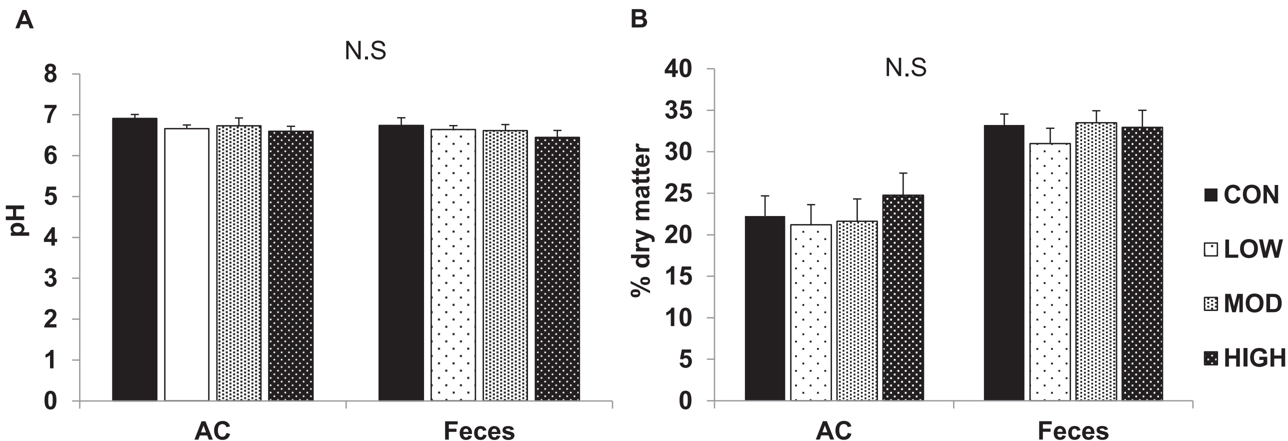

FIGURE 2 pH and dry matter (percentage dry matter) of AC contents and feces of piglets fed formula containing various concentrations of sialyllactose. Values are means ± SEMs. pH was measured immediately after removal of contents from the large intestine. Dry matter was calculated as the weight difference before and after dehydration at 80°C for 16 h. NS, not significant at $P < 0.05$. AC, ascending colon; CON, control; HIGH, high SL concentration; LOW, low SL concentration; MOD, moderate SL concentration.

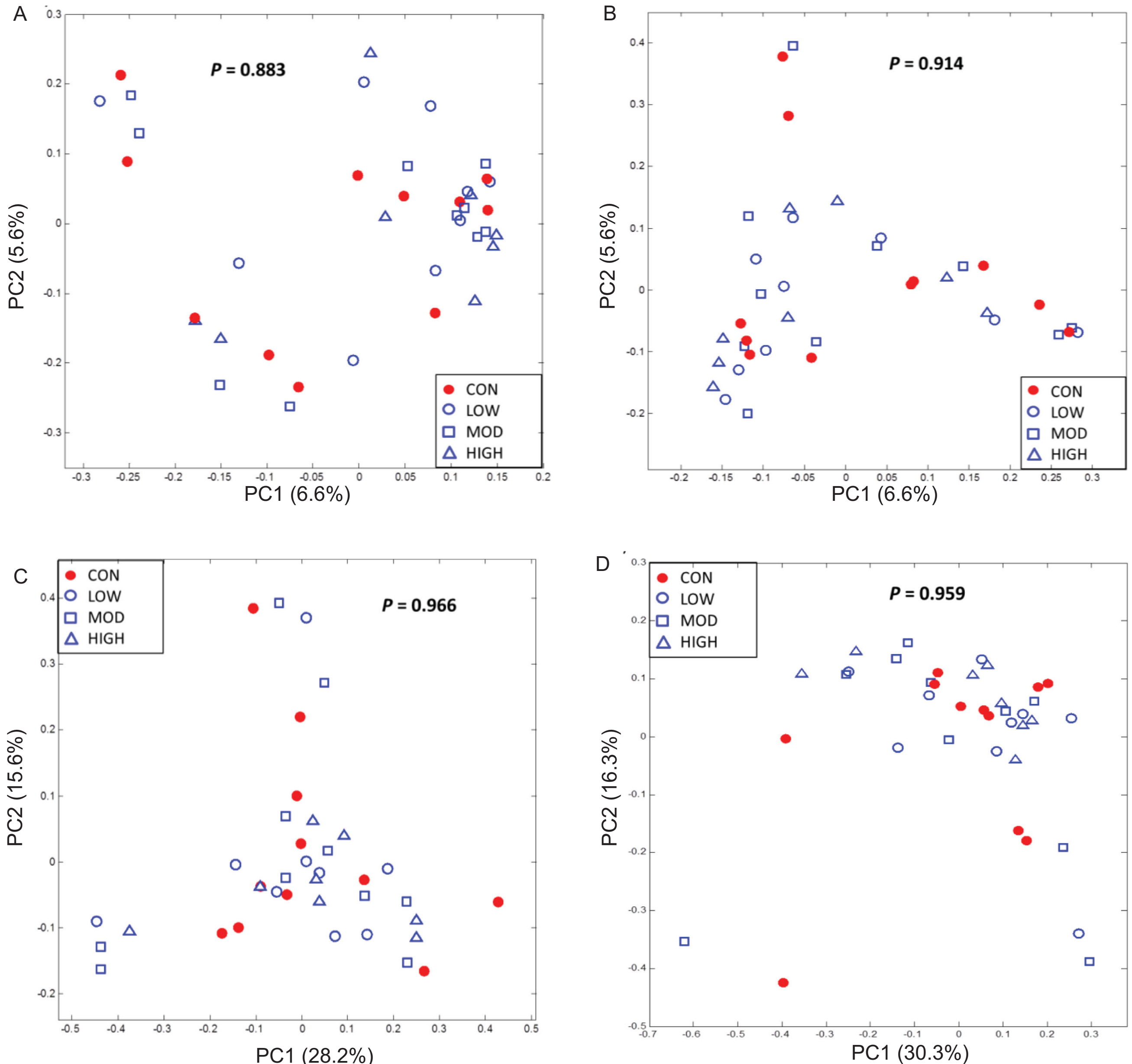

FIGURE 3 Principal coordinate analysis based on unweighted and weighted UniFrac distances generated from ascending colon contents (A and B) and feces (C and D) of piglets fed formula containing various concentrations of sialyllactose. *P* values were obtained by distance-based redundancy analysis. The percentages (in parenthesis) on each axis express the contributions of variables in accounting for the variability in a given PC. CON, control; HIGH, high SL concentration; LOW, low SL concentration; MOD, moderate SL concentration; PC, principal component.

