PAPER

Regulation of infant gut microbiota and metabolic pathways by human milk oligosaccharides: a multi-omics investigation

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The third-most solid component in breast milk is human milk oligosaccharides (HMOs)—crucial for the growth and development of infants. HMOs are divided into three main categories: fucosylated, nonfucosylated, and sialylated HMOs. However, comparative studies evaluating the variations of the impact of *in vitro* fermentation of these typical or dominant HMOs on the metabolic functions of an infant's gut microbiome remain limited. Therefore, we systematically analyzed the role of three typical HMOs—2′-fucosyl lactose, lactose-*N*-tetrose, and 3′-sialyllactose—in the structural reshaping of the infant intestinal flora; we also explored their impact on short-chain fatty acid (SCFA) production and metabolism, as well as their overall metabolic spectrum. We found that HMOs aided the growth of beneficial microorganisms, such as *Bifidobacterium*, *Lactobacillus*, and *Enterococcus*, while also reducing the number of harmful bacteria, including *Escherichia-Shigella*. Further, HMOs had a substantial impact on amino acid, purine, and lipid metabolic pathways and significantly increased SCFA levels. Correlation analyses revealed significant associations between *Bifidobacterium* and multiple lipid metabolites. Positive correlations between *Lactobacillus* and amino acid derivatives, as well as close links between *Bacteroides* and acetate production, were detected. Overall, these findings indicate that the gut flora-metabolite interplay is central to HMO function, and different HMOs variably regulate the gut microbiota composition and metabolic pathways. This study provides a theoretical basis for optimizing HMOs' fortification strategies in infant formulas.

Introduction

The World Health Organization and pediatric societies recommend that humans start nursing their babies within the first 60 min of birth, maintaining the practice until the age of 6 months.[1] Breast milk is the primary nutritional source for infants. Human milk oligosaccharides (HMOs) are a diverse and unique class of carbohydrates, constituting the third most abundant solid component in breast milk after lactose (70 g L^{-1}) and lipids (40 g L^{-1}). They are composed of five monosaccharides: D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), L-fucose (Fuc), and sialic acid (Neu5Ac). Their concentration in mature milk ranges from 10 to 15 g L^{-1}

(average 8–19 g L^{-1}), while in colostrum it reaches 20–25 g L^{-1} (average 9–22 g L^{-1}), and 4–6 g L^{-1} after 6 months. HMO concentration typically exceeds that of protein (9–12 g L^{-1}), with significant variability influenced by factors including the secreting individual's condition, ethnicity, geographic location, season, maternal nutrition and body weight, gestational age, and mode of delivery.[2] HMOs are classified into three major categories: fucosylated HMOs, non-fucosylated HMOs, and sialylated HMOs. Their synthesis follows a fundamental blueprint and carries various glycosylation epitopes with potential biological activity (Fig. 1A). Their core structure is lactose, which can undergo fucosylation *via* an α1-2 bond at the terminal Gal residue, forming 2′-fucosyl lactose (2′-FL) (Fig. 1B). Additionally, the non-reducing end of the lactose moiety connects to GlcNAc *via* a β-1,3 bond, forming lactose-*N*-trisaccharide I (LNT II). The non-reducing end of LNT II forms lactose-*N*-tetrasaccharide (LNT) by linking to Gal *via* a β-1,3 bond (Fig. 1C). Lactose can also form Neu5Ac at the Gal terminal *via* an α2-3 bond, yielding 3′-sialic acid lactose (3′-SL) (Fig. 1D).[3,4]

HMOs are mainly involved in maintaining the infant gut microbiota balance, strengthening the gastrointestinal barrier, preventing infections, and potentially supporting the immune system, brain, and cognitive development.[5] HMOs enhance

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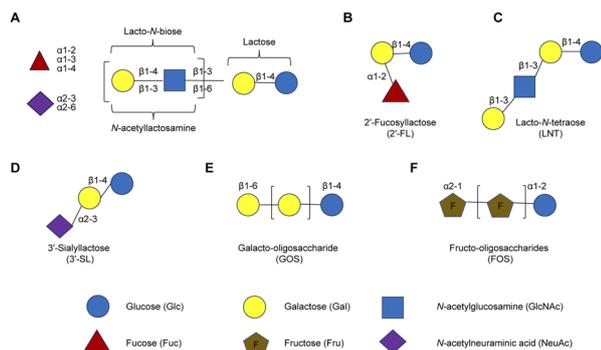† Lingling Luo and Baoyu Yang contributed equally to this study.

Fig. 1 Schematic structures of HMOs, FOS, and GOS. (A) Blueprint of HMOs structure. (B) 2′-FL. (C) LNT. (D) 3′-SL. (E) GOS. (F) FOS.

intestinal barrier integrity and immune responses by interacting with lectins. They are not directly digested or absorbed by infants; thus, they reach the colon intact, where they are selectively utilized by beneficial bacterial strains, particularly *Bifidobacteria*. The utilization of HMOs by gut microbes largely depends on their glycosidic linkage patterns and stereochemical features. Specific linkages, such as α1-2 fucosylation and α2-3/α2-6 sialylation, critically determine substrate accessibility and enzyme specificity. In addition, the degree of branching and three-dimensional conformation significantly affect the affinity of microbial glycosidases and transport systems, thereby influencing fermentation efficiency and metabolic products.[6–8] These microorganisms metabolize HMOs, converting lactose groups into short-chain fatty acids (SCFAs) such as acetate. These substances serve as nutrients for other microbial communities, thereby promoting the production of downstream metabolic products.[9] Additionally, sialic acid-containing HMOs (such as 3′-SL and 6′-SL) provide essential nutrition for nervous system development, particularly during the critical brain development stage in infants and young children. HMOs are recognized as a primary source of sialic acid for infants.[10] The functional benefits HMOs confer to developing infants are as diverse as their structural composition, making them a preferred nutritional source for infants.

