Research Article

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Effects of 2'-fucosyllactose on the composition and metabolic activity of intestinal microbiota from piglets after *in vitro* fermentation

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Abstract

Background: As indigestible carbohydrates, milk oligosaccharides possess various benefits for newborns, mainly through intestinal microbiota, among which 2'-fucosyllactose (2'-FL) is the most predominant milk oligosaccharide. However, knowledge about the fermentative characteristics of 2'-FL in the gut remains limited, especially in the small intestine. The aim of this study is to explore the differential fermentability of 2'-FL by the small and large intestinal microbiota of piglets using fructo-oligosaccharide (FOS) and lactose as controls in an *in vitro* batch fermentation experiment. During fermentation, microbial composition was characterized along with gas production and short-chain fatty acid production.

Results: 2'-Fucosyllactose showed differential fermentability in jejunal and colonic fermentation. Compared with the colon, 2'-FL produced less gas in the jejunum than in the FOS and lactose groups ($P < 0.05$). Meanwhile, 2'-FL exhibited a different influence on the microbial composition and metabolism in the jejunum and colon compared with FOS and lactose. In the jejunum, compared with the FOS and lactose groups, the 2'-FL group showed a higher abundance of *Bacteroides*, *Prevotella*, and *Blautia,* but a lower abundance of *Streptococcus* and *Lactobacillus* ($P < 0.05$), with a higher level of propionate and a lower level of lactate during fermentation ($P < 0.05$). In the colon, compared with the FOS and lactose groups, 2'-FL increased the abundance of *Blautia*, *Faecalibacterium*, and *Lachnospiraceae FCS020,* but decreased the abundance of *Prevotella_9*, *Succinivibrio*, and *Megasphaera* ($P < 0.05$) with an increase in acetate production ($P < 0.05$).

Conclusion: Overall, the results suggested that the small intestinal microbiota had the potential to ferment milk oligosaccharides. Meanwhile, in comparison with FOS and lactose, 2′-FL selectively stimulated the growth of propionate-producing bacteria in the jejunum and acetate-producing bacteria in the colon. These results demonstrated the differences in fermentation properties of 2'-FL by small and large intestinal microbiota and provided new evidence for the application of 2'-FL in optimizing gut microbiota.

Supporting information may be found in the online version of this article.

Keywords: 2'-fucosyllactose; gut microbiota; metabolites; *in vitro* fermentation; pig

INTRODUCTION

Breast milk is an essential nutritional source for neonates; it contains various proteins, lipids, and digestible and indigestible carbohydrates, which contribute to growth and development, and in particular to immune system development and intestinal microbial colonization.[1] Milk oligosaccharides are the predominant indigestible carbohydrates and are regarded as the third most abundant solid milk component.[2] Milk oligosaccharides, as bioactive components, have recently attracted increasing attention due to their prebiotic effects, such as encouraging gut microbiota, enhancing the mucosal immune system, and protecting the intestinal epithelial barrier function.[3,4] However, knowledge about fermentation of milk oligosaccharides by gut microbiota is still limited.

2'-Fucosyllactose (2'-FL), composed of lactose and fucose through an α-(1,2)-linkage, is a trisaccharide and the most abundant milk oligosaccharide, with a mean concentration of

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2.38 gL^{-1} in breast milk.[5,6] It has been reported to play a crucial role in providing benefits to breastfed newborns.[7] For instance, 2'-FL as a supplement in *in vitro* fermentation could stimulate the growth of *Bifidobacterium*, promote short-chain fatty acids (SCFAs) and lactate production, and inhibit the growth of *Escherichia coli* and *Clostridium perfringens*.[8] In mice, 2'-FL could ameliorate chemotherapy-induced intestinal mucositis by protecting intestinal epithelial cells against apoptosis.[9] A recent study has indicated that supplementation with 2'-FL could promote cognitive and structural brain development in young piglets.[10] Overall, these studies hinted that 2'-FL could be used to modulate gut microbiota and maintain gut health in pig production. Generally, 2'-FL cannot be degraded by the enzymes secreted by the host but can be utilized by the large intestinal microbiota. To date, most *in vitro* and *in vivo* studies have focused only on the effects of 2'-FL on the composition and metabolism of large intestinal microbiota.[11-13] Interestingly, accumulating evidence suggests that some specific non-digestible carbohydrates might be partially degraded in the small intestine,[14,15] such as pectin and galactooligosaccharide (GOS). Previous studies have reported that the abundant microbial population in the foregut[16,17] provides a base for the degradation of indigestible carbohydrates. However, small intestinal microbiota sampling is inconvenient, especially in humans, so knowledge of the capacity of small intestinal microbiota to ferment 2'-FL is very limited.

Organic acids, including SCFAs and lactate, are the most important products of microbial carbohydrate fermentation and are beneficial to intestinal physiological functions.[18] For instance, propionate acts as a precursor for liver gluconeogenesis,[19] butyrate provides energy for the proliferation and differentiation of colonocytes and exerts an anti-inflammation effect,[20] and microbiota-derived lactate accelerates intestinal stem cell-mediated epithelial development in a Wnt3/β-catenin-dependent manner.[21] Short-chain fatty acid concentration is associated with the structures and components of carbohydrates and gut microbial variation.[22,23] Abbeele *et al.* found that the fermentation of 2'-FL by fecal microbiota *in vitro* strongly increased acetate production with the increase in propionate and butyrate concentrations in comparison with lactose.[24] However, the acid-producing characteristics of 2'-FL fermentation by small intestinal microbiota are still unexplored.

Fiber fermentability is often evaluated using *in vitro* fermentation methods due to the advantages of *in vitro* methods over *in vivo* experiments, such as the experimental protocols being relatively simple, and the variables being easy to control. The present study therefore aimed to investigate the fermentability of 2'-FL by the small and large intestinal microbiota and to reveal the effect of 2'-FL fermentation properties on the microbial community and on metabolism. In this *in vitro* batch fermentation experiment, fructo-oligosaccharide (FOS) and lactose, common prebiotics[25] were used as the positive control groups, and jejunal and colonic microbiota from pigs were used as the inoculum to ferment 2'-FL, respectively. In addition to the cumulative gas production pH value, the microbial community and metabolite profiles were monitored during fermentation. These findings provide new insights into differential fermentation characteristics of milk oligosaccharides 2'-FL by the foregut and hindgut microbiota.

MATERIAL AND METHODS

Substrates

All the substrates used in this study were of commercial-grade level products. 2'-Fucosyllactose (purity ≥ 90%) was purchased from Shanghai Sharing Technologies Co., Ltd (Shanghai, China). Fructo-oligosaccharide was purchased from Beijing Houjia Biotechnology Co., Ltd (Beijing, China) (purity ≥ 90%; degree of polymerization: 2–7), consisting of fructose monomers linked to a terminal glucose residue via a β (2-1) glycosidic bond. Lactose (purity ≥ 99%) was purchased from Shanghai Yuanye Biotechnology Co., Ltd. One gram of FOS, lactose, or 2'-FL was dissolved in 10 mL sterile deionized water, respectively, to prepare the substrate solution. The final concentration was 1% (w/v).

Inoculum and medium preparation

The jejunal and colonic inoculums were prepared from three healthy weaned pigs (aged 42 days). The pigs were fed with a corn-soybean type commercial diet and had no antibiotics during the previous 1 month. After slaughtering, the jejunal and colonic digesta were immediately collected and stored in a sterile plastic bag pre-filled with CO_2 (Aladdin Co. Ltd, Beijing, China), respectively. After about 10 min, the digesta was transferred to the laboratory. The sample from each pig was mixed in equal proportions and then accurately weighed 200 g of the jejunal and colonic digesta by a balance (Kern PCB Z742836, weighing capacity 6000 g, accuracy: 1.0 g, precision: ±2.0 g), and was then diluted with 0.9 and 1.8 L of sterile anaerobic saline, respectively. After homogenization, the diluted mixture was filtered through four layers of sterile gauze (pore size: 150 mesh; Biosharp Co. Ltd, Heze, China) as the jejunal and colonic inoculum, respectively. All the procedures were conducted within 1 h in the anaerobic workstation (GeneScience E500, Wilmington, USA) under anaerobic conditions.

The fermentation medium was prepared according to the previously reported methods.[26,27] Briefly, the medium consisted of the basal solution, vitamin solution, bicarbonate buffer, and reducing agent solution. A liter of the basal solution contained the following components: 0.6 g KCl, 0.6 g NaCl, 0.2 g $CaCl_2{\cdot}2H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 1.46 g KH_2PO_4, 3.55 g Na_2HPO_4, 1.0 g trypticase, 0.54 g NH_4Cl, 10 mL trace elements, 10 mL hemin, 1 mL resazurin, 50 mL $NaHCO_3$, 1 mL vitamin phosphate, and 1 g cysteine-HCl. Anaerobic conditions were maintained by flushing the liquids with CO_2, and the pH value of the medium was adjusted to 6.8 ~ 7.0 with 1 $molL^{-1}$ NaOH or 1 $molL^{-1}$ HCl solution.