several bacterial taxa were modulated by the presence of SL in the diet. Proportions of Proteobacteria were highest in the AC contents of piglets fed LOW diet, intermediate in CON and HIGH, and lowest in MOD (**Figure 4**; $P < 0.05$). At the genus level, relative abundances of *Alistipes* in AC contents were highest in piglets fed HIGH diet, lowest in CON, and intermediate in LOW and MOD, whereas *Bilophila* were highest in LOW and lowest in CON, with diets MOD and HIGH being intermediate (**Table 9**). In feces, *Butyricicoccus* was highest in diet HIGH, intermediate in diets LOW and MOD, and lowest in diet CON (**Table 10**). After correction for FDR, no differences in relative abundances of bacterial phyla or genera among the diets persisted (FDR > 0.20).

Effect of diet on the α-diversity. α-Diversity was calculated with the use of QIIME after removing OTUs containing only 1 sequence and rarefying to an equal number of reads (34,500) for all samples. No significant differences were detected on observed OTUs, Chao1, Shannon, and Simpson reciprocal indexes among dietary treatment groups (**Table 11**).

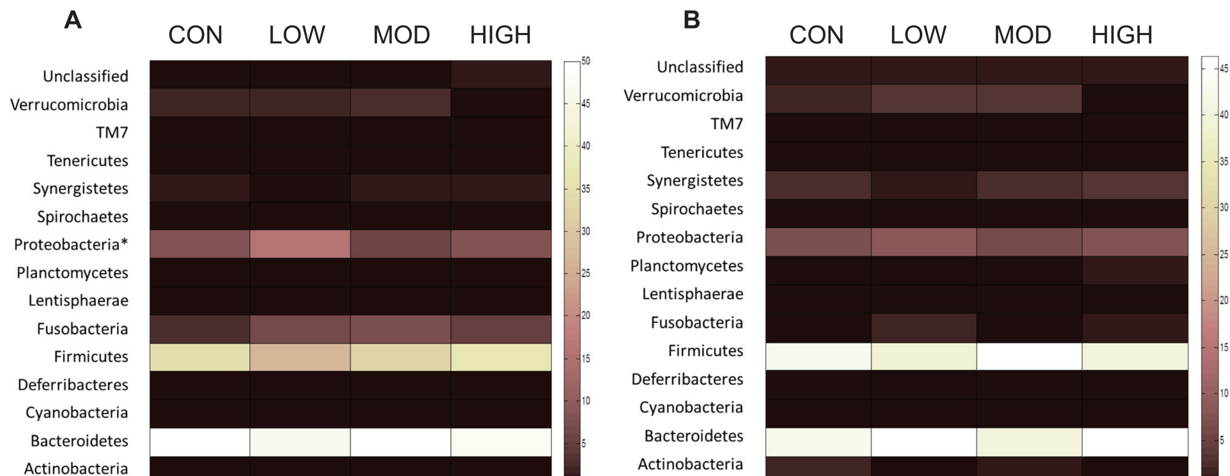

FIGURE 4 Heat maps representing relative abundances of 15 bacterial phyla in ascending colon contents (A) and feces (B) of piglets fed formula containing 0 mg/L (CON), 130 mg/L (LOW), 380 mg/L (MOD), or 760 mg/L (HIGH) SL. Relative abundances are presented on a progressive color scale with dark and light color depicting the lowest and highest abundances, respectively. *Significant differences at $P \leq 0.05$ (ANOVA). CON, control; HIGH, high SL concentration; LOW, low SL concentration; MOD, moderate SL concentration.

Discussion

MOs are complex glycans that are resistant to digestion and exert a number of important physiologic functions including shaping the composition of the gut microbiota, influencing the proliferation and maturation of intestinal cells, regulating intestinal and systemic immunity, and modulating brain structural and cognitive development (4, 19–21, 50). MOs are either absent or found in very low concentration in infant formula (10). Currently, some commercially available infant formulas contain the prebiotics GOS and PDX, which have bifidogenic effects in the infant's intestine and influence early bacterial colonization, resulting in a microbiota that closely resembles that of breastfed infants (23, 24). With technologic advances in the production of MOs, the supplementation of infant formula with these compounds has become viable. Herein, we investigated the effects of a bovine-derived modified whey enriched with SL on the growth and gut development of neonatal pigs. The results of the study demonstrated that the addition of bovine-derived SL at concentrations similar to those present in human milk to a GOS- and PDX-enriched formula was well tolerated, supported normal growth, and did not adversely affect piglet development during the first 4 wk of life. SA-containing compounds have been described as essential for the neonatal period owing to its involvement in brain development and cognition, inhibition of pathogen binding, and immunity (10, 50). In addition, several studies have demonstrated the beneficial effects of SA-containing oligosaccharides on intestinal health. Oligosaccharides (including the acidic oligosaccharides 3′SL and 6′SL) isolated from human milk induced differentiation of transformed and nontransformed human intestinal cells that directly affect the absorptive and digestive capacity of the intestine (17). In our in vivo pig study, diets were formulated to contain SL concentrations above those endogenously present in bovine milk–based human infant formula and which were similar to SL concentrations reported for mature bovine and human milk (10). SL supplementation did not significantly affect intestinal growth, morphology, goblet cell numbers, disaccharidase activity, stool consistency, and cecal and colonic content pH.

Similarly, only a few minor hematologic and histologic differences were detected with the addition of SL to formula. Coagulation (clotting) time is routinely evaluated to monitor blood clotting functions and detect coagulation disturbances. One-stage PT and aPTT are indicators of the activation of the intrinsic and extrinsic pathways, respectively, and they differ in the initial factor triggered in the coagulation pathway. Values obtained for both PT and aPTT in our study were within the normal range for young pigs. Owing to the relative insensitivity of such tests and the remarkably small change in coagulation time in the HIGH group (<2 s), the aPTT difference between the groups may not be of clinical significance. In fact, a reduction of 30% in the activity of a single factor in the coagulation cascade is needed before a noticeably prolonged time is observed in the clotting process (51). Similarly, there was a slight, but significant, increase in the activity of GLDH in the MOD group. GLDH is a mitochondrial enzyme, and is indicative of hepatocellular injury, especially in large animals (52). The use of plasma enzyme activity as a measure of hepatic injury should be interpreted cautiously. Karlsson et al. (53) did not find a correlation between the serum levels of commonly assayed enzyme activities and pathologic liver changes in newborn pigs. In addition, a reference value for GLDH activity is missing in neonatal piglets, but it has been reported to be <10 U/L in small healthy animals (53) and to range between 0.1 and 3.2 U/L in >20-kg pigs (54). Nonetheless, GLDH activity observed in CON- and SL-fed piglets was within the ranges reported for older pigs. Furthermore, the absence of a dose-response effect on GLDH and lack of abnormal values of other enzymes suggest that the change in GLDH activity in MOD animals has little physiologic significance.