Although breast milk provides all the nutrients infants need for growth, most babies worldwide rely on formula owing to various factors that hinder breastfeeding. To compensate for the deficiencies in infant formula, in recent years, galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) have been added either individually or in varying proportions as substitutes for HMOs.[11] FOS consists of a few fructose units linked by β-2,1 glycosidic bonds, with a single D-glucose unit at its non-reducing end (Fig. 1E).[12] GOS comprises a mixture of oligosaccharides with varying degrees of polymerization and bonding patterns, typically containing glucose at the reducing end and extending through multiple galactose units *via* β-1,6 or β-1,4 bonds (Fig. 1F).[13] Although FOS and GOS are added to infant formula for their prebiotic effects that mimic the biological actions of HMOs, they cannot replicate all functions of HMOs. This is because the structure of GOS and FOS (absent in breast milk) differs from that of HMOs found in human milk. Clinical studies indicate that supplementing infant formula with HMOs offers multiple benefits for infants. One study demonstrated that infants fed formula supplemented with 2′-FL, DFL, LNT, 3′-SL, and 6-SL exhibited significantly higher relative abundance of *Bifidobacteria* compared to those fed standard cow's milk-based infant formula, approaching levels seen in the breastfed reference group.[14] Other studies indicate that infants fed a formula containing a blend of 2′-FL and LNT experienced a lower incidence of respiratory tract infections.[15] Therefore, adding HMOs to infant formula to mimic breast milk is crucial for bridging the nutritional gap between infant formula and breast milk.

The establishment of the gut microbiota during infancy represents a pivotal phase in microbial community development, and the attainment of a stable microbial ecosystem within this period exerts profound and enduring effects on human health. Microbes in the gut use carbohydrate-processing enzymes to break down complex sugars into simple sugar particles, forming SCFAs and other sour substances. These compounds serve as a vital energy source for intestinal wall cells and act as key nutrients, enabling interaction between gut bacteria. This enables beneficial microorganisms to settle in our bodies, promoting their adherence to the intestinal surface.[16,17] SCFAs, primarily including acetic acid, propionic acid, and butyric acid, are saturated aliphatic organic acids composed of 1–6 carbon atom chains.[18] Furthermore, a minor fraction of HMOs undergoes absorption, subsequently serving in brain neurodevelopment or functioning as immunomodulatory factors during circulation.[19]

Recent research has increasingly relied on simulated intestinal fermentation systems to explore the mechanisms by which dietary carbohydrates affect the structure and function of intestinal flora. In one survey, the impact of different prebiotics on the intestinal flora of patients with ulcerative colitis was examined. Supplementation with 2′-FL, FOS, and GOS significantly changed the bacterial population in the gut and enhanced SCFAs synthesis. However, only 2′-FL specifically stimulated acetate production and produced the highest total SCFAs concentration after 48 h.[20] *In vitro* fermentation experiments with FOS and GOS showed that bacterial species varied greatly under different oligosaccharide treatment conditions, and a large number of SCFAs and lactic acid were produced.[17] In an *in vitro* fermentation study employing a GOS/inulin (9:1) mixture as the control, the effects of 3′-FL and LNT2 on gut microbiota composition were evaluated. 3′-FL promoted the growth of *Bacteroides* and *Enterococcus*, while LNT2 increased the proportion of *Collinsella* and *Bifidobacterium*. Both substrates stimulated the production of acetate and lactate.[16] These studies primarily investigated the impact of various dietary polysaccharides on our intestinal flora and SCFAs in a simulated *in vitro* fermentation environment. However, comparative studies evaluating the variations in the impact of *in vitro* fermentation of typical, dominant, non-fucosylated, and sialylated HMOs on the metabolic functions of an infant's gut microbiome remain limited.

Therefore, we aimed to elucidate the effects of specific HMOs (2′-FL, LNT, and 3′-SL) on infant intestinal flora, microbial by-products, and SCFAs using an *in vitro* fermentation model.

Feces from infants exclusively fed human milk were used for *in vitro* fermentation, with the three HMOs (GOS, FOS, and GOS/FOS—9 : 1) serving as positive controls. 16S rRNA gene sequencing was performed to investigate the impact of the three oligosaccharides on infant gut microbiota function. Non-targeted metabolomics was used to determine metabolic differences in infant gut microbiota induced by the three oligosaccharides and correlations. Variations in SCFAs content among the different oligosaccharide groups were chromatographically quantified and compared. Our findings provide crucial insights into how different HMOs, when fermented *in vitro*, affect bacterial populations and metabolites in the intestines of breastfed babies, offering a stronger scientific basis for incorporating these HMOs into infant nutrition products.

Materials and methods

Materials

2′-FL, LNT, and 3′-SL were supplied by DSM Co., Ltd (Shanghai, China), with >90% purity. FOS and GOS with purities of 95% and 70%, respectively, were obtained from Shanghai Yuanye Biotechnology Co., Ltd.

In vitro fermentation

Fecal samples were collected from 11 infants exclusively fed human milk aged 0–6 months. Specimens were obtained with consent from the infants' legal guardians. Infants had not received antibiotics or any other medications during the week preceding sampling, and they exhibited normal bowel movements with no constipation/diarrhea or other physical discomfort. We collected fecal samples in sterile containers and immediately froze them at −20 °C. The samples were then sent to the laboratory and stored in a refrigerator at −80 °C.

Each sample was analyzed separately, and sterile phosphate buffer was added (1×, pH 7.4) for a 1 : 3 dilution. After thorough mixing, the mixture was centrifuged at 300×g for 3 min to remove the dirt. Next, 1 mL of the fecal mixture was aseptically removed using a needle tube, mixed with HMOs, placed into a fermentation tank containing Yeast Casitone Fatty Acid medium, and incubated anaerobically at 37 °C. The control group used FOS, GOS, and a 9 : 1 ratio of GOS/FOS mixture, while the blank control only used a basic medium with fecal solution. After fermentation, the mixture was centrifuged at 12 000×g for 10 min; the supernatant and residual fluid were collected separately and prepared for subsequent analysis of metabolites and microbial content. Specimens were stored at −80 °C until further use. Each treatment included six replicates. The research protocol was approved by the Ethics Committee of Beijing Ditan Hospital; approval granted to Jing Di Lun Ke Zi [2022], no. (010)-02. Written consent forms signed by the parents of the infants were collected prior to the experiments and sample collection.

SCFAs assay

Quantitative analysis of short-chain fatty acids was performed using targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS). These substances were separated using a Waters ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm) maintained at 40 °C. The mobile phase comprised 10 mM ammonium acetate in water (solvent A) and a 50% acetonitrile–isopropanol blend (solvent B). The mobile phase flow rate during the chromatography was maintained at 0.3 mL min^{-1} each time a 2 μL sample was injected. We used the multi-reaction type negative ion monitoring mode for mass spectrometry analysis.

