



HMOs modulate immunoregulation and gut microbiota in a β -lactoglobulin-induced allergic mice model

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ABSTRACT

Milk, which contains β -lactoglobulin (β -Lg; a major allergenic protein), is the most common food allergen and one of the earliest discovered. Human milk oligosaccharides (HMOs), especially 2'-fucosyllactose (2'-FL), have multiple effects as prebiotics, including improvements to immune regulation, gut health, and cognition. In this study, we investigated the effects of HMOs on immunoregulation and intestinal flora in a β -Lg-induced milk allergy mouse model. HMOs supplementation reduced allergic symptoms, decreased serum IgE and β -Lg-specific IgE, and significantly increased levels of the anti-inflammatory cytokines IL-10, TGF- β , and IFN- γ compared to those in the β -Lg allergy group. Furthermore, HMOs and 2'-FL interventions improved richness and diversity of fecal microbiota. The relative abundance of *Lactobacillus* significantly increased, while that of *Lactococcus* and *Alistipes* significantly decreased. In summary, our study shows that HMOs and 2'-FL supplementation relieved β -Lg-induced allergies.

1. Introduction

Food allergy is a serious public health issue affecting both adults and children. Increasing evidence shows that the prevalence of food allergy has become much more prevalent over the past decades (Neau et al., 2016). The prevalence in children is as high as 6%, and in adults 1–3% (Branum & Lukacs, 2009; Kagan, 2003). The most common food allergen is milk (Jo, Garssen, Knippels, & Sandalova, 2014).

Cow's milk allergy (CMA) is an immunologically mediated adverse reaction to one or more milk proteins, usually against caseins and the two main whey proteins, α -lactalbumin and β -lactoglobulin (β -Lg). CMA can cause multiple adverse anaphylactic reactions in children (Schocker, Kull, Schwager, Behrends, & Jappe, 2019), ranging in severity from mild to life-threatening. Treg cells play an important role in maintaining the balance between immune tolerance and allergic responses (Huang, Liu, & Jan, 2010; Yamamoto, Matsunami, Komori, Hayashi, & Kadowaki, 2019). Many studies have shown that activation of allergenic and adaptive effectors and inhibition of Treg cells collectively lead to the pathogenesis of CMA (Jo et al., 2014; Kumar, Verma, Das, & Dwivedi, 2012). To date, however, treatment of food allergies has been limited by a lack of clear understanding of the molecular mechanisms involved.

Breast milk is the first diet of infants, providing essential nutrients for their growth and development as well as biologically active factors

such as immunoglobulin (Ig) and cytokines (Ballard & Morrow, 2013). Human milk oligosaccharides (HMOs), as the third largest component in human milk (Bode, 2012), promote intestinal development and the establishment of intestinal flora in infants. Many studies have documented the association between HMOs and *bifidobacteria*, which are more abundant among the gut microbiota of breast fed infants than of formula-fed infants, and which may reduce the growth of harmful bacteria (Vandenplas et al., 2018). HMOs modulate immune responses by directly affecting immune cells and cytokines and by indirectly changing the composition of intestinal flora (Goehring et al., 2016). Several clinical studies have demonstrated that infants fed formula with 2'-FL improved immune system and gut health benefits (Reverri, Devitt, Kajzer, Baggs, & Borschel, 2018). In addition, HMOs also prevent pathogens from adhering to intestinal epithelial cell receptors (Hill & Newburg, 2015).

Over the last few years, many studies have demonstrated that gut microbes play a critical role in host health, affecting many aspects of human physiology, including energy metabolism (Nicholson et al., 2012), hormone balance (Utzschneider, Kratz, Damman, & Hüller, 2016), and the immune system (Belkaid & Hand, 2014; Shi & Walker, 2004). Intestinal flora contribute to the establishment of a mucosal barrier and maintenance of intestinal homeostasis. Compared with healthy individuals, many patients with intestinal dysbiosis and allergic symptoms have lower diversity and richness of gut microbiota

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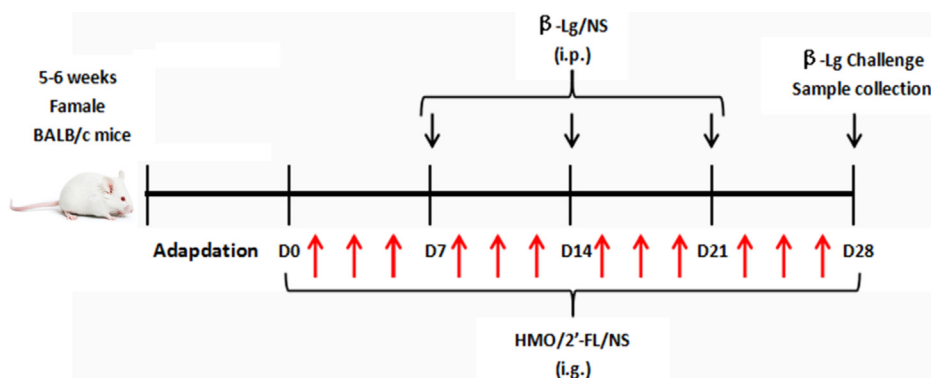


Fig. 1. The agreements used for the mice models of food allergy to β -Lg. Female BALB/c mice aged 5–6 wk were intraperitoneally injected with 0.2 mL 1 mg/mL β -Lg on days 7, 14 and 21, which was dissolved in a mixture of Freund's adjuvant and sterile saline. In parallel, mice were orally supplemented with 2'-FL (200 mg/kg, 400 mg/kg, 600 mg/kg), HMO (400 mg/kg) or NS for 4 wk. All mice were orally challenged with β -Lg on day 28, except the control group.

(Waligora-Dupriet & Butel, 2012).

Recent findings have shown that interactions between gut bacteria and the host have a momentous influence on the occurrence of autoimmune and allergic diseases (Kranich, Maslowski, & Mackay, 2011). Beneficial microbes, especially species from the phyla *Bacteroidetes*, produce factors that have beneficial anti-inflammatory effects and are distributed systemically to influence peripheral inflammatory responses. These factors positively affect the control and resolution of inflammatory and autoimmune diseases. For example, the role of short-chain fatty Acids (SCFAs) has been confirmed in inflammatory responses: SCFAs bind the G protein-coupled receptor to exert anti-inflammatory action and impact neutrophil function (Le Poul et al., 2003). In addition, specific intestinal microbes can control T-cell differentiation in the lamina propria, in which contains particularly abundant cytokine IL-17 expressing Th17 cells (Ivanov et al., 2008).