Modest changes in neutrophil and lymphocyte counts were observed in the MOD relative to the other groups. However, we did not observe a dose-response change in the number of neutrophils and lymphocytes and the values for both measures were within reference ranges (45), suggesting no immune impairment. Given the potential importance of the gut in educating cells of the immune system, more research in this area could be informative.

TABLE 9 Relative abundances of bacterial genera detected in ascending colon contents of piglets fed formula containing various concentrations of sialyllactose[1]

Genus	Family	Phylum	Concentration of sialyllactose in formula: CON (0 mg/L) ($n = 11$)	LOW (130 mg/L) ($n = 9$)	MOD (380 mg/L) ($n = 10$)	HIGH (760 mg/L) ($n = 8$)	P value
Bifidobacterium	Bifidobacteriaceae	Actinobacteria	0.06 ± 0.05	0.08 ± 0.08	0.06 ± 0.05	0 ± 0	0.7566
Eggerthella	Coriobacteriaceae	Actinobacteria	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.09 ± 0.02	0.1379
Butyricimonas	[Odoribacteraceae]	Bacteroidetes	0.85 ± 0.17	0.81 ± 0.18	0.84 ± 0.19	0.52 ± 0.15	0.5176
Odoribacter	[Odoribacteraceae]	Bacteroidetes	0.09 ± 0.03	0.32 ± 0.25	0.10 ± 0.04	0.10 ± 0.03	0.5151
[Prevotella]	[Paraprevotellaceae]	Bacteroidetes	7.66 ± 2.36	5.08 ± 1.99	4.40 ± 1.03	5.55 ± 1.35	0.4089
Bacteroides	Bacteroidaceae	Bacteroidetes	20.0 ± 5.25	18.2 ± 3.54	24.1 ± 4.01	22.4 ± 3.66	0.2738
Parabacteroides	Porphyromonadaceae	Bacteroidetes	12.6 ± 3.00	13.2 ± 3.90	10.9 ± 2.52	9.36 ± 2.34	0.7977
Prevotella	Prevotellaceae	Bacteroidetes	0.72 ± 0.45	2.50 ± 1.89	0.82 ± 0.35	0.16 ± 0.08	0.2382
Alistipes	Rikenellaceae	Bacteroidetes	0.13 ± 0.05[a]	0.18 ± 0.09[ab]	0.29 ± 0.08[ab]	0.55 ± 0.16[b]	0.0341
Christensenella	Christensenellaceae	Firmicutes	0.11 ± 0.03	0.06 ± 0.02	0.10 ± 0.03	0.08 ± 0.02	0.3831
Clostridium	Clostridiaceae	Firmicutes	0.48 ± 0.15	0.20 ± 0.08	0.59 ± 0.32	0.23 ± 0.13	0.4713
SMB53	Clostridiaceae	Firmicutes	0.04 ± 0.01	0.07 ± 0.02	0.04 ± 0.01	0.05 ± 0.02	0.5219
RFN20	Erysipelotrichaceae	Firmicutes	1.02 ± 1.02	0.52 ± 0.52	1.43 ± 1.41	0.03 ± 0.02	0.7891
[Eubacterium]	Erysipelotrichaceae	Firmicutes	0.16 ± 0.12	0.20 ± 0.08	0.10 ± 0.04	0.28 ± 0.07	0.2251
p-75-a5	Erysipelotrichaceae	Firmicutes	0.26 ± 0.16	0.07 ± 0.06	0.18 ± 0.11	0.01 ± 0.01	0.1701
Blautia	Lachnospiraceae	Firmicutes	5.96 ± 1.61	3.05 ± 0.86	5.15 ± 1.77	8.16 ± 2.66	0.2423
[Ruminococcus]	Lachnospiraceae	Firmicutes	1.14 ± 0.23	1.62 ± 0.42	1.50 ± 0.55	1.52 ± 0.57	0.8828
Dorea	Lachnospiraceae	Firmicutes	0.87 ± 0.41	0.55 ± 0.29	1.03 ± 0.41	0.47 ± 0.15	0.5612
Coprococcus	Lachnospiraceae	Firmicutes	0.26 ± 0.05	0.20 ± 0.06	0.27 ± 0.07	0.32 ± 0.09	0.6449
Clostridium	Lachnospiraceae	Firmicutes	0.07 ± 0.02	0.17 ± 0.05	0.10 ± 0.03	0.14 ± 0.07	0.2421
Lactobacillus	Lactobacillaceae	Firmicutes	6.23 ± 4.27	4.61 ± 2.01	2.46 ± 0.85	2.88 ± 1.33	0.5687
Leuconostoc	Leuconostocaceae	Firmicutes	0.35 ± 0.13	1.24 ± 0.79	1.39 ± 0.96	1.17 ± 0.45	0.5284
Oscillospira	Ruminococcaceae	Firmicutes	1.16 ± 0.22	1.57 ± 0.39	1.20 ± 0.23	3.32 ± 1.37	0.1313
Ruminococcus	Ruminococcaceae	Firmicutes	1.34 ± 0.36	0.77 ± 0.23	0.98 ± 0.19	1.19 ± 0.30	0.3378
Anaerotruncus	Ruminococcaceae	Firmicutes	0.22 ± 0.09	0.51 ± 0.17	0.24 ± 0.08	0.61 ± 0.38	0.2135
Faecalibacterium	Ruminococcaceae	Firmicutes	0.68 ± 0.30	0.29 ± 0.17	0.09 ± 0.03	0.13 ± 0.07	0.0741
Butyricicoccus	Ruminococcaceae	Firmicutes	0.03 ± 0.01	0.04 ± 0.02	0.09 ± 0.04	0.06 ± 0.01	0.2356
Lactococcus	Streptococcaceae	Firmicutes	0.44 ± 0.15	0.34 ± 0.13	0.33 ± 0.16	0.27 ± 0.18	0.7112
Streptococcus	Streptococcaceae	Firmicutes	0.04 ± 0.01	0.02 ± 0.01	0.14 ± 0.08	0.02 ± 0	0.0996
Turicibacter	Turicibacteraceae	Firmicutes	0.13 ± 0.04	0.07 ± 0.04	0.10 ± 0.03	0.20 ± 0.12	0.5348
Phascolarctobacterium	Veillonellaceae	Firmicutes	0.57 ± 0.45	1.29 ± 1.14	0.32 ± 0.19	0.53 ± 0.31	0.6845
Anaerovibrio	Veillonellaceae	Firmicutes	0 ± 0	0.15 ± 0.13	0.66 ± 0.64	0.01 ± 0.01	0.4252
Acidaminococcus	Veillonellaceae	Firmicutes	0.08 ± 0.05	0.06 ± 0.05	0.33 ± 0.23	0 ± 0	0.5096
Fusobacterium	Fusobacteriaceae	Fusobacteria	0.37 ± 0.24	0.48 ± 0.29	0.16 ± 0.09	0.13 ± 0.13	0.5627
Sutterella	Alcaligenaceae	Proteobacteria	0.08 ± 0.03	0.18 ± 0.10	0.05 ± 0.02	0.12 ± 0.05	0.3538
Desulfovibrio	Desulfovibrionaceae	Proteobacteria	1.13 ± 0.29	0.89 ± 0.33	1.04 ± 0.30	1.56 ± 0.54	0.4517
Bilophila	Desulfovibrionaceae	Proteobacteria	0.12 ± 0.02[b]	0.43 ± 0.17[a]	0.22 ± 0.05[ab]	0.21 ± 0.06[ab]	0.0287
Escherichia	Enterobacteriaceae	Proteobacteria	4.25 ± 1.28	12.6 ± 4.45	3.29 ± 0.50	5.40 ± 2.21	0.0538
Citrobacter	Enterobacteriaceae	Proteobacteria	0.10 ± 0.03	0.06 ± 0.02	0.09 ± 0.03	0.03 ± 0.02	0.0613
Pasteurella	Pasteurellaceae	Proteobacteria	0.49 ± 0.43	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.3409
Synergistes	Synergistaceae	Synergistetes	1.21 ± 0.82	0.43 ± 0.13	1.17 ± 0.80	1.20 ± 0.56	0.8587
Akkermansia	Verrucomicrobiaceae	Verrucomicrobia	1.70 ± 1.20	1.67 ± 1.19	2.92 ± 2.06	0.01 ± 0.01	0.4214

