**Bifidobacterium infantis** and 2′-fucosyllactose supplementation in early life may have potential long-term benefits on gut microbiota, intestinal development, and immune function in mice

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**ABSTRACT**

The health benefits of nutritional interventions targeting the gut microbiota in early life are transient, such as probiotics, prebiotics, and synbiotics. This study sought to determine whether supplementation with *Bifidobacterium infantis* 79 (B79), 2′-fucosyllactose (2′-FL), or both (B79+2′-FL) would lead to persistent health benefits in neonatal BALB/c mice. We found that at postnatal day (PND) 21, Ki67 and MUC2 expression increased, while total serum IgE content decreased in the B79, 2′-FL, and B79+2′-FL groups. The gut microbiota structure and composition altered as well. The levels of propionic acid, sIgA, and IL-10 increased in the 2′-FL group. Moreover, butyric acid content increased, while IL-6, IL-12p40, and tumor necrosis factor-α decreased in the B79+2′-FL group. At PND 56, Ki67 and MUC2 expression increased, whereas the gut microbiota remained altered in all 3 groups. The serum total IgG level increased only in the B79+2′-FL group. In conclusion, our study suggests that early-life supplementation with B79, 2′-FL, or their combination persistently alters the gut microbiome and promotes intestinal development; the immunomodulatory capacity of B79 and 2′-FL occurs during weaning, and their combination may persist into adulthood.

**Key words:** *Bifidobacterium infantis*, human milk oligosaccharides, intestinal development, gut microbiota, immune function

**INTRODUCTION**

Many microorganisms inhabiting the human gastrointestinal tract are known as “gut microbiota,” which play a crucial role in maintaining the host’s homeostasis (Lynch and Pedersen, 2016). Early dysbiosis of the gut microbiota leads to long-term health perturbations, demonstrating that early life is critical for building the gut microbiota composition (Vangay et al., 2015; Milani et al., 2017; Butel et al., 2018). Interventions targeting the gut microbiota have therefore attracted the attention of academic circles, such as probiotics, prebiotics, and synbiotics.

Probiotics are “live microorganisms, which, when administered in adequate amounts, confer health benefits to the host” (Hill et al., 2014, p. 506). Probiotics enhance intestinal barrier function (Camilleri, 2021), regulate immune cell balance (Wilkins and Sequoia, 2017; Eslami et al., 2020), and reduce intestinal inflammation (Xu et al., 2022) by modulating the gut microbiota composition (Wieërs et al., 2020; Wang et al., 2021). The most widely used probiotics are bifidobacteria and lactobacilli (Islam, 2016; Turroni et al., 2012; Lim and Shin, 2020). With the advent of *Bifidobacterium infantis* EVCO011, emerging evidence showed that *B. infantis* exerts a strong immunomodulatory effect (Sheil et al., 2006; Underwood et al., 2014; Henrick et al., 2021). De Andrés et al. (2018) found that supplementation with *B. infantis* R0033 increased anti-inflammatory markers IL-10, preventing inflammation and reducing inflammation-related symptoms in infants (De Andrés et al., 2018). However, the short-term and long-term outcomes of *B. infantis* supplementation in early life have not been fully clarified.

Human milk is composed of a series of nondigestible oligosaccharides consumed by a limited number of *Bifidobacterium* species to exert health-promoting effect (Zivkovic et al., 2011). Human milk oligosaccharides (HMO) are the third most abundant solid components in breast milk (Toscano et al., 2017), with 2′-fucosyllactose (2′-FL) and 3′-fucosyllactose (3′-FL) being abundantly present (Martin et al., 2016). The HMO act as substrates for *B. infantis* to facilitate the coloniza-
tion of gut microbiota and regulate immune function early in life, therefore considered as prebiotics (Bode and Jantscher-Krenn, 2012; Donovan and Comstock, 2016; Oursel et al., 2017). A symbiotic composed of *B. infantis* and HMO could regulate the gut microbiota composition, short-chain fatty acids (SCFA) generation, and immune function of healthy adults (Button et al., 2022). However, whether the symbiotics composed of HMO and *B. infantis* have potential health benefits on neonates or long-lasting effects later in life needs to be elucidated.

*Bifidobacterium infantis* B79 is a newly isolated strain from Beijing infant feces. We preliminarily confirmed the adhesion and immunomodulatory capacity of B79 in vitro. However, the immunomodulatory capacity of B79 has not been confirmed in vivo. Also, how B79 affects the gut microbiota and intestinal development remains elusive. The most abundant HMO in human milk is 2’-FL; however, only a few studies have focused on the health effects of 2’-FL or its symbiotics combined with *B. infantis*. This study aimed to investigate the short-term and long-term effects of B79 or 2’-FL supplementation in early life on intestinal development, gut microbiota, and immune function in mice during weaning and adulthood.