In vitro batch fermentation trial

The fermentation system consisted of 80 mL of the medium, 10 mL inoculum, and 10 mL fiber substrate solution. The mixture was added to the 200 mL serum bottle fitted with a butyl rubber stopper and an aluminum crimp seal, and it was then incubated at 37 °C and shaken at 100 rpm. A blank group without any fiber was used to control for background fermentation. The FOS and lactose groups were used as positive controls ($n = 4$), and the 2'-FL group was used as the treatment group ($n = 4$). The gas production at 0, 6, 12, 24, and 48 h was measured during fermentation following a method that was previously reported by Li *et al.*[28] Briefly, gas production was determined using a pressure transducer, which measured the levels of gas production at the top surface of the serum bottles with a 50 mL sterile injector. Gas production was recorded every 6 h, and then the gas was released to bring the air pressure within the bottle to 0 to facilitate the determination of gas production at the next time point. The volumes of gas production at each time point were added to determine the cumulative gas production. After fermenting for 0, 6, 12, 24, and 48 h, respectively, the serum bottle was placed on ice to stop fermentation, then 10 mL fermentation broth was collected by a 20 mL sterile injector under anaerobic conditions, and the pH value of

the fermentation broth was measured by a pH meter (Orion Star A321, ThermoFisher Scientific, Waltham, MA, USA). Finally, the samples were stored at −80 °C until analysis.

Short-chain fatty acid measurement

The SCFAs were measured according to a method that was reported previously.[29] Briefly, 1 mL of the fermentation broth sample was mixed with 200 μL of 25% metaphosphoric acid-crotonic acid and stored at −20 °C overnight. The mixture was centrifuged at 12 000 × *g* for 10 min after thawing, and the supernatant was filtered through an aqueous filter membrane (0.22 μm; Jinteng, Tianjin, China). Later, the filtered supernatant was injected into a GC-14B gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a Nukol capillary column (30 m × 0.32 mm × 0.25 μm) (Sigma Co. Ltd, St. Louis, MO, USA). The run conditions were as follows: Nitrogen was used as carrier gas at a flow rate of 0.8 $mLmin^{-1}$. The injector temperature was 220 °C; the detector temperature was 210 °C, and the oven temperature was 150 °C.

Lactate measurement

Lactate concentrations were determined using Ultimate 3000 high-performance liquid chromatography (HPLC) (ThermoFisher Scientific, Waltham, MA, USA) coupled to a UV detector. Briefly, 0.6 mL of 50% metaphosphoric acid was mixed with 0.6 mL fermentation broth and stored at −20 °C overnight. After thawing, the mixture was centrifuged at 12 000 × *g* for 15 min. The supernatant was filtered through an aqueous filter membrane (0.22 μm) (Jinteng) and injected into HPLC. Chromatographic column: Zorbax SB-Aq 80 A (4.6 × 150 mm, 5 μm) (ThermoFisher Scientific, Waltham, MA, USA); mobile phase A: 20 mM KH_2PO_4 buffer; mobile phase B: acetonitrile; flow rate: 0.5 $mLmin^{-1}$; detection wavelength: 210 nm; injection volume: 20 μL; column temperature: 25 °C.

DNA extraction, MiSeq sequencing, and data processing

The total microbial genomic DNA was extracted from the fermentation broth following the method reported by Dai *et al.*[27] Briefly, 3 mL fermentation broth was added to a 2 mL sterile tube, centrifuging at 9000 × *g* for 5 min at 4 °C, and discarding the supernatant and collecting bacterial cells. The total genomic DNA of bacterial cells was extracted using the bead-beating and phenol-chloroform extraction method as described in a previous study.[30] After phenol-chloroform extraction, DNA was precipitated with 75% ethanol and re-suspended in 50 μL of Tris-HCl/Ethylene Diamine Tetraacetic acid (EDTA), buffer, and stored at −80 °C until further processing.

The V3-V4 regions of the 16S rRNA gene were amplified using the universal primers: 338 Forward: 5′-barcode-ACTCCTACGGGAGGCAGCAG-3′ and 806 Reverse: 5′-GGACTACHVGGGTWTCTAAT-3′, where 'barcode' is an eight-base sequence unique to each sample.[31] The amplicons were purified using a DNA gel extraction kit according to the manufacturer's instructions (Thermo Fisher Scientific). The purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 250) on an Illumina MiSeq platform (Biozeron Co. Ltd, Shanghai, China) according to standard protocols.[32] The minimum number of sequencing data was 30 000 reads per sample.

The raw sequence data generated from 16S rRNA MiSeq sequencing were demultiplexed and quality-filtered using QIIME2.[33] The 250 bp reads were truncated at any site receiving an average quality score less than 20 over a 10 bp sliding window, discarding the truncated reads that were shorter than 50 bp. Reads with exact barcode matching, two nucleotide mismatch in primer matching, or containing ambiguous characters were removed. Only sequences that overlapped longer than 10 bp were assembled according to their overlap sequence. Reads that could not be assembled were discarded. The high-quality sequences were clustered into operational taxonomic units (OTUs) with 97% similarity cut-off using UPARSE (version 7.1, http://drive5.com/uparse/),[34] and chimeric sequences were identified and removed using UCHIME.[35] The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed by Ribosomal Database Project (RDP) Classifier against the silva (SSU119) 16S rRNA database using a confidence threshold of 70%.[36] Microbial diversity (including Shannon diversity indices and ACE richness estimators) and the principal coordinate analysis (PCoA) based on the Bray–Curtis distance were conducted by using an online tool, microbiomeAnalyst (https://www.microbiomeanalyst.ca).[37]

Statistical analysis

The data were analyzed using SPSS Statistics 20.0 software (SPSS Inc., Chicago, IL, USA). The differences in the microbial diversity indexes and bacterial abundance (at the phylum, genus, and OTU levels) among different groups at the same time point were analyzed by the Kruskal–Wallis analysis of variance (ANOVA) performed on ranks for non-parametric data with the Benjamini–Hochberg false discovery rate (FDR) multiple-testing correction.[38] The data for accumulative gas production, pH value, and microbial metabolite concentrations were analyzed by the Bonferroni *post hoc* test for parametric data using a one-way ANOVA, and $P < 0.05$ was considered statistically significant. Data were expressed as means ± standard errors of the mean (SEMs).

RESULTS

Gas production

Cumulative gas production was determined at 0, 6, 12, 24, and 48 h during *in vitro* fermentation. Compared with the FOS and lactose groups, the cumulative gas production in the 2'-FL group was the lowest during the whole *in vitro* fermentation by the jejunal inoculum ($P < 0.05$, Fig. 1A), and the cumulative gas was higher in the lactose group than FOS group at 6 and 12 h of fermentation ($P < 0.05$, Fig. 1A). Similar results were observed during *in vitro* fermentation by the colonic inoculum, and the cumulative gas in the lactose group was higher than FOS group ($P < 0.05$, Fig. 1B). Moreover, the blank group showed the lowest gas production both in the jejunal and colonic fermentation *in vitro* (Fig. 1).

pH value and organic acids production

In the jejunal fermentation broth, the pH value of the fermentation broth in the 2'-FL group was the highest among the three groups ($P < 0.05$, Fig. 2A). Meanwhile, the pH value of the fermentation broth in the FOS group was higher than the lactose group at 6,12, and 24 h during *in vitro* fermentation ($P < 0.05$, Fig. 2A). As for organic acid production in the jejunal fermentation broth, the acetate, butyrate, total SCFA, and lactate were higher in the lactose and FOS groups than the in 2'-FL group ($P < 0.05$, Fig. 2B,D–F), while propionate was higher in the 2'-FL group than the FOS and lactose groups ($P < 0.05$, Fig. 2C) during *in vitro* fermentation. In the colonic fermentation broth, the 2'-FL group had the highest pH value at 6 and 12 h of fermentation and the lowest pH value at 48 h among the three groups except for the blank group ($P < 0.05$, Fig. 2G). As for the organic acids production in the colonic fermentation broth, the acetate, propionate, butyrate, and total SCFA concentrations

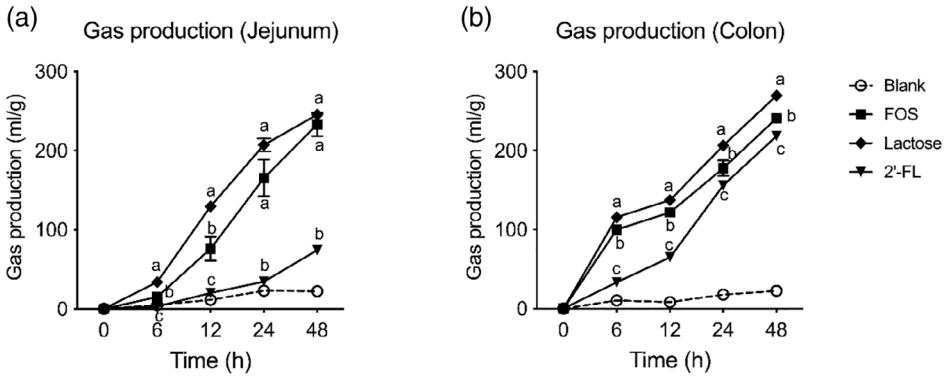

Figure 1. Cumulative gas production during *in vitro* fermentation. (a) Gas production in the jejunum. (b) Gas production in the colon. The data were expressed as means ± SEMs ($n = 4$). The different superscript letters (a–c) at the same time point (6, 12, 24, or 48 h) indicated significant differences among groups except the blank group ($P < 0.05$). FOS, fructo-oligosaccharides. 2'-FL, 2'-fucosyllactose.