CMA affects the absorption and utilization of high-quality milk proteins by infants. However, to our knowledge, there are no data on HMOs as potential protective factors against the development of β -Lg-induced allergy. Our previous work showed that β -Lg-induced allergy is characterized by inflammatory cell infiltration into the colons of sensitized mice (Wang et al., 2018). The present study aimed to investigate whether HMOs, 2'-FL among them, can modulate immune responses in a β -Lg-induced mouse mode of milk allergy. Findings from this study will contribute to a better understanding of the protective effects of HMOs in allergic diseases and provide new insights into a potential approach to relieve CMA.

2. Materials and methods

2.1. Human milk oligosaccharides

The 2'-FL tested in this study was obtained from Sharing Technologies (Shanghai, China). HMOs were extracted from the breast milk of 32 volunteers according to established methods (Ward, 2009). Mothers were asked to collect breast milk when their infants were aged 3 mo. To reduce differences between individuals, the breast milk samples were mixed, and their contents were quantified by Liquid Chromatography-Mass Spectrometry (LC-MS) as described by others (W. Zhang et al., 2019). HMOs composition in the samples included 2'-FL (58.75%), 3'-FL (4.97%), 3'-SL (1.88%), 6'-SL (3.26%), and LNDFH II (5.92%).

2.2. Experimental animals

Female BALB/c mice aged 5–6 weeks were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (certificate number: 11400700318316; Beijing, China). Upon arrival, all mice were randomly allocated to the control or experimental groups: C, control group; A- β -Lg, allergic group; HMO, human milk oligosaccharide group; L-2'-FL, low-dose group (200 mg/kg); M-2'-FL, middle-dose

group (400 mg/kg); H-2'-FL, high-dose group (600 mg/kg). Mice were kept in a specific-pathogen-free animal facility for 1 week under a 12 h light/dark cycle; free access to food and water was allowed. Animals used in this study were cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 85–23,1996), and all animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the guidelines of Northeast Agricultural University (Harbin, China).

2.3. Animal model

BALB/c mice were randomly sub-divided into 6 groups. All mice except the control group were intraperitoneally injected with 0.2 mL 1 mg/mL β -Lg dissolved in a mixture of Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA) on days 7, 14 and 21 (Adel-Patient et al., 2005; Fu et al., 2019). From day 1 to day 28, the mice in the HMO and 2'-FL-treated groups were supplied 0.2 mL of the corresponding oligosaccharide three times per week by oral gavage. Mice from the control and β -Lg allergic groups were treated with sterile saline. On day 28, all mice were orally challenged with β -Lg (20 mg/mouse), except those in the control group. After wards, blood, fecal, and tissue samples from each mouse were immediately collected for further analysis (Fig. 1).

2.4. Symptom valuation

The antiallergic effects of HMOs were investigated as follows:

1. After 1 h of β -Lg challenge, hypersensitivity symptoms were evaluated using a scoring standard (Table 1).
2. Animal body weight in all groups was monitored once weekly during the experiment.
3. After sacrifice, mouse colons were collected and their lengths measured (Park et al., 2018).
4. The colons and lungs of experimental mice were removed on day 28, fixed in 10% neutral formalin, and embedded in paraffin. Colon sections were stained with hematoxylin and eosin for observation of intestinal mucosal integrity and lymphocytic infiltration. All analyses were performed under a microscope at 10 $\times$ magnification (Park et al., 2018).

Table 1
Scoring methods of anaphylactic symptoms.

Anaphylactic symptoms	Grade
no symptoms	0
pilar erect, scratching and rubbing around the nose and head	1
decreased activity, puffiness around the eyes and mouth	2
static activity time exceeds 1 min	3
Arching from the back, cyanosis around the mouth and the tail	4
Convulsion or death	5

- The mice were weighed before being sacrificed. The thymus and spleen were immediately excised surgically and weighed. An immune organ index was calculated according to the following formula:

$$\text{thymus or spleen indices (mg/g)} = \frac{\text{thymus or spleen weight (mg)}}{\text{body weight (g)}}$$

2.5. Measurement of cytokines and immunoglobulin

Serum samples were collected to assay cytokines and immunoglobulin (Ig). After 1 h of β -Lg challenge, blood samples were collected from the eyeballs and centrifuged at 3000 rpm for 10 min at 4 °C to obtain serum, and then stored at −20 °C before analysis. Levels of serum IFN- γ , TGF- β , IL-10, total IgE (eBioscience, San Diego, CA, USA) and β -Lg-specific IgE (Cheng Lin, Beijing, China) were quantified using ELISA Kits according to the manufacturer's instructions.

2.6. RNA isolation and quantitative real-time PCR

After mice were sacrificed on day 28, their colons were dissected, immersed in RNAlater (Sigma-Aldrich), and stored at −80 °C until further processing. Total RNA extraction was carried out using a Total RNA kit (Tiangen, Beijing, China) with colon tissue and reverse-transcribed to cDNA by an RNA template with FastQuantcDNA. cDNA was amplified using a RealMasterMix (SYBR Green) kit (Invitrogen, Carlsbad, CA, USA) and run in the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Thermal cycling conditions for all reactions were as follows: 1 cycle at 95 °C for 15 min, 40 cycles at 95 °C for 10 s, and 60 °C for 30 s. Primers are listed in Table 2. Relative levels of gene expression were calculated by reference to the level of GAPDH using the $\Delta\Delta\text{CT}$ method (Livak & Schmittgen, 2001).

2.7. SCFA determination and quantification

Short-chain fatty acid analysis was conducted as described elsewhere (de Theije et al., 2014). Briefly, mouse fecal supernatant was analyzed using a gas chromatography-mass spectrometer (GC-MS) and acetic, propionic, butyric, *iso*-butyric, valeric, *iso*-valeric and caproic acid concentrations were quantitated (based on a 4-methyl valeric acid internal standard).