**MATERIALS AND METHODS**

**Experiment Design**

Twenty pregnant mice were randomly divided into control, B79, 2’-FL, and B79 + 2’-FL groups according to the weight difference within 0.5 g (n = 5). Pups were gavaged from birth to 21 d after birth and 10 mice were killed on postnatal day (PND) 21 for the collection of samples (n = 10). The remaining pups were intraperitoneally injected 4 times with ovalbumin (OVA) on PND 22, 36, 42, and 49, respectively. The remaining mice were slaughtered on PND 56, and samples were collected (Figure 1A).

**Mice**

The mice were purchased from Liaoning Changsheng Biotechnology Co. Ltd. (SCXK-2020-0001, Liaoning, PR China) and kept in an individual ventilated cage system. The ambient temperature was 25 ± 1°C and the humidity was 55 ± 5% under a 12-h light-dark cycle with free access to water and food. Mice rearing cages, drinking bottles, bedding, feed, and sterile clothing for researchers were all sterilized by autoclaving (205.8 kPa, 132°C, 10 min). As used bedding, spruce wood shavings (Chengdu Dossy Experimental Animals Co. Ltd.) were provided.

The pregnant mice of all groups gave birth on the same day (November 29, 2020), all the pups were the same age and received intervention at the same time. The pregnant mice gave birth to 29 pups in the control group, 32 pups in the B79 group, 36 pups in the 2’-FL group, and 32 pups in the B79 + 2’-FL group. The pups included both males and females, who lived in the same cage with their mother for 21 d. After that, they were caged according to group and sex. Because this study was not similar other studies (such as in the metabolism field), where sex could have a greater effect on the results, this manuscript combines 2 sexes in the same group for analysis. Furthermore, the results by re-analyzing the tested all indicators based on sex were consistent with the present manuscript.

The experimental protocols were approved by the West China School of Public Health Medical Ethics Committee of Sichuan University (approved number: SYXK 2018-001). All experimental procedures were performed in accordance with the Guidelines for Animal Experiments at the West China School of Public Health, Sichuan University (Sichuan, PR China), which comply with the National Research Council’s Guide for the Care and Use of Laboratory Animals.

Briefly, this study follows the 3R principle. (1) Reduction: because the present study needed to collect samples at PND 21 and 56 to get available results, total 20 pregnant mice have to be used. Twenty is the minimum number to get sufficient samples at each time point. (2) Replacement: we conducted this study based on the immunomodulatory abilities of B79 and 2’-FL confirmed in vitro. In vivo studies were used to simulate physiological conditions to make the study more complete. (3) Refinement: all personnel involved with the care and use of animals are adequately educated, trained, and qualified in the basic principles of laboratory animal science to help ensure high-quality science and animal well-being. All efforts were made to minimize suffering.

**Bifidobacterium infantis 79 and 2’-FL Treatment.** *Bifidobacterium infantis* 79 and 2’-FL were produced by Chr. Hansen (Chr. Hansen, Hoersholm, Denmark) and were procured by the Mengniu Dairy (Group) Co. Ltd. (Inner Mongolia of China). The freeze-dried live B79 (2.4 × 10¹⁷ cfu/g) were dissolved in sterile saline to prepare bacterial suspensions. The daily intake of live B79 was estimated to be 10⁷ cfu/mouse from PND 0 to 7, 10⁸ cfu/mouse from PND 7 to 14, and 10⁹ cfu/mouse from PND 14 to 21. The intervention dose of HMO is usually 7 g/L, but when a component of HMO is used alone, the intervention dose is 70 g/L (Musilova et al., 2017; Izumi et al., 2019). Therefore, the gavage dose of 2’-FL was 70 g/L in this study. The gavage volume was 10 μL from PND 0 to
7, 100 μL from PND 7 to 14, and 200 μL from PND 14 to 21.

**OVA Treatment.** The mice were injected intraperitoneally with 40 μg of OVA (Sigma-Aldrich, St. Louis, MO) and 4 mg of Imject Alum (Thermo Scientific, Waltham, MA) on PND 22, 36, 42, and 49 (Cheng et al., 2019; Figure 1A).

**Immunohistochemistry.** The paraffin-embedded ileum and colon were cut into 5-μm widths, deparaffinized with xylene, and hydrated with absolute ethanol and 70 to 90% alcohol. The slides were heated in a water bath at 95°C for 40 min using citrate antigen retrieval solution (pH 6.0; Beijing Solarbio Science & Technology Co. Ltd, Beijing, China). After cooling down, the slides were incubated with 3% H₂O₂ in the dark for 15 min and washed thrice with PBS. Then, the slides were permeated with a horseradish peroxidase-conjugated secondary antibody (1:200 dilution; Gene company limited, Hong Kong, China) for 45 min at 37°C, washed thrice with PBS, stained with DAB (Gene Co. Ltd, Hong Kong, China) for 1 to 5 min, and then counter-stained with hematoxylin. The slides were scanned using the DP73 digital microphotographic device (Olympus, Tokyo, Japan), and ten 200× magnified images from each slide were selected from left to right. The positive staining cells were counted using a blind method by an expert.