16S rRNA amplicon sequencing analysis of gut microbiota

We used bacterial samples in the feces to extract all genomic DNA, using a commercially available kit, following the manufacturer's instructions. We employed F515 and 806R primers to amplify the V3–V4 hypervariable region within the 16S rRNA gene, followed by electrophoresis on a 2% agarose gel to verify the accuracy of the resulting amplicons. We used the NovaSeq 6000 PE250 system (Novogene Co., Ltd, Beijing, China) for further sequencing. Bioinformatic processing and diversity analyses (α- and β-diversity) were performed using the QIIME2 pipeline, while data visualization was accomplished with R software packages.

Non-targeted metabolomics

Non-targeted metabolomics analysis of the samples was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). We used a Hypersil GOLD column for the separation (100 × 2.1 mm, 1.9 microns), which was maintained at 40 °C, at a flow rate of 0.2 mL min^{-1}. This moving medium consisted of two parts: a 0.1% formic acid aqueous solution (solvent A) and methanol (solvent B). Gradient elution was performed as follows: 0–1.5 min, 98% solvent A and 2% solvent B; 1.5–3.0 min, linear change to 15% solvent A and 85% solvent B; 3–10 min, linear change to 0% solvent A and 100% solvent B; 10.1–12 min, return to the initial composition of 98% solvent A and 2% solvent B for re-equilibration. In our mass spectrometry analysis, we used both positive and negative ion modes of electrospray ionization, and the collected data ranged from 100 to 1500 m/z. The ESI ion source in our laboratory was set up as follows: injection voltage, 3.5 kV; sheath gas flow, 35 psi; auxiliary gas flow, 10 L min^{-1}; ion transfer tube temperature, 320 °C; ion injection RF level, 60; and auxiliary gas heater temperature, maintained at 350 °C, able to switch between positive and negative ion modes. MS/MS sequential acquisition was the triggered scan.

Statistical analysis

All data are presented as mean ± standard deviation. We used GraphPad Prism 10 (GraphPad Software, Inc., CA, USA) and Origin 2022 (Origin Lab Corporation, USA) for statistical analysis and drawing. A P-value < 0.05 indicates statistical significance. To further analyze metabolome data, we used MetaboAnalyst 6.0, Novogene Cloud Platform, and Maiwei Cloud Platform. We also used Spearman's correlation analysis to examine the correlation between the changed metabolites and the types of microorganisms in the feces.

Results and discussion

Gut microbiota composition following *in vitro* fermentation of different HMO types

Alpha and beta diversity analyses. To assess the impact of various HMOs on the variety and depth of the microbial ecosystem during *in vitro* fermentation, we conducted an α-diversity assessment. The results showed that introducing different HMOs into the fermentation process significantly modified the total number of species and the overall richness of the microbial populations. The Chao 1 index was higher in the HMO groups (2′-FL, LNT, 3′-SL) than in the positive control groups (GOS, FOS; 9 : 1); however, we found no significant differences compared with the untreated control group (Fig. 2A). Furthermore, the overall microbial richness in the 2′-FL, LNT, and 3′-SL groups was comparable, with no significant differences. The number of microorganisms in the LNT group was slightly higher than that in the 2′-FL and 3′-SL groups, although the difference was not substantial. The Shannon index increased with increasing species diversity; a comparison of HMO and Blank control groups revealed no significant difference in their Shannon indices (Fig. 2B). While similar numbers of microorganisms were found in the 2′-FL, LNT, and 3′-SL treated groups, the 3′-SL group had slightly more microorganisms than the LNT and 2′-FL groups. Principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) showed that the microbial community structure was different among the groups that were administered the oligosaccharides (Fig. 2C and D). The HMOs (2′-FL, LNT, 3′-SL) and Blank groups exhibited distinct community compositions. The positive control groups (GOS, FOS, and GOS/FOS; 9 : 1) exhibited distinct clustering patterns from those of the HMO and Blank groups. The 2′-FL, LNT, and 3′-SL groups were mutually segregated, indicating significant differences in their microbial community compositions.

Analysis of species composition following fermentation of different HMOs. The human gut microbiome plays a crucial role in human health; it is easily and dynamically influenced by factors such as the environment and diet. The initial stage of growth is crucial for an infant's immune system development, as it ensures metabolic stability and protects the mucosal barrier.[21] Fig. 3A displays the impact of seven treatments on fermented gut microbiota phyla after 36 h. *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidota* were the predominant phyla. In contrast to the Blank group, the HMO and positive control groups exhibited higher levels of *Firmicutes* and *Actinobacteria*, along with reduced counts of *Proteobacteria* and *Bacteroidetes*. The 3′-SL group had the highest proportion of *Firmicutes*, while the LNT group had the highest proportion of *Actinobacteria*. *Firmicutes* exhibits a strong ability to break down carbohydrates and produces butyrate, which plays an important role in promoting health, as a metabolic by-product.[22] The increased proportion of *Proteobacteria*, primarily comprising pathogenic bacteria, leads to host nutritional and metabolic disorders as well as intestinal ecological imbalance.[23] A series of digestive diseases, such as inflammation of the stomach and intestines, appendix infections, and Crohn's disease, are closely related to the *Proteobacteria* family.[24]

