Ameliorating effect of 2'-Fucosyllactose and 6'Sialyllactose on lipopolysaccharide-induced intestinal inflammation

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ABSTRACT

Human milk oligosaccharides (HMO) affect gut microbiota during neonatal development, particularly with respect to the immune system. Bovine milk-based infant formulas have low oligosaccharide contents. Thus, efforts to fortify infant formulas with HMO are being undertaken. Two major HMO, 2'-fucosyllactose (2'-FL) and 6'Sialyllactose (6'SL), exert anti-inflammatory effects; however, the associations between anti-inflammatory effects induced by 2'-FL and 6'SL co-treatment and gut microbiota composition and metabolite modulation remain unclear. Therefore, in this study, we evaluated the effects of a mixture of these HMO. To determine the optimal HMO ratio for anti-inflammatory effects and elucidate its mode of action, LPS-induced inflammatory HT-29 epithelial cells and intestinal inflamed suckling mice were treated with various mixtures of 2'-FL and 6'SL. 2'-FL:6'SL ratio of 5:1 was identified as the most effective pre-treatment HMO mixture in vitro; thus, this ratio was selected and used for low, middle, and high-dose treatments for subsequent in vivo studies. In vivo, high-dose HMO treatment restored LPS-induced inflammation symptoms, such as body weight loss, colon length reduction, histological structural damage, and intestinal gene expression related to inflammatory responses. High-dose HMO was the only treatment that modulated the major phyla Bacteroidetes and Firmicutes and the genera *Ihubacter*, *Mageeibacillus*, and *Saccharofermentans*. These changes in microbial composition were correlated with intestinal inflammation-related gene expression and short-chain fatty acid production. To our knowledge, our study is the first to report the effects of *Ihubacter*, *Mageeibacillus*, and *Saccharofermentans* on short chain fatty acid levels, which can subsequently affect inflammatory cytokine and tight junction protein levels. Conclusively, the HMO mixture exerted anti-inflammatory effects through changes in microbiota and metabolite production. These findings suggested that supplementation of infant formula with HMO may benefit formula-fed infants by forming unique microbiota contributing to neonatal development.

Key Words: Human milk oligosaccharides, 2'-fucosyllactose, 6'Sialyllactose, gut microbiota

INTRODUCTION

In early stages of life, infants primarily rely on mother’s milk as the main source of nutrients and dietary glycans. Breast milk provides prebiotic glycans that stimulate the growth of beneficial colonic microbes with anti-microbial and immune modulatory effects to reduce inflammation (Fragkou et al., 2021). These glycans are referred to as human milk oligosaccharides (HMO) (Kellman et al., 2022), which are complex free oligosaccharides with mixtures of >250 oligosaccharides and are present at concentrations of 5–15 g/L in human milk (Kostopoulos et al., 2020). The metabolites of HMO produced by gut bacteria affect immune modulation or reduce systemic inflammation by inducing the immune response or controlling cytokine levels, respectively. The concentration of milk oligosaccharides in bovine milk, a material used for infant formula, is significantly lower than that in human milk. Particularly, the concentrations of fucosyl oligosaccharides including 2'-fucosyllactose (2'-FL; Fucα1–2Galβ1–4Glc) are in trace levels in bovine milk, whereas they are rather abundant in human milk (Urashima et al., 2013). Considering that milk oligosaccharides have crucial biological functions; it is concluded that some HMO should be supplemented into infant formulas when they are commercially available at the industrial level.

2'-FL is the most abundant HMO in secretor donor milk and reduces LPS-induced colitis symptoms by increasing the abundance of bacteria such as *Akkermansia*, and producing propionate and butyrate, which are the short chain fatty acids (SCFA) known to

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reduce inflammatory cytokine levels (Lv et al., 2023). 6′-Sialyllactose (6′-SL; Neu5Acα2–6Galβ1–3Glc) is the most abundant sialyl HMO in human milk and attenuates intestinal damage by inhibiting toll-like receptor 4 (TLR4) signaling activation, thus preventing proinflammatory cytokine expression (Huang et al., 2023). Co-treatment with 2′-FL and 6′-SL prevents necrotizing enterocolitis (NEC) in NEC-induced mice through TLR4 signaling inhibition (Sodhi et al., 2021). The immune modulation effects, such as inhibition of TLR4 signaling, are reportedly derived by HMO directly (Cheng et al., 2021; Rosa et al., 2022) or by the bacterial metabolites of HMO (Chen et al., 2023; Huang et al., 2023; Lv et al., 2023). However, to our knowledge, the associations between anti-inflammatory effects induced by 2′-FL and 6′-SL co-treatment, gut microbiota composition, and metabolite modulation have not yet been reported. Thus, this study aimed to elucidate the association of TLR4 signaling, gut microbiota composition, and SCFA production with the anti-inflammatory effects of 2′-FL and 6′-SL co-treatment.