**Fecal DNA Extraction and Bacterial Quantitation**

Fresh mice fecal samples were collected and frozen at −80°C on PND 21 and 56. The total fecal DNA
was extracted using the TIANamp Stool DNA Kit (Tiangen Biotech Co. Ltd., Beijing, China), strictly following the manufacturer’s instructions. Quantitative PCR (qPCR) was performed using TaKaRa SYBR Premix Ex TaqII (Takara Biomedical Technology Co. Ltd., Beijing, China) by real-time fluorescence qPCR instrument (Bio-Rad Laboratories, Shanghai, China). We performed colony counting experiments on <i>Bifidobacterium</i> and <i>B. infantis</i> standard strains (ATCC, Mansarice, VA), and conducted gradient dilution of the bacterial suspension to determine the corresponding cycle threshold (Ct) values. According to log<sub>10</sub> cfu and Ct value, the log<sub>10</sub> cfu-Ct value standard curve was drawn (Supplemental Table S1; https://doi.org/10.7910/dvn/hws8fl; Luo, 2023). When the Ct value is greater than 35, it can be regarded as negative expression, and log<sub>10</sub> 5 = 5 is calculated according to the standard curve. Therefore, we set log<sub>10</sub> 5 as the detection limit. The concentration of fecal <i>Bifidobacterium</i> and <i>B. infantis</i> in feces was calculated based on the Ct value. The reaction system is shown in Supplemental Table S2 (https://doi.org/10.7910/dvn/hws8fl; Luo, 2023). The primer sequences of <i>Bifidobacterium</i> and <i>B. infantis</i> are shown in Supplemental Table S3 (https://doi.org/10.7910/dvn/hws8fl; Luo, 2023). The reaction program is shown in Supplemental Table S4 (https://doi.org/10.7910/dvn/hws8fl; Luo, 2023).

**Amplification and Sequencing of Genes Encoding 16S rRNA**

After extracting the fecal DNA, PCR amplification was performed on the 16S rDNA V3–V4 region. Single-end sequencing was performed using a small fragment library and the lon S5 XL sequencing platform. The sequences were clustered into operational taxonomic units (OTU) with 97% identity, and the OTU sequence and Silva132 database were annotated for species. An evolutionary tree was constructed by comparing representative sequences, and QIIME scripts were used to calculate α-diversity indices (Ace, Chao1, Shannon, and Simpson). The mean relative abundance of species at the phylum and genus levels is shown in bar graphs. Principal coordinate analysis was conducted using QIIME 1.9.1 based on weighted Unifrac.

**Detection of Cecal SCFA Content**

The supernatants from 100 mg of homogenized cecal samples were obtained by centrifugation (13,400 × g, 10 min at 4°C). A gas chromatograph-mass spectrometer Agilent 7890 A/5975 C (Agilent Technologies, Palo Alto, CA) was used for quantification. Herein, 4-methyl valeric acid was used as an internal standard.

**Detection of Cecal sIgA, Serum Total IgE, and IgG**

The cecal contents and serum of mice were collected on PND 21 and 56 and frozen at −80°C. The cecal slgA and serum total IgE and IgG content were measured using Elabscience ELISA kits (Elabscience Biotechnology Co. Ltd., Hubei, PR China) in strict accordance with the manufacturer’s instructions. The cecal contents (50 mg) were weighed and added to 200 μL of PBS. The supernatant was collected after centrifugation at 1,000 × g twice for 5 min at 4°C, and the cecal slgA content was determined.

**Detection of Cytokine mRNA Expression in Spleen**

The spleen samples were collected on PND 21 and 56 and frozen at −80°C. The total RNA was extracted from the mouse spleen using the Foregene Biological Animal Tissue RNA Extraction Kit (Foregene Biotechnology Co. Ltd., Sichuan, PR China) strictly according to the manufacturer’s instructions. The mouse spleen RNA was reverse transcribed into cDNA by a PCR instrument using Biorad’s iscript cDNA synthesis kit (Bio-Rad Laboratories, Shanghai, China). The reverse-transcription reaction system is shown in Supplemental Table S5 (https://doi.org/10.7910/dvn/hws8fl; Luo, 2023). The reaction program is mentioned in Supplemental Table S6 (https://doi.org/10.7910/dvn/hws8fl; Luo, 2023). The qPCR was performed using the Biorad SsoAdvanced Universal SYBR Green Supermix kit by real-time fluorescence qPCR instrument (Bio-Rad Laboratories, Shanghai, China). The expression of the target gene was calculated using the 2<sup>ΔΔCt</sup> relative quantification method with β-actin as a reference. The reaction system is mentioned in Supplemental Table S7 (https://doi.org/10.7910/dvn/hws8fl; Luo, 2023). The primer sequence of β-action, IL-6, IL-10, IL-12p40, and tumor necrosis factor-α (TNF-α) are shown in Supplemental Table S8 (https://doi.org/10.7910/dvn/hws8fl; Luo, 2023). The qPCR was performed using the Biorad SsoAdvanced Universal SYBR Green Supermix kit by real-time fluorescence qPCR instrument (Bio-Rad Laboratories, Shanghai, China). The expression of the target gene was calculated using the 2<sup>ΔΔCt</sup> relative quantification method with β-actin as a reference. The reaction system is mentioned in Supplemental Table S9 (https://doi.org/10.7910/dvn/hws8fl; Luo, 2023).

