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Dietary GOS supplementation mitigates peanut-induced allergic responses through the gut microbiota–SCFA–barrier axis

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ABSTRACT: Galacto-oligosaccharides (GOS) are common prebiotics, and further research is necessary to determine whether they exert positive effects on peanut allergy and to elucidate the underlying mechanisms. In our study, mice received GOS at doses corresponding to 0.5%, 2%, and 5% of the human-equivalent dose. The objective was to clarify the impact of GOS on intestinal barrier function, immune regulation, and microbial composition. Results demonstrated that medium and high doses of GOS significantly alleviated allergic symptoms and physiological indicators. This was achieved by restoring Th1/Th2 balance through suppression of Th2 cytokines (IL-4, IL-13) and promotion of IFN- γ production, while also enhancing intestinal barrier function via increased secretion of sIgA and tight junction proteins (ZO-1, Occludin). Gut microbiota analysis revealed that GOS increased microbial diversity and modulated community structure at both the phylum and genus levels. Notably, GOS enriched butyrate-producing bacteria, such as *Coprococcus* and *Ruminococcus*, which correlated with a significant increase in short-chain fatty acids, particularly butyrate and acetate. These findings suggest that GOS may alleviate peanut allergy by remodeling the gut microbiota, promoting the growth of butyrate-producing genera, and increasing the production of immunomodulatory metabolites, thereby improving intestinal barrier integrity and immune regulation.

Keywords: Galactose oligosaccharide; Peanut allergy; Immune response; Intestinal barrier; Gut microbiota

1. Introduction

Peanut allergy is a common type of food allergy, predominantly manifesting in the pediatric demographic, wherein approximately 80% of affected individuals are beset with a persistent allergy to peanuts^[1]. Empirical evidence has recently illuminated an escalating incidence of peanut allergies, with estimates suggesting that 1-2% of the populace is now afflicted^[2]. Peanuts are imbued with a complex array of allergens, numbering 18, of which Ara h 1, Ara h 2, Ara h 3, and Ara h 6 are prominently associated with allergic reactions^[3]. The allergenic potential of peanuts is subject to modulation by various processing techniques, the composition of food matrices, and the stability of allergenic proteins during digestion. Thermal processing of peanuts, as evidenced by degranulation studies, has elucidated that roasted peanuts exhibit an augmented allergenicity

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relative to their raw counterparts, while boiled peanuts are associated with a diminished allergenic potential^[4].

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Transitioning to the discussion of the gastrointestinal tract, it is pertinent to note that this organ represents the most expansive immunological environment within the human body, harboring a rich and diverse microbial ecosystem. This symbiotic relationship between the intestinal microbiota and the host immune system is pivotal in upholding gut health and immunological homeostasis. Disruption of this microbial equilibrium, however, can precipitate immune dysregulation, thereby heightening the propensity for sensitization. Additionally, the integrity of the intestinal barrier function is inextricably linked to allergenicity, with current research underscoring the ability of peanut proteins to penetrate the intestinal epithelial barrier^[9], compromise epithelial cell viability, and disrupt barrier integrity, potentially implicating various cellular and molecular components such as goblet cells, Occludin, Claudin-1, Human Junctional Adhesion Molecule-A (JAM-A), and Zonula Occludens-1 (ZO-1)^[10]. Moreover, it has been posited that alterations in the intestinal flora may precede disease onset, with variations in the flora capable of modulating systemic effects through secretions like short-chain fatty acids (SCFAs)^[11, 12] and influencing allergic responses by regulating Th1/Th2 cytokines and allergic mediators, including Chemokine ligand 11 (CCL11)^[13-15]. Thus, the preservation of intestinal health emerges as a critical factor in the prophylaxis and management of allergic conditions.

Galactooligosaccharides (GOS), a widely recognized prebiotic, typically comprise 2 to 5 units of polymerization^[16] and have been documented in numerous studies to ameliorate intestinal barrier function, facilitate the absorption of intestinal minerals such as iron ions, and bolster the resilience of the intestinal microbiota^[17]. Recently, GOS has been implicated in the rectification of intestinal barrier damage instigated by dietary advanced glycation end products and in alleviating ovalbumin-induced allergic dermatitis by redressing the perturbed state of the intestinal microbiota^[18-20] have proposed that dietary intervention with a combination of 0.5% scGOS/lcFOS can enhance the safety profile of subcutaneous peanut immunotherapy by inhibiting mast cell degranulation. GOS selectively modulate the gut microbiota by reducing the abundance of potentially pro-inflammatory genera while increasing beneficial bacterial populations such as *Lactobacillus* and *Olsenella*^[21].

