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Research Article

The synergies effect of 3′-sialyllactose and 2′-fucosyllactose on the attenuation of food allergy symptoms in a mouse model

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Abstract: The functional components known as milk oligosaccharides are crucial in the milk of diverse animals. Increasing evidence of immunomodulatory effects of milk oligosaccharides suggests that they may have some therapeutic potential in allergy. Here, we assess the effect of 3′-sialyllactose (3′-SL) on symptomatology and immune responses in a *β*-lactoglobulin (*β*-Lg)-sensitized mouse model of food allergy. Furthermore, 3′-SL mixed with 2′-fucosyllactose (2′-FL) at weight ratios of 1:6 and 1 000:1 to explore whether they had a synergies effect on alleviate sensitization. The results showed that 3′-SL and the mixture of 3′-SL and 2′-FL can regulate immune response and intestinal homeostasis. The intervention of 3′-SL and its mixture with 2′-FL can alleviate the symptoms of dermatitis, significantly reduce the serum specific-immunoglobulin (Ig) E (s-IgE) and specific-IgG1 (s-IgG1) levels ($P < 0.05$), reduce the accumulation of mast cells in the jejunum and maintain the diversity of the intestinal flora. 3′-SL could alleviate sensitization in a dose-dependent manner, and 3′-SL and 2′-FL have a synergies effect in reducing allergy. This study presented here opens the possibility that 3′-SL and the mixture of 3′-SL and 2′-FL in the form of nutritional ingredients could be used as adjuncts to existing treatment approaches to relieve food allergy in infants.

Keywords: 3′-sialyllactose; 2′-fucosyllactose; food allergy; immune response; intestinal homeostasis

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1 Introduction

Oligosaccharides, one of the most important components of milk, are oligomeric carbohydrates consisting of 2 to 10 monosaccharides linked by glycosidic bonds[1]. Human milk oligosaccharides (HMOs) are the third most abundant nutrient in human milk, with concentrations of 20–23 g/L in colostrum and 12–14 g/L in mature milk[2]. Although soluble oligosaccharides are not unique to breast milk and are found in the milk of almost all mammals, their content varied from species to species. The oligosaccharide content of bovine milk was lower than that of human milk, with oligosaccharide concentrations of 1–2 g/L and 0.05 g/L in human colostrum and mature milk respectively[3] and 0.03–0.06 g/L in bovine mature milk. Despite of the differences in the total amount of oligosaccharides, there are also differences in specific types of milk oligosaccharides between species. In our previous study, 30, 42, 32, 34 and 35 types of oligosaccharides were found in bovine, caprine, ovine, camel and human milk, with camel milk having the highest number of common oligosaccharides with the most closest abundance with human milk[4]. However, human milk is rich in 2′-fucosyllactose (2′-FL), whereas camel milk is rich in 3′-sialyllactose (3′-SL) and the ratio of 2′-FL and 3′-SL was the most different between camel (1:1 000) and human milk (6:1)[4].

Milk oligosaccharides have many potential health benefits, such as influencing host–microbe interactions and immune system regulation. The vast majority of undigested HMOs reach the large intestine, where they provide selective substrates for specific intestinal bacteria, modulate the immune system and prevent epithelial adhesion of intestinal pathogens. For example, HMOs have been shown to promote the growth of beneficial bifidobacteria in the intestinal tract and prevent pathogen adhesion[5–6]. Intestinal bacteria can act directly or indirectly on the immune system by stimulating natural immunity and controlling inflammatory responses, as well as by inducing adaptive immune responses and tolerance to the environment. At the same time, HMOs directly strengthen the intestinal epithelial barrier and enhance Th1 and Th17 cell effector responses[7]. In addition, milk oligosaccharides are believed to be beneficial for brain development and cognitive improvement function. Sialic acid in milk oligosaccharides had been reported to function in brain development and improves the function of the cognitive system[8]. Oral administration of 2′-FL alone was also found to enhance cognitive performance in both childhood and adulthood of rat model[9].

In addition, milk oligosaccharides, as a kind of functional ingredient to which infants are initially exposed, have been proven to have the potential to attenuate allergic reactions. For example, oral treatment with 2′-FL and 6′-sialyllactose (6′-SL) could alleviate anaphylactic symptoms induced by oral ovalbumin challenge in sensitized mice[10]. Li et al.[11] showed that 2′-FL could reduce the expression of pro-inflammatory factors interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) in mRNA and protein levels by inhibiting the toll-like receptor 4 (TLR4)/nuclear factor κB (NF-κB) pathway and alleviating allergic symptoms in sensitized mice. However, only 2′-FL and 6′-SL have been proven to

potentially attenuate food allergies. 3′-SL, which is the most abundant in animal milk, has not been confirmed whether it has an effect on attenuating allergic reactions. In addition, these studies only investigated the effects of single fucosylated oligosaccharides or single sialylated oligosaccharides on alleviating allergic symptoms. It is not clear whether the two oligosaccharides have synergistic or antagonistic effects in alleviating food allergy with different ratios.

Therefore, the aim of this study was to assess the effect of 3′-SL on modulating symptomatology and immune responses by using a *β*-lactoglobulin (*β*-Lg)-sensitized mouse model. Furthermore, the mixture of 2′-FL and 3′-SL, which mixed according to the concentration ratio of oligosaccharides in human milk and animal milk, was also explored by intragastric administration in the *β*-Lg-sensitized mouse model. The allergic degree of mice under different intervention amounts and mixture ratios of milk oligosaccharides was compared through apparent indexes (specific dermatitis lesion degree, allergic symptom score and tissue section) and biochemical indexes (serum specific antibody, cytokine content and intestinal flora), which could reflect the effect of 3′-SL and the mixture of 2′-FL and 3′-SL in attenuating food allergy. In addition, the mechanism of milk oligosaccharide in improving allergic symptoms *in vivo* was explored through the expression of genes related to TLR4/NF-κB pathway. This study aims to investigate the feasibility of 3′-SL in the relieve of food allergy, which may provide guidance on the improvement of infant formula for the babies who are sensitized to bovine milk proteins.