After the use of HMOs during fermentation, the proportion of *Klebsiella*, *Clostridium_sensu_stricto_1*, and *Escherichia-Shigella* became smaller, while the number of *Enterococcus* and *Bifidobacterium* increased compared with that in the Blank control (Fig. 3B). The HMO group exhibited a reduced percentage of *Escherichia-Shigella*, in contrast to the positive control. *Bifidobacterium*, a Gram-positive bacterium, is a key probiotic belonging to the phylum *Actinobacteria* that is crucial for human gut health. Its biological effects encompass modulation of serum cholesterol levels, prevention of gastrointestinal disorders, regulation of immune responses, and potential anti-cancer activity.[25] Early-life diversity of *Bifidobacterium* species is associated with beneficial long-term health effects, whereas reduced relative abundance is linked to diseases such as atopic dermatitis, allergies, and childhood obesity.[26] In infant intestines, *Bifidobacterium* uses a special lactate dehydrogenase to turn aromatic amino acids such as tryptophan, phenylalanine, and tyrosine into a product similar to lactic acid. This metabolic process preserves gut homeostasis while modulating systemic immunity.[27] HMO degradation by *Bifidobacteria* depends on species-specific glycoside hydrolase (GH) repertoires and associated transport proteins. Fucosidases from the GH29 and GH95 families play a key role in releasing fucose from fucosylated human milk oligosaccharides and are crucial for the degradation of 2′-FL.[28] The sialidases in GH33 mainly act on salivary HMOs
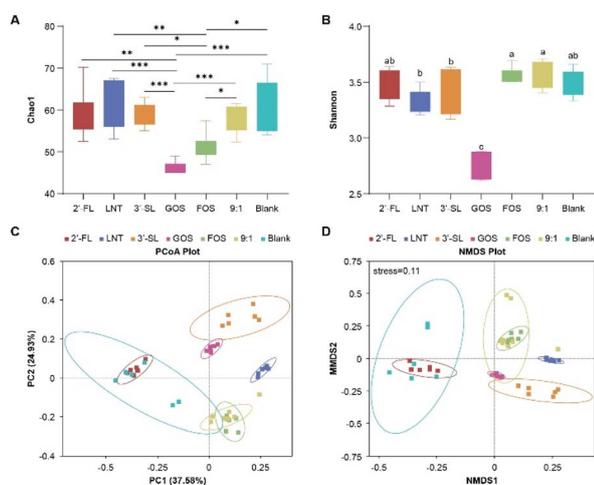
Fig. 2 Analyses of α- and β-diversity. (A) Chao 1 index. (B) Shannon index. (C) PCoA analysis. (D) NMDS analysis.
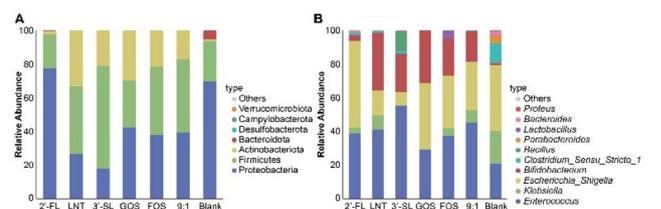
Fig. 3 Distribution of gut microbiota at the phylum (A) and genus (B) levels.

(3′-SL and 6′-SL) to release sialic acid;[29] GH2, GH20, GH42, GH112, and GH136 are all members of glycoside hydrolase families involved in the LNT metabolic pathway. For example, a member of GH136 and GH20 can act on the β1-3 bond in LNT to produce lacto-N-biose (LNB) and lactose (Lac), which can then be metabolized by GH2, GH20, and GH42. In addition, GH2 and GH42 can utilize the residual lactose from oligosaccharide metabolism, linking its utilization to 2′-FL, LNT, GOS, and free lactose. Moreover, GH112 specifically catalyzes the phosphorolysis of LNB and galacto-N-biose (GNB).[30] *Enterococci* live naturally in the human digestive system. One of them, *Enterococcus faecalis*, helps to break down long fatty acids, including alpha-linolenic acid, and other compounds such as non-adenosine nucleotides.[31] Notably, the FOS group exhibited greater *Lactobacillus* concentrations compared with those in the Blank control group, aligning with prior research findings.[32] *Lactobacillus* belongs to the *Firmicutes* family and is an important probiotic in the human large intestine. It significantly influences immune system regulation and gastrointestinal disease prevention.[33] Research has shown that HMO supplements change the composition and proportion of the gut microbiota, aiding in the maintenance of digestive system health.

Linear discriminant analysis scores of gut microbiota. We employed the Linear Enzyme-Specific Analysis (LEfSe) method to identify microorganisms whose numbers changed significantly following fermentation with different types of HMOs. Based on LDA > 2 and $p < 0.05$, the genera contributing to the inter-group differences were evaluated (Fig. 4A). Across all groups, we found 16 significant differences at the genus level, with 3, 7, and 6 major differences in bacteria in the HMO, Blank, and positive control groups, respectively.

Fig. 4(B–G) displays the dominant gut microbial proportions across experimental groups. In contrast to the Blank control group, both the HMO and positive control groups exhibited a substantial boost in *Bifidobacteria* levels, while *Clostridium-*

sensu-stricto-1 populations were substantially reduced. A comparison of the three HMO groups revealed that the LNT group had the largest number of *Bifidobacteria*, while the GOS group ranked second. Previous studies have found that fermentation of HMOs and GOS with newborn babies' feces in the laboratory resulted in more *Bifidobacteria*.[34] *Bifidobacteria* produce organic acids that help maintain a healthy gut flora composition, inhibit the reproduction of harmful microorganisms, and improve digestive and immune functions.[25] *Clostridium-sensu-stricto-1*, a Gram-positive bacterium, showed a considerable increase in abundance during gut microbiota dysbiosis. Previous studies have demonstrated its marked enrichment in neonatal mouse models of necrotizing enterocolitis, irritable bowel syndrome, and leukemia.[35] Compared with the Blank, 2′-FL, and LNT groups, the 3′-SL group exhibited significantly promoted growth of *Lactobacillus*—a particularly powerful bacterium that reduces intestinal inflammation and improves intestinal barrier strength.[36] *Bacteroides* were substantially more abundant in the 2′-FL group than in the other groups. *Escherichia-Shigella* bacteria were significantly fewer in the HMO than in the Blank control groups. *Escherichia-Shigella* are the primary diarrhea-causing pathogens in infants and young children, potentially leading to developmental delays.[37] Addition of HMOs to all groups resulted in a significantly decreased number of *Escherichia-Shigella* compared with that in the Blank control group. This indicates that HMOs, especially 3′-SL, can suppress harmful bacteria. The proportion of *Enterococcus* in all test groups was much higher than that in the Blank control group. *Enterococcus* is a beneficial microorganism that helps to ferment several foods, such as cheese and sausages. They can also help maintain the balance of bacteria in the gut and reduce inflammation.[38] Furthermore, *Enterococci*, such as *E. faecalis M-74* and *E. durans KLDS*, reduce cholesterol levels.[39,40] Overall, HMOs can help in maintaining a healthy digestive system by allowing good microorganisms to grow more while preventing the growth of harmful bacteria.

Subsequently, microbial function prediction was performed using PICRUSt. For each group, we used functional annotations and sample abundance data in the database to determine the top 10 functional items at each annotation level. Then, based on the selected terms, we prepared a bar graph to demonstrate their relative functional abundances. Excluding unclassified groups, the primary functional features of each group included membrane transport, carbohydrate metabolism, amino acid metabolism, and replication and repair (Fig. 4H).