2.8. 16S rDNA gene sequencing of gut microbiota

The 16S rDNA gene amplicon sequencing was performed on the IlluminaMiSeq platform (Harbin Bio-Technology Co. Ltd., Harbin, China) as described previously. Bioinformatics analysis of sequencing data was carried out with the Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso et al., 2010).

2.9. Statistical analysis

Statistical significance of data was assessed by one-way ANOVA with SPSS version 24 (SPSS, Inc., Chicago, IL, USA). All data are expressed as means $\pm$ standard error of the mean. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. 2'-FL reduces allergic symptoms of β -Lg-induced mice

As shown in Fig. 2A, after the oral β -Lg challenge, the allergic symptoms score of the β -Lg allergic group was 3.60 ± 0.89 , significantly higher than that of the control group ($P < 0.05$). Intervention effects in the HMO and 2'-FL high-dose groups were more obvious than those in other groups. The scores of allergic symptoms in the HMO group and 2'-FL high-dose group were both 1 ± 0.71 , indicating that HMOs could relieve allergic symptoms and that 2'-FL would played a major role in the HMOs treatment.

As shown in Fig. 2B, compared with control group, the β -Lg allergic group (14.93 ± 0.09 g) showed an obvious decrease in body weight after the injection of β -Lg. Body weight in the HMO and 2'-FL groups had not increased significantly compared with that in the β -Lg allergic group by day 7. At day 14, the body weight of the β -Lg allergic group continued to decrease, while those of the HMO and 2'-FL groups began to increase. At day 28, the body weight of the HMO group had increased remarkably; the body weights of the 2'-FL groups also increased, and the body weight of the 2'-FL high-dose group was closest to that of the control group.

Furthermore, in the β -Lg allergic group (7.10 ± 0.79 cm), colon length was significantly shorter than in the control group (9.26 ± 0.59 cm) ($P < 0.05$). This contraction of colon length could be prevented via different interventions. Colon lengths were 8.50 ± 0.70 cm, 8.20 ± 0.83 cm, and 8.10 ± 0.51 cm in the 2'-FL high-dose, middle-dose, and low-dose groups, respectively. In the 2'-FL high-dose group, colon length significantly increased ($P < 0.05$) compared with β -Lg allergic group. The above results showed that 2'-FL supplementation effectively mitigated the decreased colon length induced by β -Lg (Fig. 2C).

Photomicrographs of hematoxylin and eosin (H&E)-stained colon and lung sections are shown in Fig. 2(D and E). In the β -Lg allergic group, the colon mucosal lining was destroyed, which was accompanied by obvious infiltration of inflammatory cells, alveolar wall thickening of the lung tissue, vasodilatation, and hemorrhage. However, the colon mucosal injury and hemorrhage were alleviated in 2'-FL-treated mice. The results show that 2'-FL has a protective effect on allergies caused by β -Lg.

As shown in Table 3, compared with those in the control group, the thymus indexes (3.69 ± 0.15 mg/g) and spleen indexes (4.81 ± 0.26 mg/g) in the β -Lg allergic group were significantly decreased ($P < 0.05$). However, the immune organ indexes were significantly increased in each 2'-FL treatment group and in the HMO group ($P < 0.05$), especially in the HMO and 2'-FL high-dose groups. These results show that HMOs may have a protective effect on the growth of the spleen and thymus in β -Lg-allergic mice.

3.2. Cytokines and immunoglobulin

The levels of cytokines are represented in Fig. 3(A–C). Compared with those in the control group, the levels of IFN- γ , IL-10, and TGF- β in the β -Lg-allergic group were significantly reduced ($P < 0.05$). Conversely, the levels of these cytokines were significantly increased in the HMO and 2'-FL treatment groups ($P < 0.05$). Furthermore, 2'-FL

Table 2
Designed primer sequences for mRNA Expression of Target Genes.

Gene	Forward (5' to 3')	Reverse (5' to 3')
GAPDH	5'-AGAAGGTGGTGAAGCAGGCATC-3'	5'-CGAAGGTGGAAGAGTGGGAGTTG-3'
Foxp3	5'-AAGAATGCCATCCGCCACAACC-3'	5'-GGCGTTGGCTCCTCTTCTTGC-3'
TGF- β	5'-GGCATCGCTCATCTCCACAGTG-3'	5'-AGGCAACAGGTCAAGTCGTTCTTC-3'
IL-10	5'-CTGCTATGCTGCCTGCTCTTACTG-3'	5'-ATGTGGCTCTGGCCGACTGG-3'