2 Materials and methods

2.1 Materials

2′-FL and 3′-SL were purchased from Shanghai Hepu Biotechnology Co., Ltd. Calcipotriol hydrate (MC903 reagent) and *β*-Lg were purchased from Sigma-Aldrich. Female BALB/c (three-week-old) mice were purchased from Charles River Laboratories (Suzhou, China) with license number JN. No. 20211115b0901226[442], and all animal experiments complied with the ARRIVE guidelines. IL-2, IL-4, IL-6, IL-10, TNF-α, *β*-hexosaminidase (*β*-Hex) enzyme linked immunosorbent assay (ELISA) kits (Mlbio, Shanghai, China) were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. The FastPure® Cell/Tissue Total RNA Isolation Kit V2 (Vazyme, Nanjing, China) and quantitative real-time PCR (qPCR) reverse transcription kit, Equalbit dsDNA HS Assay Kit (Vazyme, Nanjing, China), and ChamQ™ Universal SYBR® qPCR Master Mix (Vazyme, Nanjing, China) were purchased from Vazyme Biotech Co., Ltd. TIANamp Stool DNA Kit was purchased from Tiangen Biotech (Beijing) Co., Ltd. All other experimental reagents were analytically pure and purchased from Sinopharm Chemical Reagent Co., Ltd.

2.2 Construction of mouse allergy model

We employed a model in which mice were epicutaneously sensitized to a food antigen *β*-Lg on a developing atopic dermatitis-like skin lesion induced by topical treatment with the vitamin D analog MC903 based on a previous study[12]. Specific experimental groupings are shown in Table 1.

The mice were randomly divided into nine groups (n = 10) (Table 1): control group (CON), MC903 group (MC903, inducing atopic dermatitis, a chronic allergic disease), administration of *β*-Lg allergy after MC903 group (POS), low-dose 3′-SL group (L-SL), medium-dose 3′-SL group (M-SL), high-dose 3′-SL group (H-SL), medium-dose 2′-FL group (M-FL), medium-dose of the mixture of 2′-FL and 3′-SL with the proportion of oligosaccharide content in camel milk group (M-CR, 1:1 000, *m*/*m*) and medium-dose of the mixture of 2′-FL and 3′-SL with the proportion of oligosaccharide content in human milk group (M-HR, 6:1, *m*/*m*). The ratio of 2′-FL and 3′-SL for M-CR and M-HR was referred to our previous study, in which the ratio of 2′-FL and 3′-SL ranging from 1:1 000 to 6:1 from camel milk, bovine milk, goat milk, and human milk[4]. Therefore, we choose the lowest and highest ratio in this study to check the anti-allergenicity effect. The dosage was set by referring to previous study[11] as the additional amount of galactose and fructose-oligosaccharide (189.6–309.6 μg/kg bw) in commercially available infant milk powder. Three dose gradients of low (200 μg/g bw), medium (400 μg/g bw) and high (600 μg/g bw) were set respectively to assess whether 3′-SL had an effect on food allergy symptoms and the effect was dose dependent. Four groups of different oligosaccharide interventions were set at the same dose (M-FL, M-SL, M-CR and M-HR) to assess whether there was a synergies effect of 3′-SL and 2′-FL in the alleviation of food allergies. The total amount of mixture was all set at a medium dose (400 μg/g bw) level.

For CON group, only phosphate buffered saline (PBS) solution was applied to the ears of move every day. For MC903 group, mice were treated daily with 0.1 mmol/L MC903 of ethyl alcohol (EtOH) solution applied to the ears and after natural air drying, PBS solution without *β*-Lg was applied to the ears in mice. For POS group, mice were treated daily with 0.1 mmol/L MC903 of EtOH

Table 1 Grouping and treatment of experimental animals.

Group	Intragastric treatment	Sensitization treatment	
		Sensitization stage	Challenge stage
CON	Normal saline	20 μL PBS	PBS
MC903	Normal saline	20 μL, 0.1 mmol/L MC903 in ethanol + PBS	PBS
POS	Normal saline	20 μL, 0.1 mmol/L MC903 in ethanol + 0.02 mL/g bw 10 g/L *β*-Lg in PBS	0.02 mL/g bw 250 g/L *β*-Lg in PBS
L-SL	200 μg/g bw 3′-SL		
M-SL	400 μg/g bw 3′-SL		
H-SL	600 μg/g bw 3′-SL		
M-FL	400 μg/g bw 2′-FL		
M-CR	400 μg/g bw (2′-FL:3′-SL = 1:1 000, *m*/*m*)		
M-HR	400 μg/g bw (2′-FL:3′-SL = 6:1, *m*/*m*)		

solution applied to the ears, and then, PBS solution containing 10 g/L β-Lg was applied to the ears after ears were dried by natural air. These treatments were continued for 14 days. After sensitization stage, on the morning of day 15, PBS solution containing 250 g/L β-Lg was given to the mice except for CON and MC903 group for the first challenge. In the afternoon of day 18, PBS solution containing 250 g/L β-Lg was given to the mice except for CON and MC903 group for the second challenge. For CON and MC903 group, PBS solution without β-Lg was intragastric for both challenges. Specific intragastric samples and corresponding doses of each group are shown in Table 1.

After the second challenge, anaphylactic responses were evaluated according to the scoring form of mouse anaphylactic symptoms (Table 2) within 20–40 min. The mice were sacrificed on the second day after the challenge. Their serum, spleen, small intestine, colon and feces in the colon were collected for further analysis.

Table 2 The scoring form of mouse anaphylactic symptoms.