In light of the evidence suggesting that roasted peanuts are more likely to provoke severe allergic reactions compared to boiled peanuts, uncertainty persists regarding the impact of GOS on allergic reactions

elicited by the roasted peanut matrix and the underlying mechanisms. To address this lacuna in knowledge, the roasted peanut matrix was employed as the sensitizing agent to establish a sensitized BALB/c murine model. In alignment with recommended daily GOS intake, a dosing regimen of 0.5%, 2%, and 5% GOS was designed to assess the influence of GOS. The subsequent analysis was conducted with a focus on alterations in intestinal immunity and microbiota composition, thereby providing a theoretical substratum for the application of GOS across diverse domains including food, biological, and medical sciences.

2. Materials and methods

2.1. Materials

Peanuts were purchased from local supermarkets; BALB/c mice (female, 3-4 weeks) were purchased from Hebei Medical University; Etiology Toxin from *Vibrio Cholerae* etiology toxin was purchased from Shanghai Maokang Biotechnology Co. Ltd; Galactooligosaccharides (GOS) were purchased from Shanghai Yuanye Biotechnology Co. Ltd; 4% paraformaldehyde was purchased from Beijing Labgic Technology Co. Ltd; Paraffin was purchased from Shanghai Moqian Biotechnology Co. Ltd; HE staining kit and Tris-HCl were purchased from Beijing Solarbio Science & Technology Co. Ltd. CD8a monoclonal Antibody, CD4 monoclonal Antibody, IFN gamma (IFN- γ) monoclonal antibody, Interleukin 4 (IL-4) monoclonal antibody Purchased from Anolen (Beijing) Biotechnology Co. Ltd; NaCl, KCl, Na₂HPO₄ and KH₂PO₄ were purchased from Tianjin Bodi Chemical Co. Ltd. Mouse secretory immunoglobulin kits sIgA, IL-4, IL-13, IFN- γ , Occludin, ZO-1 and Immunoglobulins E were purchased from Shanghai MLBIO Biotechnology Co. Ltd. The chemical reagents used are of analytical grade or above.

2.2. Preparation of peanut extract (PE)

Preheat the oven to 150 °C in advance. Place the peanuts in the oven and roast them for 10 min. After roasting, remove the peanuts and let them cool to room temperature. Shell the peanuts, and grind them into a fine powder using a grinder. Measure out exactly 10 g of the peanut powder and mix it homogeneously with 100 mL of a Tris-HCl buffer solution (50mM Tris, adjust pH=7.2 with HCl). Incubate the mixture at 4 °C for 4 h, ensuring continuous stirring. After the incubation period, centrifuge the mixture at 13000 rpm for 15 min. Carefully collect the supernatant fraction and subject it to lyophilization to obtain a concentrated preparation of peanut protein powder.

2.3. Experimental design and sample collection

Figure 1-A illustrates the experimental design in which 3- to 4-week-old female BALB/c mice were randomly allocated into six distinct groups: the Control (CK) group, Peanut Allergy (PA) group, Galactooligosaccharides (GOS) group, PA combined with Low GOS (PA+LG) group, PA combined with Moderate GOS (PA+MG) group, and PA combined with High GOS (PA+HG) group, each comprising eight mice. Throughout the study, the CK and PA groups were provided with standard feed, whereas the GOS, PA+LG, PA+MG, and PA+HG groups were fed diets supplemented with varying concentrations of GOS, specifically at 5.0%, 0.5%, 2.0%, and 5.0% respectively. The PA group, PA+LG group, PA+MG group, and

PA+HG group were orally administered with 5 mg of PE and 10 µg of cholera toxin (CT) adjuvant once a week. On the 35th day, the PA group, PA+LG group, PA+MG group, and PA+HG group were given high-dose stimulation of 10 mg PE by gavage. All animal procedures were conducted in strict adherence to the National Research Council's guidelines for the care and use of laboratory animals.

Following each weekly sensitization gavage (on days 7, 14, 21, and 28) and the final high-dose challenge on day 35, the mice were monitored for allergic responses, and their body temperatures were recorded at 0, 30, and 60 minutes post-gavage. After the second temperature measurement(30min), fecal and blood samples were collected using sterile centrifuge tubes. The fecal samples were rapidly immersed in liquid nitrogen for freezing and subsequently stored at -80 °C. The blood samples were kept at 4 °C overnight, centrifuged at 3000 rpm for 10 min the following day, and the separated serum was stored at -80 °C. On the 35th day, mice were stimulated with high doses of PE, the allergic reactions and body temperatures were documented using the same protocol, and fecal and serum samples were collected. Thereafter, the mice were humanely euthanized, and the spleen, thymus, cecal contents, ileum, and other small intestinal tissues were excised and placed in sterile containers.