**Statistical Analyses**

Statistical analyses were performed using IBM SPSS Statistics v.20.0. All data are expressed as the mean ± standard deviation. For data with a normal distribution, a one-way ANOVA was used if variances were uniform, and a comparison between groups was performed using the Tukey test. Kruskal-Wallis rank-sum test is used when normality is not satisfied, and a comparison between groups was performed using the Nemenyi test. P < 0.05 indicated statistical significance.
RESULTS

Body Weight

The BW of mice was measured at PND 21 and 56 to observe the growth of mice. There was no significant difference in the BW between groups at PND 21 and 56 (Figure 1B and C).

Immunohistochemical Analysis of Ki67 and MUC2 Expression in the Intestine

In describing intestinal development, we used Ki67 and MUC2 to determine intestinal epithelial cells’ proliferation and differentiation degree. At PND 21, the number of Ki67-positive cells in the ileum and colon of the B79, 2'-FL, and B79+2'-FL groups was significantly increased compared with the control group (P < 0.01, Figure 2A). At PND 56, the number of Ki67-positive cells in the ileum of the 2'-FL group and colon of the B79 and B79+2'-FL groups was significantly increased compared with the control group (P < 0.05, Figure 2B).

At PND 21, the number of MUC2-positive cells in the ileum of the 2'-FL group was significantly increased compared with the control group (P < 0.001, Figure 3A). At PND 56, the number of MUC2-positive cells in the ileum and colon of the 2'-FL and B79+2'-FL groups was significantly increased compared with the control group (P < 0.05, Figure 3B).

Diversity of Gut Microbiota

The α-diversity indices and principal coordinate analysis (PCoA) were used to describe the structure of gut microbiota at different stages based on 16S rRNA amplicon sequencing. At PND 21, the Chao1 and ACE indices of the B79 and B79+2'-FL groups were significantly decreased compared with the control group (P < 0.01, Figure 4A). At PND 56, the Chao1, ACE,
Shannon, and Simpson indices of the B79 group were significantly increased \((P < 0.01, P < 0.01, P < 0.001,\) and \(P < 0.001,\) respectively). In contrast, the Chao1 and ACE indices of the B79+2'-FL group were significantly increased compared with the control group \((P < 0.001,\) Figure 4B). The PCoA based on weighted UniFrac distance revealed that each group’s gut microbiota community structure was significantly distinguished on both axes at PND 21 and 56 (Figure 4C).

**Composition of Gut Microbiota**

The gut microbiota composition was analyzed at the phylum and genus levels. At PND 21 and 56, the dominant bacteria in the gut microbiota were Bacteroidota, Firmicutes, Proteobacteria, and Campylobacterota. At PND 21, the mean relative abundance of Proteobacteria in the B79 and B79+2'-FL groups was significantly decreased compared with the control group \((P < 0.001,\) Figure 5A). The mean relative abundance of Firmicutes in the 2'-FL group was significantly increased, while Bacteroidota was significantly decreased compared with the control group \((P < 0.05,\) Figure 5A). At PND 56, the mean relative abundance of Bacteroidota in all groups was significantly increased compared with the control group \((P < 0.05,\) Figure 5A). The mean relative abundance of Firmicutes in the B79 group was significantly decreased compared with the control group \((P < 0.01,\) Figure 5A). The mean relative abundance of Campylobacterota in the B79+2'-FL group was significantly decreased compared with the control group \((P < 0.05,\) Figure 5A).