Variations in SCFAs following *in vitro* fermentation of different HMOs

Increasing evidence has suggested that metabolites produced by gut bacteria, such as signaling molecules, have widespread effects on our entire body. Moreover, SCFAs affect the production of immune mediators, as well as the activation and differentiation of immune cells.[34,41] Following co-fermentation of diverse HMOs using infant fecal bacteria *in vitro* (Fig. 5), we analyzed the resulting SCFAs levels. During fermentation, the gut microbiota produces SCFAs, with acetate produced most
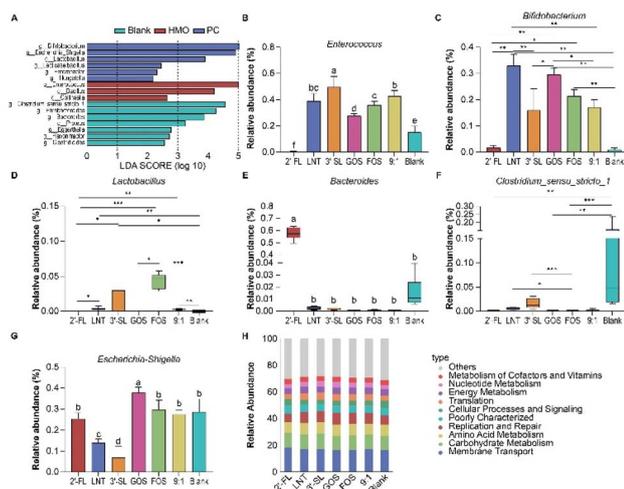

Fig. 4 LEfSe analysis plot (LDA > 2) and functional prediction plot. (A) LDA score plot. (B) *Enterococcus*. (C) *Bifidobacterium*. (D) *Lactobacillus*. (E) *Bacteroides*. (F) *Clostridium-sensu-stricto-1*. (G) *Escherichia-Shigella*. (H) Functionality Forecast Chart.
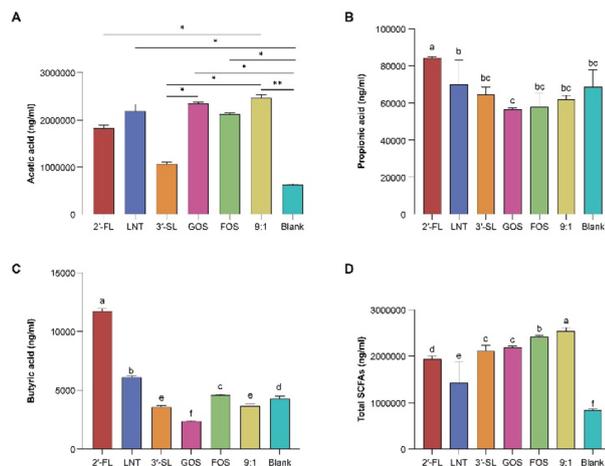
Fig. 5 Comparison of SCFAs content following the *in vitro* co-fermentation of different oligosaccharides with infant feces. (A) Acetic acid. (B) Propionic acid. (C) Butyric acid. (D) Total SCFAs.

abundantly, followed by propionate and butyrate. These SCFAs not only serve as important energy substrates for colon epithelial cells but also participate in regulating gut barrier integrity and mucosal immunity (such as anti-inflammatory responses); some SCFAs can be absorbed through the colon epithelium into the portal circulation and exert systemic metabolic and immune regulatory effects.[42] *In vitro* fermentation, animal models, and clinical studies consistently show that HMOs with different structures differ in their efficiency of microbial utilization and SCFA production, and the microbial metabolites driven by HMOs are closely related to the host's immune-metabolic status.[43]

In the experimental group that received HMOs, acetate concentrations markedly increased relative to the Blank control group. Meanwhile, the LNT cohort outpaced both the 2′-FL and 3′-SL groups regarding acetate levels (Fig. 4A). Research has shown that acetic acid can cross the blood–brain barrier and is an important source of energy for our nervous system and body functions. It regulates lipid and insulin signaling pathways and participates in body fat and cholesterol synthesis.[17,44] The 2′-FL group had the greatest amounts of propionic and butyric acids following 36 h of fermentation, greatly surpassing those of the Blank and other prebiotic groups (Fig. 5B and C). Propionic acid is another key SCFA within our digestive system. Studies have indicated that this compound undergoes metabolism in the liver, where it may fuel glucose production and inhibit cholesterol formation.[45] Butyric acid reduces the risk of colon cancer as it provides energy for the metabolism of intestinal epithelial cells.[46] Moreover, butyrate suppresses inflammation by preventing NF-κB activation and dampening pro-inflammatory cytokine synthesis. It also regulates microglial activity and neurotransmission, thereby aiding in the alleviation of stress and anxiety behaviors.[47] HMO addition increased the production of SCFAs after fermentation compared with that of the Blank control group counterpart (Fig. 4D). Overall, the fermentation results indicated that HMOs and other polysaccharides generate large quantities

of SCFAs, which modulate the intestinal microenvironment and thereby promote host gut health.

Effects of HMO fermentation on infant and toddler fecal metabolites

Differential metabolite analysis. To further elucidate the impact of HMOs on metabolites, we analyzed the variable metabolite profiles of the HMOs fermentation supernatants *via* non-targeted metabolomics. Metabolite yields were measured for the 7 sample groups following 36 h of fermentation, identifying 3074 active compounds. Principal component analysis results clearly distinguished each oligosaccharide group from the Blank control group, revealing that fermenting with different oligosaccharides had a substantial impact on infant gut microbial metabolites (Fig. 6A and B). Orthogonal partial least squares discriminant analysis revealed that, under both positive and negative ion modes, the samples clustered in their respective groups, with no inter-group overlap, indicating significant differences in the metabolites produced by the fermentation of different oligosaccharide groups (Fig. 6C and D).

We further quantified metabolites across the seven groups using the following criteria: VIP > 1, $p < 0.05$, and FC ≥ 2 or ≤ 0.5 (Fig. 7A–E). Fig. 7F shows the classification of metabolites, which are distributed as follows: lipids and lipid molecules accounted for 32.8%, organic acids and their derivatives accounted for 21.49%, organic heterocyclic compounds accounted for 16.12%, aromatic compounds accounted for 8.03%, organic oxides accounted for 6.63%, and phenylpropanoids and polyketones accounted for 6.07%.