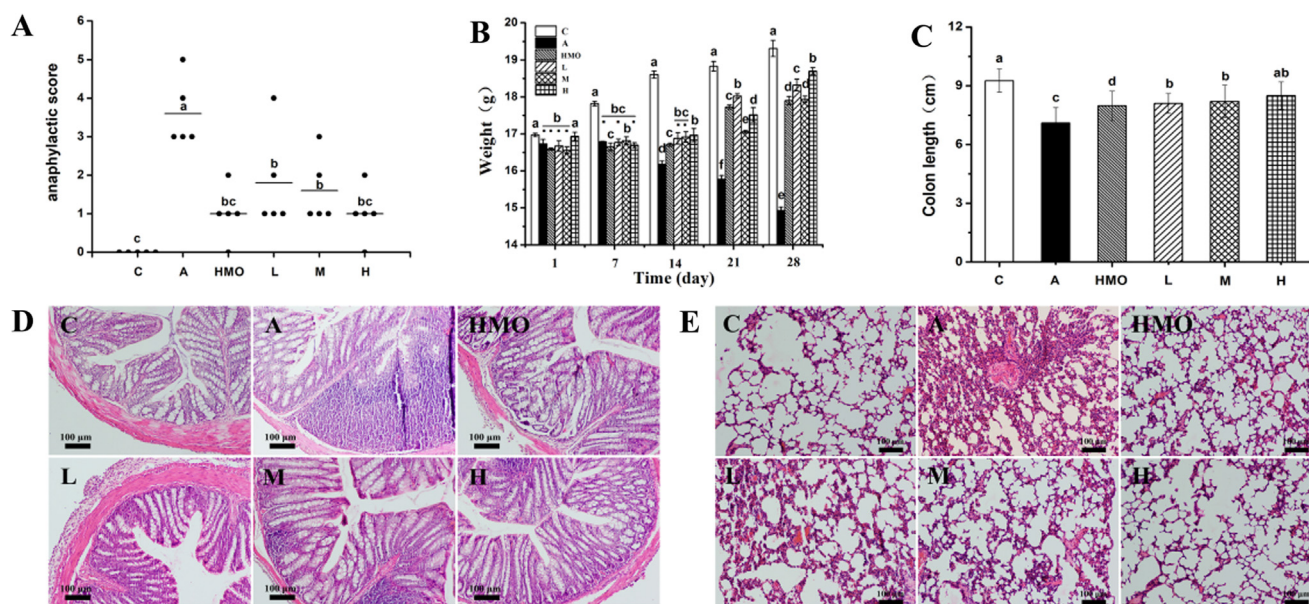


Fig. 2. Effects of HMOs on allergic symptoms score (A), weight (B), colon length (C), colon (D) and lung (E) histology in β -Lg sensitized mice. C, Control group; A, β -Lg allergic group; HMO, human milk oligosaccharide group (400 mg/kg); L, 2'-Fucosyllactose low-dose group (200 mg/kg); M, 2'-Fucosyllactose middle-dose group (400 mg/kg); H, 2'-Fucosyllactose high-dose group (600 mg/kg). Data are expressed as the mean $\pm$ SD (n = 10). Significant differences ($P < 0.05$) between the groups are indicated with different letters above the data.

Table 3

Effect of 2'-FL on thymus and spleen indices in mice.

Group	Spleen index (mg/g)	Thymus index (mg/g)
C	5.24 $\pm$ 0.31 ^a	3.98 $\pm$ 0.23 ^b
A	4.74 $\pm$ 0.34 ^b	3.69 $\pm$ 0.15 ^c
HMO	5.40 $\pm$ 0.20 ^a	4.30 $\pm$ 0.24 ^a
L	5.25 $\pm$ 0.15 ^a	4.09 $\pm$ 0.20 ^{ab}
M	5.28 $\pm$ 0.17 ^a	4.16 $\pm$ 0.20 ^{ab}
H	5.38 $\pm$ 0.20 ^a	4.26 $\pm$ 0.21 ^a

C, Control group; A, β -Lg allergic group; HMO, human milk oligosaccharide group (400 mg/kg); L, 2'-Fucosyllactose low-dose group (200 mg/kg); M, 2'-Fucosyllactose middle-dose group (400 mg/kg); H, 2'-Fucosyllactose high-dose group (600 mg/kg). Data are expressed as the mean $\pm$ SD (n = 10). Significant differences ($P < 0.05$) between the groups are indicated with different letters above the data.

improves cytokine decline in a dose-dependent manner. It should be noted that treatment with 2'-FL at 600 mg/kg maximally improved cytokine levels in the mice induced by β -Lg.

Immunoglobulin levels are represented in Fig. 3D and Fig. 4A. The concentrations of IgE and β -Lg-specific IgE in the β -Lg-allergic group (173.10 $\pm$ 3.36 ng/mL and 94.56 $\pm$ 0.63 μ g/L, respectively) were significantly higher than those in the control group (119.00 $\pm$ 2.30 ng/mL and 20.24 $\pm$ 0.42 μ g/L, respectively) ($P < 0.05$). Moreover, serum IgE and β -Lg-specific IgE concentrations in allergic mice treated with 2'-FL and HMOs were significantly reduced ($P < 0.05$). These results suggest that 2'-FL attenuated the allergic inflammatory response.

3.3. IL-10, TGF- β and Foxp3 mRNA expression in colon tissue

To further explore the effects of different interventions on Treg responses in the colon, the mRNA expression levels of Treg-specific transcription factors were measured (Fig. 4B). Compared with those in control group, expression levels of Treg-associated cytokines (TGF- β and IL-10) and transcription factors (Foxp3) were significantly lower in the β -Lg-allergic group ($P < 0.05$), while the expression of TGF- β , IL-10, and Foxp3 notably increased in the HMO and 2'-FL groups.

3.4. SCFA

The level of acetic acid in the feces of β -Lg-sensitized mice was significantly increased ($P < 0.05$), while the levels of propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid were significantly decreased ($P < 0.05$) (Fig. 5). Different concentrations of 2'-FL and HMOs effectively lowered the acetic acid content in the intestinal tracts of allergic mice. In particular, the acetic acid levels in the 2'-FL high-dose and HMO groups were not significantly different from that in the control group ($P > 0.05$). High-dose 2'-FL treatment significantly promoted the metabolism of propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid compared with middle- and low-dose treatments. There were no significant differences in levels of hexanoic acid after treatment with any concentration of 2'-FL or HMOs, as illustrated in Fig. 5.

3.5. Structural comparison of fecal microbiota among different groups

As shown in Table 4, the number of high-quality sequences in each group was 32548–42928, and the average length of these sequences was 455.8–462.0 bp. The 2'-FL middle- and high-dose groups were closest to the control group, with 300 and 296 OTUs, respectively.

To further explore the diversity of microbial species and their relative abundances, microbiome composition was analyzed at the phylum, family, and genus levels (Fig. 6A). *Firmicutes* (42.80–58.8%), *Bacteroidetes* (34.3–45.9%), *Actinobacteria* (1.20–4.60%), and *Proteobacteria* (0.8–4.2%) ranked among the top 4 intestinal microorganisms in each group, with their sequences totaling more than 93%. The composition of bacteria in the gut differed in each group.