were higher in both FOS and lactose groups than in the 2'-FL group at 6 and 12 h of fermentation ($P < 0.05$, Fig. 2H–K). At 24 and 48 h of fermentation, the 2'-FL group had the highest acetate and total SCFA concentrations among the three groups ($P < 0.05$, Fig. 2H,K); the FOS group had the highest propionate level compared with both the lactose group and 2'-FL group ($P < 0.05$, Fig. 2I); for butyrate, the lactose group had the higher level than the FOS group, and the lowest level was in the 2'-FL group ($P < 0.05$, Fig. 2J). The lactate concentration in the 2'-FL group was lower than in the FOS and lactose groups at 6 h of fermentation ($P < 0.05$, Fig. 2L). Thus, these results indicated that 2'-FL might be fermented by different jejunal and colonic bacteria to yield organic acids compared with FOS and lactose.

Microbial community structure and composition

The changes in the microbial community in the fermentation broth among the three groups during *in vitro* fermentation were determined using the 16S rRNA gene high-throughput sequencing. After sequencing, a total of 1 568 310 and 1 568 983 reads were obtained from all the samples from the jejunal and colonic fermentation broth, respectively. For the jejunal sequencing data, the numbers of minimum, maximum, and average reads are 29 559, 30 197, and 30 159 per sample, respectively. For the colonic sequencing data, the numbers of minimum, maximum and average reads are 30 133, 30 190, and 30 159 per sample, respectively. The rarefaction curves of all the samples showed an approached plateau (Fig. S1), suggesting that the obtained reads were enough for further analysis. During *in vitro* jejunal fermentation, the abundance-based coverage estimator (ACE) index of the microbiota was higher in the 2'-FL group than in the FOS and lactose groups at 12, 24, and 48 h ($P < 0.05$, Fig. 3A). Meanwhile, the 2'-FL group had a higher Shannon index than in the FOS and lactose groups at 6, 12, 24, and 48 h of fermentation ($P < 0.05$, Fig. 3B). Moreover, the PCoA based on Bray–Curtis distance showed a significant cluster between 2'-FL group and the other groups in microbial composition (Fig. 3C). During *in vitro* colonic fermentation, at 12 h, the ACE index of the microbiota was higher in lactose group than the FOS group ($P < 0.05$, Fig. 3D); at 48 h, the 2'-FL and lactose groups showed higher ACE index of the microbiota compared with the FOS group ($P < 0.05$, Fig. 3D). Meanwhile, the 2'-FL group had a higher Shannon index than FOS and lactose groups both at 6 and 12 h of fermentation ($P < 0.05$, Fig. 3E). The PCoA based on Bray-Curtis distance also showed a significant separation of microbial communities between the 2'-FL group and the other groups (Fig. 3F). Overall, these results revealed that, compared with FOS and lactose, 2'-FL had a differential impact on the jejunal and colonic microbial structure during *in vitro* fermentation.

The relative abundances of bacteria at the phylum and genus levels were analyzed to explore the changes in microbial composition during *in vitro* fermentation further. At the phylum level, a total of five phyla were detected in the jejunal fermentation broth (Fig. 4A) and six phyla were detected in the colonic fermentation broth (Fig. 4C). During *in vitro* jejunal fermentation, Firmicutes and Proteobacteria were the dominant bacterial groups among the phyla that were detected (Fig. 4A), with lower abundance of Firmicutes (17.30 ± 1.63% at 6 h, 28.57 ± 3.72% at 12 h, 23.85 ± 1.78% at 24 h, and 22.04 ± 4.51% at 48 h of fermentation) and higher abundance of Proteobacteria (82.54 ± 1.61% at 6 h, 70.29 ± 3.48% at 12 h, 55.28 ± 3.82% at 24 h, and 30.47 ± 2.87% at 48 h of fermentation) in the 2'-FL group compared with lactose groups ($P < 0.05$, Fig. 5A). Additionally, the 2'-FL group had a higher abundance of Bacteroidota (0.66 ± 0.14% at 12 h, 11.65 ± 2.53% at 24 h, and 31.70 ± 2.75% at 48 h of fermentation) and Fusobacteriota (0.06 ± 0.03% at 6 h, 0.38 ± 0.13% at 12 h, 8.75 ± 1.59% at 24 h, and 11.11 ± 2.26% at 48 h of fermentation), and a lower abundance of Actinobacteriota (0.05 ± 0.02% at 6 h, 0.08 ± 0.02% at 12 h, 0.47 ± 0.24% at 24 h, and 4.66 ± 2.42% at 48 h of fermentation) in comparison with the FOS and lactose groups ($P < 0.05$, Fig. 5A). During *in vitro* colonic fermentation, Firmicutes and Bacteroidota were the two dominant phyla in the 2'-FL group compared with the FOS and lactose groups, accounting for 95% of the total sequences (Fig. 4C), with a higher abundance of Firmicutes (43.32 ± 5.56% at 6 h, 50.05 ± 3.92% at 12 h, and 52.62 ± 6.89% at 24 h of fermentation) and lower abundance of Bacteroidota (53.40 ± 5.64% at 6 h, 46.11 ± 3.63% at 12 h, and 43.61 ± 7.53% at 24 h of fermentation) and Proteobacteria (1.06 ± 0.11% at 6 h, 1.16 ± 0.12% at 12 h, 1.50 ± 0.51% at 24 h, and 1.51 ± 0.31% at 48 h of fermentation) ($P < 0.05$, Fig. 5B).

The relative abundance of the top 20 genera in the jejunal and colonic fermentation broth at the genus level is depicted in Fig. 4B, D, respectively. During *in vitro* jejunal fermentation, *Clostridium sensu stricto 1* (21.29%), *Streptococcus* (18.49%), and *Citrobacter* (17.55%) were the predominant genera in the three groups (Fig. 4B). The differential analysis showed that compared with the FOS group or lactose group, the 2'-FL group had a higher relative abundance of *Citrobacter* (41.34 ± 0.81% at 6 h, 37.12 ± 1.41% at 12 h, 24.94 ± 2.59% at 24 h, and 17.34 ± 2.63% at