VIP value plot of differentially expressed metabolites. The VIP plot revealed upregulated or downregulated metabolites
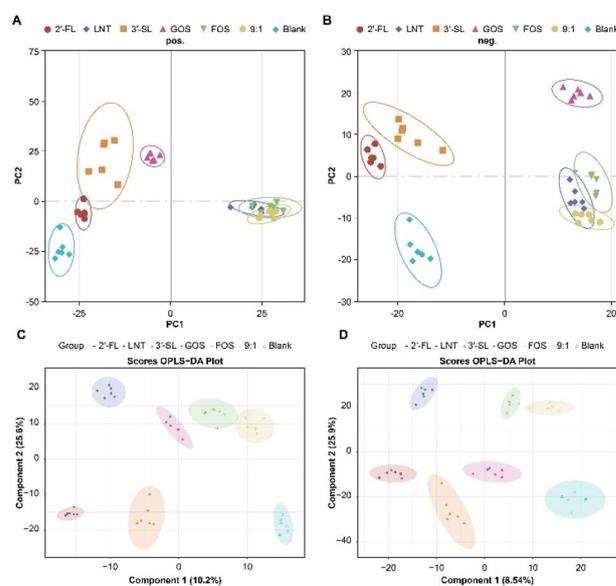

Fig. 6 Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) plots following *in vitro* co-fermentation of various oligosaccharides with infant feces. (A) PCA plot in positive ion mode. (B) PCA plot in negative ion mode. (C) OPLS-DA plot in positive ion mode. (D) OPLS-DA plot in negative ion mode.
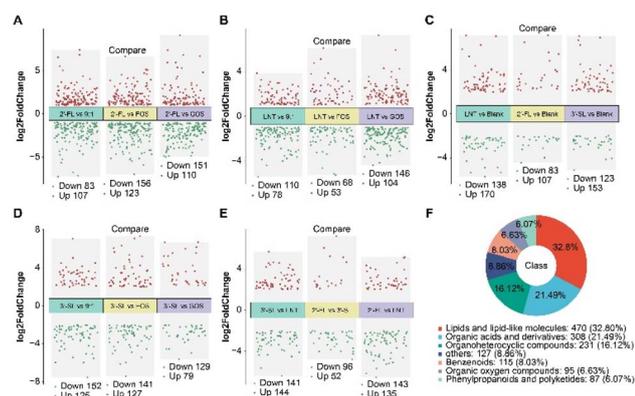
Fig. 7 Volcano plots and classification statistics of differentially expressed compounds between oligosaccharide groups and the Blank control. (A) 2′-FL vs. 9 : 1, 2′-FL vs. FOS, 2′-FL vs. GOS. (B) LNT vs. 9 : 1, LNT vs. FOS, LNT vs. GOS. (C) 3′-SL vs. Blank, 2′-FL vs. Blank, 3′-SL vs. Blank. (D) 3′-SL vs. 9 : 1, 3′-SL vs. FOS, 3′-SL vs. GOS. (E) 3′-SL vs. LNT, 2′-FL vs. 3′-SL, 2′-FL vs. LNT. (F) Classification statistics.

across the comparison groups (see SI Fig. S1 and 2). In the 2′-FL vs. Blank comparison, histidine–proline was an active cyclic dipeptide, which is widely distributed in human and animal tissues as well as bodily fluids, exhibiting significant effects in anti-diabetic, antioxidant, and anti-obesity activities.[48] Within mammalian cells, 3′-Deoxyinosine undergoes conversion into the active form cordycepin triphosphate, demonstrating anti-inflammatory properties.[49] Lyso-phosphatidylcholine (14 : 1(9Z)/0 : 0) (LysoPC[14 : 1{9Z}/0 : 0]) is a type of lysophosphatidylcholine; as the most abundant lysophosphatidylglycerol in human blood, it exhibits anti-inflammatory and antioxidant effects in the body. It is also associated with conditions such as type 2 diabetes.[50]

Indole-3-propionic acid (IPA), a tryptophan metabolite generated by gut bacteria, was used in the LNT vs. Blank analysis. IPA supplementation improves glycemia and insulin sensitivity, reduces hepatic lipid production and inflammatory factors, treats gut microbial problems, maintains the intestinal barrier, and lowers gut immunological responses.[51]

Compared to the Blank control group, the 3′-SL group exhibited adenosine-5′-monophosphate (AMP) participation in purine nucleotide and adenosine-triphosphate (ATP) metabolism. AMP ameliorates impaired glucose regulation in hypertensive stroke rats exhibiting insulin resistance without obesity.[52] Conjugated linoleic acids represent a group of unsaturated fats that have been shown to reduce cancer, diabetes, obesity, and arteriosclerosis risks.[53]

In a GOS vs. Blank comparison, L-urobilin was detected to be a bile pigment formed by intestinal bacteria during the heme degradation pathway that can be absorbed via the portal vein.[54] In the FOS vs. Blank experiment, cordycepin safeguarded against diverse pathologies, including acute lung injury, asthma, rheumatoid arthritis, Parkinson's disease, hepatitis, and atherosclerosis. Cordycepin can also affect several signaling pathways, including NF-κB, RIP2/Caspase-1, Akt/GSK-3β/p70S6K, TGF-β/Smads, and Nrf2/HO-1.[55] Valyl-alanine-

alanine, a tripeptide extracted from milk, has been shown to effectively reduce high blood pressure and have anti-inflammatory effects in animal and human trials. It also mimics insulin in promoting lipogenesis and preventing adipocyte inflammation.[56] In a 9 : 1 vs. Blank comparison, N-decanoylglycine was found to be a naturally occurring lipid present in various mammalian tissues that is associated with disrupted lipid oxidation.[57]

In the 2′-FL vs. LNT groups, glycocholic acid 3-sulfate is a bile acid sulfate with hepatoprotective effects.[58] Inecalcitol, a vitamin D3 analog, modulates cellular differentiation and apoptosis by activating the vitamin D receptor; hence, it is considered a potential antitumor drug candidate.[59] N-Oleoyl-L-serine is an endogenous N-acyl molecule, present in the bone tissue, that promotes osteoblast proliferation, regulates bone remodeling, and prevents osteoporosis.[60] Choline is an essential nutrient that serves as a precursor of the neurotransmitter acetylcholine, supporting hepatic lipid metabolism, cognitive function, and embryonic brain development.[61] Pristimerin is a quinine-type terpenoid that exhibited biological and pharmacological activities, including anticancer, antioxidant, antibacterial, and anti-inflammatory effects in a 2′-FL vs. 3′-SL comparison.[62] In the LNT vs. 3′-SL groups, tigogenin inhibited the proliferation of synovial cells and induce apoptosis under laboratory conditions, potentially aiding in the treatment of rheumatoid arthritis. Furthermore, tigogenin suppresses the proliferation of breast and prostate cancer cells and exhibits immunomodulatory properties.[63] Creatine is synthesized in a two-step reaction involving arginine, glycine, and methionine. It provides energy to the brain and can help enhance short-term memory and cognitive abilities.[64] Glycocholic acid, a conjugate of glycine and bile acids, improves the absorption of fat-soluble vitamins when orally supplemented under conditions of bile acid deficiency or impaired bile acid synthesis.[65] Ramiprilat inhibits angiotensin-converting enzyme to manage hypertension.[66]