The relative abundance of different bacterial families in different groups is illustrated in Fig. 6B. In the β -Lg-allergic group, *Streptococcaceae* was the main family (41.10%), followed by *Muribacaceae*, *Rikenellaceae*, *Lachnospiraceae*, and *Lactobacillaceae*. These five families accounted for 86.30% of the community population. However, the major bacterial families changed with HMOs and 2'-FL interventions. The abundance of *Streptococcaceae* in the HMO, 2'-FL low-dose, 2'-FL middle-dose, and 2'-FL high-dose groups decreased by 40.70%, 10.70%, 38.10%, and 40.90%, respectively, while abundance of *Lachnospiraceae* increased in the HMO group compared with that in the β -Lg-allergic

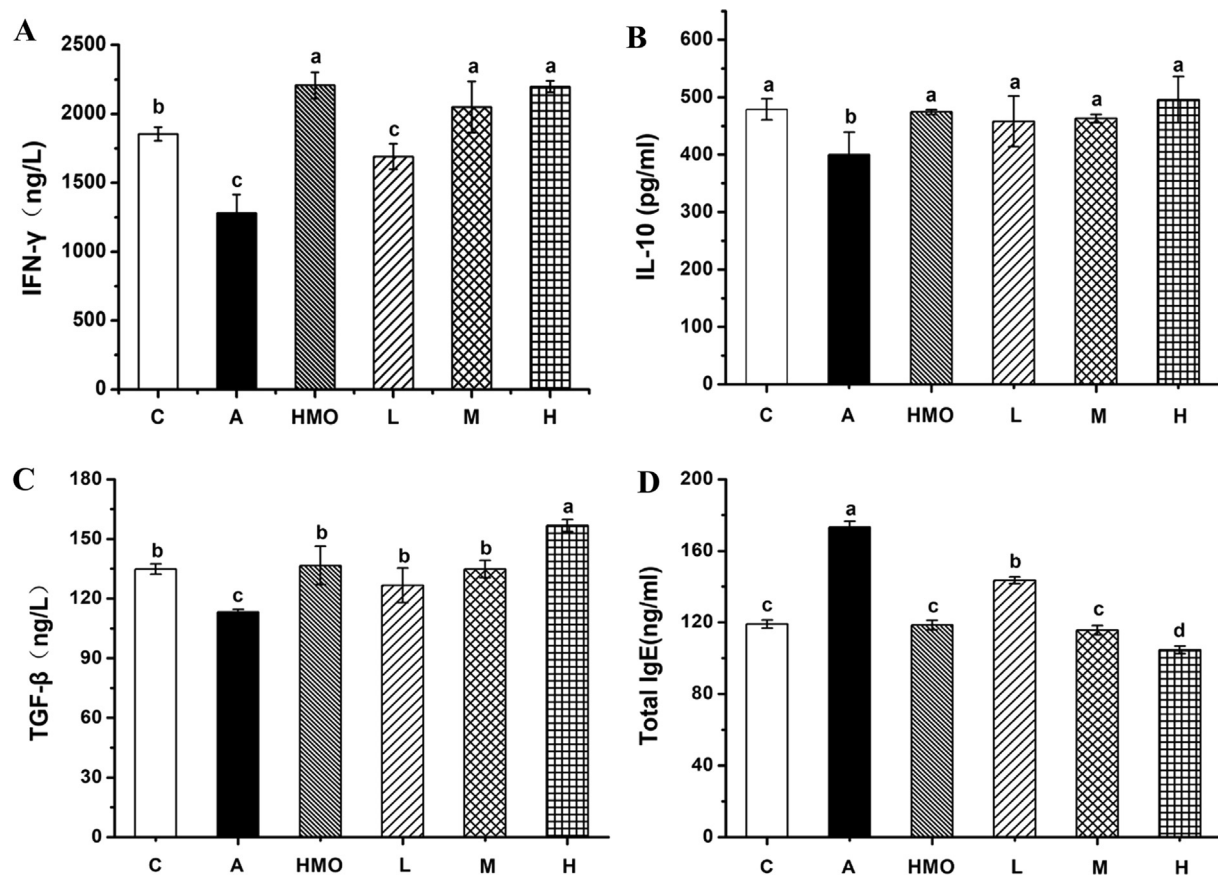


Fig. 3. Effects of HMOs on the secretion of cytokines (A: IFN- γ ; B: IL-10; C: TGF- β) and IgE (D) in serum. C, Control group; A, β -Lg allergic group; HMO, human milk oligosaccharide group (400 mg/kg); L, 2'-Fucosyllactose low-dose group (200 mg/kg); M, 2'-Fucosyllactose middle-dose group (400 mg/kg); H, 2'-Fucosyllactose high-dose group (600 mg/kg). Data are expressed as the mean $\pm$ SD ($n = 10$). Significant differences ($P < 0.05$) between the groups are indicated with different letters above the data.

group ($P < 0.05$).

To further analyze changes in intestinal flora in each group, the relative abundances of the 14 most abundant bacterial groups at genus level were selected for analysis (Fig. 6C). There was a relatively high abundance of unclassified *Muribaculaceae* in each group. *Lactobacillus* is a microorganism beneficial to host health; it helps maintain human health and regulate immune function. Compared with that in the

control group, a decline in the relative abundance of *Lactobacillus* and a rise in the relative abundances of *Lactococcus* and *Alistipes* were noted in the β -Lg-allergic group. The interventions had the opposite effect, with HMOs and 2'-FL high-dose treatments producing the most pronounced changes.