At the genus level, the mean relative abundance of Alloprevotella, Prevotellaceae_UCG-001, Parabacteroides, and Roseburia was significantly increased in the B79 group \((P < 0.01, P < 0.01, P < 0.05,\) and \(P < 0.01,\) respectively), while Candidatus_Saccharo-
nas (P < 0.01), Alistipes, Lachnoclostridium, Oscillo-
libacter, Desulfovibrio, and Rikenellaceae_RC9_gut_-
group (P < 0.05) significantly decreased compared with the
control group at PND 21. The mean relative abundance of
Lachnospiraceae_NK4A136_group, Alistipes, and
Odoribacter in the 2'-FL group was significantly
decreased compared with the control group at PND 21
(P < 0.001, P < 0.01, and P < 0.05, respectively). The
mean relative abundance of Alloprevotella, Prevotella-
aceae_UCG-001, Colidextribacter, Roseburia, and Bifido-
bacterium was significantly increased in the B79+2-FL
group (P < 0.01, P < 0.01, P < 0.01, P < 0.01, and
P < 0.05, respectively). At the same time, Lactobacillus,
Alistipes, Oscillibacter, Desulfovibrio, and Rikenellaceae
RC9 gut group significantly decreased compared with the control group at PND 21 (\(P < 0.05, P < 0.01, P < 0.001\), and \(P < 0.05\), respectively, Figure 5B).

At PND 56, the mean relative abundance of *Alloprevotella*, *Faecalibacterium*, *Clostridium_sensu_stricto_1*, and *Bifidobacterium* was significantly increased in the B79 group (\(P < 0.01, P < 0.001, P < 0.05, \) and \(P < 0.05\), respectively). At the same time, *Lactobacillus* and *Odoribacter* significantly decreased compared with the control group (\(P < 0.001\)). The mean relative abundance of *Alloprevotella* and *Candidatus_Saccharimonas* was significantly increased in the 2ʹ-FL group (\(P < 0.001\)).
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0.05 and \(P < 0.001\), respectively). At the same time, *Lactobacillus*, *Alistipes*, *Odoribacter*, and *Anaeroplasma* significantly decreased compared with the control group (\(P < 0.01, P < 0.01, P < 0.001\), and \(P < 0.05\), respectively). The mean relative abundance of *Blautia* was significantly increased in the B79+2'-FL group (\(P < 0.01\)), while *Lactobacillus* and *Odoribacter* significantly decreased compared with the control group (\(P < 0.001\), Figure 5B).

**Fecal Bifidobacterium and B. infantis Concentration by qPCR**

The mean relative abundance of *Bifidobacterium* was changed by 16S rRNA amplicon sequencing. Therefore, we used qPCR to quantify further the concentration of *Bifidobacterium* and *B. infantis* in feces. At PND 21, compared with the control group, the fecal *Bifidobacterium* and *B. infantis* concentrations of the B79 and B79+2'-FL groups were significantly increased (\(P < 0.001\)). Meanwhile, the fecal *Bifidobacterium* and *B. infantis* concentrations in the B79+2'-FL group were significantly higher than B79 (\(P < 0.05\), Figure 6). At PND 56, there was no significant difference between the groups’ fecal *Bifidobacterium* and *B. infantis* concentrations (Figure 6).

**Modifications of Cecal SCFA Content**

To detect the modification of gut microbiota metabolite profiles, fecal samples were analyzed by GC-MS to quantify SCFA. At PND 21, compared with the control group, the acetic acid content was significantly decreased in the B79 group (\(P < 0.01\)), the propionic acid content was significantly increased in the 2'-FL group (\(P < 0.05\)), and the butyric acid content was significantly increased in the B79+2'-FL group (\(P < 0.05\)). At PND 56, there was no significant difference in the SCFA content between groups (Figure 7).

**Immunoglobulin Levels in Cecal Supernatants and Serum**

At PND 21, compared with the control group, the cecal sIgA levels in the 2'-FL group were significantly increased (\(P < 0.05\)), the serum total IgE levels in all groups were significantly decreased (\(P < 0.001, P < 0.05\), and \(P < 0.01\), respectively, Figure 8A). At PND 56, the serum total IgG levels in the B79+2'-FL group were significantly increased compared with the control group (\(P < 0.001\)). The cecal sIgA and serum total IgE levels between groups showed no significant difference between the groups (Figure 8B).

**mRNA Expression of Cytokines in Spleen**

At PND 21, compared with the control group, the IL-10 mRNA expression in the 2'-FL group was significantly increased (\(P < 0.001\)), the IL-6, IL-12p40, and TNF-\(\alpha\) mRNA expression in the B79+2'-FL group was significantly decreased (\(P < 0.05\), Figure 9A). At PND 56, there was no significant difference in the mRNA expression of cytokines between groups (Figure 9b).