Heatmaps comparing the relative abundances of the top 30 metabolites in each group further revealed differences in metabolite profiles across the treatment groups (Fig. 8). Both 2′-FL and 3′-SL exhibited a significant upward trend in multiple organic acid, such as 2-hydroxy-3-methylvalerate and azelaic acids, and amino acid derivatives (e.g., Glu-Pro, prolyl-methionine), whereas the LNT group exhibited enrichment in certain lipid metabolites (e.g., ethyl linoleate, cis-4,7,10,13,16-docosapentaenoic acid; Fig. 8J). These findings indicate the considerably divergent regulatory effects of different oligosaccharides on gut metabolic products. The significant upregulation of organic acids and amino acid derivatives in the 2′-FL and 3′-SL groups indicates the potential of these HMOs in promoting SCFA production and nitrogen metabolism activity. 2′-FL and sialic acid-containing HMOs (e.g., 3′-SL) have been reported to be effectively utilized by the gut microbiota to produce SCFAs, promoting host gut health and immune regulation.[67] The enrichment of lipid metabolites in the LNT group suggests a potential mechanism involving fatty acid metabolic pathways. LNT is readily utilized by Bifidobacterium sp.,
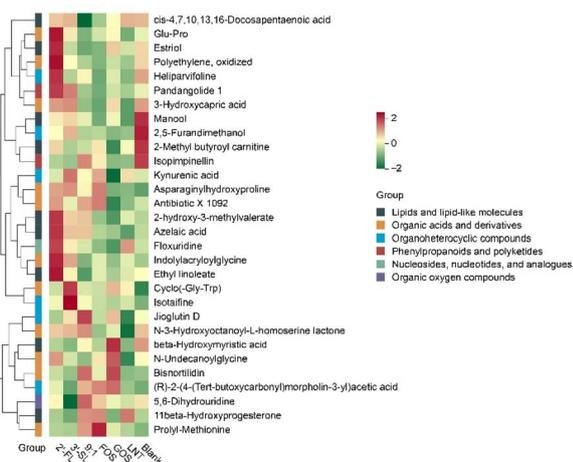
Fig. 8 Metabolite cluster analysis diagram.

promoting lipid metabolism-related pathways during fermentation.[68]

Differential metabolite pathway analysis. We conducted an in-depth analysis of the metabolites in different experimental groups and found that the metabolic pathways in each group mainly involved amino acid metabolism (Fig. 9). Important pathways included: arginine and proline metabolism; alanine, aspartic acid, and glutamate metabolism; tyrosine metabolism;

cysteine and methionine metabolism; histidine metabolism; and tryptophan metabolism. These activated pathways in turn involve glutathione metabolism, steroid hormone synthesis, purine metabolism, linoleic acid processing, cAMP signaling pathways, as well as nicotinate and nicotinamide metabolism.

Arginine and proline metabolisms are frequently associated with immunoregulation, nitric oxide production, and regulation of cell cycle progression.[69,70] Tryptophan, an essential amino acid that the human body cannot produce, is the basis of proteins and the original material for many biologically active substances. The tryptophan metabolic pathway or tryptophan metabolites hold therapeutic potential for conditions such as inflammation, metabolic disorders, depression and mood disorders, and cancer.[71] Alanine is a key precursor of glucose synthesis and is important for hepatic glucose synthesis from non-carbohydrate precursors.[72] Glutamate serves as a substrate for protein synthesis and is metabolized by the liver through gluconeogenesis to produce glucose. Aspartate is a primary energy source for ATP production via regulating the production of intermediates in the tricarboxylic acid cycle.[73] Linoleic acid has therapeutic effects against inflammatory diseases.[74]

Correlation analysis between gut microbiota and SCFAs, as well as gut microbiota and non-target metabolites. Spearman's correlation analysis was employed to elucidate the interplay between metabolites and gut microbiota and establish connections between key microbial classifications—specifically at the phylum and genus levels—and SCFAs. This approach allowed us to determine which microorganisms played a role in driving fluctuations in SCFA concentrations. Subsequently, Spearman's correlation analysis between genus-level gut microbiota and the top 100 metabolites was conducted (Fig. 10). At the phylum level, the results indicated that acetate production was primarily driven by *Bacteroidetes*. The high occurrence of this microorganism suggests a strong positive correlation
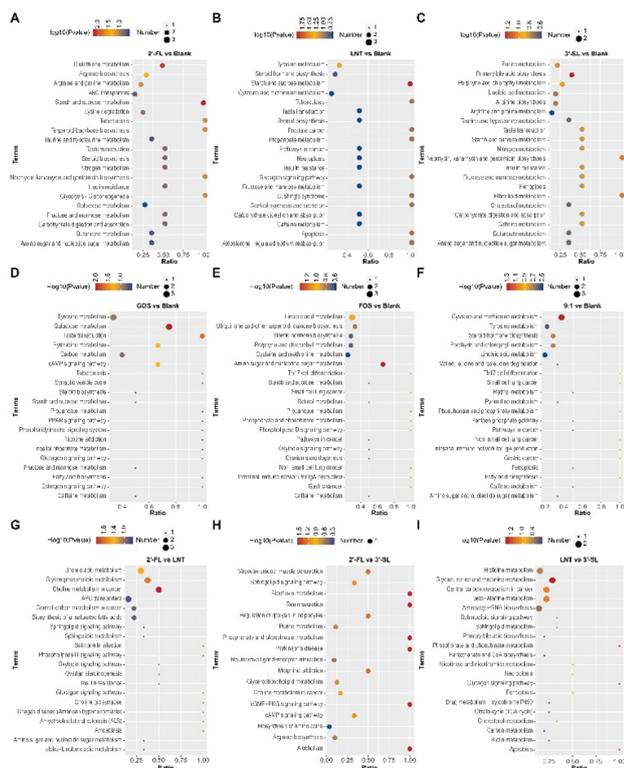