In this study, an LPS-induced intestinal inflammation mouse model was used to assess the effects of 2′-FL and 6′-SL co-treatment on gut inflammation by evaluating intestine structural damage, inflammatory cytokine expression, gut microbiota composition, and SCFA production. In breast milk, approximately 70% of oligosaccharides are fucosylated and the remaining 30% are sialylated (Kostopoulos et al., 2020). 2′-FL and 6′-SL are known as the most abundant fucosylated or sialylated HMO, even considering concentration variations among secretor type and lactation period. Previous studies (Facinelli et al., 2019; Sodhi et al., 2021) suggesting the efficacy of 2′-FL and 6′-SL mixtures against intestinal inflammation support the potential use of these combinations as promising interventions for fortifying infant formulas. Therefore, 2′-FL and 6′-SL co-treatment was selected as an HMO treatment in this study. The optimal 2′-FL:6′-SL ratio of 5:1 was determined using an HT-29 intestinal in vitro model. This ratio was a mix of 2 mg/mL 2′-FL, which is generally recognized as safe (GRN No. 571), and 0.4 mg/mL 6′-SL, which is lower than the maximum approved industrial level of 0.7 mg/mL (Turck et al., 2022). Moreover, mean 2′-FL concentrations were 5 times higher than 6′-SL concentrations in >500 donated human milk samples, indicating that the 5:1 mixture is a promising supplement to make infant formula compositions more similar to that of breast milk (Thurl et al., 2017). Hence, this ratio was selected for HMO treatment in further in vivo studies. The concentration used in the first in vivo study was equivalent to the dosage used in the in vitro model. However, this dosage was lower than that reported in previous literature supporting the anti-inflammatory effects of HMO (Lv et al., 2023), suggesting that our treatment did not fully exhibit its effects. Therefore, a second in vivo study was conducted with 3 HMO concentrations; low, medium, and high. The low dosage was equivalent to that used in the first in vivo study, and the high dosage was derived from the animal equivalent dose calculation based on body surface area (Nair and Jacob, 2016).

MATERIALS AND METHODS

HMO preparation

For this study, 2′-FL and 6′-SL were purchased from Jennewein Biotechnologie GmbH (Rheinbreitbach, Germany). Samples were acquired as spray-dried powders containing ≥90% (wt/wt) of 2′-FL or 6′-SL.

Cell culture

HT-29 human colorectal adenocarcinoma cells were obtained from the Korean Cell Line Bank (Seoul, South Korea). The cells were maintained in culture dishes containing Roswell Park Institute 1640 medium (Gibco, Dublin, Ireland) supplemented with 10% fetal bovine serum (HyClone, MA, USA) and 1% penicillin/streptomycin (GE Healthcare, Chicago, IL, USA). Cells were incubated in a humid atmosphere (37°C, 5% CO2).

Anti-inflammatory activity evaluation in HT-29 cells

The cells were seeded at a density of 5 × 10⁵ cells/well in 12-well plates. After 24 h of incubation, the cells were pretreated with HMO mixtures prepared in Roswell Park Institute 1640 medium. Groups treated with 2 mg/mL 2′-FL mixed with 0.4 mg/mL 6′-SL were designated as 5:1; those treated with 0.4 mg/mL 2′-FL mixed with 0.4 mg/mL 6′-SL were designated as 1:1; and those treated with 0.4 mg/mL 2′-FL mixed with 2 mg/mL 6′-SL were designated as 1:5. After HMO treatment, the cells were incubated for 24 h (37°C, 5% CO2). To stimulate inflammatory responses, 1 μg/mL LPS (Sigma–Aldrich) diluted with Roswell Park Institute 1640 medium was added and incubated for 24 h (37°C, 5% CO2). Total mRNA was extracted using TRIzol reagent (Life Technologies, CA, USA) following the manufacturer’s instructions. The concentration and purity of the extracted RNA were assessed using a NanoDrop spectrophotometer (BioTek, Winooski, VT, USA) and adjusted to a final concentration of 0.1 μg/μL. cDNA was synthesized using a reverse transcription kit (Thermo Fisher Scientific). The PCR cycling conditions were 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Subsequently, reverse transcription quantita-
tive real-time PCR (RT-qPCR) was performed using the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-rad, Hercules, CA, USA). Targeted genes were quantified using a MG2 × qPCR Master Mix (SYBR Green) (MGmed, South Korea). The RT-qPCR cycling conditions were as follows: initial denaturation cycle at 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 s, annealing at 55–70°C for 30 s, and extension at 70°C for 5 s. Finally, the mRNA expression levels of each targeted gene were analyzed and normalized to the internal standard gene, GAPDH, using Bio-Rad CFX Maestro (Bio-Rad Laboratories, Hercules, CA, USA). Primer sequences used in this study are listed in Table 1.