**DISCUSSION**

Whether probiotics can significantly alter the gut microbiota structure is still conflicting. Previous studies have suggested that probiotics only exist transiently in the intestine, which may not significantly and persistently alter the gut microbiota structure in terms of \(\alpha\)-diversity indices (Derrien and van Hylckama Vlieg, 2015; Zmora et al., 2018). Conversely, some researchers hold a different point of view (Ferrario et al., 2014; Wang et al., 2015; Kristensen et al., 2016; Laursen et al., 2017). García Rodenas et al. (2016) observed that phylogenetic distance significantly differed from the control group infants after supplementation with *L. reuteri* DSM 17938. Kato-Kataoka et al. (2016) found an increase in observed fecal species in healthy adults after supplementation with *Lactobacillus casei* strain *Shirota*. However, these studies detected the gut microbiota structure immediately or later after probiotics intervention, lacking long-term research evidence. The latest research demonstrated that changes in the gut microbiota structure of infants caused by *B. infantis* EVC001 are still detectable one year after the intervention, providing evidence for the long-term effects of *B. infantis* on the gut microbiota structure (O’Brien et al., 2022). We observed that the \(\alpha\)-diversity indices of the B79 group were significantly increased, and samples of each group were separated based on PCoA analysis after discontinuation of intervention for 5 weeks. The results demonstrated that supplementation of B79 in early life had a persistent effect on altering the gut microbiota structure in mice, which was in continuity with the latest research and our previous study (Cheng et al., 2019).

As for the HMO and synbiotics, whether they can significantly alter the gut microbiota structure lacks research evidence. Studies similar to this one used different types of HMOs or synbiotics, resulting in the limited extrapolation of the results (Cheng et al., 2016; Mischke et al., 2018; Alliet et al., 2022; Cerdó et al., 2022). We observed that samples in the 2'-FL and B79+2'-FL groups were significantly separated from that in the control group, regardless of mice at PND 56.
Furthermore, the α-diversity indices of the B79 + 2'-FL groups were changed significantly. These results indicated that HMO and synbiotics composed of B. infantis and HMO have the potential to affect the gut microbiota structure as well persistently.

The conclusion about how probiotics, HMO, and synbiotics shape the gut microbiota composition was relatively consistent. Contemporary research indicates that at the end of these interventions, fecal Bifidobacterium concentration (or relative abundance) increased significantly (Charbonneau et al., 2013; Bazanella et al., 2017; Frese et al., 2017; Alcon-Giner et al., 2022). We observed that fecal Bifidobacterium and B. infantis concentrations of the B79 and B79+2'-FL groups increased significantly at the end of the intervention. At the same time, there were no statistical differences between groups after the discontinuation of intervention for 5 weeks, which is in accordance with the current knowledge. With the advent of research, only specific Bifidobacterium strains can utilize HMO.

Figure 6. Fecal Bifidobacterium concentration of mice at postnatal d 21 (A) and 56 (B), and fecal Bifidobacterium infantis concentration of mice at postnatal d 21 (C) and 56 (D) (log10, n = 6). B79 = Bifidobacterium infantis 79; 2'-FL = 2'-fucosyllactose; B79+2'-FL = combination. Error bars indicate SE. *P < 0.05. ***P < 0.001. n = 6/group.
Sakanaka et al. (2019) revealed that the utilization of 2ʹ-FL by *Bifidobacterium* was mediated by FL transporter-1, mainly expressed in *B. infantis* and *B. breve*. However, the fecal *Bifidobacterium* and *B. infantis* concentrations of 2ʹ-FL-treated mice did not increase significantly in this study. We speculated that the lack of *B. infantis*, a 2ʹ-FL specific utilizer, inhabited in the intestine of conventional BALB/c pups might be the possible reason.

Gut microbiota affects infant intestinal homeostasis and maturation (Shi et al., 2017). The proliferation of intestinal epithelial cells (IEC) in conventional specific pathogen-free mice was significantly increased compared with germ-free mice, confirming the significance of gut microbiota in early intestinal development (Hayes et al., 2018). The *Bifidobacterium* flagellum, metabolites (such as SCFA), and lactic acid all promote IEC proliferation (Diao et al., 2017; Lee et al., 2018; O'Connell Motherway et al., 2019). The 2ʹ-FL promotes IEC differentiation by increasing MUC2 expression via the TLR4 pathway (Yao et al., 2022). However, it is still unknown how the *B. infantis* and 2ʹ-FL combination affect the IEC’s development. We found that Ki67 expression significantly increased in all 3 groups, and MUC2 expression increased in the 2ʹ-FL and B79+2ʹ-FL groups; all those effects were persistent into adulthood in mice. Early-life supplementation with B79, 2ʹ-FL or their combination, promoted intestinal development in mice, which could also last into adulthood.