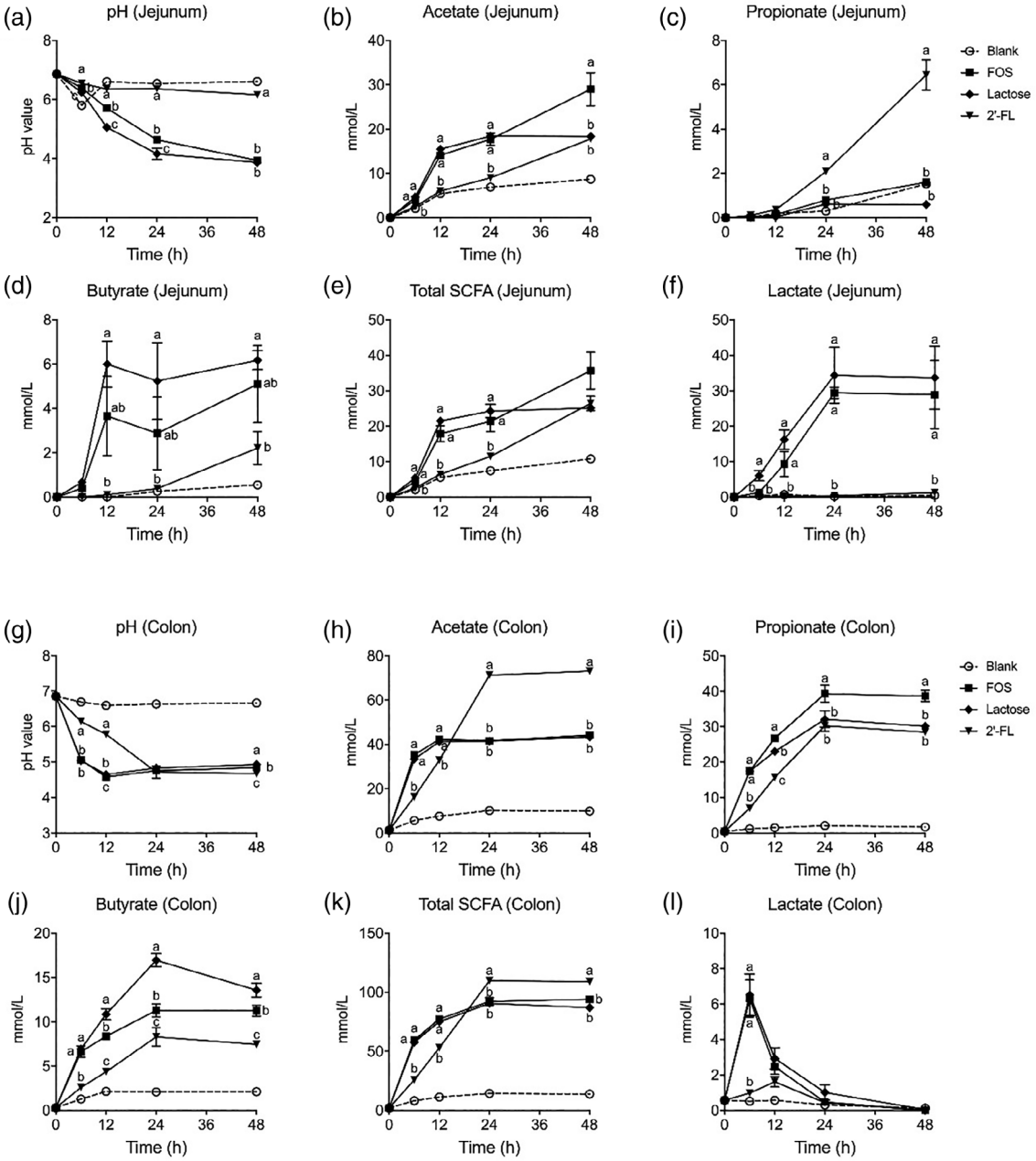

Figure 2. The pH value, and SCFA and lactate production during *in vitro* fermentation: (a) pH of jejunal fermentation broth; (b–e) SCFA concentration in jejunal fermentation broth; (f) Lactate concentration in jejunal fermentation broth; (g) pH of colonic fermentation broth. (h–k) SCFA concentration in colonic fermentation broth. (l) Lactate concentration in colonic fermentation broth. The data are expressed as means ± SEMs ($n = 4$). The different superscript letters (a–c) at the same time point (6, 12, 24, or 48 h) indicated significant differences among groups except the blank group ($P < 0.05$). SCFA, short-chain fatty acids. FOS, fructo-oligosaccharides. 2'-FL, 2'-fucosyllactose.

48 h of fermentation), *Klebsiella* (15.80 ± 0.75% at 6 h, 7.71 ± 0.88% at 24 h, and 4.45 ± 0.38% at 48 h of fermentation), *Escherichia-Shigella* (7.73 ± 0.88% at 6 h, 8.30 ± 1.28% at 12 h, 9.83 ± 3.65% at 24 h, and 3.67 ± 0.48% at 48 h of fermentation), *Fusobacterium* (0.06 ± 0.04% at 6 h, 0.37 ± 0.12% at 12 h, 8.74 ± 1.58% at 24 h, and 11.1 ± 2.26% at 48 h of fermentation), *Bacteroides* (2.85 ± 0.79% at 24 h and 15.95 ± 3.35% at 48 h of fermentation), *Prevotella* (0.13 ± 0.03% at 12 h, 1.93 ± 1.20% at 24 h, and 12.45 ± 2.97% at 48 h of fermentation), and *Blautia* (0.06 ± 0.03% at 12 h, 0.91 ± 0.57% at 24 h, and 8.20 ± 1.83% at 48 h of fermentation), but a lower relative abundance of *Streptococcus* (4.22 ± 0.15% at 6 h, 1.52 ± 0.66% at 12 h, 1.50 ± 0.28% at 24 h, and 0.59 ± 0.16% at 48 h of fermentation) and *Lactobacillus* (0.35 ± 0.17% at 6 h, 0.33 ± 0.08% at 24 h, and 0.10 ± 0.04% at 48 h of fermentation) ($P < 0.05$, Fig. 6A). During *in vitro* colonic fermentation, the primary dominant genera in the three groups were *Prevotella_9* (43.59%), *Blautia* (8.17%), and *Prevotella* (7.18%) (Fig. 4D), and *Prevotella_9* and *Prevotella* are reported to be two different species at the moment.[39] Meanwhile, in comparison with the FOS or lactose group, the relative abundance of *Blautia* (10.56 ± 1.76% at 6 h, 13.87 ± 1.82% at 12 h, 19.29 ± 4.69% at 24 h, and 19.89 ± 4.06% at 48 h of fermentation), *Roseburia* (5.77 ± 0.95% at 6 h, 6.68 ± 0.74% at 12 h, and 3.58 ± 0.89% at 48 h of fermentation), *Faecalibacterium* (4.41 ± 0.67% at 6 h and

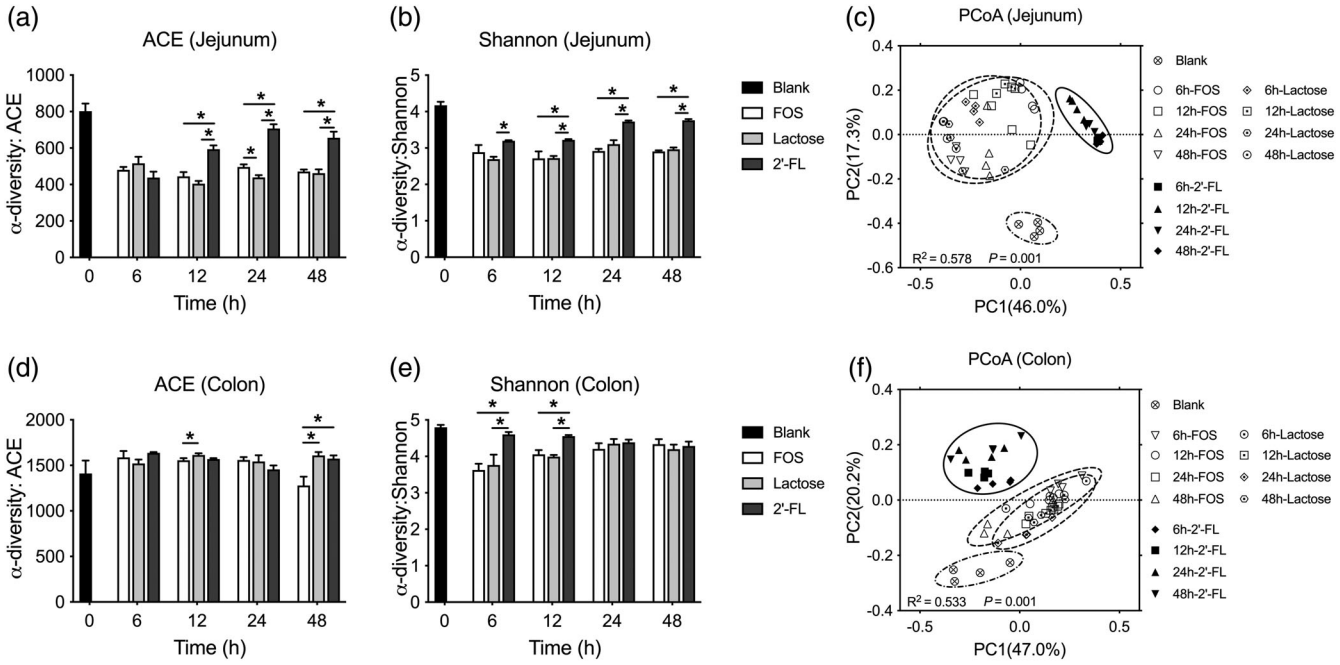

Figure 3. The microbial community structure in fermentation broth. Alpha diversity: ACE (a) and Shannon index (b) in jejunal fermentation broth. The data are expressed as means ± SEMs ($n = 4$), $^*P < 0.05$. (c) The principal coordinate analysis (PCoA) of microbiota is based on the Bray–Curtis distance in jejunal fermentation broth. Circles with solid or dashed lines indicate a significant distinction between the 2'-FL group and the FOS or lactose group using permutational multivariate analysis of variance (PERMANOVA) ($P < 0.05$). The alpha diversity: ACE (d) and Shannon index (e) in colonic fermentation broth. The data were expressed as means ± SEMs ($n = 4$), $^*P < 0.05$. (f) The PCoA of microbiota based on the Bray–Curtis distance in colonic fermentation broth. Circles with solid or dashed lines indicate a significant distinction between the 2'-FL group and the FOS or the lactose group using permutational multivariate analysis of variance (PERMANOVA) analysis ($P < 0.05$). FOS, fructo-oligosaccharides. 2'-FL, 2'-fucosyllactose.