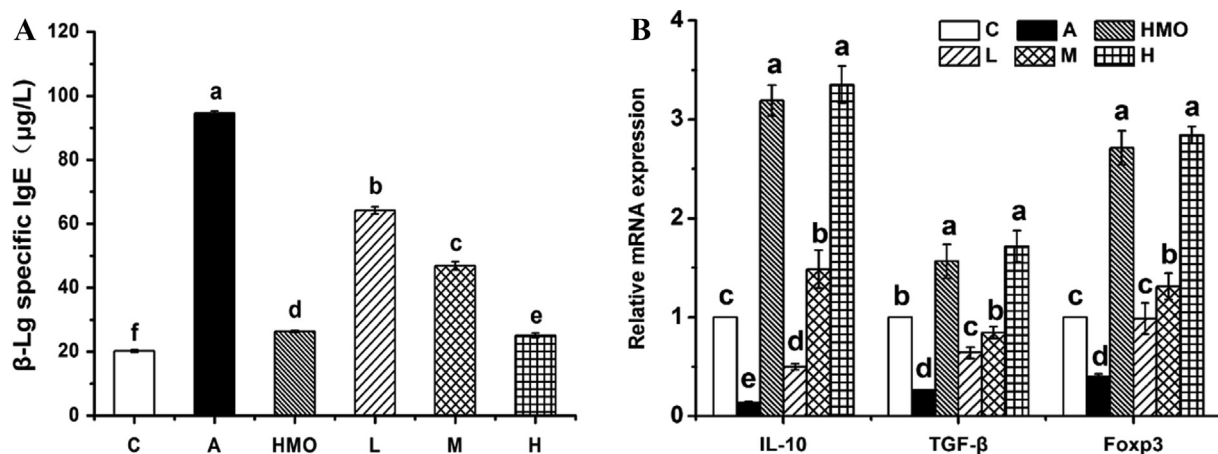


Fig. 4. Effects of HMOs on the secretion of β -Lg specific IgE in serum (A). mRNA levels of IL-10 TGF- β and Foxp3 in the colon were determined with RT-PCR (B). C, Control group; A, β -Lg allergic group; HMO, human milk oligosaccharide group (400 mg/kg); L, 2'-Fucosyllactose low-dose group (200 mg/kg); M, 2'-Fucosyllactose middle-dose group (400 mg/kg); H, 2'-Fucosyllactose high-dose group (600 mg/kg). Data are expressed as the mean $\pm$ SD ($n = 10$). Significant differences ($P < 0.05$) between the groups are indicated with different letters above the data.

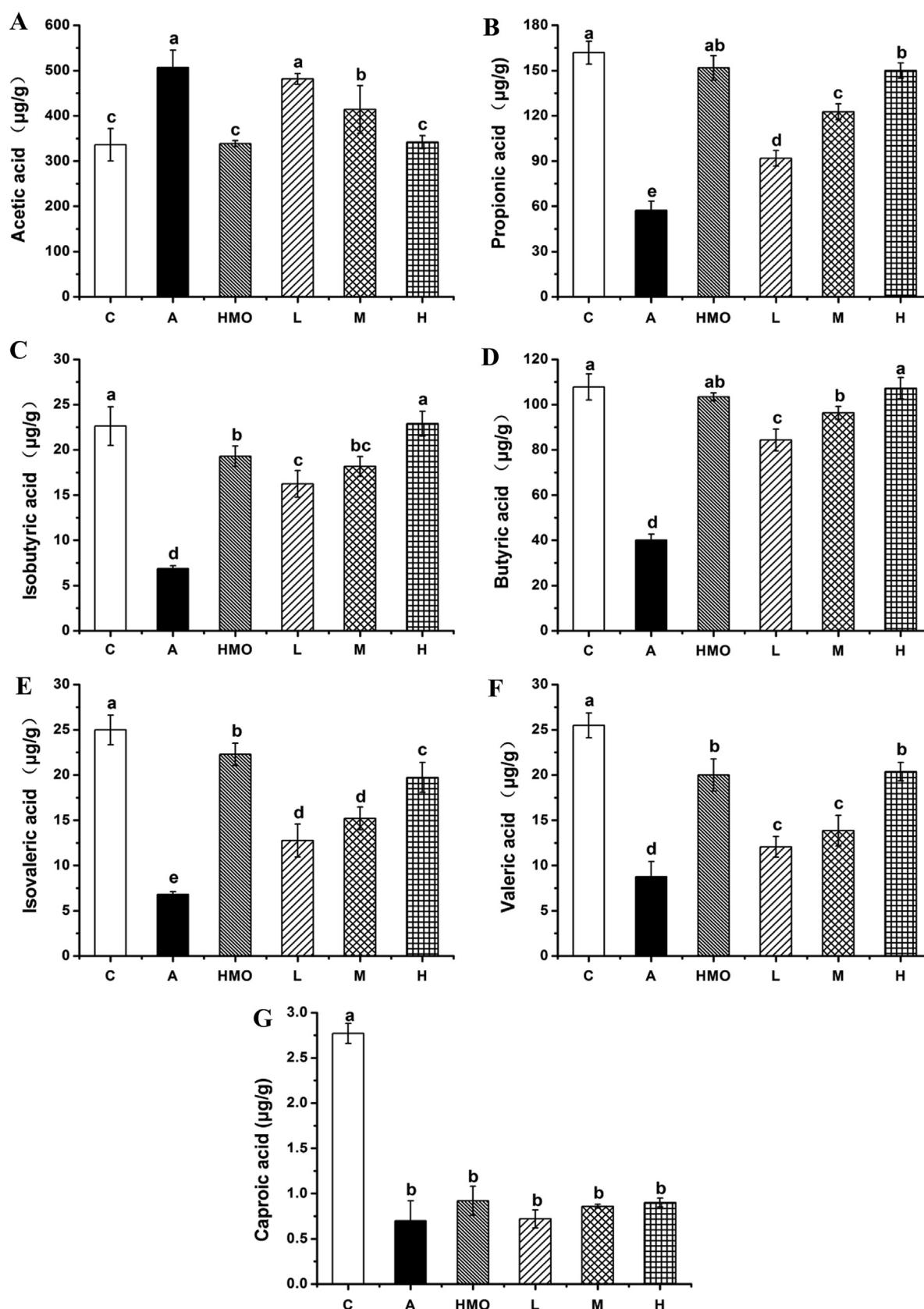


Fig. 5. The concentration of acetic acid (A), propionic acid (B), isobutyric acid (C), butyric acid (D), isovaleric acid (E), valeric acid (F) and caproic acid (G) in the mouse feces of different groups. C, Control group; A, β -Lg allergic group; HMO, human milk oligosaccharide group (400 mg/kg); L, 2'-Fucosyllactose low-dose group (200 mg/kg); M, 2'-Fucosyllactose middle-dose group (400 mg/kg); H, 2'-Fucosyllactose high-dose group (600 mg/kg). Data are expressed as the mean $\pm$ SD (n = 10). Significant differences ($P < 0.05$) between the groups are indicated with different letters above the data.

Table 4
Sample information and sequence abundance in mouse fecal samples.