Accumulating evidence suggests that gut microbiota is fundamental to immunological development. Early gut microbiome dysbiosis is associated with acute and chronic immune dysregulation, such as allergy, colic, and atopic wheezing (Rhoads et al., 2018). Therefore, interventions targeting the gut microbiota are increasingly used in treating immune diseases. It is now well established that these interventions can affect immune response by regulating T-cell differentiation. For instance, *Bifidobacterium* reduced serum IgE levels by promoting Th1 cell generation from naive CD4⁺ T cells (Henrick et al., 2021). Human milk oligosaccharides reduced the serum IgE level and elevated IL-10 by promoting Th1 and Treg cell generation (Xiao et al., 2019). Synbiotics reduced serum IgE, IL-6, IL-12, and TNF-α levels by inhibiting the differentiation of naive CD4⁺ T cells into Th2 and Th17 (van Esch et al., 2016). We found that at PND 21, the serum total IgE content of the B79, 2ʹ-FL, and B79+2ʹ-FL groups significantly decreased, the IL-10 levels of the 2ʹ-FL group significantly increased, and the IL-6, IL-12, and TNF-α levels of the B79+2ʹ-FL group significantly decreased. These results confirmed that the supplementation of *B. infantis*, 2ʹ-FL, and their synbiotics in early life could regulate immune function. We speculated that the gut microbiota and their metabolites might be the underlying mechanisms.

Short-chain fatty acids are produced by the gut microbiota’s fermentation of nondigestible carbohydrates and have been known to exhibit immunomodulatory functions, including acetate, propionate, and butyrate (Hooper et al., 2012). The SCFA can facilitate naive T-cell differentiation by regulating the cellular energy status (Atarashi et al., 2008; Delgoffe et al., 2009). Berod
et al. (2014) found propionic acid inhibits de novo fatty acid synthesis by deactivating acetyl-CoA carboxylase 1, promoting Treg differentiation and IL-10 production. Studies revealed that butyric acid could induce Treg differentiation and produce secretory immunoglobulin A (sIgA) by class switch recombination under the induction of IL-10 (Cao et al., 2015; Melcher et al., 2022). The formula milk supplemented with HMO increased IL-10 and fecal sIgA in infants by promoting SCFA generation, indicating that the immunomodulatory effects of HMO are accomplished by SCFA (Vandenplas et al., 2018). Another study illustrated that synbiotics composed of *Bifidobacterium* and fructooligosaccharide (FOS) significantly increased butyric acid content while reducing IL-6 and TNF-α levels (Hinrichsen et al., 2021).

The present study observed that the contents of propionic acid, IL-10, and sIgA in the 2′-FL group were significantly increased at weaning. The abundance of *Roseburia* and butyrate acid content significantly increased, while the expression of IL-6, IL-12, and TNF-α significantly decreased in the B79+2′-FL group. We speculated that 2′-FL facilitated Treg cell differentiation by promoting the generation of propionic acid, which increased IL-10 levels and induced B cells to produce sIgA. The combination of B79 and 2′-FL may promote butyric acid generation by increasing the abundance of *Roseburia*, which reduces the levels of Th17-related cytokines IL-6 and TNF-α. Surprisingly, although other immune-related indicators were similar among groups, the antigen-induced serum total IgG content of B79 and 2′-FL treated mice was significantly increased in adulthood.

To the best of our knowledge, no other studies have observed that synbiotics composed of *B. infantis* and 2′-FL could promote long-term IgG content. These results indicated that early life-specific *Bifidobacterium* and HMO could differently promote the host’s early-life immune development by regulating gut microbiota and SCFA production, especially the significantly increased propionic acid, butyrate acid, and their producers. In addition, we observed that the long-lasting modulatory effects of B79 on immune function manifested only when combined with 2′-FL. Our previous studies have demonstrated that *Bifidobacterium bifidum* TMC3115 treatment during the perinatal and postnatal period could protect neonatal mice from IgE-mediated allergy and DSS-induced colitis during adulthood, indicating the long-term health benefits of this strain (Cheng et al., 2019). These results indicated that the strain-specificity
**Figure 9.** The mRNA expression of cytokines in spleens of mice at PND 21 and PND 56. (A) The mRNA expression of IL-6, IL-10, IL-12p40, and tumor necrosis factor-α (TNF-α) in spleens of mice at PND 21. (B) The mRNA expression of IL-6, IL-10, IL-12p40, and TNF-α in spleens of mice at PND 56. The 2 ends of each horizontal line represent the 2 compared groups. *P < 0.05. **P < 0.01. ***P < 0.001. B79 = *Bifidobacterium infantis* 79; 2’-FL = 2’-fucosyllactose; B79+2’-FL = combination. PND = postnatal day. n = 6/group.
of *Bifidobacterium* exerted health benefits on the host when used on its own or combined with other substances.

It is worth noting that there are still many uncertainties to extrapolate the observed health benefits of *B. infantis* and 2'-FL in the tested mice, although *B. infantis* and 2'-FL have confirmed to have the potent health benefits in the present study. Therefore, it is necessary to verify the effectiveness of B79 and 2'-FL in human beings in the future.