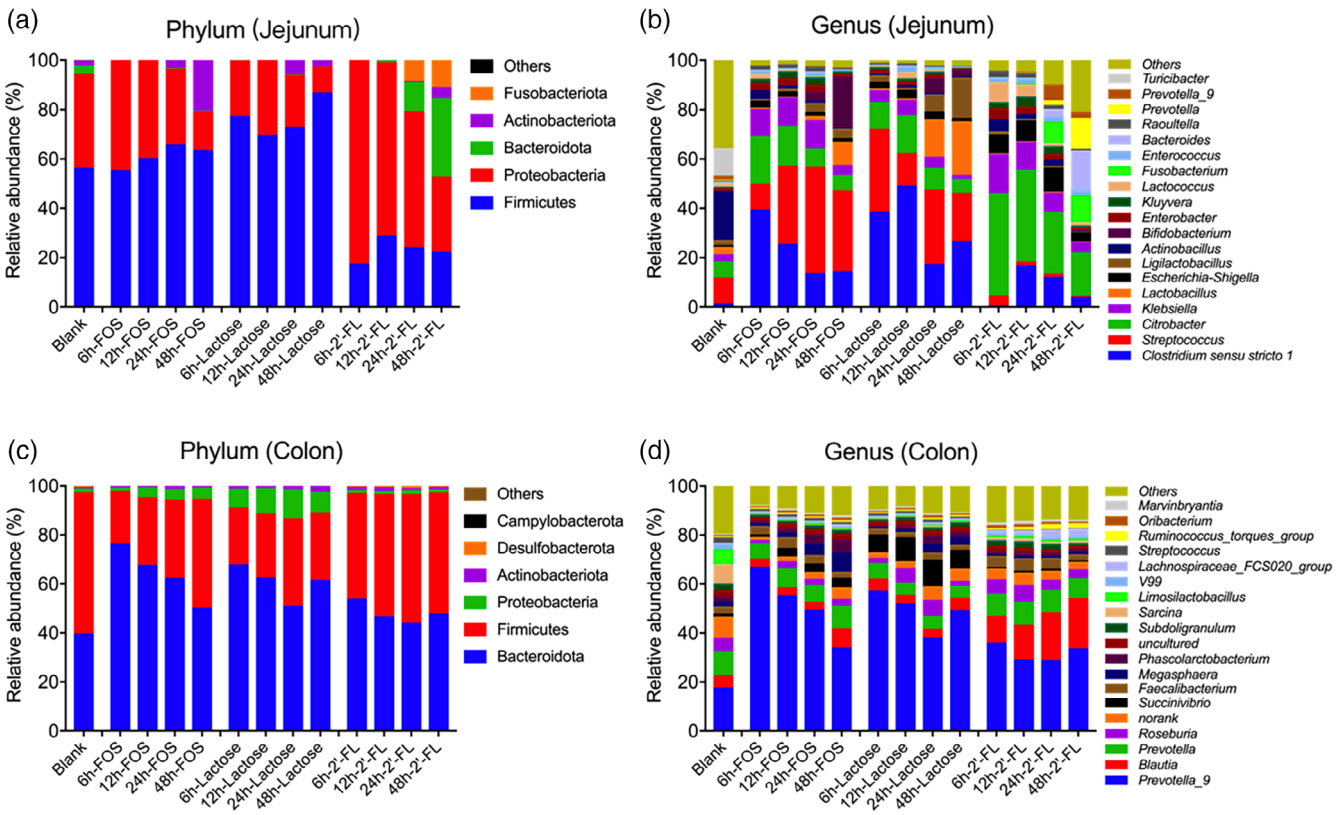

Figure 4. The microbiota composition at the phylum and genus levels in fermentation broth, respectively. The relative abundance of bacteria at the phylum (a) and genus (b) levels in jejunal fermentation broth. The relative abundance of bacteria at the phylum (c) and genus (d) levels in colonic fermentation broth. The data were expressed as means. FOS, fructo-oligosaccharides. 2'-FL, 2'-fucosyllactose.

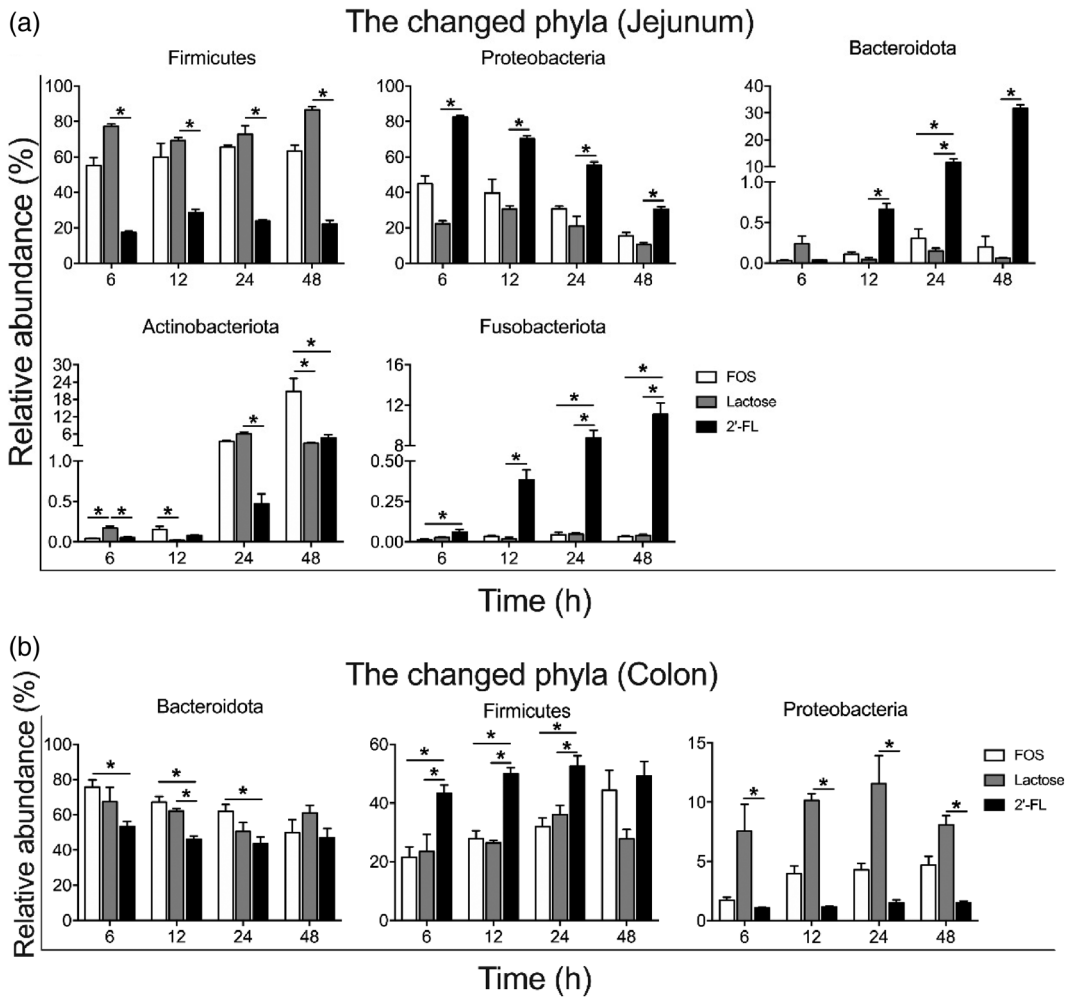

Figure 5. The significantly changed phyla in the jejunal (a) and colonic (b) fermentation broth. The data are expressed as means ± SEMs ($n = 4$), *$P < 0.05$. FOS, fructo-oligosaccharides. 2'-FL, 2′-fucosyllactose.

4.96 ± 0.56% at 12 h of fermentation), *Subdoligranulum* (2.26 ± 0.18% at 6 h and 2.76 ± 1.23% at 12 h of fermentation), and *Lachnospiraceae FCS020 group* (0.87 ± 0.08% at 6 h, 1.13 ± 0.16% at 12 h, 2.81 ± 1.33% at 24 h, and 2.82 ± 0.92% at 48 h of fermentation) was higher, whereas the relative abundance of *Prevotella_9* (35.44 ± 6.72% at 6 h, 28.53 ± 3.4% at 12 h, and 28.18 ± 8.97% at 24 h of fermentation), *Succinivibrio* (0.53 ± 0.07% at 6 h, 0.69 ± 0.08% at 12 h, 0.69 ± 0.08% at 24 h, and 0.4 ± 0.09% at 48 h of fermentation), *Megasphaera* (0.68 ± 0.09% at 12 h, 0.87 ± 0.2% at 24 h, and 0.84 ± 0.52% at 48 h of fermentation), and *Streptococcus* (0.21 ± 0.03% at 6 h, 0.26 ± 0.03% at 12 h, 0.23 ± 0.1% at 24 h, and 0.25 ± 0.06% at 48 h of fermentation) were lower in the 2'-FL group ($P < 0.05$, Fig. 6B).

DISCUSSION

Breast milk containing indigestible carbohydrates, like milk oligosaccharides, is considered to be an important source of nutrition for neonates, providing unique benefits to promote the colonization of beneficial bacteria and to improve intestinal health and host immunity.[40] 2'-Fucosyllactose is the most abundant fucosyloligosaccharide, comprising more than 30% of the total human milk oligosaccharides (HMOs).[6] In this study, lactose and FOS were used as control groups to evaluate the effects of 2'-FL on the microbial community and metabolites in the jejunum and colon of pigs *in vitro*. The results showed that the gas and SCFA production had no distinct increase in the blank group during the fermentation, suggesting that fiber or carbohydrates were the main substrates to drive the fermentation, and also that the fiber types may be the primary factors to impact the characteristics of fermentation. In comparison with lactose and FOS, 2'-FL altered the microbial composition and metabolism by increasing different types of acid-producing bacteria and SCFA selectively in the jejunum and colon. Moreover, the fermentation efficiency of 2'-FL was lower than lactose and FOS in the jejunum and colon. For the fermentation of FOS and lactose, they showed similar results on the microbial composition and metabolism, which may be attributed to the similar chemical composition and structure. Overall, these results provided insights into the fermentation characteristics of 2'-FL by the intestinal microbiota of pigs *in vitro*.