Group	Number of OTUs	Number of sequences	Average length (bp)
C	293	42,928	458.7
A	260	32,548	462.0
HMO	287	39,404	455.8
L	242	36,532	460.9
M	300	40,712	456.5
H	296	41,041	456.2

C, Control group; A, β -Lg allergic group; HMO, human milk oligosaccharide group (400 mg/kg); L, 2'-Fucosyllactose low-dose group (200 mg/kg); M, 2'-Fucosyllactose middle-dose group (400 mg/kg); H, 2'-Fucosyllactose high-dose group (600 mg/kg). The number of valid sequences and OTUs are expressed as all samples in the group.

4. Discussion

Some studies have demonstrated that HMOs can alleviate allergic reactions and regulate intestinal flora, and 2'-FL in particular has been shown to reduce symptoms of ovalbumin (OVA) allergies in sensitized mice (Castillo-Courtade et al., 2015). The 2'-FL content in breast milk is negatively correlated with the incidence of infant diarrhea due to food allergic (Morrow et al., 2004). However, the benefit of HMOs in β -Lg-induced allergy is rarely reported. Therefore, the purpose of this study was to investigate the ability of HMOs, especially 2'-FL, to alleviate β -Lg-induced allergy.

In our study, we showed that intraperitoneal injection of β -Lg in

mice significantly reduced body weight, immune organ index, and levels of the cytokines IL-10, TGF- β , and IFN- γ . Moreover, the total serum IgE and β -Lg-specific IgE content of β -Lg-sensitized mice reached 173.10 ± 3.36 ng/mL and 94.56 ± 0.63 μ g/L, respectively, higher than those of control mice (119.00 ± 2.30 ng/mL and 20.24 ± 0.42 μ g/L, respectively; $P < 0.05$). The above results clearly show that β -Lg suppressed the immune function of BALB/c mice, indicating successful establishment of the immunosuppressive model. After intervention with HMOs and different concentrations of 2'-FL, body weight, colon length, and allergy symptoms of the mice improved. Of these treatments, HMOs and high-dose 2'-FL had the most significant relief effects. These results are consistent with the findings of Zhang et al. (2017).

CMA is generally characterized by a Th2-dominant immune response, and Treg cells were also involved in the development of allergies (Adel-Patient et al., 2005; Kumar et al., 2012). Treg cells play an important role in maintaining autoimmune tolerance (Gomes-Santos et al., 2012; Ray, Khare, Krishnamoorthy, Qi, & Ray, 2010). Treg cells can be divided into natural Treg cells and inducible Treg cells based on their source (Workman, Szymczak-Workman, Collison, Pillai, & Vignali, 2009). Sakaguchi first confirmed that Treg cells were involved in immune regulation through inhibiting secretion of the cytokines IL-10 and TGF- β (Sakaguchi, Sakaguchi, Asano, Itoh, & Toda, 1995). Under normal circumstances, TGF- β can suppress proliferation of effector T cells and induce T cells to differentiate into Treg cells; however, when the body suffers from inflammation, Treg cell proliferation is inhibited (Peck & Mellins, 2009; Sewell, North, Webster, & Farrant, 1997;