Score	Anaphylactic symptoms
0	Asymptomatic
1	Scratching frequency increases
2	Swollen eyes and mouth, reduced movement, inactivity, increased breathing rate
3	Dyspnea and cyanosis of taste
4	Inactivity, muscle contraction and spasm
5	Shock or death

2.3 Measurement of specific-immunoglobulin (Ig) E (s-IgE) and specific-IgG1 (s-IgG1) levels

The serum s-IgE and s-IgG1 levels were measured by ELISA. Briefly, covered the 96-well plate with 100 μL β-Lg solution (10 μg/mL) and incubated it overnight at 4 °C. The 96-well plates were sealed with blocking solution (PBST containing 2% bovine serum albumin) at 37 °C for 2 h after washing with 300 μL PBST. Then 100 μL serum samples (diluted 1:5 for IgE and diluted 1:10 for IgG1) were added and incubated at 37 °C for 2 h after the wells were washed again with 300 μL PBST for four times. Washed the well with 300 μL PBST, and then added the horseradish peroxidase (HRP)-conjugated IgE antibody (100 μL, 1:1 000 diluted, Thermo, Massachusetts, USA) or HRP-conjugated IgG1 antibody (100 μL, 1:5 000 diluted, Abcam, Shanghai, China) following by incubation at 37 °C for 2 h. Washed the plate again with 100 μL tetramethylbenzidine (TMB) chromogenic solution (Beyotime, Shanghai, China) and added freshly prepared TMB chromogenic solution following by incubation at 37 °C for 20 min for color development. Finally, added 2 mol/L H_2SO_4 (50 μL per well) to stop the colorimetric reaction and read absorbance photometric value at 450 nm by using Multiskan Sky Microplate Spectrophotometer (Thermo, Massachusetts, USA).

2.4 Histological analysis

Samples of the small intestine were placed in the tissue fixative containing 4% paraformaldehyde. The samples were embedded in paraffin to make a 5-micron paraffin section. The histopathological assessment of the samples was carried out with hematoxylin and eosin (H&E) and toluidine blue (TB) staining. The image was observed by an inverted biological microscope (Eclipse Ti, Nikon Co., Japan).

2.5 Measurement of cytokine evaluation

According to the manufacturer's instructions, the serum cytokines (β-Hex, IL-2, IL-4, IL-6, IL-10 and TNF-α) concentrations were measured by commercial ELISA kits.

2.6 Determination of mRNA in spleen by qPCR

Six splenetic samples for each group were analyzed by using qPCR through mRNA-specific primers. mRNA-specific primers of splenetic sample are *TLR4*, myeloid differentiation protein 88 (*MyD88*), tumor necrosis factor receptor activator 6 (*TRAF6*) and NF-κB. The forward and reverse primer sequences are shown in Table 3. Total RNA extracted from samples with FastPure® Cell/Tissue Total RNA Isolation Kit V2. Total RNA samples were adjusted uniformly to 500 ng/μL using NanoDrop (Thermo, Massachusetts, USA). The cDNA was synthesized using HiScript® II Q Select RT SuperMix for qPCR (Thermo, Massachusetts, USA). The test samples were used as templates for qPCR using mRNA-specific primers and ChamQ™ Universal SYBR® qPCR Master Mix (Vazyme, Nanjing, China) in Roche LightCycler 96 (Roche, Basel, Switzerland). Results were normalized by folds with *β-actin* and calculated using the $2^{-\Delta\Delta Ct}$ algorithm.

2.7 Microbiota 16S rRNA gene sequencing

Microbial DNA of colon contents was extracted by using the TIANamp Stool DNA Kit. The DNA concentration was determined by Equalbit dsDNA HS Assay Kit. Primers (341F and 806R) were used to amplify the V3–V4 region of the microbial 16S rRNA gene under PCR conditions. PCR products were extracted by agarose gel electrophoresis, purified by magnetic beads, quantified by NanoDrop (Thermo, Massachusetts, USA), and were sequenced at Illumina MiSeq/Novaseq (Illumina, San Diego, USA).

2.8 Statistical analysis

All the data were analyzed using software SPSS 25.0. Means were compared among groups using one-way analysis of variance (ANOVA). Duncan test was used when the variance was uniform and Tamhane test was used when the variance was not uniform. The results were expressed as mean ± standard deviation ($\bar{x} \pm s$). When the $P < 0.05$, the difference between groups was considered statistically significant.

Table 3 The sequence of mRNA-specific primers.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
TLR4	ATGGCATGGCTTACACCACC	GAGGCCAATTTTGTCTCCACA
MyD88	TCATGTTCTCCATACCCTTGGT	AAACTGCGAGTGGGGTCAG
TRAF6	AAAGCGAGAGATTCTTTCCCTG	ACTGGGGACAATTCACTAGAGC
NF-κB	ATGGCAGACGATGATCCCTAC	TGTTGACAGTGGTATTTCTGGTG

3 Results

3.1 Degree of specific dermatitis and the changes in body weight during modeling

Flow chart of animal experiment was shown in Figure 1A. As shown in Figure 1B, the application of MC903 ethanol solution (MC903 group) to the ear of mice caused specific ear skin dermatitis lesions in the sensitization phase. The POS group had even server atopic dermatitis with darker pink color ears, drier cochlea, thicker keratinization, more obvious skin lines and keratosis caused by crusting than CON group. By observing the specific ear dermatitis lesions in mice, we found that the intervention of H-SL and the mixture of 3′-SL and 2′-FL can alleviate the symptoms of dermatitis in mice.

Changes in body weight were monitored throughout the modeling period. As shown in Figure 1C, the growth rate of mice in MC903 group and POS group were significantly reduced compared with the CON group in the sensitization stage ($P < 0.05$), and the average body weight of mice in the MC903 group, POS group and M-FL group even lower than the initial body weight. However, the body weight of mice in M-SL, H-SL, M-CR and M-HR group increased slightly during the sensitization stage, while the L-SL group remained almost unchanged. During the intervention group, the growth rate of mice in the M-HR group was the highest compared to other treated groups. After being challenged with a large dose of β-Lg allergen, the body weight of mice in POS group and M-FL group continued to decrease, while the body weight of mice in H-SL, M-HR and M-CR group increased gradually.