Cumulative gas production is one of the indicators to evaluate the substrate fermentation efficiency by bacteria. In the present study, the cumulative gas produced by 2'-FL fermentation was lower than lactose and FOS in the jejunum and colon. These findings were consistent with results of the previous study, which reported that the total gas production of 2'-FL fermentation was lower than FOS and lactose fermentation by preterm infant fecal microbiota during the first 4 weeks of life,[41] indicating that 2'-FL

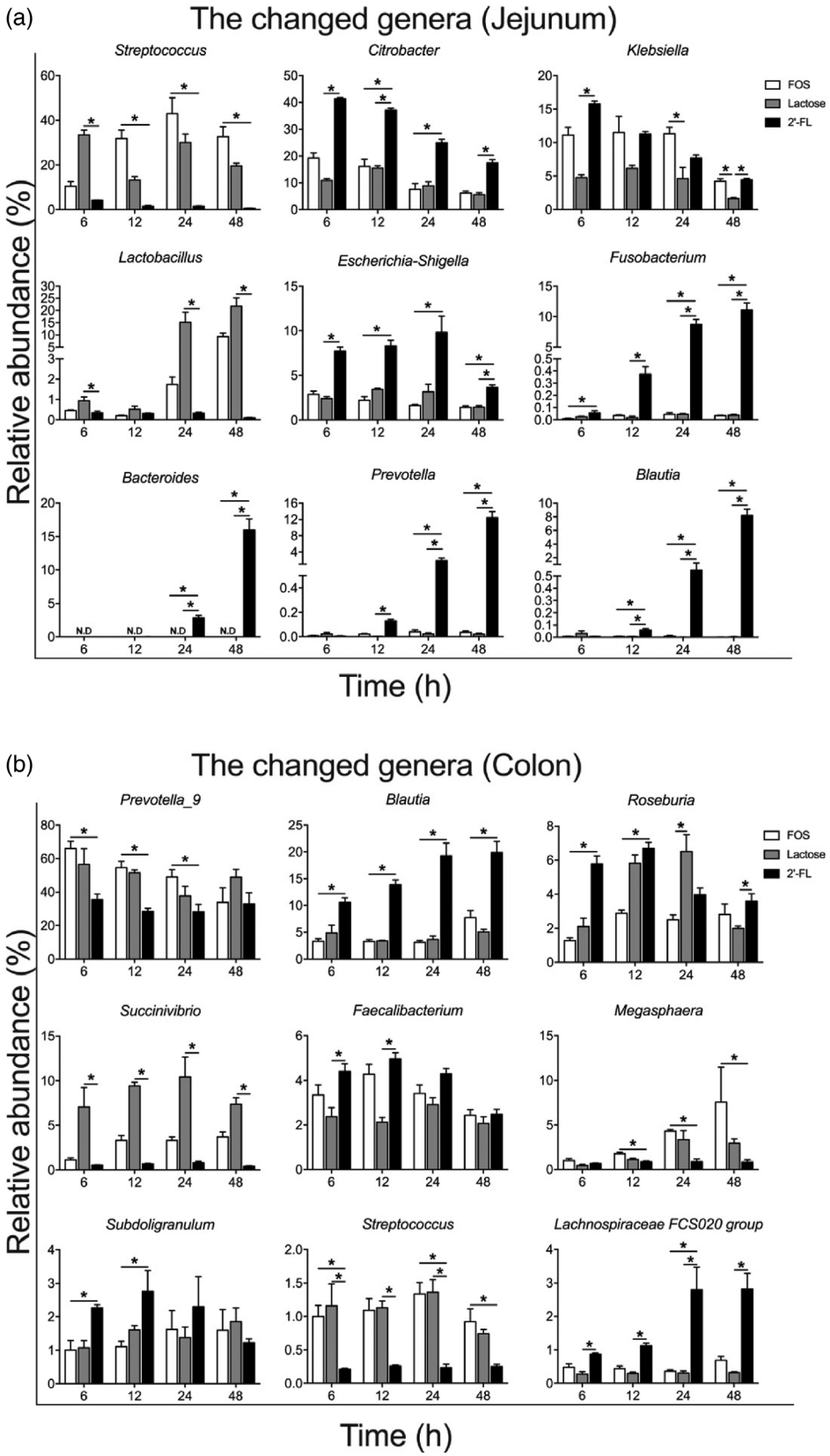

Figure 6. The significantly changed genera in the jejunal (a) and colonic (b) fermentation broth. The data are expressed as means ± SEMs ($n = 4$), *$P < 0.05$. FOS, fructo-oligosaccharides. 2'-FL, 2'-fucosyllactose.

could not be utilized easily by bacteria in comparison with lactose and FOS due to different chemical composition and structure among the three substrates, such as monosaccharide types and glycosidic bond.[42] However, a recent study has reported no significant differences in gas production among different types of HMOs fermented by infant fecal inoculum.[43] These results indicated that the fermentation efficiency of HMOs was associated with other factors, such as microbial composition. It is well known that, in the small intestine of growing pigs, especially in the jejunum, *Lactobacillus* is the most dominant genus, and the number of *Bifidobacteria* is low. It has been reported that *Bifidobacterium* spp. could utilize 2'-FL,[44] and *Lactobacillus* spp. could directly uptake FOS and lactose,[45,46] which also explained these results in gas production. Compared with the jejunum, the gas production in the colonic inoculum also increased, which might be due to the differences in population and species of bacteria in the small and large intestines, as microbes in the colon have a higher density and release diverse carbohydrate-active enzymes to degrade 2'-FL.[16,47]

Short-chain fatty acids and lactate, the primary microbial metabolites of carbohydrate fermentation, play a key role in protecting gut health and are often used to evaluate the prebiotic properties of fermentation substrates. Acetate, propionate, and butyrate account for more than 90–95% of total SCFAs,[42] with a theoretical molar ratio of 3:1:1 in the gut.[48] Generally, FOS and 2'-FL cannot be degraded by the enzymes secreted by the host and are mainly utilized by the microbiota in the large intestine. During jejunal fermentation *in vitro*, a higher propionate concentration was produced in the 2'-FL group, and FOS and lactose were fermented to produce more acetate and butyrate, respectively. These findings suggest that the small intestinal microbiota have the capacity to ferment oligosaccharides, and 2'-FL has a stronger propionate-producing capacity. However, the generation of these metabolites depends on the carbohydrate types and microbiota composition.[49] Both FOS and lactose positive controls showed a higher level of lactate production, which might be attributed to the abundance of *Lactobacillus* and *Streptococcus* in the small intestine. *Lactobacillus* and *Streptococcus* have been reported to be the dominant genera in the small intestine, and they prefer to utilize simple carbohydrates to produce lactate.[16,17]

In the present study, 2'-FL produced higher levels of acetate. Propionate and butyrate were higher in the FOS and lactose groups, respectively, during colonic fermentation *in vitro*. The generation of different types of SCFA is strongly associated with the monosaccharide types. It is reported that acetate and butyrate are produced through the fermentation of aldehyde monosaccharides (such as glucose, galactose, and mannose) whereas propionate is produced mainly through the fermentation of ketone monosaccharides (such as fructose, arabinose, and tagatose).[22] This phenomenon could be attributed to the unique monosaccharide components of different types of oligosaccharides. Our findings were consistent with a previous study on infants, where 2'-FL and lactose were fermented by infant fecal microbiota to yield a high proportion of acetate and butyrate, respectively.[24] However, these findings were different from the findings for jejunal fermentation *in vitro*, probably due to the abundance of more complex acid-producing bacteria in the large intestine than in the small intestine.[50] In the present study, the acetate level in the 2'-FL group was low during jejunum fermentation but was high during colonic fermentation. The conversion of acetate in the jejunum and colon may be associated with the composition and high density of bacterial cells in the colon, which contributed to enhancing the fermentation efficiency of 2'-FL and further promoting acetate production. The lactate level was also higher in the FOS and lactose groups at the fermentation prophase, but lactate concentration decreased with the progression of fermentation. Lactate, as an intermediate, could be converted into propionate and butyrate by microbes.[51] The higher level of propionate and butyrate in the FOS and lactose groups could therefore be attributed to lactate conversion by the cross-feeding between the lactate-producing bacteria and lactate-utilizing bacteria, respectively.[52,53] Short-chain fatty acid provides many benefits for the host, such as supplying an additional energy source for intestinal epithelial cells, suppressing potential pathogen colonization, stimulating epithelial proliferation, and enhancing tight junction formation.[54] Overall, these results indicated that milk oligosaccharide 2'-FL could maintain gut health like other prebiotics.