**CONCLUSIONS**

Herein, we observed that early-life supplementation with B79, 2'-FL, and their combination significantly enhanced intestinal development. At the same time, the B79 treatment exerted limited immunomodulatory capacity in weaned mice, 2'-FL enhanced immune function in weaned mice, and the novel synbiotics composed of B79 and 2'-FL enabled its immunomodulatory capacity on weaned mice to persist into adulthood. Although the underlying mechanisms deserve further scrutiny, the health benefits of B79 and 2'-FL might be acquired by regulating the gut microbiota and its primary metabolites, SCFA, especially presenting as the increased propionic acid, butyrate acid, and their producers. Clinical trials are warranted to determine whether B79 and 2'-FL supplementation early in life exert long-term health benefits.

**ACKNOWLEDGMENTS**

This study was supported by the National Natural Science Foundation of China (Grant number: 81973042) and National Science Foundation for Young Scientists of China (Grant number: 82003453, 82204037) and China Postdoctoral Science Foundation (Grant number: 2022M712228). The authors thank Beijing Novo-gene Co., Ltd. for assisting with the next-generation sequencing, and Jun Gao from the Department of Toxicological Inspection, Sichuan center for Disease Prevention and Control, Chengdu, China, are truly appreciated for the immunohistochemical analysis. The 16S rRNA sequencing data reported in this study were submitted to the Sequence Read Archive (SRA) under the BioProject ID PRJNA888835 in NCBI (https://www.ncbi.nlm.nih.gov/sra/PRJNA888835). The original data for this study can be found at: https://data.mendeley.com/datasets/tcc7hftfdy/draft?a=e60c1543-2ed0-49c5-a4b9-7e2716c7b1e6. The authors have not stated any conflicts of interest.

**REFERENCES**


Luo et al.: *B. infantis* and 2'-FUCOSYLLACTOSE SUPPLEMENTATION 7474


van Esch, B. C., S. Abbring, M. A. Diks, G. M. Dingjan, L. F. Hart- 
hoorn, A. P. Vos, and J. Garszen. 2016. Post-sensitization admin-
istration of non-digestible oligosaccharides and Bifidobacteri-
um longum subsp. infantis in vitro and in humanised mice. Be-

Vandenplas, Y., B. Berger, V. P. Carnielli, J. Kazyczk, H. Lagström,
M. Sanchez Luna, N. Migacheva, J. M. Moselemans, J. C. Picaud,
M. Possner, A. Singhal, and M. Wabitsch. 2018. Human milk oli-
gosaccharides: 2′-fucosyllactose (2′-FL) and lacto-N-neotetraose 
3390/nu10091161.

Vangay, P., T. Ward, J. S. Gerber, and D. Knights. 2015. Antibiotics,
pediatric dysbiosis, and disease. Cell Host Microbe 17:553–564. 

Takahashi, K. Nomoto, and Y. Yamashiro. 2015. Intestinal micro-
biora profiles of healthy pre-school and school-age children and ef-
https://doi.org/10.1159/000441066.

Microbiota: An Effective Method to Improve Immunity. Molecules 

Wieers, G., L. Belkhir, R. Enaud, S. Leclercq, J. M. Philippart de Foy, 

Wilkins, T., and J. Selhoos. 2017. Probiotics for Gastrointestinal 

Xiao, L., W. R. van De Worp, R. Stassen, C. van Mastrigt, N.
Kettelaar, B. Stahl, B. Blijenbreg, S. A. Overbeek, G. Folkerts, 
promote immune tolerance via direct interactions with human 
ger.org/10.1002/eji.201847971.

Xu, L., B. Liu, L. Huang, Z. Li, Y. Cheng, Y. Tian, G. Pan, H. Li, 
and Their Metabolites Ameliorate the Symptoms of Inflamma-
tory Bowel Diseases in a Colitis Mouse Model. Microbiol. Spectr. 

Yao, Q., L. Fan, N. Zheng, C. Blecker, V. Delcenserie, H. Li, and J.
Wang. 2022. 2′-Fucosyllactose Ameliorates Inflammatory Bowel 
Disease Induced by Modulating Gut Microbiota and Promoting MUC2 
822020.

Human milk glycobiome and its impact on the infant gastroin-
testinal microbiota. Proc. Natl. Acad. Sci. USA 108(supple-

Zmora, N., G. Zilberman-Schapira, J. Suez, U. Mor, D. Dori-Bachash, 
S. Bashirades, E. Kotler, M. Zir, D. Regev-Lehavi, R. B. Brik, 
S. Federici, Y. Cohen, R. Linesvky, D. Rothschild, A. E. Moor, 
S. Ben-Moshe, A. Harmelin, S. Itzkovitz, N. Maharshak, O. Shi- 
bolet, H. Shapiro, M. Pevsner-Fischer, I. Sharon, Z. Halpern, E. 
Segal, and E. Elina. 2018. Personalized gut mucosal colonization 
resistance to empiric probiotics is associated with unique host and 
1016/j.cell.2018.08.041.

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