3.2 The concentration of s-IgE and s-IgG1 in the serum challenged by β-Lg

The s-IgE and s-IgG1 levels in mouse serum after allergen challenge were shown in Figures 2A and B. The s-IgE and s-IgG1 levels in the serum of POS group were significantly increased ($P < 0.05$) compared with the control. The intervention of 3′-SL significantly reduced the s-IgE and s-IgG1 levels of mouse serum, and this efficacy was dose-dependent. Among the groups with different doses of 3′-SL intervention, the s-IgE and s-IgG1 in the serum of H-SL group were the lowest, followed by M-SL group and L-SL groups. Among the groups with different oligosaccharides at the same dose, the s-IgE and s-IgG1 levels in the serum of M-HR group were the lowest, followed by M-CR group, which were all lower than M-SL group. The levels of s-IgE and s-IgG1 suggested 3′-SL as well as the combination of 3′-SL and 2′-FL had effect on the attenuation of allergy.

3.3 Anaphylactic symptoms and activation of mast cells

After being challenged with a large dose of β-Lg, the mice appeared to have obvious anaphylactic symptoms, including scratching the head, slow movement, diarrhea, and decreased rectal temperature, as shown in Figure 2C. The anaphylactic symptom scores of the mice were significantly higher in the POS group than the CON group and the MC903 group ($P < 0.05$), indicating that food allergy model was successfully established in this study. The intervention of 3′-SL and the mixture of 3′-SL and 2′-FL could alleviate the allergic symptoms of mice based on the anaphylactic symptom scores. M-HR group had the lowest anaphylactic symptom score, followed by the H-SL group, but there was no statistically significant difference in the scores between the two groups ($P > 0.05$). The anaphylactic symptom score of M-FL group and M-SL groups were in the middle, which were all lower than L-SL and POS group. However, there was no significant difference in allergic symptoms between L-SL group and POS group ($P > 0.05$).

We assessed the mast activation of cell by measuring β-Hex levels in mice serum and observed degranulation of mast cell. The levels of β-Hex in the mice serum of each group is shown in Figure 2D. The level of β-Hex in the serum of mice in the POS group was significantly higher than that in the CON group and the MC903 group ($P < 0.05$). The serum levels of β-Hex in the oligosaccharides treated group were all significantly decreased ($P < 0.05$) compared with the POS group, except for the M-CR group and the L-SL group ($P > 0.05$). Among the different oligosaccharides and combination group, the levels of serum β-Hex were lower in the H-SL and M-FL groups than in M-SL and M-HR groups.

The jejunum tissue structure of mice was investigated by H&E staining. The jejunum of mice in the CON group had a complete morphological structure (Figure 3A), while the jejunum of mice in the POS group had severe villous cell necrosis and shedding. The necrosis and shedding of villous cells of the jejunum of the mice was also observed in the L-SL group, which had no significant

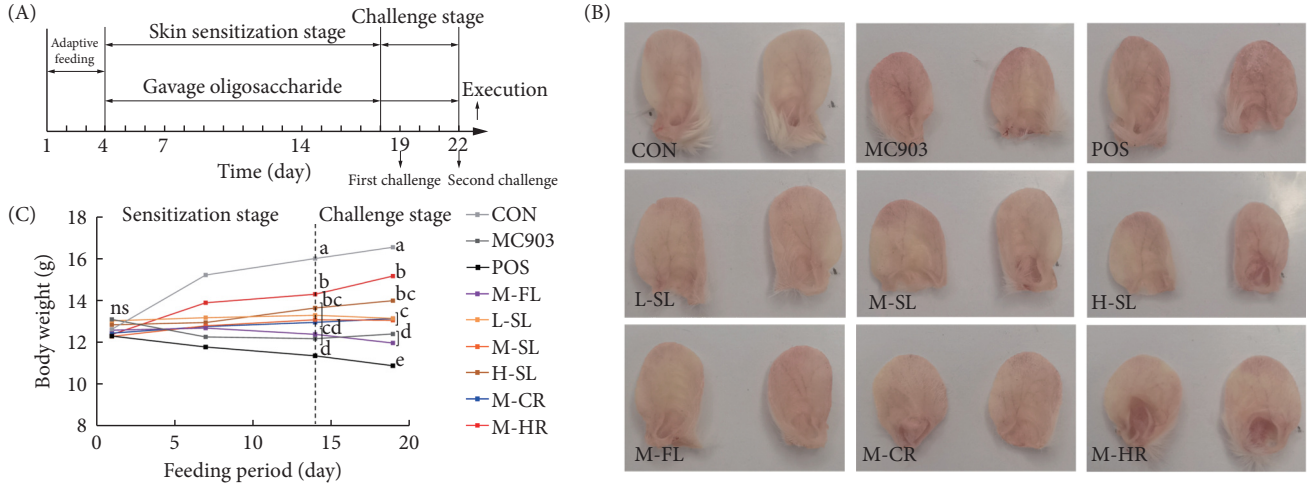

Figure 1 Experimental workflow and allergy symptom results of different challenge groups (n = 10). (A) Flow chart of animal experiment; (B) degree of ear specific dermatitis in mice; (C) trends of body weight in mice. Different lowercase letters indicate significant differences ($P < 0.05$), ns indicates no significant difference. CON, blank group; MC903, adjuvant group; POS, positive group; M-FL, medium-dose 2′-FL group; L-SL, low-dose 3′-SLgroup; M-SL, medium-dose 3′-SL group; H-SL, high-dose 3′-SL group; M-CR, camel milk ratio group; M-HR, breast milk ratio group.