**LPS-induced mouse colitis model**

The animal experiments were approved by the Institutional Animal Care and Use Committee of Korea University (KUIACUC-2020–0066). All animal experiments were performed in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for reporting research using animals. Two-week-old male C57BL/6N mice purchased from Young Bio (Sungnam, South Korea) were acclimatized for 1 week at 25°C with a 12 h light/dark cycle and ad libitum access to food and water. For the first animal experiment, 20 mice were randomly divided into the following 4 groups: Control (Con), LPS treated only (LPS), and HMO mixture treated groups (HMO-only treated [HMO] at 0.3 mg/g, and HMO pretreated at 0.3 mg/g by daily oral gavage for 2 weeks [LPS+HMO]);

<table>
<thead>
<tr>
<th>Target Species</th>
<th>Gene</th>
<th>Sequence</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
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<td>Human</td>
<td><em>IL1β</em></td>
<td>F: 5′-ATG CCA CCT TTT GAC AGT GAT G-3′</td>
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<tr>
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<td><em>IL6</em></td>
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<tr>
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<tr>
<td>Human</td>
<td><em>iNOS</em></td>
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<tr>
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<td><em>GAPDH</em></td>
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<tr>
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</tr>
<tr>
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<tr>
<td>Mouse</td>
<td><em>Muc2</em></td>
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<td>F: 5′-ATG TCC AGT TGA AGG GTT GTG TGA G-3′</td>
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1. *Ocl*: Occludin.
2. *Cldn4*: Claudin4
Con and LPS groups were gavaged with saline. HMO mixtures were prepared by adding 2'-FL (0.25 mg/g) and 6'-SL (0.05 mg/g) in saline. After 10 d of oral gavage, the LPS and LPS+HMO groups were intraperitoneally injected with LPS (1 mg/kg) for 5 d to induce intestinal inflammation, and the Con and HMO groups were injected with saline. During intraperitoneal injection, daily oral gavage was performed simultaneously. After 24 h of fasting, all mice were euthanized using CO2 inhalation. Blood samples were collected via cardiac puncture and centrifuged at 13000 × g for 15 min at 4°C to extract serum, which was stored at −20°C for a maximum of 2 weeks for further analysis. After excision, the colons were washed with PBS for length measurements, and the colon tissues were stored in liquid nitrogen for further analysis. The ileum was washed with PBS and severed longitudinally into 2 pieces. One piece was fixed in 10% neutral-buffered formalin (Sigma–Aldrich, USA) for hematoxylin and eosin staining, and the other was stored in liquid nitrogen for further analysis. For the second animal study, 25 mice were randomly divided into the following 5 groups: Con, LPS, and HMO mixture treated groups (HMO pretreated at low dose [HMO-L] at 0.3 mg/g, HMO pretreated at medium dose [HMO-M] at 0.6 mg/g, and HMO pretreated at high dose [HMO-H] at 3 mg/g by daily oral gavage for 2 weeks); the Con and LPS groups were gavaged with saline. HMO mixtures were prepared by adding the following: 2'-FL (0.25 mg/g) and 6'-SL (0.05 mg/g) in saline for HMO-L; 2'-FL (0.5 mg/g) and 6'-SL (0.1 mg/g) in saline for HMO-M; and 2'-FL (2.5 mg/g) and 6'-SL (0.5 mg/g) for HMO-H. After 10 d of oral gavage, all groups except the Con group were intraperitoneally injected with LPS (1 mg/kg) for 5 d to induce intestinal inflammation, and the Con group was injected with saline. During intraperitoneal injection, daily oral gavage was performed simultaneously. Subsequent procedures were similar to those used in the first animal experiment.

**Serum analysis**

Serum glucose (Embiel, Gunpo, South Korea) and IL-10 ELISA kits (Koma Biotech, Seoul, South Korea) were used according to the manufacturers’ instructions.

**Histological analysis of intestinal inflammation**

Ileal tissues were fixed in 10% (vol/vol) neutral formalin and embedded in paraffin (Oh et al., 2020). Then, 5-µM sections were sliced and stained with hematoxylin-eosin. Observations were performed using a light microscope (Olympus CKX41, magnification 100 × ) (Olympus, Tokyo, Japan).

**RNA extraction and RT-qPCR**

Animal tissues were homogenized using a Tissue-Tearor homogenizer (BioSpec, Oklahoma, USA). Total mRNA from the homogenized samples was extracted using TRIzol reagent (Life Technologies, CA, USA) following the manufacturer’s instructions. Subsequent procedures were similar to those used to evaluate the anti-inflammatory activity in HT-29 cells.