[1]Values are means ± SEMs. Labeled means in a row without a common superscript letter differ at $P \leq 0.05$ (ANOVA). Only the genera with mean relative abundances ≥0.05% were analyzed. Taxa that have brackets around the names are proposed taxonomies that greengenes recommend based on the whole genome phylogeny, but not officially recognized by Bergey's manual of systematic bacteriology. CON, control; HIGH, high SL concentration; LOW, low SL concentration; MOD, moderate SL concentration.

Although previous studies have reported microbial composition changes with the addition of higher doses of SL to the diet, we observed that the addition of SL-enriched bovine-derived modified whey to a milk replacer containing PDX + GOS had only minor effects on the gut microbiota relative to CON formula. The only statistically significant change was higher fecal density of *C. perfringens* from animals fed SL at the MOD (380 mg/L) SL concentration relative to CON and HIGH. Among the factors that may explain differences between the findings reported herein and previous reports are the presence of PDX and GOS in all dietary treatments and the lower SL dose used. The relevance of this finding is unclear, but it is worth noting that there was no dose response in *C. perfringens* abundance among the groups. Furthermore, the presence of *C. perfringens* in the pig intestine is not unusual. Bacteria from the *Clostridium* genus (Firmicutes phylum) are commonly found during the first 2 wk of life in the nursing piglet intestine, although its abundance decreases after weaning (55). *C. perfringens* is found in high abundance in the microbiota of sow-reared piglets; however, its presence was not correlated with enteritis (56, 57).

TABLE 10 Relative abundances of bacterial genera detected in feces of piglets fed formula containing various concentrations of sialyllactose[1]