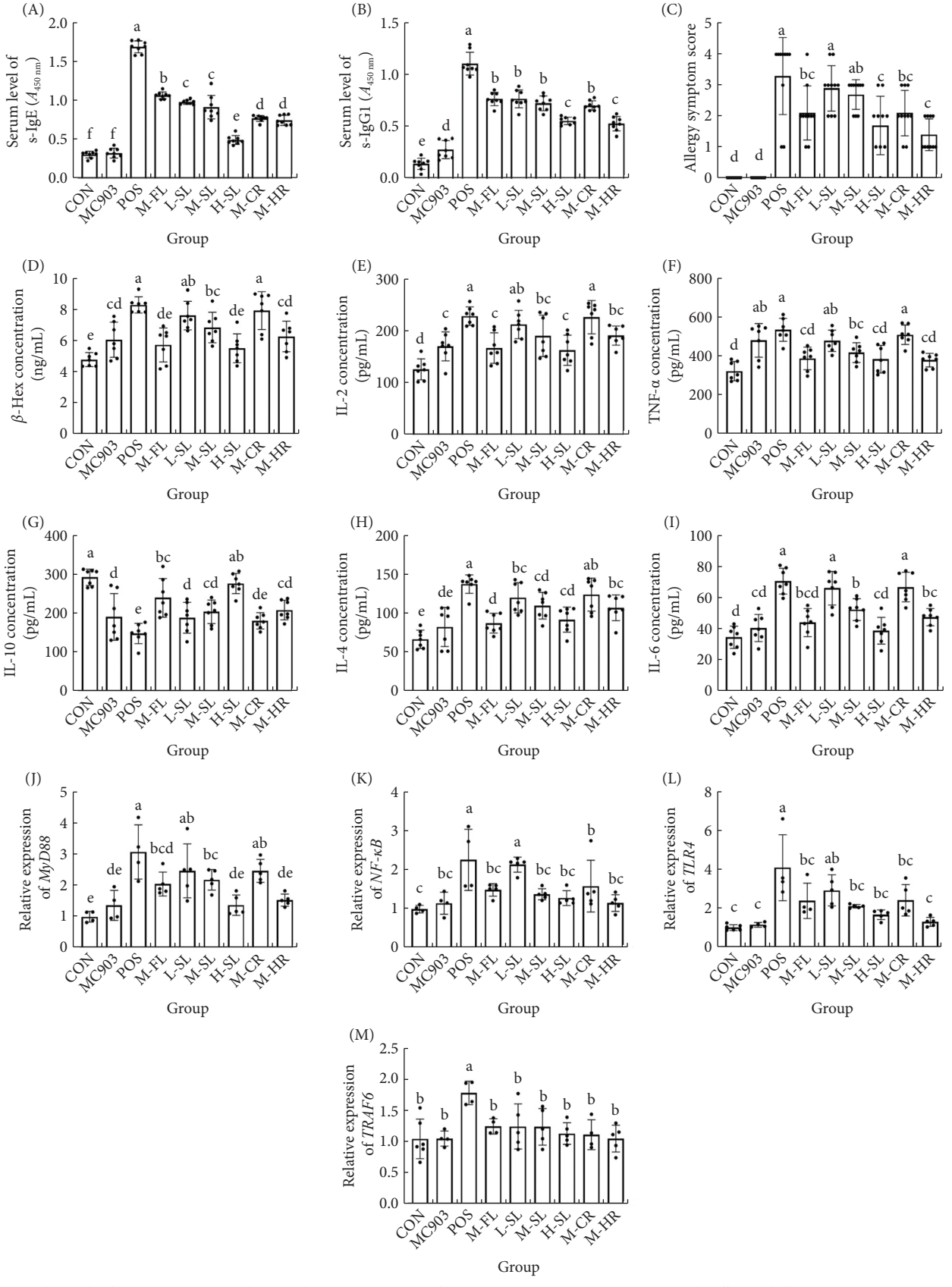

Figure 2 The levels of serum antibody, cytokines and mRNA expression of TLR4 pathway-related genes in mice with different challenge groups. (A) Serum level of s-IgE; (B) serum level of s-IgG1; (C) allergy symptom score; (D) serum level of β-hexosaminidase (β-Hex); (E) serum level of IL-2; (F) serum level of TNF-α; (G) serum level of IL-10; (H) serum level of IL-4; (I) serum level of IL-6; (J) relative expression of *MyD88*; (K) relative expression of *NF-κB*; (L) relative expression of *TLR4*; (M) relative expression of *TRAF6*. Different lowercase letters indicate significant differences ($P < 0.05$).