**Fecal SCFA analysis**

Feces were collected immediately after defecation and snap-frozen in liquid nitrogen. Frozen samples were homogenized and used for GC coupled with MS, to determine the fecal SCFA content (Oh et al., 2020). Briefly, 10 mg fecal samples were combined with 100 µL crotonic acid, 50 µL HCl, and 200 µL ether, and then homogenized and centrifuged at 1000 × g for 10 min. Supernatants were transferred to vials and 16 µL N-tert-butylidimethylsilyl-N-methyltrifluoroacetamide (Sigma–Aldrich, USA) was added. After mixing, vials were sealed and heated at 80°C for 20 min and then kept in room temperature for 48 h. Samples were placed in a 6890N Network GC System (Agilent Technologies, California, USA) with a HP-5MS column (30 m, 0.25 mm, 0.25 µm) and 5973 Network Mass Selective Detector (Agilent Technologies, USA). Helium (99.9999% purity) was used as a delivery gas at a flow rate of 1.2 mL/min. The head pressure was 97 kPa and the split was 20:1. The inlet and transfer line temperatures were 250 and 260°C, respectively. The following temperature program was used: 60°C (3 min), 60–120°C (5°C min), 120–300°C (20°C min). One microliter of sample was injected with 30 min of run time. SCFA concentrations were qualified by comparing their peak areas with those of the standards.

**Fecal genomic DNA isolation and next-generation sequencing**

Fecal genomic DNA was extracted using a QIAAmp DNA stool Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer’s instructions (Eor et al., 2021). The amount of double-stranded DNA was quantified using Quant-IT PicoGreen (Invitrogen, Madison, USA). Fecal metagenomic DNA samples were subjected to next-generation sequencing (NGS; Macrogen Inc., Seoul, South Korea). PCR amplification was performed for the V3 and V4 regions using the following universal primers: V3-F:5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3'; V4-R:5'-GTC TCG TGG CTC CCG AGA TGT GTA TAA GAG ACA GGA CTA CHV

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GGG TAT CTA ATC C-3′. Metagenomic libraries were prepared using a Nextera XT kit following the Illumina MiSeq protocol (Deb et al., 2020). Sequencing was performed using an Illumina MiSeq machine (MiSeq 2500) using 2 × 300 bp paired-end chemistry on multiplexed pooled samples.

**Taxonomic analysis of next-generation sequences**

Raw NGS data were analyzed using Quantitative Insights into Microbial Ecology2 (QIIME2). The contigs sequences were imported via qiime tools import command using paired end demultiplexed format. DADA2 plugins were used for contig quality control and chimera removal (command: q2-dada2 denoise-paired). For taxonomic assignment, a Greengenes database classifier was used with the following commands: qiime feature-classifier classify-sklearn, qiime metadata tabulate, and qiime taxa barplot.

**Statistical analyses**

Statistical analyses were performed using IBM SPSS Statistics software (version 25.0; IBM, Armonk, NY, USA). One-way ANOVA was used to analyze statistical differences between the mean values. Statistical significance was set at \( P < 0.05 \). All figures were generated using GraphPad Prism 9.0 (GraphPad Software, Boston, MA, USA). Correlation-based analyses and visualizations were performed using R Studio (RStudio, United States) and related packages.

**RESULTS**

**Effects of 2′-FL and 6′-SL mixtures with different ratios on LPS-induced inflammation in HT-29 intestinal epithelial cells**

To identify the optimum 2′-FL:6′-SL ratio, HMO mixtures were treated in an LPS-induced inflammatory cell model. The gene expression of inflammatory response related markers \( \text{IL1}\beta, \text{IL6}, \text{IL8}, \text{CD14}, \text{iNOS}, \text{ZO1}, \text{MUC2}, \text{Ocl} \), and \( \text{MUC2} \) was determined using RT-qPCR (Figure 1). LPS induction of inflammatory responses elevated \( \text{IL1}\beta, \text{IL6}, \text{IL8}, \text{CD14}, \) and \( \text{iNOS} \) gene expression levels; whereas, HMO-only treatments did not exhibit any elevation \( (P < 0.05) \) (Figures 1a–e). Pre-treatment of LPS-induced cells with 5:1 and 1:5 HMO mixtures normalized \( \text{IL1}\beta, \text{IL6}, \text{IL8}, \text{CD14}, \) and \( \text{iNOS} \) levels by downregulating gene expression by at least 11.3-fold \( (P < 0.05) \) (Figures 1a–e). Pre-treatment with the 1:1 HMO mixture also normalized \( \text{IL6}, \text{IL8}, \text{CD14}, \) and \( \text{iNOS} \) expression by downregulating gene expression by at least 9.8-fold; whereas, this pretreatment only reduced \( \text{IL1}\beta \) expression by 12-fold, which resulted in higher expression levels than that in the Con group \( (P < 0.05) \) (Figures 1a–e). Moreover, none of the treatments affected \( \text{ZO1} \) tight junction protein expression; only the 5:1 pre-treatment increased \( \text{MUC2} \) expression by 0.56-fold, resulting in higher levels than those in the Con group \( (P < 0.05) \) (Figures 1f and g). Based on the effects of the gut barrier-related markers, a 5:1 ratio was selected as the optimal HMO mixture ratio for further experiments.