2.4. Determination of organ index

On the 35th day, mice were stimulated with high doses of PE, mice immune organs spleen and thymus were collected and quickly weighed and calculated the immune organ index.

2.5. Cytokine assays

Dilute the serum obtained from high-dose stimulation of mice in section 2.3 with PBS at a ratio of 1:100 (v/v). Subsequently, utilize an enzyme-linked immunosorbent assay (ELISA) kit to quantify the concentrations of serum immunoglobulin E (IgE), interleukin-4 (IL-4), interleukin-13 (IL-13), and interferon-gamma (IFN-γ).

2.6. Detection of immune cell typing

Grind and filter the spleen dissected in section 2.3 through a 200-mesh stainless steel sieve. During the grinding process, wash the cells with 3 mL of RPMI 1640 complete culture medium supplemented with 10% fetal bovine serum. Transfer the collected cells into a centrifuge tube and incubate with 3 mL of red blood cell lysis buffer for 5 min. Centrifuge at 1000 rpm for 5 min, discard the supernatant, and collect the cell pellet. Resuspend the spleen cells in 2 mL of pH 7.4 Phosphate-buffered saline (PBS), centrifuge again at 1000 rpm for 5 min, and discard the supernatant. Repeat the centrifugation step once more.

Subsequently, add the cell pellet to 5 mL of pre-cooled RPMI 1640 medium containing 10% fetal bovine serum to create a homogeneous spleen cell suspension. For viability assessment, perform trypan blue staining by mixing 200 µL of cell suspension with 200 µL of trypan blue. Count the live cells under a low-power microscope; the suspension can be used if the viability is above 98%. Adjust the spleen cell suspension density to 1×10^6 cells/mL using RPMI 1640 with 10% fetal bovine serum.

Take 100 µL of the cell suspension and transfer it to a centrifuge tube. Add 1 µL of Fluorescein

isothiocyanate (FITC)-conjugated anti-CD4 and 1 μ L of P-phycoerythrin (PE)-conjugated anti-CD8a antibodies. Incubate the mixture at 4°C in the dark for 30 min, followed by centrifugation at 1500 rpm for 5 min. Wash the cells with 1 mL of PBS, centrifuge again at 1500 rpm for 5 min, discard the supernatant, and resuspend the cells in 500 μ L of PBS. Fix the cells by adding 200 μ L of 1% paraformaldehyde and incubating at 4°C in the dark for 30 min.

After fixation, add 200 μ L of membrane disruptor (0.3% Triton X-100) and centrifuge at 1500 rpm for 5 min. Wash the cells once more with 1 mL of PBS. Then, add either 1 μ L of PE-conjugated anti-IFN- γ or 5 μ L of PE-conjugated anti-IL4 antibody separately, and incubate at 4°C in the dark for 30 min. Centrifuge at 1500 rpm for 5 min, wash with 1 mL of PBS, centrifuge again at 1500 rpm for 5 min, discard the supernatant, and resuspend the cells in 500 μ L of PBS for flow cytometry analysis.

2.7. Histopathological observation

The 2 cm ileal tissue was soaked in 4% paraformaldehyde for 24 hours. Firstly, the samples were dehydrated with 75%, 85%, 95%, 95%, 100%, and 100% ethanol, treated with xylene until transparent, and immersed in molten wax (58 °C). The embedded paraffin samples were subsequently cut into 8 μ m thin sections with a microknife and then baked (60 °C), dewaxed, rehydrated, and stained with hematoxylin. The samples were then washed, differentiated (1% HCL ethanol solution), washed, dehydrated (75% and 85% ethanol), and stained with eosin staining solution. Finally, the dye was washed off the sample, dehydrated (95% ethanol and 100% ethanol), and sealed transparently.

2.8. Intestinal Permeability and sIgA Assay

A 5 cm small intestine tissue was taken, placed on a glass plate, and the intestinal villi were scraped until only the casing remained. Intestinal villi were extracted with normal saline at room temperature for 1 h, centrifuged at 3000 rpm for 5 min, and the supernatant was taken for the determination of the sIgA content by ELISA. Twenty milligrams of small intestine and 100 μ L of high-efficiency tissue lysis solution were mixed and evenly split in an ice water bath for 1 h, centrifuged (4 °C, 3000 rpm) for 20 min, and the supernatant was taken for the determination of the tight junction protein content by ELISA.