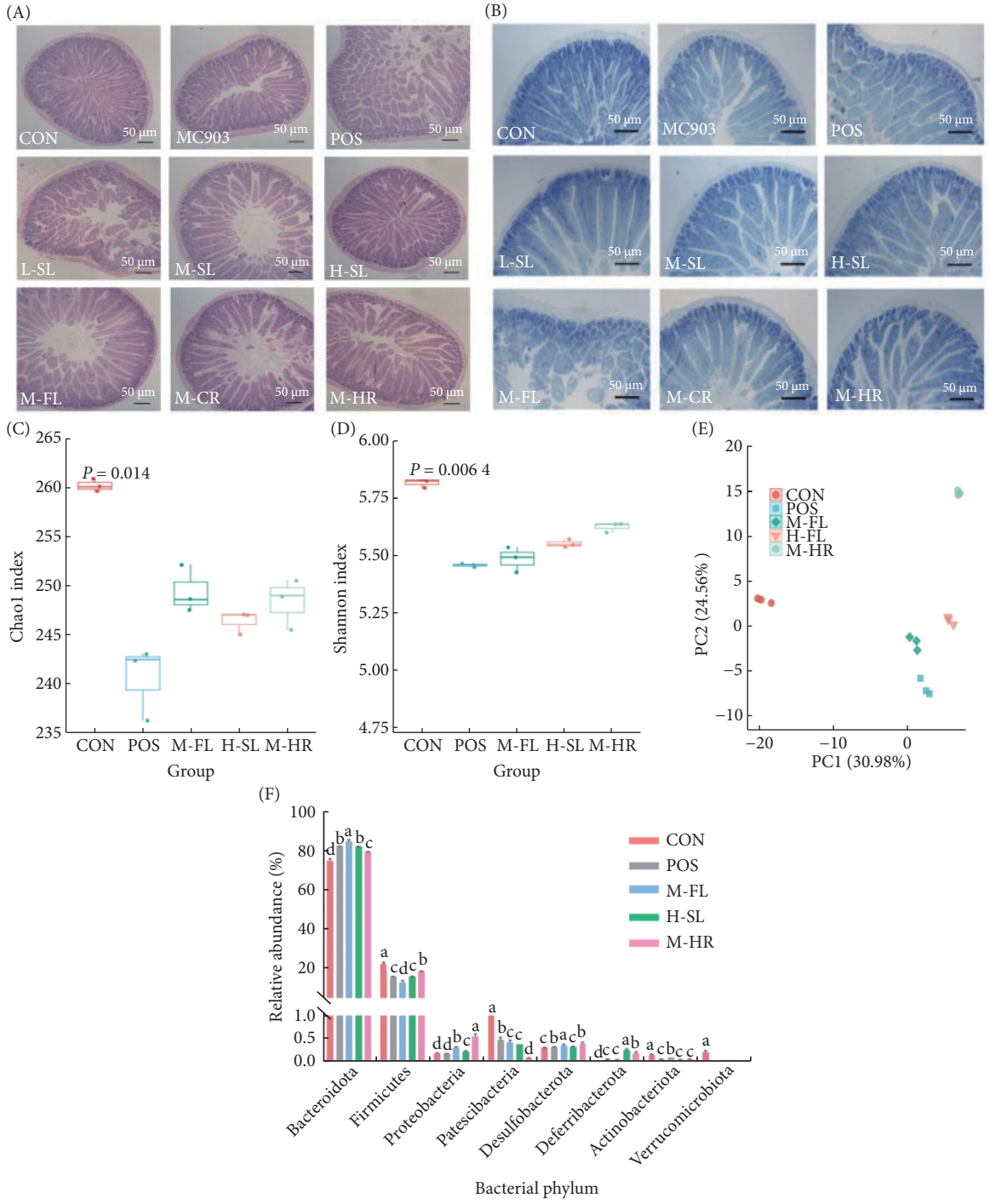

Figure 3 The damage of intestine tissue and changes of gut microbiota in the mice with different challenges. (A) Typical hematoxylin and eosin (H&E) and (B) toluidine blue (TB) stained jejunum sections of mice (magnification, × 40) and differences in gut microbiota of mice with different oral challenges; (C) Chao1 index; (D) Shannon index; (E) Principal component analysis (PCA) based on the Bray-Curtis distance (β-diversity); (F) Species differences at phylum level of intestinal flora. Different lowercase letters indicate significant differences ($P < 0.05$).

difference from the mice in POS group. However, the jejunal inflammation of the mice in the other oligosaccharide treated groups were significantly improved, especially for H-SL and M-HR, with the structure of the small intestinal villi kept intact and a few shed of villous cells.

Next, TB staining was used to observe the mast cell granules in the jejunum tissue of mice (Figure 3B). The mast cells in the jejunum of mice had no obvious aggregation phenomenon in the CON group, while the number of jejunal mast cells significantly increased in the POS group. The intervention of 3′-SL and the mixture of 3′-SL and 2′-FL reduced the accumulation of mast cells in the jejunum of mice. In the intervention group, there was less mast cell aggregation in the jejunum of mice in the H-SL, M-FL and M-HR groups compared with the POS group.

3.4 The secretion levels of cytokines in the serum

In addition, we evaluated the immune response of mice by the secretion levels of cytokines in the serum of mice (Figures 2E–I).

The expression levels of pro-inflammatory factors (IL-2, TNF-α, IL-4, and IL-6) in the serum of mice in the POS group were significantly up-regulated compared with CON group ($P < 0.05$). The pro-inflammatory factors in the serum of the M-FL, M-SL, H-SL and M-HR were significantly reduced ($P < 0.05$) compared to POS group, whereas there were no significant changes in M-CR group and the L-SL group ($P > 0.05$). On the other hand, the level of anti-inflammatory factor IL-10 in the serum of the mice in the POS group was significantly down-regulated compared with CON group ($P < 0.05$). The intervention of H-SL and the M-HR could increase the level of IL-10 in the serum of mice.

3.5 Allergy induced activation on the TLR4/NF-κB pathway

To further investigate the mechanism of the allergy reduction effect of oligosaccharides, we investigated the expression of genes related to TLR4/NF-κB pathway. Activation of the TLR4/NF-κB pathway in allergic immune responses is shown in Figures 2J–M. The expression of *TRL4*, *MyD88*, *TRAF6* and *NF-κB* mRNA in mice of the POS group was significantly up-regulated than the CON group ($P < 0.05$), whereas they were significantly lower in 3′-SL, 2′-FL, and the mixture of 3′-SL and 2′-FL groups than POS group.

3.6 Gut microbiota in allergy mice

The gut microbes in the mice of the three groups (M-FL, L-SL and M-HR), which showed better desensitization effects in the previous indicators, were selected for further 16S rRNA gene sequencing analysis. According to the Chao1 index and Shannon index, the α-diversity of the intestinal flora of the POS group was significantly lower than that of the mice in the CON group (Figures 3C and D, $P < 0.05$), but they were considerably increased in M-FL, L-SL and M-HR groups compared to POS group.