**Effects of 2′-FL and 6′-SL mixture on intestinal inflammation on LPS-induced colitis suckling mice**

For the in vivo evaluation, the 2′-FL and 6′-SL mixture (5:1) was used as a treatment in an LPS-induced colitis mouse model. Serum IL-10 levels were measured using commercial ELISA kits, and the levels of the intestinal inflammation markers \( \text{Ocl}, \text{Zo1}, \text{Muc2}, \text{Il1}\beta, \text{Il6}, \) and \( \text{Cox2} \) were measured using RT-qPCR (Figure 2). Despite having no effect on colon length, LPS injection reduced body weight, indicating induction of intestinal inflammation \( (P < 0.05) \) (Figures 2a and b). However, LPS+HMO treatment did not ameliorate LPS-induced body weight loss \( (P < 0.05) \) (Figures 2a and b). LPS injection also elevated the levels of the inflammation markers serum \( \text{Il1}\beta, \text{Il6}, \) and \( \text{Cox2} \) \( (P < 0.05) \) (Figures 2c–f). Moreover, LPS induction reduced expression of the intestinal tight junction proteins \( \text{Ocl} \) and \( \text{Zo1} \) by at least 0.46-fold, and LPS+HMO treatment normalized \( \text{Ocl} \) expression by 0.62-fold \( (P < 0.05) \) (Figures 2g and h). Neither LPS nor LPS+HMO treatment affected \( \text{Muc2} \) expression (Figure 2i).

**Effects of 2′-FL and 6′-SL mixture with different dosages on growth performance and intestinal structures of LPS-induced colitis suckling mice**

The effects of high doses of the HMO mixture were evaluated (Figure 3). The dosage used in the first animal study was equivalent to that of HMO-L; whereas, the other doses used (i.e., HMO-M and HMO-H) were 2 and 10-times higher than that of HMO-L, respectively. LPS injection induced body weight loss, which was normalized by a 2-week treatment with HMO-M \( (P < 0.05) \) (Figure 3a). Other growth performance factors, such as food and water intake, were not affected (Figures 3b and c). The LPS-induced reduction in colon length was alleviated by all HMO treatments, indicating protective effects of HMO against LPS-induced inflammation \( (P < 0.05) \) (Figure 3d). Moreover, the ileum hematoxylin
and eosin staining results showed intestinal epithelial and crypt damage in the LPS group; whereas, the histological structures improved in all the HMO treatment groups (Figure 3h). The cecal weight, serum glucose levels, and serum IL-10 levels showed no differences after LPS or HMO treatment (Figures 3e–g). Overall, these results suggested that HMO exerted protective effects against LPS-induced intestinal inflammation.

**Effects of 2′-FL and 6′-SL mixture with different dosages on intestinal inflammation-related genes expression in LPS-induced colitis suckling mice**

The effects of the HMO dose on the levels of intestinal inflammation-related biomarkers were evaluated using RT-qPCR (Figure 4). LPS-induced inflammation increased ileal Tlr4, Il6, and Il1β expression levels, which were downregulated by at least 0.37-fold in all HMO-treated groups ($P < 0.05$) (Figures 4a–c). Il8 expression was not affected by LPS induction; however, it was reduced by HMO treatment ($P < 0.05$) (Figure 4d). The gene expression levels of the tight junction proteins Zo1 and Cldn4 were reduced by LPS-induced inflammation and ameliorated by HMO-H treatment, with a minimum of a 0.39-fold increase ($P < 0.05$) (Figures 4e–f). HMO-L and HMO-M treatments did not affect tight junction protein expression, implying that dosage is an important factor in optimizing the effects of HMO. Downregulated colonic gene expression of Tlr4, Il6, and Il1β in the LPS group was recovered by HMO-M and HMO-H treatments, with at least a 0.52-fold increase ($P < 0.05$) (Figure 5). These results indicated that HMO treatment, particularly HMO-H, effectively prevented LPS-induced inflammatory damage in the intestine.

**Effect of 2′-FL and 6′-SL mixtures on gut microbial composition and SCFA production in LPS-induced colitis suckling mice determined using fecal analysis**

The effects of HMO dosages on gut microbial compositions were assessed via NGS (Figure 6). The major phyla were Bacteroidetes, Firmicutes, and Verrucomicrobia (Figure 6a). LPS increased the Bacteroidetes abundance and decreased the Firmicutes abundance; whereas, HMO-H significantly reduced the Bacteroidetes abundance and increased the Firmicutes abundance, thus increasing the Firmicutes/Bacteroidetes ($F/B$) ratio ($P < 0.05$) (Figures 6c–f). The Verrucomicrobia abundance remained unaltered among treatments (Figure 6e). The major genera detected were *Bacteroides*, *Muribaculum*, *Alistipes*, *Lactobacillus*, *Kineothrix*, and *Akkermansia* (Figure 6b). Among the observed genera, the abundances of *Ihubacter*, *Mageeibacillus*, *Saccharofermentans*, and *Petrocella* varied among treatments (Figures 6g–j). HMO-H was the only treatment that significantly elevated the abundances of *Ihubacter*, *Mageeibacillus*, and *Saccharofermentans* to higher levels than those in the Con and LPS groups ($P < 0.05$) (Figures 6g–i). The *Petrocella* abundance was also higher in the HMO-H group than in the Con group ($P < 0.05$) (Figure 6j). Moreover, HMO-H was the only...
treatment that elevated the production of all SCFA, as determined using GC ($P < 0.05$) (Figures 6k–m). Thus, LPS-induced inflammation modified the gut microbiota composition and SCFA production, which were also modulated by HMO-H treatment.