Carbohydrate substrates can modulate the gut microbiota structure and composition in a structure-dependent manner.[23] In comparison with FOS and lactose, the structure of 2'-FL is complex with two glycosidic bonds (β1-4 and α1-2) and three monosaccharides (fucose, glucose, and galactose).[6] Thus, it was inferred that 2'-FL modulated the microbiota differentially in comparison with FOS and lactose. Notably, 2'-FL increased the microbiota diversity (ACE and Shannon indices) and altered the microbial structure during jejunal and colonic fermentation. The complex carbohydrates could effectively structure gut microbial consortia and maintain diversity by providing more abundant substrates.[55] These results were consistent with a previous study on humans, reporting that the complex carbohydrate substrate could promote greater diversity than simple homopolymers and achieve health benefits by increasing gut microbiota diversity.[56]

Furthermore, 2'-FL differentially stimulated the growth of different types of bacterial groups in the jejunum and colon *in vitro*. For instance, during jejunal fermentation, 2'-FL increased the abundance of Bacteroidetes but decreased the abundance of Firmicutes, which might be due to the increase in *Bacteroides* and *Prevotella* and the decrease in *Streptococcus* and *Lactobacillus*. *Bacteroides* and *Prevotella* are the primary complex polysaccharide degraders in the gut and release various carbohydrate-active enzymes to degrade 2'-FL.[57] These bacteria are also considered to be the main propionate producers in the intestine.[52] *Streptococcus* and *Lactobacillus* in Firmicutes are the predominant genera in the small intestine and are driven by a rapid uptake and conversion of simple carbohydrates.[17] These bacteria pose different preferences for different oligosaccharides. Some previous studies on functional genomes have reported that *Streptococcus* and *Lactobacillus* could uptake and degrade FOS as the energy source directly.[45] Moreover, *Streptococcus* and *Lactobacillus* are recognized as the primary lactate producers, which utilize simple carbohydrates to produce lactate.[58,59] In this study, a higher level of lactate was observed in the FOS and lactose groups. However, in comparison with FOS and lactose, 2'-FL increased the abundance of Firmicutes and decreased the abundance of Bacteroidetes during colonic fermentation, which was the opposite to the results in the jejunum. These results could be attributed to the increase of *Blautia* and *Roseburia* in Firmicutes and the decrease of *Prevotell_9* in Bacteroidetes in the 2'-FL group. Notably, 2'-FL fermentation increased the abundance of the genus *Blautia* in the jejunum, suggesting that *Blautia* might be a specialized degrader of 2'-FL. It is reported that fucose from the degradation of 2'-FL by the microbiota was released into intestinal tract as substrates mediating the growth of *Blautia*.[60] From *in vitro* co-culture assays, *Blautia*, and particularly *Blautia wexlerae*, has been reported to utilize fucose released from 2'-FL by *Bifidobacterium bifidum*, which

possessed extracellular GH95 α-L-fucosidase.[60] In this study, the abundance of *Megasphaera*, a common lactate-utilizing bacteria in the gut, was increased in the FOS group, which could convert lactate to propionate through the acrylate pathway.[51] A previous study reported that, under carbon-limited steady-state conditions, lactate was converted to butyrate rather than propionate by *Megasphaera*.[51] In the current study, the higher levels of propionate and butyrate were produced by fermenting FOS and lactose, respectively, and lactate sharply declined after 6 h of fermentation. Thus, we speculated that the increased *Megasphaera* might contribute to higher propionate and butyrate in the FOS and lactose groups, respectively. Overall, 2'-FL could influence the composition of the bacteria community in the small and large intestines compared with FOS and lactose. However, the utilization mechanism of 2'-FL by microbes is still unclear due to the diversity and complexity of the gut microbiota. Hence, the relationship between specific bacteria and 2'-FL degradation needs further investigation.

CONCLUSION

In summary, the present study systematically revealed the *in vitro* fermentability properties of the milk oligosaccharides 2'-FL by the jejunal and colonic microbiota from piglets. Compared with FOS and lactose, 2'-FL showed differential influences on microbial composition and metabolism in the small and large intestines. The small intestinal microbes showed a potential ability to ferment 2'-FL with a higher propionate. In contrast, 2'-FL was utilized by the large intestinal microbes to produce more acetate than FOS and lactose after 24 and 48 h fermentation. These findings provided new prospects for modulating microbial community and SCFA production to exert health benefits on newborns by administering milk oligosaccharides.

AUTHOR CONTRIBUTIONS

Yanxin Ye participated in the experimental design and performed the experiment and analyzed the data. Yanan Zhang was responsible for data analysis and drafted the manuscript. Jiaqing Guo, Mengting Wang, Xuan Li and Yuting Ren assisted in the experiment procedures. Weiyun Zhu conducted the study design. Kaifan Yu supervised the concept, contributed to the study design, reviewed and edited the manuscript, and participated the entire research process. All authors have read and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Andreas NJ, Kampmann B and Mehring Le-Doare K, Human breast milk: a review on its composition and bioactivity. *Early Hum Dev* **91**:629–635 (2015).
2 Casado B, Affolter M and Kussmann M, OMICS-rooted studies of milk proteins, oligosaccharides and lipids. *J Proteomics* **73**:196–208 (2009).
3 Xiao M, Ren X, Yu Y, Gao W, Zhu C, Sun H *et al.*, Fucose-containing bacterial exopolysaccharides: sources, biological activities, and food applications. *Food Chem X* **13**:100233 (2022).
4 Natividad JM, Rytz A, Keddani S, Bergonzelli G and Garcia-Rodenas CL, Blends of human Milk oligosaccharides confer intestinal epithelial barrier protection in vitro. *Nutrients* **12**:3047 (2020).
5 Erney RM, Malone WT, Skelding MB, Marcon AA, Kleman-Leyer KM, O'Ryan ML *et al.*, Variability of human milk neutral oligosaccharides in a diverse population. *J Pediatr Gastroenterol Nutr* **30**:181–192 (2000).
6 Zhu Y, Wan L, Li W, Ni D, Zhang W, Yan X *et al.*, Recent advances on 2′-fucosyllactose: physiological properties, applications, and production approaches. *Crit Rev Food Sci Nutr* **62**:2083–2092 (2022).
7 Lewis ZT, Totten SM, Smilowitz JT, Popovic M, Parker E, Lemay DG *et al.*, Maternal fucosyltransferase 2 status affects the gut bifidobacterial communities of breastfed infants. *Microbiome* **3**:13 (2015).
8 Yu ZT, Chen C, Kling DE, Liu B, McCoy JM, Merighi M *et al.*, The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota. *Glycobiology* **23**:169–177 (2013).
9 Zhao G, Williams J, Washington MK, Yang Y, Long J, Townsend SD *et al.*, 2'-Fucosyllactose ameliorates chemotherapy-induced intestinal mucositis by protecting intestinal epithelial cells against apoptosis. *Cell Mol Gastroenterol Hepatol* **13**:441–457 (2022).
10 Sutkus LT, Joung S, Hirvonen J, Jensen HM, Ouwehand AC, Mukherjea R *et al.*, Influence of 2'-Fucosyllactose and Bifidobacterium longum subspecies infantis supplementation on cognitive and structural brain development in young pigs. *Front Neurosci* **16**: 860368 (2022).
11 Natividad JM, Marsaux B, Rodenas CLG, Rytz A, Vandevijver G, Marzorati M *et al.*, Human Milk oligosaccharides and lactose differentially affect infant gut microbiota and intestinal barrier in vitro. *Nutrients* **14:**2546 (2022).
12 Iribarren C, Magnusson MK, Vigsnaes LK, Aziz I, Amundsen ID, Suligoj T *et al.*, The Effects of Human Milk Oligosaccharides on Gut Microbiota, Metabolite Profiles and Host Mucosal Response in Patients with Irritable Bowel Syndrome. *Nutrients* **13**:3836 (2021).
13 Kong C, Faas MM, de Vos P and Akkerman R, Impact of dietary fibers in infant formulas on gut microbiota and the intestinal immune barrier. *Food Funct* **11**:9445–9467 (2020).
14 Ferreira-Lazarte A, Moreno FJ, Cueva C, Gil-Sanchez I and Villamiel M, Behaviour of citrus pectin during its gastrointestinal digestion and fermentation in a dynamic simulator (simgi(R)). *Carbohydr Polym* **207**:382–390 (2019).
15 van Trijp MPH, Rosch C, An R, Keshtkar S, Logtenberg MJ, Hermes GDA *et al.*, Fermentation kinetics of selected dietary fibers by human small intestinal microbiota depend on the type of fiber and subject. *Mol Nutr Food Res* **64**:e2000455 (2020).
16 Mu C, Yang Y, Su Y, Zoetendal EG and Zhu W, Differences in microbiota membership along the gastrointestinal tract of piglets and their differential alterations following an early-life antibiotic intervention. *Front Microbiol* **8**:797 (2017).
17 Zoetendal EG, Raes J, van den Bogert B, Arumugam M, Booijink CC, Troost FJ *et al.*, The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. *ISME J* **6**: 1415–1426 (2012).
18 Koh A, De Vadder F, Kovatcheva-Datchary P and Backhed F, From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. *Cell* **165**:1332–1345 (2016).
19 Yu K, Zhang Y, Chen H and Zhu W, Hepatic metabolomic and transcriptomic responses induced by cecal infusion of sodium propionate in a fistula pig model. *J Agric Food Chem* **67**:13073–13081 (2019).
20 Martin-Gallausiaux C, Marinelli L, Blottiere HM, Larraufie P and Lapaque N, SCFA: mechanisms and functional importance in the gut. *Proc Nutr Soc* **80**:37–49 (2021).
21 Lee YS, Kim TY, Kim Y, Lee SH, Kim S, Kang SW *et al.*, Microbiota-derived lactate accelerates intestinal stem-cell-mediated epithelial development. *Cell Host Microbe* **24**:833–846 (2018).