Principal component analysis (PCA) was used for β-diversity analysis of the intestinal flora of mice (Figure 3E). The separation along the first principal component shows the distance between the flora composition of allergic mice and healthy mice (the CON group). All the oligosaccharide intervened groups were well separated from that of CON group, of which H-SL group and the M-HR group were much more significantly different from the POS group ($P < 0.05$). Separating along the second principal component, we observed that the microbiota composition of mice in the H-SL and M-FL groups was closer to that of healthy mice than POS and M-HR groups.

With respect to the species composition of gut microbiota at the phylum level of mice (Figure 3F). Bacteroidota and Firmicutes were the two dominant intestinal flora in the CON group, which accounted for 98.05% of the total microbita. The abundance of Bacteroidota was all increased in the gut of allergic mice, and the abundance of Firmicutes was decreased. The elevation level of Bacteroidota and reduction level of Firmicutes in M-FL group were much more than that of POS group. No significant differences were found in Bacteroidota and Firmicutes between POS and H-SL group. Only M-HR group had the closest abundance of Bacteroidota and Firmicutes compared to CON group.

4 Discussion

The "Dual Allergen Exposure Hypothesis" proposes that early exposure to allergens and persistent inflammation resulting from a compromised skin barrier may induce and exacerbate food allergies. In this study, topical treatment of mouse skin with the vitamin D derivative MC903 resulted in an impaired skin barrier. Consistent with the results of Noti et al.[12], application of MC903 ethanol solution to the ear of mice would cause specific dermatitis lesions of the ear skin (Figure 1B). Specific dermatitis lesions could affect the growth rate of mice, resulting in stunted growth and even body weight loss in severe cases (Figure 1C). The group with more severe ear inflammation, the more affected the growth rate of mice by atopic dermatitis. This means that early skin barrier damage and early exposure to allergens may affect infant growth and development. In fact, studies have shown that persistent inflammation caused by specific diseases can lead to growth retardation in children[13].

During the challenge stage, the average body weight of the mice in the POS group and the M-FL group continued to decrease, which may be due to the stimulation of large doses of β-Lg to induce an immune response in the mice, resulting in allergic symptoms such as dyspnea and diarrhea, which in turn affects the normal activities and food intake of mice, resulting in body weight loss. In addition, we found that the group with a higher degree of atopic dermatitis after sensitization treatment had a more severe allergic reaction after the high-dose β-Lg challenge, which is consistent with a previous study[14]. Thus, protecting early skin barrier function is very important to maintain body health. However, the body weight loss of mice intervened with 3′-SL and the mixture of 3′-SL and 2′-FL were significantly reduced in atopic dermatitis lesions (Figure 1C). The reduction of body weight loss of oligosaccharides administration group was in line with previous study which showed that the 2′-FL intervention was effective in alleviating body weight loss due to β-Lg-induced allergy[15]. Other studies also reported that dietary supplementation with oligosaccharides had been shown to reduce the risk of developing allergic disease and suppresses acute allergic symptoms, such as allergic skin responses and food allergy, in animal and clinical studies[16–18].

As foreign antigens penetrate the damaged mice skin barrier, dendritic cells recognize the antigen and activate Th2 cells. The activated Th2 cells secreted IL-4, IL-6, and atopic-related cytokines, of which IL-4 could active B cells to secrete IgE[19]. The reduction of 3′-SL and mixture of 3′-SL and 2′-FL intervention on IgE and IgG1 levels in the serum of allergic mice was consistent with a previous study, which showed feeding a mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides led to a decrease in total levels of IgE and IgG1 in serum of infants at high risk of allergy[20]. The relatively low concentration of Th2 cytokines (IL-4) in 3′-SL and the mixture of 3′-SL and 2′-FL groups were also in line with a previous study[21]. On the other hand, IL-10 is an important anti-inflammatory factor that inhibits the proliferation and differentiation of Th1 cells and suppresses the release of pro-inflammatory factors, significantly attenuating the IgE-dependent allergic response in mice[22–23]. The increase of anti-inflammatory factor IL-10 in 3′-SL and the mixture of 3′-SL and 2′-FL groups agreed with a previous study, which reported that lacto-*N*-neotetraose intervention increased spontaneous IL-10 production and reduced inflammatory cytokine production from peritoneal T cells[24]. Another study also reported that increase of IL-10 in 6′-SL-treated animals sensitized by ovalbumin[10].

The relatively low level of serum IgE and IgG1 and Th2 cytokine IL-4, as well as the high level of Th1 cytokine IL-10 in the allergic mice administered with oligosaccharides suggested that 3′-SL and the mixture of 3′-SL and 2′-FL could alleviate the allergic reaction in

mice through balancing Th1 and Th2 cell related immune response, and the alleviation effect of oligosaccharides mixture were more significant than a single type of oligosaccharide, indicating that there is a certain synergistic effect of 2′-FL and 3′-SL .

Activation of TLR4 and its signaling pathway plays an important role in the pathogenesis of various immune diseases or inflammatory responses. TLR4 could recognize relevant ligands and trigger the high expression of downstream signaling pathways. MyD88 is a major ligand of the TLR4 pathway. The activation of TLR4 stimulated the recruitment of TRAF6, and lead to the phosphorylation of NF-κB and nuclear translocation, which subsequently activated pro-inflammatory mediators and stimulated the release of inflammatory factors[25–26]. Previous studies have confirmed that β-Lg stimulation could significantly upregulate the TLR4 pathway[27]. Kang et al.[28] found that oral administration of 3′-SL could inhibit the expression of related cytokines by regulating NF-κB in ear tissues, alleviating AD-like symptoms due to house dust mite and 2,4-dinitrochlorobenzene. Another study showed that 3′-SL had anti-inflammatory activity, mediated by NF-κB, reduction of IL-12 and IL-8 expression in Caco-2 cells, and stimulates the anti-inflammatory nuclear receptor peroxisome proliferative activated receptor γ[29]. The significantly reduction in the expression levels of the TLR4/NF-κB pathway related proteins in allergic mice administered with different oligosaccharides were consistent with previous studies. These results suggested that the effect of 3′-SL and the mixture of 3′-SL and 2′-FL on the alleviation of allergy symptom in mice model was related to the suppression on the inflammation factors of TLR4/NF-κB pathway.