2.9. Determination of SCFA in intestinal contents

The collected intestinal contents were frozen for 48 h and then dried in a vacuum dryer. Twenty mg of dried sample were taken into a centrifuge tube and 200 μ L of 70% ethanol was added and then extracted for 4 h at room temperature before centrifuged at 3000 rpm for 10 min. The 50 μ L of the supernatant and 10 μ L of the internal standard were taken as samples to be tested, and 3% pyridine, 250 mM 1-EDC•HCl, and 20 mM 2-NPH •HCl were added 50 μ L each, and incubated in a water bath at 60 °C for 20 min. At the end of the reaction, 34 μ L of the mixture of KOH and methanol (15% KOH: methanol = 4:1) was added to the sample and then reacted in a 60°C-water bath for 20 min. Then samples above were then cooled to room temperature and extracted by adding 500 μ L 0.5 mol phosphoric acid solution and 1 mL of ether mixture for 3 min, centrifuged at 3000 rpm for 10 min. Take ether layer, add 2 mL of water vibration for 3 min, centrifuge at

3000 rpm for 10 min. After standing, take the ether layer and blow dry with nitrogen. Redissolved in 250 μ L methanol and injected by HPLC. The concentrations of acetate, propionate, and butyrate in the colonic contents were analyzed by HPLC.

2.10. Determination of the intestinal flora in the feces

Extract DNA from the microbial content within mouse fecal samples that were collected following the high-dose stimulation protocol outlined in Section 2.3. The concentration of the extracted DNA was quantified by Nanodrop, and its quality was assessed by 1.2% agarose gel electrophoresis. The 16S rRNA gene, containing the V3-V4 region, was amplified using the primers F: ACTCCTACGGGAGGCAGCA and R: GGACTACHVGGGTWTCTAAT. Subsequently, microbial diversity analysis was performed. Sequences with 100% similarity were clustered into the same operational taxonomic unit (ASV).

2.11. Statistical analysis

All data in this paper were analysed using the SPSS26 statistical software package, and all values were expressed as mean $\pm$ standard deviation, $P < 0.05$ was considered statistically significant. All the graphs involved in this paper are drawn by Origin Pro 2023, Science Slides, QIIME2 and GraphPad Prism 9.

For gut microbiota analysis, α -diversity differences were assessed by Kruskal-Wallis test, and β -diversity was evaluated using PERMANOVA based on Bray-Curtis distances. All analyses were performed using SPSS 26.0 and QIIME2. $P < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Effect of the GOS on Food Allergy Symptoms and Serological Analysis

In the present study, BALB/c mice were sensitized via oral gavage with a preparation of roasted peanut protein conjugated with CT adjuvant, while GOS were administered as a dietary intervention (Figure 1a). Throughout the course of the experiment, continuous monitoring of the mice's peanut-specific IgE concentrations and allergic manifestations was conducted (Figure 1b-e). The results indicated that the CK group did not manifest appreciable allergic symptoms over the entire duration of the study. Contrarily, the PA group demonstrated a progressive onset of allergic reactions, characterized by erect hair, diminished coat luster, mild trauma to the anterior paws, as well as an increase in the frequency of ear scratching, nasal obstruction, and sneezing, in correlation with the number of gavage administrations. Similarly, the GOS supplement group exhibited symptoms of sneezing and ear scratching, albeit at a reduced frequency compared to the PA group (Figure 1c). The thermal and dietary profiles of the mice during the intervention phase are detailed in Supplementary Figure s-1 (a, b). As depicted in Supplementary Figure s-1 (a), a transient decrease followed by an increase in abdominal temperature was noted in the PA group within the first hour post-sensitization, whereas the body temperatures of the other groups remained comparatively stable. Supplementary Figure s-1 (b) suggests that the dietary intake of the GOS group and the combined PA+HG group exhibited a trend towards stabilization throughout the experimental duration. Consequently, the administration of GOS did not induce toxicity or adverse effects in the experimental mice.