			Concentration of sialyllactose in formula				
Genus	Family	Phylum	CON (0 mg/L) (n = 11)	LOW (130 mg/L) (n = 9)	MOD (380 mg/L) (n = 10)	HIGH (760 mg/L) (n = 8)	P values
Bifidobacterium	Bifidobacteriaceae	Actinobacteria	0.08 ± 0.07	0.02 ± 0.02	0.24 ± 0.16	0 ± 0	0.2710
Eggerthella	Coriobacteriaceae	Actinobacteria	0.37 ± 0.14	0.16 ± 0.06	0.18 ± 0.04	0.24 ± 0.04	0.3496
Butyricimonas	[Odoribacteraceae]	Bacteroidetes	0.69 ± 0.13	0.85 ± 0.24	0.76 ± 0.26	0.90 ± 0.31	0.9630
Odoribacter	[Odoribacteraceae]	Bacteroidetes	0.39 ± 0.23	0.23 ± 0.13	0.21 ± 0.14	0.14 ± 0.05	0.8768
[Prevotella]	[Paraprevotellaceae]	Bacteroidetes	4.10 ± 1.41[b]	2.01 ± 1.05[a,b]	0.62 ± 0.20[a]	2.13 ± 0.72[a,b]	0.0455
Bacteroides	Bacteroidaceae	Bacteroidetes	16.1 ± 3.64	21.7 ± 4.10	20.6 ± 4.68	24.1 ± 5.08	0.0541
Parabacteroides	Porphyromonadaceae	Bacteroidetes	10.5 ± 3.03	12.5 ± 3.83	9.11 ± 1.66	9.15 ± 2.82	0.7478
Prevotella	Prevotellaceae	Bacteroidetes	0.52 ± 0.22	0.75 ± 0.33	0.46 ± 0.28	0.33 ± 0.18	0.6304
Alistipes	Rikenellaceae	Bacteroidetes	0.64 ± 0.25	0.43 ± 0.16	0.76 ± 0.20	0.94 ± 0.29	0.2557
Christensenella	Christensenellaceae	Firmicutes	0.35 ± 0.09	0.21 ± 0.05	0.35 ± 0.07	0.39 ± 0.25	0.4295
Clostridium	Clostridiaceae	Firmicutes	0.87 ± 0.25	1.09 ± 0.57	2.05 ± 1.17	1.03 ± 0.63	0.4564
SMB53	Clostridiaceae	Firmicutes	0.07 ± 0.03	0.06 ± 0.02	0.05 ± 0.02	0.05 ± 0.01	0.9838
[Eubacterium]	Erysipelotrichaceae	Firmicutes	0.29 ± 0.16[a,b]	0.34 ± 0.11[a,b]	0.13 ± 0.05[a]	0.70 ± 0.26[b]	0.0236
p-75-a5	Erysipelotrichaceae	Firmicutes	2.45 ± 2.14	0.11 ± 0.07	2.08 ± 1.99	0.01 ± 0.01	0.2833
[Ruminococcus]	Lachnospiraceae	Firmicutes	1.46 ± 0.33	2.16 ± 0.54	2.42 ± 0.83	1.39 ± 0.53	0.3824
Blautia	Lachnospiraceae	Firmicutes	4.90 ± 0.94	4.22 ± 1.44	4.32 ± 1.46	7.40 ± 3.08	0.5165
Clostridium	Lachnospiraceae	Firmicutes	0.28 ± 0.14	0.31 ± 0.12	0.23 ± 0.05	0.35 ± 0.09	0.7577
Coprococcus	Lachnospiraceae	Firmicutes	0.26 ± 0.08	0.28 ± 0.08	0.23 ± 0.07	0.21 ± 0.05	0.9057
Dorea	Lachnospiraceae	Firmicutes	0.80 ± 0.34	0.67 ± 0.39	0.85 ± 0.22	0.44 ± 0.13	0.7124
Lactobacillus	Lactobacillaceae	Firmicutes	1.87 ± 0.61	2.32 ± 0.81	2.12 ± 1.05	1.12 ± 0.42	0.6332
Leuconostoc	Leuconostocaceae	Firmicutes	1.33 ± 0.70	1.15 ± 0.42	0.74 ± 0.40	0.70 ± 0.26	0.7906
Anaerotruncus	Ruminococcaceae	Firmicutes	0.32 ± 0.09	0.68 ± 0.16	0.61 ± 0.18	0.65 ± 0.15	0.0554
Butyricicoccus	Ruminococcaceae	Firmicutes	0.03 ± 0.01[a]	0.05 ± 0.02[a,b]	0.04 ± 0.01[a,b]	0.15 ± 0.07[b]	0.0239
Faecalibacterium	Ruminococcaceae	Firmicutes	0.64 ± 0.28	0.16 ± 0.05	0.21 ± 0.15	0.23 ± 0.10	0.2503
Oscillospira	Ruminococcaceae	Firmicutes	2.99 ± 0.62	2.38 ± 0.33	3.42 ± 1.04	4.00 ± 1.45	0.6158
Ruminococcus	Ruminococcaceae	Firmicutes	2.39 ± 0.66	1.95 ± 0.37	2.15 ± 0.43	1.72 ± 0.39	0.4842
Lactococcus	Streptococcaceae	Firmicutes	1.13 ± 0.38	0.96 ± 0.63	0.61 ± 0.34	0.18 ± 0.08	0.0619
Streptococcus	Streptococcaceae	Firmicutes	0.07 ± 0.03	0.05 ± 0.03	0.04 ± 0.01	0.02 ± 0.01	0.5977
Turicibacter	Turicibacteraceae	Firmicutes	0.38 ± 0.11	0.26 ± 0.11	0.76 ± 0.50	0.60 ± 0.37	0.7676
Acidaminococcus	Veillonellaceae	Firmicutes	0.15 ± 0.12	0.18 ± 0.18	0.37 ± 0.28	0 ± 0	0.8525
Megasphaera	Veillonellaceae	Firmicutes	0.03 ± 0.02	0.04 ± 0.02	0.05 ± 0.03	0.11 ± 0.09	0.5973
Phascolarctobacterium	Veillonellaceae	Firmicutes	0.65 ± 0.42	0.63 ± 0.35	0.27 ± 0.13	0.52 ± 0.27	0.7991
Bilophila	Desulfovibrionaceae	Proteobacteria	0.31 ± 0.10	0.65 ± 0.17	0.48 ± 0.11	0.58 ± 0.23	0.2002
Desulfovibrio	Desulfovibrionaceae	Proteobacteria	0.91 ± 0.19	1.22 ± 0.38	1.27 ± 0.28	3.07 ± 1.76	0.2497
Escherichia	Enterobacteriaceae	Proteobacteria	4.33 ± 1.14	5.10 ± 1.00	3.22 ± 1.03	3.04 ± 0.98	0.3927
Synergistes	Synergistaceae	Synergistetes	2.35 ± 1.58	0.86 ± 0.23	2.44 ± 1.04	2.71 ± 0.95	0.5715
Akkermansia	Verrucomicrobiaceae	Verrucomicrobia	1.72 ± 1.25	3.13 ± 2.46	2.97 ± 2.03	0.03 ± 0.02	0.1049

[1]Data are means ± SEMs. Labelled means in a row without a common superscript letter differ, $P \leq 0.05$ (ANOVA). Only the genera with mean relative abundances ≥ 0.05% were analyzed. Taxa that have brackets around the names are proposed taxonomies that greengenes recommend based on the whole genome phylogeny, but not officially recognized by Bergey's manual of systematic bacteriology. CON, control; HIGH, high SL concentration; LOW, low SL concentration; MOD, moderate SL concentration.

The addition of prebiotics such as PDX and GOS to formula has produced changes in microbial communities in human infants, rats, and piglets. Mika et al. (58) demonstrated an increase in *Lactobacillus* spp. abundance in rats fed diets supplemented with GOS and PDX at 7 g/kg diet for 4 wk. In piglets, the addition of PDX and GOS to formula (4 g/L at a 1:1 ratio) increased the number of *Parabacteroides* and *Lactobacillus* species in colonic contents relative to control formula (59). Infants fed formula with a prebiotic blend of PDX and GOS (4 g/L total at a 1:1 ratio) for 60 d had increased total *Bifidobacterium* counts and a gut microbial profile that more closely resembled that of breastfed infants (23). Thus, PDX and GOS at the concentration present in the diets may have resulted in similar gut bacterial communities among the groups.