22 Wang M, Wichienchot S, He X, Fu X, Huang Q and Zhang B, In vitro colonic fermentation of dietary fibers: fermentation rate, short-chain fatty acid production and changes in microbiota. *Trends Food Sci Tech* **88**:1–9 (2019).
23 Payling L, Fraser K, Loveday SM, Sims I, Roy N and McNabb W, The effects of carbohydrate structure on the composition and functionality of the human gut microbiota. *Trends Food Sci Tech* **97**:233–248 (2020).
24 Van den Abbeele P, Sprenger N, Ghyselinck J, Marsaux B, Marzorati M and Rochat F, A comparison of the in vitro effects of 2'Fucosyllactose and lactose on the composition and activity of gut microbiota from infants and toddlers. *Nutrients* **13**:726 (2021).
25 Holscher HD, Dietary fiber and prebiotics and the gastrointestinal microbiota. *Gut Microbes* **8**:172–184 (2017).
26 Williams BA, Bosch MW, Boer H, Verstegen M and Tamminga S, An in vitro batch culture method to assess potential fermentability of feed ingredients for monogastric diets. *Anim Feed Sci Tech* **123–124**:445-462 (2005).
27 Dai ZL, Zhang J, Wu GY and Zhu WY, Utilization of amino acids by bacteria from the pig small intestine. *Amino Acids* **39**:1201–1215 (2010).
28 Li Y, Hou Z, Shi Q, Cheng Y and Zhu W, Methane production from different parts of corn Stover via a simple Co-culture of an anaerobic fungus and methanogen. *Front Bioeng Biotechnol* **8**:314 (2020).
29 Zhang Y, Yu K, Chen H, Su Y and Zhu W, Caecal infusion of the short-chain fatty acid propionate affects the microbiota and expression of inflammatory cytokines in the colon in a fistula pig model. *J Microbial Biotechnol* **11**:859–868 (2018).
30 Zoetendal EG, Heilig HG, Klaassens ES, Booijink CC, Kleerebezem M, Smidt H *et al*., Isolation of DNA from bacterial samples of the human gastrointestinal tract. *Nat Protoc* **1**:870–873 (2006).
31 Mao S, Zhang M, Liu J and Zhu W, Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function. *Sci Rep* **5**:16116 (2015).
32 Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N *et al*., Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* **6**:1621–1624 (2012).
33 Hall M and Beiko RG, 16S rRNA gene analysis with QIIME2. *Methods Mol Biol* **1849**:113–129 (2018).
34 Edgar RC, UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* **10**:996–998 (2013).
35 Edgar RC, Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**:2460–2461 (2010).
36 Amato KR, Yeoman CJ, Kent A, Righini N, Carbonero F, Estrada A *et al*., Habitat degradation impacts black howler monkey (Alouatta pigra) gastrointestinal microbiomes. *ISME J* **7**:1344–1353 (2013).
37 Chong J, Liu P, Zhou G and Xia J, Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat Protoc* **15**:799–821 (2020).
38 Benjamini Y and Hochberg Y, Controlling the false discovery rate: a practical and powerful approach to multiple testing, *journal of the Royal Statistical Society. Series B: Methodological* **57**:289–300 (1995).
39 Hishiya N, Uno K, Nakano A, Konishi M, Higashi S, Eguchi S *et al*., Association between the gut microbiome and organic acid profiles in a Japanese population with HIV infection. *J Infect Chemother* (2023).
40 Zhang B, Li LQ, Liu F and Wu JY, Human milk oligosaccharides and infant gut microbiota: molecular structures, utilization strategies and immune function. *Carbohydr Polym* **276**:118738 (2022).
41 Wang X, Li J, Li N, Guan K, Yin D, Zhang H *et al*., Evolution of intestinal gases and fecal short-chain fatty acids produced in vitro by preterm infant gut microbiota during the first 4 weeks of life. *Front Pediatr* **9**:726193 (2021).
42 Pi Y, Hu J, Bai Y, Wang Z, Wu Y, Ye H *et al*., Effects of dietary fibers with different physicochemical properties on fermentation kinetics and microbial composition by fecal inoculum from lactating sows in vitro. *J Sci Food Agric* **101**:907–917 (2021).
43 Li H, Lane JA, Chen J, Lu Z, Wang H, Dhital S *et al*., In vitro fermentation of human milk oligosaccharides by individual Bifidobacterium longum-dominant infant fecal inocula. *Carbohydr Polym* **287**:119322 (2022).
44 Cheng L, Kiewiet MBG, Logtenberg MJ, Groeneveld A, Nauta A, Schols HA *et al*., Effects of different human Milk oligosaccharides on growth of Bifidobacteria in monoculture and Co-culture with Faecalibacterium prausnitzii. *Front Microbiol* **11**:569700 (2020).
45 Barrangou R, Altermann E, Hutkins R, Cano R and Klaenhammer TR, Functional and comparative genomic analyses of an operon involved in fructooligosaccharide utilization by lactobacillus acidophilus. *Proc Natl Acad Sci U S A* **100**:8957–8962 (2003).
46 Zhao X and Ganzle MG, Genetic and phenotypic analysis of carbohydrate metabolism and transport in lactobacillus reuteri. *Int J Food Microbiol* **272**:12–21 (2018).
47 El Kaoutari A, Armougom F, Gordon JI, Raoult D and Henrissat B, The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. *Nat Rev Microbiol* **11**:497–504 (2013).
48 Fernandes J, Su W, Rahat-Rozenbloom S, Wolever TM and Comelli EM, Adiposity, gut microbiota and faecal short chain fatty acids are linked in adult humans. *Nutr Diabetes* **4**:e121 (2014).
49 Bridgman SL, Azad MB, Field CJ, Haqq AM, Becker AB, Mandhane PJ *et al*., Fecal short-chain fatty acid variations by breastfeeding status in infants at 4 months: differences in relative versus absolute concentrations. *Front Nutr* **4**:11 (2017).
50 Zhang Y, Mu C, Liu S and Zhu W, Dietary citrus pectin drives more ileal microbial protein metabolism and stronger fecal carbohydrate fermentation over fructo-oligosaccharide in growing pigs. *Anim Nutr* **11**:252–263 (2022).
51 Prabhu R, Altman E and Eiteman MA, Lactate and acrylate metabolism by Megasphaera elsdenii under batch and steady-state conditions. *Appl Environ Microbiol* **78**:8564–8570 (2012).
52 Louis P and Flint HJ, Formation of propionate and butyrate by the human colonic microbiota. *Environ Microbiol* **19**:29–41 (2017).
53 Duncan SH, Louis P and Flint HJ, Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. *Appl Environ Microbiol* **70**:5810–5817 (2004).
54 Pieper R, Kroger S, Richter JF, Wang J, Martin L, Bindelle J *et al*., Fermentable fiber ameliorates fermentable protein-induced changes in microbial ecology, but not the mucosal response, in the colon of piglets. *J Nutr* **142**:661–667 (2012).
55 Yao T, Chen MH and Lindemann SR, Structurally complex carbohydrates maintain diversity in gut-derived microbial consortia under high dilution pressure. *FEMS Microbiol Ecol* **96**:fiaa158 (2020).
56 Chung WSF, Walker AW, Vermeiren J, Sheridan PO, Bosscher D, Garcia-Campayo V *et al*., Impact of carbohydrate substrate complexity on the diversity of the human colonic microbiota. *FEMS Microbiol Ecol* **95**:fiy201 (2019).
57 Flint HJ, Scott KP, Duncan SH, Louis P and Forano E, Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* **3**:289–306 (2012).
58 Hartemink R, Quataert MC, van Laere KM, Nout MJ and Rombouts FM, Degradation and fermentation of fructo-oligosaccharides by oral streptococci. *J Appl Bacteriol* **79**:551–557 (1995).
59 Pham VT, Lacroix C, Braegger CP and Chassard C, Early colonization of functional groups of microbes in the infant gut. *Environ Microbiol* **18**:2246–2258 (2016).
60 Horigome A, Hashikura N, Yoshida K, Xiao JZ and Odamaki T, 2'-Fucosyllactose increases the abundance of Blautia in the presence of extracellular Fucosidase-possessing bacteria. *Front Microbiol* **13**:913624 (2022).