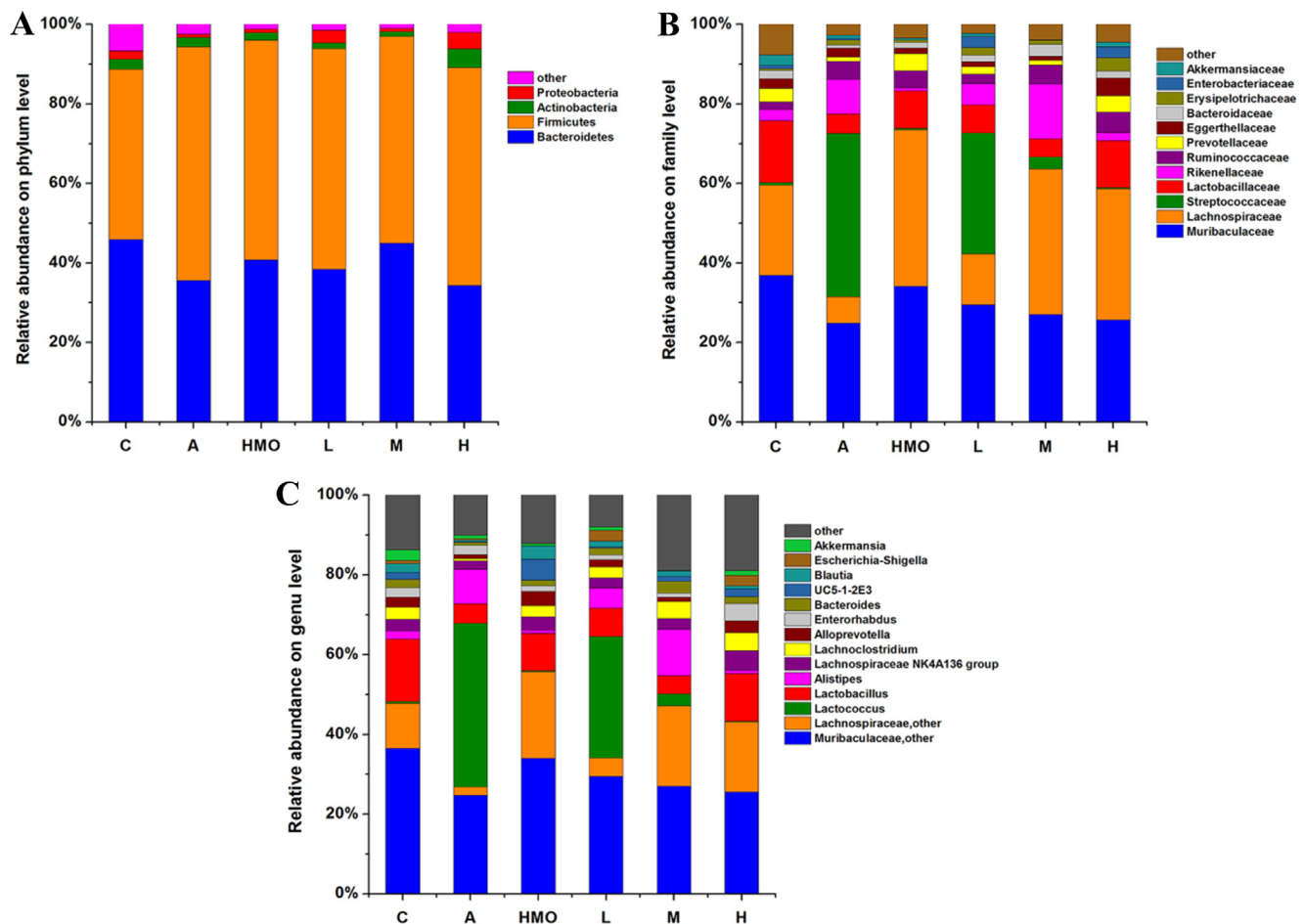


Fig. 6. Comparisons of fecal bacterial communities at phylum (A), family (B) and genus (C) levels in all groups. C, Control group; A, β -Lg allergic group; HMO, human milk oligosaccharide group (400 mg/kg); L, 2'-Fucosyllactose low-dose group (200 mg/kg); M, 2'-Fucosyllactose middle-dose group (400 mg/kg); H, 2'-Fucosyllactose high-dose group (600 mg/kg).

Yamashita, Takahashi, Tanaka, Nagai, & Inagaki, 2012). Activation of Treg cells reportedly contributes to tolerance of cow's milk allergens (Jo et al., 2014). Our experimental results show that the expression levels of TGF- β , IL-10, and Foxp3 in the β -Lg-allergic group were reduced compared to those in the control group. After ingestion of HMOs and different concentrations of 2'-FL, the concentrations of IL-10 and TGF- β increased, ($P < 0.05$). Of the treatments, HMOs and high-dose 2'-FL had the best effects. This was consistent with the results of Foks' research (Foks et al., 2011).

SCFA can improve the function of the intestinal barrier (Fukuda et al., 2011), lower the pH of the colon, and help prevent inflammation (Kim, 2018). In recent years, it has become increasingly distinct that the composition of the intestinal flora and its metabolic activity affect the development of allergies (Wopereis, Oozeer, Knipping, Belzer, & Knol, 2014). Low levels of isopropyl acid in the feces have been associated with the occurrence of food allergies (Sandin, Bråbäck, Norin, & Björkstén, 2009).

Studies on SCFA in feces of allergic and healthy infants have shown that the contents of propionic acid, butyric acid and isobutyric acid were significantly reduced, while the content of acetic acid was significantly increased in feces of allergic infants compared to levels in feces of healthy infants (Böttcher, Nordin, Sandin, Midtvedt, & Björkstén, 2000). A study on the potential role of SCFA in preventing allergies, found that butyrate and propionate levels were negatively correlated with the prevalence of allergies in children (Roudit et al., 2019). This is similar to the results of our study. In this work, high levels of acetic acid were detected in the feces of the β -Lg-allergic group, while the abundance of other short-chain fatty acids showed an obvious downward trend, consistently with the observations of Böttcher et al. (2000). The above data illustrates that HMOs may alleviate allergies indirectly by affecting SCFA composition.

Many studies have reported that allergies can cause intestinal flora disorders. Intestinal flora play important roles in the metabolism, immune system, and energy utilization (Caesar, Fåk, & Bäckhed, 2010). The balance of the gut microbiota affects the gut barrier, which is closely related to the development of certain acute and chronic diseases (Sekiroy, Russell, Antunes, & Finlay, 2010). At the family level, the *Streptococcaceae* family (belonging to *Firmicutes* phylum), *Muribaculaceae* family, and *Rikenellaceae* family (belonging to *Bacteroidetes* phylum) were the main gut microbiota in β -Lg-sensitized mice. Similar to the family-level analysis, studies at the genus level also showed significant enrichment in β -lg-sensitized mice of *Lactococcus*, unclassified *Muribaculaceae*, and *Alistipes*. Prior reports have associated high abundances of *Lactococci* with development of certain diseases, such as endocarditis (Rostagno, Pecile, & Stefano, 2012), cholangitis (Shimizu et al., 2019), and peritonitis (Lee et al., 2014). After HMOs intervention, *Lactococcus* abundance in the allergic group decreased markedly.

Muribaculaceae, a bacterial family previously called S24-7, belongs to the order *Bacteroides*, and can promote intestinal metabolism and participate in the innate immunity system (Chen et al., 2018; Ormerod et al., 2016). *Alistipes* is usually related to infections, especially abdominal and systemic infections (Boente et al., 2010). After treatment with HMOs, the abundances of unclassified *Lachnospiraceae* and *Lactobacillus* increased, while those of *Lactococcus* and *Alistipes* decreased. These results indicate that increased beneficial bacteria such as *Lactobacillus* could reduce the occurrence of intestinal diseases, suggesting that HMOs improved intestinal flora structure to relieve allergies induced by β -lg.

5. Conclusion

In this work, the effects of HMOs and 2'-FL on the immune mechanisms and intestinal flora in a β -Lg-sensitized mouse models was evaluated. HMOs significantly reduced inflammatory responses, improved the structure of gut flora and repaired the damaged intestinal

mucosal. To our knowledge, our work is the first report that 2'-FL relieves on β -Lg allergy. Further research on the mechanism of these anti-allergic effects will be necessary to determine the therapeutic potential of HMOs, and consumption of HMOs requires further clinical studies.

6. Ethics statement

All animal experiments were conducted according to the guidelines of the Laboratory Animal Ethics Association of the Northeast Agricultural University. Furthermore, volunteers who donated their human milk were informed prior to initiating the study and their participation was considered.

CRedit authorship contribution statement

Tingting Liu: Validation, Formal analysis, Investigation, Writing - original draft. **Peiyao Chen:** Investigation, Validation, Writing - review & editing. **Maliha Munir:** Writing - review & editing. **Libo Liu:** Conceptualization, Methodology, Supervision, Project administration. **Chun Li:** Conceptualization, Methodology, Validation, Writing - review & editing, Project administration. **Aili Li:** Resources, Supervision. **Hengfang Fu:** Investigation.

Declaration of Competing Interest

The authors declare no competing financial interest.

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