Other prebiotics, including SA-containing oligosaccharides, have been tested in formula for their ability to modulate the gut microbiota. Jacobi et al. (16) observed significant changes in the colonic microbial ecology in neonatal piglets fed formula supplemented solely (no other prebiotics) with 6′SL, but not 3′SL, at 4 g/L. The most significant change observed was an increase in bacterial species that are capable of metabolizing 6′SL, namely *Collinsella aerofaciens* (phylum Actinobacteria), genera *Ruminococcus* and *Faecalibacterium* (phylum Firmicutes), and genus *Prevotella* (phylum Bacteroidetes), in 6′SL-fed piglets relative to controls (16). Tarr et al. (60) demonstrated that dietary supplementation with 5% 3′SL or 6′SL mitigated stress-induced changes in the gut microbial population. In human infants, the addition of 10 g/L bovine MO enriched in 3′SL and 6′SL resulted in a moderate change in the microbiota, which included lower Clostridia counts relative to formula without bovine MO (26). As described earlier, the concentration of SL used herein was significantly lower than in other studies, which may

TABLE 11 Diversity measures obtained from the microbiota in ascending colon contents and feces of piglets fed formula containing various concentrations of sialyllactose[1]

	Concentration of sialyllactose in formula				
	CON (0 mg/L) (n = 11)	LOW (130 mg/L) (n = 9)	MOD (380 mg/L) (n = 10)	HIGH (760 mg/L) (n = 8)	P value
Ascending colon					
Observed OTUs	3386 ± 257	3205 ± 230	3541 ± 170	3521 ± 264	0.6331
Shannon	7.1 ± 0.3	6.5 ± 0.4	7.0 ± 0.3	7.1 ± 0.4	0.3625
Simpson reciprocal	26.6 ± 4.7	16.9 ± 3.4	27.7 ± 6.3	26.4 ± 4.9	0.2931
Chao1	6368 ± 529	6313 ± 354	7061 ± 387	6667 ± 545	0.5228
Feces					
Observed OTUs	3717 ± 277	3788 ± 126	3881 ± 313	3704 ± 134	0.8358
Shannon	7.4 ± 0.3	7.3 ± 0.2	7.4 ± 0.3	7.5 ± 0.2	0.9648
Simpson reciprocal	36.3 ± 7.2	27.1 ± 4.3	34.1 ± 5.6	29.5 ± 5.6	0.3494
Chao1	6934 ± 706	7408 ± 381	7572 ± 781	6677 ± 456	0.6267

[1]Values are means ± SEMs. No significant differences were detected at $P < 0.05$ (ANOVA). CON, control; HIGH, high SL concentration; LOW, low SL concentration; MOD, moderate SL concentration; OTU, operational taxonomic unit.

explain differences in the extent to which SL affected the gut microbiota. No effect of SL on VFA concentrations was observed. Previous studies have shown that although 6′SL is fermented by the human infant microbiome, it produced lower concentrations of SCFAs compared with GOS, LNnT, or 2′FL (61). Therefore, small effects of SL fermentation were likely not detectable owing to the presence of PDX and GOS in all diets.

Currently, most infant formulas include prebiotics, but the addition of MOs has been limited by the lack of availability and high cost of these compounds. However, a few clinical trials have been conducted to test the tolerance and safety of MO supplementation in formula. Marriage et al. (62) reported that the addition of GOS + 0.2 or 1.0 g/L of 2′FL (total amount of oligosaccharide was 2.4 g/L) for 4 mo was well tolerated, and that infant growth and 2′FL uptake were similar to those observed in exclusively breastfed infants (62). Similarly, a clinical study testing the safety of the addition of 2′FL (1.0 g/L) and LNnT (0.5 g/L) to a cow milk–based formula showed that HMO supplementation supported age-appropriate growth and was safe (6). Our study confirms that adding dietary SL at ≤760 mg/L to formula containing prebiotics is well tolerated by neonatal piglets. We demonstrated that it supported weight gain and did not have any adverse effect on the intestine or other organ weights and structure. The addition of SL to a prebiotic-containing diet did not affect overall microbiota structure and diversity in AC contents and feces, but had minor effects on the relative abundances of specific microbes. We hypothesize that the lack of distinct changes in the microbiota and VFAs can be attributed to the presence of prebiotics, specifically PDX and GOS, in the base formula. However, our group recently reported that the combination of prebiotics and SL affected the development of the prefrontal cortex and corpus callosum in these piglets (27), suggesting that SL-containing oligosaccharides have the potential to influence brain development without detrimentally affecting the development of other organ systems in the piglet.

Although the piglet is considered to be one of the best preclinical animal models for the human infant (28, 29), a limitation of the study is that the native microbiome of the piglet differs from that of the human infant, thus it is not possible to directly extrapolate microbiome findings to infants. Therefore, future studies with humanized gnotobiotic piglets could better inform SL-induced changes in microbiome structure and function. Another limitation is the use of a complex SL-enriched whey fraction. As pure forms of 3′SL and 6′SL become available, future research should seek to investigate their unique roles in neonatal development. Lastly, the presence of PDX and GOS in the formulas likely masked potential SL-specific outcomes on microbiome composition or VFA concentrations; however, the goal of this experiment was to investigate the impact of SL in the presence of prebiotics present in commercially available infant formula.

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References

1. Fong B, Ma K, McJarrow P. Quantification of bovine milk oligosaccharides using liquid chromatography-selected reaction monitoring-mass spectrometry. J Agric Food Chem 2011;59:9788–95.
2. Sprenger N, Lee LY, De Castro CA, Steenhout P, Thakkar SK. Longitudinal change of selected human milk oligosaccharides and association to infants' growth: an observatory single center, longitudinal cohort study. PLoS One 2017;12:e0171814.
3. Davis EC, Wang M, Donovan SM. The role of early life nutrition in the establishment of gastrointestinal microbial composition and function. Gut Microbes 2017;8:143–71.
4. Donovan SM, Comstock SS. Human milk oligosaccharides influence neonatal mucosal and systemic immunity. Ann Nutr Metab 2016;69(Suppl) 2:42–51.
5. Wang B. Sialic acid is an essential nutrient for brain development and cognition. Annu Rev Nutr 2009;29:177–222.