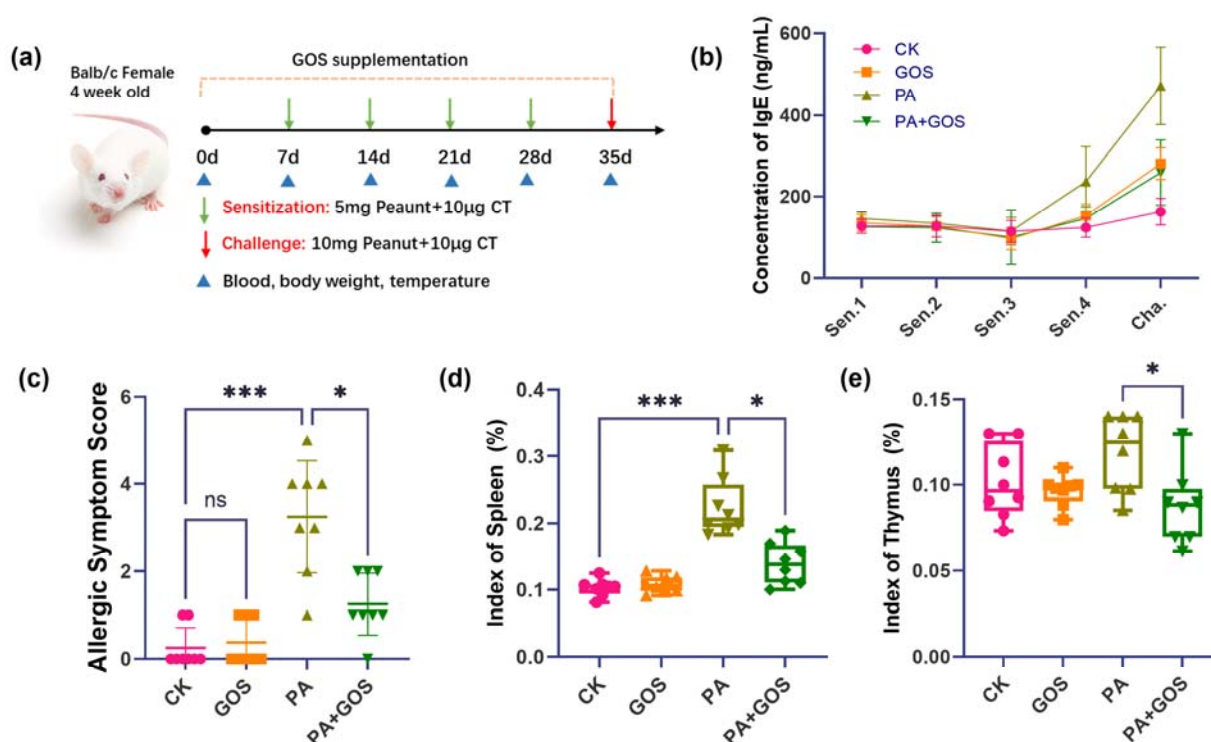


Fig.1 Effect of the GOS on organ indexes and IgE level of peanut allergy mice. (a) The immunization protocol of BALB/c mouse food allergy model; changes in the specific-IgE level of individual mice ($n=8$); (c) system scores (d) Spleen and (e) thymus were obtained from mice 1h after high-dose stimulation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the indicated control.

The measurement of organ index can to some extent reflect the immune status of the body. In order to reveal the effect of GOS on the immune status of peanut allergic mice, thymus index and spleen index were measured in this study. As shown in Figure 1 (d), compared with the CK group, the spleen index of mice in the PA group was significantly increased ($P < 0.05$), indicating that the spleen of mice in the PA group was significantly enlarged. Meanwhile, the thymus index has also increased. Adopting a 5.0% GOS diet can reduce the thymus index ($P < 0.05$) and spleen index ($P < 0.05$) of PA group mice. Research has shown that GOS has an immune enhancing effect on mice in response to inflammation and can restore the spleen index of peanut allergic mice.

IgE is a characteristic indicator of peanut allergic reactions. Compared with the CK group, the supplementation of GOS significantly reduced the serum IgE concentration of peanut allergic mice in the last two weeks of the experimental period ($P < 0.05$) (see Figure 1b). Allergies are typically associated with increased concentrations of eosinophils and IgE antibodies, with IL-5 cytokine concentration playing an important role in IgE formation and eosinophil development^[22]. Therefore, the possible explanation for the decrease in specific IgE concentration in the PA+GOS group may be related to the concentration of IL-5 cytokines. The slight increase in IgE levels in the non-allergic group also indicates the possibility of other immune disorders, or the possibility of mice developing allergies to long-term exposure to bedding and basal feed.