Mast cells are important effector cells in food allergy and are present between the submucosa and lamina propria of the intestine. Mast cell aggregation is closely related to the severity of inflammation when the body is allergic. When an allergic reaction occurs in the organism, mast cells release allergic mediators such as β-Hex leading to bronchoconstriction, vasodilation, increased vascular permeability and aggregation of inflammatory cells, resulting in allergic symptoms such as tissue edema, stomach pain and diarrhea[30]. The attenuation of H-SL, M-FL and M-HR groups in mast cell aggregation of mouse jejunum as well as reduced levels of β-Hex (Figure 2) suggested both 3′-SL and 2′-FL could reduce mucosal mast cell activation. Kang et al.[28] observed that oral administration of 3′-SL alleviated intradermal mast cell infiltration of mice with allergic contact dermatitis. The antigen-activation of mast cells in the intestine may result in the diarrhea[31]. Therefore, the relief of 3′-SL and its mixture with 2′-FL on allergic symptoms may be related to the inhibition of mast cell activation and reduction in the secretion of β-Hex.

Furthermore, the intestine can efficiently absorb nutrients, water, and electrolytes from food, which constitutes a tight barrier against harmful substances and pathogens and their toxins from the external environment. The damage and dysfunction of the intestinal barrier are related to the susceptibility and aggravation of various autoimmune and inflammatory diseases, including food allergies. 3′-SL and its mixture with 2′-FL at certain doses could keep the structural integrity of jejunal tissue and reduce the inflammatory conditions such as necrosis and detachment of villous cells in POS mice. HMOs had been reported to directly strengthen the intestinal epithelial barrier and protect the host from pathogens[7]. Salivary acidified HMOs could balance the dynamic homeostasis of intestinal cells[32]. As the destruction of intestinal barrier may cause the leakage of harmful substance and thus induce the inflammation or allergy induced immune response[33], the protection of 3′-SL and its mixture with 2′-FL at certain doses on the intestinal barrier was probably related to the reduction in the allergy symptom. The differences in the protection effect between 3′-SL and 3′-SL and 2′-FL mixture might be due to differences in the manner or extent of absorption of different oligosaccharides, which in turn leads to differences in the degree of protection of the intestinal barrier by different oligosaccharides. Acid oligosaccharides pass through enterocytes only via a nonspecific paracellular pathway, and neutral oligosaccharides pass through enterocytes via a paracellular or transcellular pathway involving a receptor pathway[34]. The differences in the intestinal barrier protection between 3′-SL and 2′-FL still need further studies to confirm.

The symbiotic microbiota plays a crucial role in food allergies[35]. There is growing evidence that the gut microbiota early in life is linked to allergic disease[36]. Dysbiosis can be detected before the onset of clinical signs of allergy, and there is a causal relationship between disturbances in microbial colonization early in life and susceptibility to allergic disease later in life[37–38]. At 1 week, 1 month, and 3 months[39] of infants, lower gut microbiota diversity was associated with eczema at 18 and 24 months, and food allergy at 1 year. Recent studies have shown that colonizing the guts of germ-free mice with gut microbiota from healthy infants reduces the chance of cow's milk allergy in mice[40]. It could be seen that there was a close relationship between the homeostasis of intestinal flora and the development of allergic diseases in infants and young children. The decreased richness and diversity of gut microbiota of H-SL group suggested that the attenuation of H-SL on the food allergy is not related to regulation of gut microbiota; whereas the increased richness and diversity of gut microbiota of M-HR group (3′-SL and 2′-FL mixture groups formulated in proportion to the content of human milk oligosaccharides) indicated that the alleviation effect of 3′-SL and 2′-FL could be involved in their function in regulating the destabilized gut microbiome in allergic mice. The higher level of Shannon index in M-HR group suggesting that 3′-SL and 2′-FL had synergetic effect on the regulation of gut microbiota. Based on the results of clinical indicators and Th2 related cytokines, this synergetic effect may be highly related to the ratio between 3′-SL and 2′-FL as the function of different oligosaccharides on regulating intestinal microbes and influencing intestinal permeability are different[41–42]. Therefore, the effect of mixture of 3′-SL and 2′-FL with different ratio on the attenuation of food induced allergy deserved further exploration.

5 Conclusion

In general, both 3′-SL and the combination of 3′-SL and 2′-FL showed the positive effect on the relief of food allergy, however, the effect differed between different groups. 3′-SL could relieve allergy in a dose-dependent manner with H-SL group having the most relieve effect. However, M-SL group showed lower attenuation effect compared to M-HR group, suggesting that the combination of 3′-SL and 2′-FL at the ratio of 1:6 have a synergistic effect in reducing allergy. On the other hand, the M-CR group with the combination of 3′-SL and 2′-FL at ratio of 1 000:1 didn't have the synergistic effect. The reduction effect of 3′-SL and the mixture of 3′-SL and 2′-FL on allergic symptoms could be related to their function in inhibiting the aggregation and secretion of β-Hex of mast cell, regulating the inflammatory response of the body as an immune regulatory factor, as well as inhibiting TLR4/NF-κB

signaling pathway and thus inhibiting the expression of pro-inflammatory factors. In addition, the mixture of 3′-SL and 2′-FL could also reduce allergic symptoms by regulating the diversity and structure of intestinal flora; whereas the 3′-SL didn't have positive effect on the recovery of allergy induced decreasing in the richness of the gut microbiota. This study presented here open the possibility that 3′-SL and the mixture of 3′-SL and 2′-FL in the form of nutritional ingredients could be used as adjuncts to reduce the severity of symptoms, and provides a new effective way to relieve food allergy in infants.

Conflict of interest

The authors declare that they have no conflict of interest.

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