6. Puccio G, Alliet P, Cajozzo C, Janssens E, Corsello G, Sprenger N, Wernimont S, Egli D, Gosoniu L, Steenhout P. Effects of infant formula with human milk oligosaccharides on growth and morbidity: a randomized multicenter trial. J Pediatr Gastroenterol Nutr 2017;64:624–31.
7. Bode L. Human milk oligosaccharides: every baby needs a sugar mama. Glycobiology 2012;22:1147–62.
8. Niñonuevo MR, Perkins PD, Francis J, Lamotte LM, LoCascio RG, Freeman SL, Mills DA, German JB, Grimm R, Lebrilla CB. Daily variations in oligosaccharides of human milk determined by microfluidic chips and mass spectrometry. J Agric Food Chem 2008;56:618–26.
9. McGuire MK, Meehan CL, McGuire MA, Williams JE, Foster J, Sellen DW, Kamau-Mbuthia EW, Kamundia EW, Mbugua S, Moore SE et al. What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically. Am J Clin Nutr 2017;105:1086–100.
10. ten Bruggencate SJ, Bovee-Oudenhoven IM, Feitsma AL, van Hoffen E, Schoterman MH. Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides. Nutr Rev 2014;72:377–89.
11. Chaturvedi P, Warren CD, Buescher CR, Pickering LK, Newburg DS. Survival of human milk oligosaccharides in the intestine of infants. Adv Exp Med Biol 2001;501:315–23.
12. DiFilippo E, Bettonvil M, Willems RH, Braber S, Fink-Gremmels J, Jeurink PV, Schoterman MH, Gruppen H, Schols HA. Oligosaccharides in urine, blood, and feces of piglets fed milk replacer containing galacto-oligosaccharides. J Agric Food Chem 2015;63:10862–72.
13. Marcobal A, Barboza M, Froehlich JW, Block DE, German JB, Lebrilla CB, Mills DA. Consumption of human milk oligosaccharides by gut-related microbes. J Agric Food Chem 2010;58:5334–40.
14. Wiese M, Khakimov B, Nielsen S, Sørensen H, van den Berg F, Nielsen DS. CoMiniGut—a small volume *in vitro* colon model for the screening of gut microbial fermentation processes. Peer J 2018;6:e4268.
15. Fuhrer A, Sprenger N, Kurakevich E, Borsig L, Chassard C, Hennet T. Milk sialyllactose influences colitis in mice through selective intestinal bacterial colonization. J Exp Med 2010;207:2843–54.
16. Jacobi SK, Yatsunenko T, Li D, Dasgupta S, Yu RK, Berg BM, Chichlowski M, Odle J. Dietary isomers of sialyllactose increase ganglioside sialic acid concentrations in the corpus callosum and cerebellum and modulate the colonic microbiota of formula-fed piglets. J Nutr 2016;146:200–8.
17. Kuntz S, Rudloff S, Kunz C. Oligosaccharides from human milk influence growth-related characteristics of intestinally transformed and non-transformed intestinal cells. Br J Nutr 2008;99:462–71.
18. Kuntz S, Kunz C, Rudloff S. Oligosaccharides from human milk induce growth arrest via G2/M by influencing growth-related cell cycle genes in intestinal epithelial cells. Br J Nutr 2009;101:1306–15.
19. Holscher HD, Davis SR, Tappenden KA. Human milk oligosaccharides influence maturation of human intestinal Caco-2Bbe and HT-29 cell lines. J Nutr 2014;144:586–91.
20. Goldberg RF, Austen WG Jr, Zhang X, Munene G, Mostafa G, Biswas S, McCormack M, Eberlin KR, Nguyen JT, Tatlidede HS et al. Intestinal alkaline phosphatase is a gut mucosal defense factor maintained by enteral nutrition. Proc Natl Acad Sci USA 2008;105:3551–6.
21. Berding K, Wang M, Monaco MH, Alexander LS, Mudd AT, Chichlowski M, Waworuntu RV, Berg BM, Miller MJ, Dilger RN et al. Prebiotics and bioactive milk fractions affect gut development, microbiota, and neurotransmitter expression in piglets. J Pediatr Gastroenterol Nutr 2016;63:688–97.
22. Bhatia S, Prabhu PN, Benefiel AC, Miller MJ, Chow J, Davis SR, Gaskins HR. Galacto-oligosaccharides may directly enhance intestinal barrier function through the modulation of goblet cells. Mol Nutr Food Res 2015;59:566–73.
23. Scalabrin DM, Mitmesser SH, Welling GW, Harris CL, Marunycz JD, Walker DC, Bos NA, Tölkkö S, Salminen S, Vanderhoof JA. New prebiotic blend of polydextrose and galacto-oligosaccharides has a bifidogenic effect in young infants. J Pediatr Gastroenterol Nutr 2012;54:343–52.
24. Ashley C, Johnston WH, Harris CL, Stolz SI, Wampler JL, Berseth CL. Growth and tolerance of infants fed formula supplemented with polydextrose (PDX) and/or galactooligosaccharides (GOS): double-blind, randomized, controlled trial. Nutr J 2012;11:38.
25. Goehring KC, Marriage BJ, Oliver JS, Wilder JA, Barrett EG, Buck RH. Similar to those who are breastfed, infants fed a formula containing 2′-fucosyllactose have lower inflammatory cytokines in a randomized controlled trial. J Nutr 2016;146:2559–66.
26. Meli F, Puccio G, Cajozzo C, Ricottone GL, Pecquet S, Sprenger N, Steenhout P. Growth and safety evaluation of infant formulae containing oligosaccharides derived from bovine milk: a randomized, double-blind, noninferiority trial. BMC Pediatr 2014;14:306.
27. Mudd AT, Fleming SA, Labhart B, Chichlowski M, Berg BM, Donovan SM, Dilger RN. Dietary sialyllactose influences sialic acid concentrations in the prefrontal cortex and magnetic resonance imaging measures in corpus callosum of young pigs. Nutrients 2017;9:1297.
28. Odle J, Lin X, Jacobi SK, Kim SW, Stahl CH. The suckling piglet as an agrimedical model for the study of pediatric nutrition and metabolism. Annu Rev Anim Biosci 2014;2:419–44.
29. Mudd AT, Dilger RN. Early-life nutrition and neurodevelopment: use of the piglet as a translational model. Adv Nutr 2017;8:92–104.
30. National Research Council. Guide for the care and use of laboratory animals. 8th ed. Washington (DC): National Academies Press; 2011.
31. Monaco MH, Kashtanov DO, Wang M, Walker DC, Rai D, Jouni ZE, Miller MJ, Donovan SM. Addition of polydextrose and galactooligosaccharide to formula does not affect bacterial translocation in the neonatal piglet. J Pediatr Gastroenterol Nutr 2011;52:210–16.
32. Croix JA, Carbonero F, Nava GM, Russell M, Greenberg E, Gaskins HR. On the relationship between sialomucin and sulfomucin expression and hydrogenotrophic microbes in the human colonic mucosa. PLoS One 2011;6:e24447.
33. Hartke JL, Monaco MH, Wheeler MB, Donovan SM. Effect of a short-term fast on intestinal disaccharidase activity and villus morphology of piglets suckling insulin-like growth factor-I transgenic sows. J Anim Sci 2005;83:2404–13.
34. Dudley MA, Jahoor F, Burrin DG, Reeds PJ. Brush-border disaccharidase synthesis in infant pigs measured in vivo with [$^2H_3$]leucine. Am J Physiol 1994;267:G1128–34.
35. Li M, Bauer LL, Chen X, Engfer M, Kuhlenschmidt TB, Kuhlenschmidt MS, Fahey GC Jr, Donovan SM. Microbial composition and in vitro fermentation patterns of human milk oligosaccharides and prebiotics differ between formula-fed and sow-reared piglets. J Nutr 2012;142:681–9.
36. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 2013;41(1):e1.
37. Muturi EJ, Donthu RK, Fields CJ, Moise IK, Kim CH. Effect of pesticides on microbial communities in container aquatic habitats. Sci Rep 2017;7:44565.
38. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol 2013;79:5112–20.
39. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI et al. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 2010;7:335–6.
40. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 2006;72:5069–72.
41. Price MN, Dehal PS, Arkin AP. FastTree 2 – approximately maximum-likelihood trees for large alignments. PLoS One 2010;5:e9490.
42. Lozupone C, Hamady M, Knight R. UniFrac – an online tool for comparing microbial community diversity in a phylogenetic context. BMC Bioinformatics 2006;7:371.
43. Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM et al. The ribosomal database project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 2009;37:D141–5.
44. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner HH. Vegan: community