Fig. 9 Metabolic pathway enrichment maps for differential metabolites across comparison groups. (A) 2′-FL vs. Blank. (B) LNT vs. Blank. (C) 3′-SL vs. Blank. (D) GOS vs. Blank. (E) FOS vs. Blank. (F) 9 : 1 vs. Blank. (G) 2′-FL vs. LNT. (H) 2′-FL vs. 3′-SL. (I) LNT vs. 3′-SL.
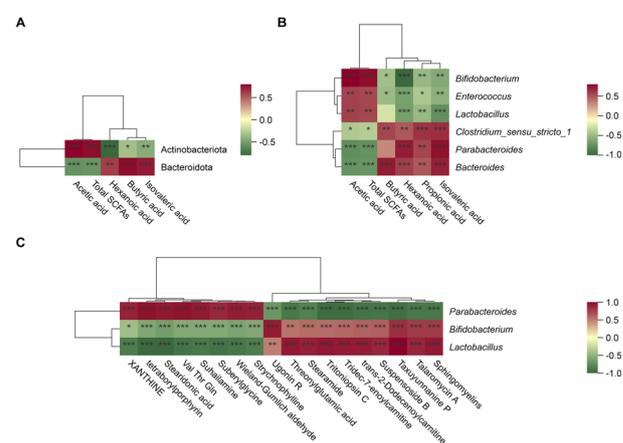

Fig. 10 Correlation analysis of gut microbiota and metabolites. (A) Correlation analysis between gut microbiota at the phylum level and short-chain fatty acids. (B) Correlation analysis between gut microbiota at the genus level and short-chain fatty acids. (C) Correlation between gut microbiota at the genus level and metabolites green indicates negative correlation; red indicates positive correlation. ***p < 0.001; **p < 0.01; *p < 0.05.

with acetate levels and total concentration of SCFAs ($p < 0.001$). This is consistent with previous reports that identified *Bacteroides* as a major source of acetate.[75] Increased propionic and butyric acid concentrations are significantly associated with the function of beneficial bacteria such as *Bifidobacterium*, *Lactobacillus*, and *Enterococcus*. These bacterial genera not only utilize HMOs as substrates but also promote the production of butyrate-producing bacteria through cross-feeding effects.[76] In contrast, the phylum *Actinobacteriota* showed a negative correlation with acetate and total SCFAs, while positively correlating with butyrate, suggesting that it may exert dual regulatory effects across different metabolic pathways. These findings indicate that distinct gut bacteria exhibit varying preferences for fermentable substrates.

Bifidobacteria showed significant positive correlations with polyphenol derivatives (*e.g.*, ugonin R), amino acid metabolites (*e.g.*, threonlyglutamic acid), and lipid metabolites (*e.g.*, tridec-7-enoylcarnitine, sphingomyelins), consistent with their known ability to produce SCFAs through carbohydrate fermentation, regulate lipid metabolism, and improve intestinal homeostasis.[77] *Lactobacillus* exhibits a positive correlation with lipid-related metabolites (*e.g.*, suspensoside B, taxyumanine P, and sphingomyelins), suggesting a potential role in membrane lipid metabolism, energy utilization, and immune regulation;[78] *Parabacteroides* showed a positive correlation with purine metabolites such as xanthine, suggesting a potential involvement in purine metabolism pathways, consistent with previous findings that *Parabacteroides* can regulate intestinal immune function through purine metabolites.[79] *Parabacteroides* also exhibited significant positive correlations with polyunsaturated fatty acids (*e.g.*, stearidonic acid) and amino acid derivatives (*e.g.*, suberylglycine and suhailamine), suggesting a potential involvement in regulating lipid metabolic pathways. This process provides energy substrates for the host while maintaining nitrogen metabolism balance.[80] Overall, our findings demonstrated functional complementarity among different microbial communities in metabolite utilization and revealed the synergistic effects exerted by probiotics through multiple metabolic pathways, thereby promoting host health.

Conclusions

We investigated the role of three oligosaccharides—2′-FL, LNT, and 3′-SL—in the structural reshaping of infant intestinal flora and explored their impact on SCFA production and metabolism. The results showed that HMOs can greatly increase the number of beneficial bacteria, including *Bifibacterium* and *Lactobacillus*, while also inhibiting the growth of harmful bacteria, such as *Escherichia-Shigella*. We found that LNT approximately doubled the abundance of *Bifidobacteria*, while markedly elevating acetate levels to an average of 6.1 mg mL^{-1}. The 2′-FL group exhibited superior propionic and butyric acid production, averaging 84.37 and 11.74 mg mL^{-1}, respectively, whereas the strongest promotion of lactobacilli was detected in the 3′-SL group. Metabolites with differential expression were mainly concentrated on amino acid and lipid metabolic pathways, such as alanine, aspartate, and glutamate metabolism; tryptophan

metabolism; and fatty acid synthesis pathways. Furthermore, correlation analysis revealed significant positive correlations among *Bifidobacterium* polyphenol derivatives, including ugonin R ($p < 0.001$), amino acid metabolites (such as threonlyglutamic acid ($p < 0.01$)), and lipid metabolites (such as tridec-7-enoylcarnitine and sphingomyelins ($p < 0.001$)). We found a strong positive correlation between *Lactobacillus* and lipids ($p < 0.001$), while *Bacteroides* was significantly associated with purine metabolites ($p < 0.001$). These results indicate a close functional connection between the microbial communities in the human gut and their metabolic products. Overall, this research highlights the distinct ways by which HMOs govern metabolic processes in infants' digestive systems, paving the way for more scientifically sound development and practical use of HMOs in baby formula.

Author contributions

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplementary information (SI): Fig. S1: VIP values of differential metabolites (Top 25). (A) 2′-FL *vs.* Blank. (B) LNT *vs.* Blank. (C) 3′-SL *vs.* Blank. (D) GOS *vs.* Blank. (E) FOS *vs.* Blank. (F) 9 : 1 *vs.* Blank. Fig. S2: VIP values of differential metabolites (Top 25). (A) 2′-FL *vs.* LNT. (B) 2′-FL *vs.* 3′-SL. (C) LNT *vs.* 3′-SL. See DOI: https://doi.org/10.1039/d5ra08644b.

Acknowledgements

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