Correlations between gut microbiota compositions, intestinal gene expression, and SCFA in LPS-induced colitis suckling mice treated with 2′-FL and 6′-SL mixtures

Spearman’s correlation analysis was conducted to determine the relationship between the NGS, RT-qPCR, and GC results (Figure 7). Actinobacteria and Bacteroidetes were positively correlated with the levels of the proinflammatory biomarkers $Il6$, $Il8$, and $Tir4$;
whereas, Firmicutes were inversely proportional to \( \text{Il6} \) expression \((P < 0.05)\) (Figure 7a). The ileal \( \text{Cldn4} \) level was proportional to the Verrucomicrobia abundance and negatively correlated with Bacteroidetes \((P < 0.05)\) (Figure 7a). Among the major genera, \( \text{Ihubacter} \), \( \text{Mageeibacillus} \), \( \text{Pseudoclostridium} \), \( \text{Ruminiclostridium} \), \( \text{Saccharofermentans} \), \( \text{Anaerotruncus} \), and \( \text{Neglecta} \) were negatively correlated with the levels of the intestinal proinflammatory markers \( \text{Il6} \), \( \text{Il8} \), \( \text{Il1β} \), and \( \text{Tlr4} \) \((P < 0.05)\) (Figure 7b). The level of the ileal tight junction protein \( \text{Zo1} \) was proportional to \( \text{Mageeibacillus} \), \( \text{Saccharofermentans} \), \( \text{Petrocella} \), and \( \text{Absiella} \) \((P < 0.05)\) (Figure 7b). Hence, HMO-H treatment altered the microbiota composition, thereby promoting the anti-inflammatory activities of HMO-H. Furthermore, among the major phyla, Firmicutes were positively correlated with propionate and butyrate production; whereas, Bacteroidetes and Deferrribacteres were negatively correlated with SCFA production \((P < 0.05)\) (Figure 7c). Among the major genera, \( \text{Ihubacter} \), \( \text{Mageeibacillus} \), \( \text{Pseudoclostridium} \), and \( \text{Saccharofermentans} \) were positively correlated with SCFA production; whereas, \( \text{Clostridium} \), \( \text{Muricomes} \), \( \text{Murimonas} \), \( \text{Absiella} \), and \( \text{Parasutterella} \) were inversely correlated with SCFA production \((P < 0.05)\) (Figure 7d). These results suggested that changes in the microbiota composition may have been related to changes in the amount of SCFA.

**DISCUSSION**

In this study, the potential benefits of incorporating 2'-FL and 6'-SL into infant formula were assessed in
LPS-induced intestinal inflammation models. Gastrointestinal inflammation is a state of colonic inflammation throughout the gastrointestinal tract that results from pathogens, damaged cells, or irritants (Oz et al., 2016). LPS, a bacterial endotoxin, can enter the human body through consumption of LPS-containing food supplements or absorption of LPS produced by gut bacteria (Wassenaar and Zimmermann, 2018). An overabundance of bacterial LPS triggers chronic inflammation, through which an abnormal colonic microbiome is formed, eventually impairing development of the immune, digestive, and neural systems (Fedirko et al., 2017; Indrio et al., 2022). Upon entering the body, the complex of LPS with TLR4, mediated by the action of the CD14 protein, triggers downstream signaling of adaptor molecules, such as MyD88, which induce nuclear factor kappa B (NF-κB) or Janus Kinase signaling (Yesudhas et al., 2014). NF-κB induces inflammatory responses through activities of proinflammatory cytokines such as IL6, IL8, and IL1β (Liu et al., 2017). Janus Kinase induces proinflammatory responses by triggering iNOS and COX2 activities (Kwon et al., 2013). In this study, the epithelial cells, the inflammation of which was induced by LPS, were treated with different ratios of 2′-FL and 6′-SL mixtures to determine the effects on TLR4 signaling-related gene expression (Figure 1). All the HMO ratios suppressed LPS-induced elevation of the proinflammatory response markers IL6, IL8, IL1β, CD14, and iNOS. However, only the 5:1 mixture was effective in upregulating MUC2 expression. MUC2 encodes an intestinal-type secretory mucin that fortifies the gut barrier integrity and prevents pathogen entry into the intestine (Johansson and Hansson, 2016). In addition, as this was the only ratio that exhibited

![Figure 4. Effects of 2′-FL and 6′-SL mixture on gene expression of ileal inflammation related markers in an LPS-induced intestinal inflammation mouse model. Ileal gene expression of: (a) Tlr4, (b) Il6, (c) Il1β, (d) Il8, (e) Zo1, and (f) Cldn4. Data are expressed as the mean ± SE (n = 5). Mean values in the same series with different lowercase letters are significantly different (P < 0.05).](#)
the potential to modify both proinflammatory signaling and intestinal barrier-related gene expression, that was selected as the optimal HMO treatment for further evaluations.