ecology package [Internet]. R package version 2.3-1; 2015. Available from: https://cran.r-project.org/web/packages/vegan/vegan.pdf.
45. Ventrella D, Dondi F, Barone F, Serafini F, Elmi A, Giunti M, Romagnoli N, Forni M, Bacci ML. The biomedical piglet: establishing reference intervals for haematology and clinical chemistry parameters of two age groups with and without iron supplementation. BMC Vet Res 2017;13:23–30.
46. Rispat G, Slaoui M, Weber D, Salemink P, Bethoux C, Shrivastava R. Hematologic and plasma biochemical values for healthy Yucatan micropigs. Lab Animal 1993;27:368–73.
47. Merck and the Merck Veterinary Manual. Serum biochemical references ranges [Internet]. Available from: https://www.merckvetmanual.com/special-subjects/reference-guides/serum-biochemical-reference-ranges.
48. Cooper CA, Moraes LE, Murray JD, Owens SD. Hematologic and biochemical reference intervals for specific pathogen free 6-week-old Hampshire-Yorkshire crossbred pigs. J Anim Sci Biotechnol 2014;5:5.
49. Houle VM, Schroeder EA, Odle J, Donovan SM. Small intestinal disaccharidase activity and ileal villus height are increased in piglets consuming formula containing recombinant human insulin-like growth factor-I. Pediatr Res 1997;42:78–86.
50. Wang B. Molecular mechanism underlying sialic acid as an essential nutrient for brain development and cognition. Adv Nutr 2012;3:465S–72S.
51. Hall RL, Everds NE. Principles of clinical pathology for toxicology studies. In: Hayes AW Kruger CL, editors. Hayes' principles and methods of toxicology. 6th ed. Boca Raton, FL: CRC Press, Taylor & Francis Group; 2008. p. 1306–45.
52. GLDH [Internet]. eClinPath. Cornell University College of Veterinary Medicine. Available from: http://www.eclinpath.com/chemistry/liver/liver-injury/glutamate-dehydrogenase/.
53. Karlsson M, Satas S, Stone J, Porter H, Thoresen M. Liver enzymes cannot be used to predict liver damage after global hypoxia-ischemia in a neonatal pig model. Neonatology 2009;96:211–18.
54. Mainka S, Dänicke S, Böhme H, Wolff J, Matthes S, Flachowsky G. Comparative studies on the effect of ergot contaminated feed on performance and health of piglets and chickens. Arch Anim Nutr 2005;59:81–9.
55. Frese SA, Parker K, Calvert CC, Mills DA. Diet shapes the gut microbiome of pigs during nursing and weaning. Microbiome 2015;3:28.
56. Baker AA, Davis E, Rehberger T, Rosener D. Prevalence and diversity of toxigenic *Clostridium perfringens* and *Clostridium difficile* among swine herds in the Midwest. Appl Environ Microbiol 2010;76:2961–7.
57. Farzan A, Kircanski J, DeLay J, Soltes G, Songer JG, Friendship R, Prescott JF. An investigation into the association between cpb2-encoding Clostridium perfringens type A and diarrhea in neonatal piglets. Can J Vet Res 2013;77:45–53.
58. Mika A, Day HE, Martinez A, Rumian NL, Greenwood BN, Chichlowski M, Berg BM, Fleshner M. Early life diets with prebiotics and bioactive milk fractions attenuate the impact of stress on learned helplessness behaviours and alter gene expression within neural circuits important for stress resistance. Eur J Neurosci 2017;45:342–57.
59. Hoeflinger JL, Kashtanov DO, Cox SB, Dowd SE, Jouni ZE, Donovan SM, Miller MJ. Characterization of the intestinal lactobacilli community following galactooligosaccharides and polydextrose supplementation in the neonatal piglet. PLoS One 2015;10(8):e0135494.
60. Tarr AJ, Galley JD, Fisher SE, Chichlowski M, Berg BM, Bailey MT. The prebiotics 3′sialyllactose and 6′sialyllactose diminish stressor-induced anxiety-like behavior and colonic microbiota alterations: evidence for effects on the gut-brain axis. Brain Behav Immun 2015;50:166–77.
61. Vester Boler BM, Rossoni Serao MC, Faber TA, Bauer LL, Chow J, Murphy MR, Fahey GC Jr. In vitro fermentation characteristics of select nondigestible oligosaccharides by infant fecal inocula. J Agric Food Chem 2013;61:2109–19.
62. Marriage BJ, Buck RH, Goehring KC, Oliver JS, Williams JA. Infants fed a lower calorie formula with 2′FL show growth and 2′FL uptake like breast-fed infants. J Pediatr Gastroenterol Nutr 2015;61:649–58.