In our first animal studies, HMO treatment was effective in reducing the ileal Il1β, Il6, and Cox2 expressions in LPS-induced suckling mice (Figures 2d–f). Furthermore, HMO treatment fully recovered Ocl expression comparable to that in the Con group, indicating the intestinal protective effects of HMO. However, HMO treatment did not change LPS-induced Muc2 expression, despite MUC2 enhancement in the HT-29 inflammatory cell model by pretreatment with HMO (Figures 1g and 2i). These results do not align with a previous report in which HMO treatment recovered Muc2 impairment in NEC-induced suckling mice (Wu et al., 2019). This may be explained, at least in part, by the difference in the experimental conditions, including doses and treatment periods. Previous studies used considerably higher LPS and HMO doses than those in our studies, which may have resulted in more significant recovery of Muc2+ epithelial cells in the colon. In addition, the gavage of HMO was not effective in alleviating LPS-induced weight loss nor modulating colon length and serum inflammatory cytokine levels, implying that HMO did not fully exhibit their protective effects (Figures 2a–c). In contrast to our findings, 2′-FL treatment significantly attenuated body weight loss and inflammatory cytokine levels in an LPS-induced mouse model (Lv et al., 2023). This previous study also used much higher HMO doses than that used in our studies. Therefore, we conducted additional studies with various HMO treatment doses in LPS-induced suckling mice (Figures 3–7). HMO treatment effectively ameliorated LPS-induced body weight loss, colon length reduction, and ileal structural damage (Figure 3). Moreover, HMO treatment downregulated the gene expressions of Tlr4, Il6, and Il1β in both the ileum and colon, indicating the anti-inflammatory potentials of HMO (Figures 4 and 5). These results align with a previous report, in which sialyl HMO reduced Tlr4, Il1β, and Il6 levels in NEC-induced suckling mice (Zhang et al., 2021). Notably, the expression levels of Zo1 and Cldn4 were only significantly enhanced in the HMO-H treatment group, emphasizing the importance of HMO dosages for achieving desirable effects (Figures 4e and f).

Long-term exposure to LPS-induced inflammation may contribute to abnormal gut microbiota profiles, consequently leading to altered production of metabolites, such as SCFA. Therefore, the gut microbiome and SCFA profiles were analyzed (Figure 6). LPS injection increased the Bacteroidetes abundance and decreased the Firmicutes abundance (Figures 6c and d). Only the HMO-H treatment recovered the abundance of both phyla, with a corresponding increase in the F/B ratio (Figure 6f). The F/B ratio is associated with gut homeostasis, and its changes are linked to pathologies, such as obesity (high F/B ratio) and dysbiotic conditions (low F/B ratio) (Stojanov et al., 2020). A reduction in the F/B ratio induced by LPS cause gut inflammation, due to increase of some Bacteroidetes species that synthesize LPS in the gut microbiome (D’Hennezel et al., 2017). Only the HMO-H treatment increased the F/B ratio, indicating alleviation of gut dysbiosis and inflammation. Moreover, Bacteroidetes and expression of the ileal proinflammatory cytokines Il6 and Il8, and colonic Il8 were positively correlated, thereby supporting the possibility that a reduction in the Bacteroidetes...
Figure 6. Fecal analysis of LPS-induced intestinal inflammation mice treated with different 2′-FL and 6′-SL mixture dosages. (a) Community bar plot at the phylum level, (b) Community heat map at the genus level. Major phyla relative abundance (%) of: (c) Bacteroidetes, (d) Firmicutes, and (e) Verrucomicrobia. (f) Ratio of Bacteroidetes/Firmicutes. Relative abundance (%) of: (g) Ihubacter, (h) Mageebacillus, (i) Saccharofermentans, and (j) Petrocella. Amount of short-chain fatty acids produced: (k) acetate, (l) propionate, and (m) butyrate. Data are expressed as the mean ± SE (n = 5). Mean values in the same series with different lowercase letters are significantly different (P < 0.05).
abundance contributed to the anti-inflammatory effects of HMO-H (Figure 7a). Furthermore, this was the only treatment which increased the abundances of *Ihubacter*, *Mageeibacillus*, and *Saccharofermentans*, all of which belong to phylum Firmicutes (Figures 6g–i). The Firmicutes abundance was inversely correlated with ileal and colonic inflammation-related genes such as *Il8*, *Il6*, *Il1β*, or *Tlr4* and positively correlated with *Zo1* levels (Figure 7b). Thus, HMO-H-related gut microbiota composition and modulation of inflammatory responses may have influenced each other. A negative correlation between *Ihubacter* abundance in the colonic microflora and the proinflammatory cytokine *IL6* levels has been reported (Han et al., 2022); however, to our knowledge, associations between this abundance and the expression level of these tight junction proteins or other metabolites have not yet been reported.

SCFA are the major metabolites produced by gut bacteria as end products of indigestible polysaccharide fermentation (Lv et al., 2023). Acetate, propionate, and butyrate are the major SCFA that regulate intestinal homeostasis. Acetate reduces the gut pH and contributes to creating an acidic environment that promotes growth of beneficial bacteria (Fukuda et al., 2011). Propionate contributes to maintaining intestinal homeostasis by promoting epithelial cell migration and wound closure, thereby improving epithelial turnover and protecting against ulcer formation (Bilotta et al., 2021). Butyrate effectively protects the intestinal epithelial barrier integrity by modifying tight junction protein expression and intestinal mucus production (Siddiqui and Cresci, 2021). Only the HMO-H treatment increased acetate, propionate, and butyrate production to levels higher than those observed in the LPS group (Figures 6k–m). Notably, only this decreased the Bacteroidetes abundance, which was negatively correlated to SCFA levels (Figure 7c). Moreover, HMO-H increased the abundances of *Ihubacter*, *Mageeibacillus*, and *Saccharofermentans* (all belonging to phylum Firmicutes), which was directly proportional to SCFA levels (Figures 7d). These results indicated that the gut microbiota, the composition of which was influenced by HMO treatment, may have contributed to the increase in SCFA production. TLR4 signaling modulation through intestinal flora shifting is the main pathway for the anti-inflammatory effects of HMO (Cheng et

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**Figure 7.** Spearman’s correlation analysis of major gut microbiota composition, short-chain fatty acids, and intestinal inflammation-related gene expression. Spearman’s correlations between: (a) major phyla and intestinal gene expression; (b) major genera and intestinal gene expression; (c) major phyla and short chain fatty acids; and (d) major genera and short chain fatty acids. Data are expressed as mean values (n = 5). ***P < 0.001, **P < 0.01, *P < 0.05.
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al., 2021; Rosa et al., 2022). In addition, our results suggested that changes in gut microbiota composition due to HMO-H influenced SCFA production and modulated intestinal inflammatory signaling. Previous studies have demonstrated the anti-inflammatory effects exerted by sole treatment with acetate, propionate, or butyrate in intestinal inflammation models through the downregulation of inflammatory cytokine expression and upregulation of tight junction protein expression (Tedelind et al., 2007, Tong et al., 2016, Chen et al., 2018). Therefore, the increased SCFA production induced by HMO-H treatment may have regulated the inflammatory responses, which subsequently subsided the LPS-induced symptoms.

Enhancement of SCFA production and reduction of the inflammation response are triggered by HMO treatment (Liu et al., 2020, Lv et al., 2023). However, to our knowledge, our study is the first to show that *Ihubacter* and *Mageeibacillus* (all belonging to phylum Firmicutes) were associated with both SCFA production and regulation of inflammatory factors. The species of *Ihubacter* and *Mageeibacillus* detected in this study were *Ihubacter massiliensis* (a human gut-isolated strain) and *Mageeibacillus indolicus* (a human genital-isolated strain), respectively (Austin et al., 2015, Ndongo et al., 2016). Therefore, the relationships between HMO treatment and these microflora that were present in both humans and mice are meaningful observations before clinical studies. Given that there is the significant species difference in gut microflora (strains, distribution, activities, etc.) between mice and humans, further clarification is necessary in clinical studies.

**CONCLUSION**

A high-dose treatment with a 2′-FL:6′-SL ratio of 5:1 restored LPS-induced inflammatory symptoms, such as body weight loss, colon length reduction, and damage to the histological structure of the ileum. Furthermore, the LPS-induced elevation of intestinal gene expression during inflammatory responses was suppressed by all HMO dosages. However, only HMO-H treatment enhanced *Cldn4* expression, implying that optimal dose selection is crucial for maximum efficacy. HMO-H was also the only treatment that recovered LPS-induced changes in phyla such as Bacteroidetes and Firmicutes. HMO-H-induced changes in the intestinal flora may have attenuated inflammation by increasing SCFA, which consequently affected the expression of genes involved in inflammatory signaling. The protective effects of HMO mainly involved TLR4 signaling and gut microbiota shifts. Furthermore, this study contributes to deciphering the metabolic activities of *Ihubacter* and *Mageeibacillus*, which are bacterial genera for which available literature is scarce. This study underscores the potential significance of incorporating HMO into infant formula, as they may reduce inflammation and minimize developmental differences between formula-fed and breastfed infants, thus potentially improving the overall health and well-being of formula-fed children.

**Notes**

Conflict of interest The authors have not stated any conflicts of interest.

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