3.2 Analysis of Immune Cell Subpopulations and Cytokine Production

Helper T cells (Th cells) are key participants in adaptive immunity and are crucial for almost all adaptive immune responses. The differentiation level of cells in the spleens of mice in each group was determined by flow cytometry, and the results are shown in Figure 2 (a). Compared with the CK group, the proportion of Th2 cells increased in the PA group, while the proportion of Th1 cells decreased. Compared with the PA group, the proportion of Th1 cells in the PA+LG group and PA+MG group remained relatively stable, while the proportion of Th1 cells increased in the PA+HG group; At the same time, the proportion of Th2 cells was inhibited in the PA+LG group, PA+MG group, and PA+HG group, with the inhibitory effect of medium dose GOS being the most significant. The regulatory effect of GOS on Th1/Th2 is shown in Figure 2 (b). Compared with the PA group, the Th1/Th2 ratio of the PA+LG group remained basically unchanged, while the Th1/Th2 ratio of the PA+MG group and the PA+HG group significantly increased. Therefore, the regulatory mechanism of GOS on Th cells may be dose-dependent. Low doses of GOS can inhibit the differentiation of Th2 cells, while when the dose reaches a certain level, it can induce the differentiation of Th1 cells.

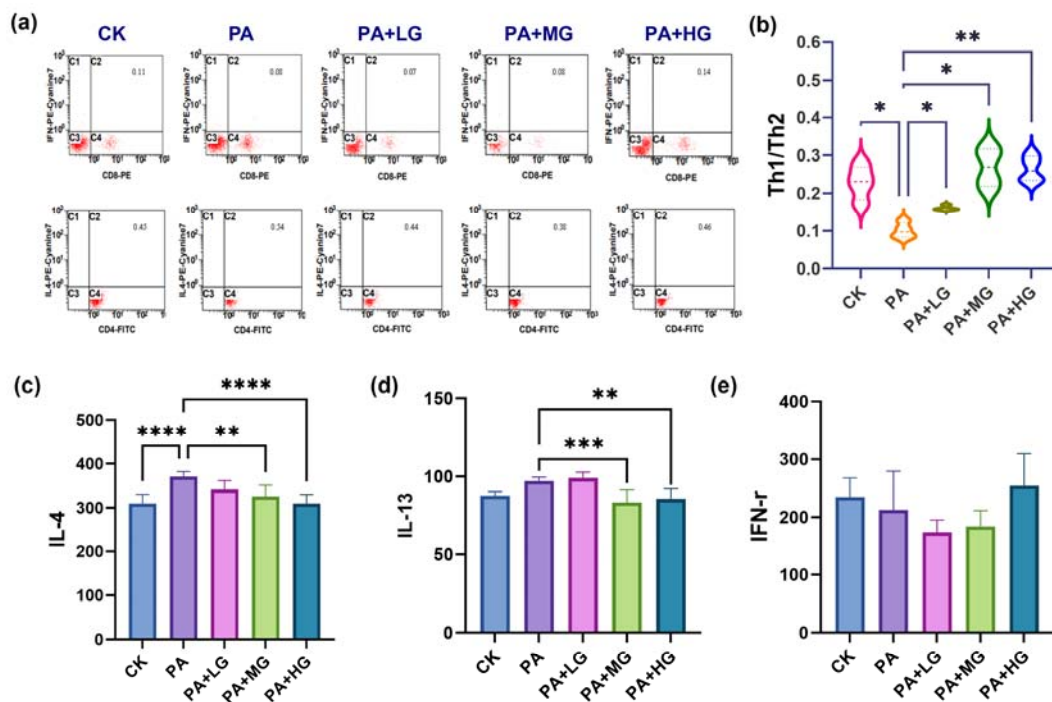


Fig. 2 Cell differentiation and cytokine determination of GOS supplemented peanut allergic mice. (a) and (b) the proportion of Th1 and Th2 in spleen was measured by Flow cytometry. (c), (d) and (e) were the contents of IL-4, IL-13 and IFN- γ in serum, respectively. The results were obtained from 8 mice in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the indicated control.

The serum cytokine levels of mice supplemented and not supplemented with GOS were detected by enzyme-linked immunosorbent assay (ELISA) (Figure 2c-e). The cytokine secretion results showed that the concentration of IL-4 in the PA group was significantly higher than that in the CK group ($P < 0.05$), while there was no statistically significant difference between the CK group, PA+MG group, and PA+HG group ($P > 0.05$) (Figure 2c). In addition, moderate doses of GOS significantly reduced peanut induced IL-13 levels ($P < 0.05$) (Figure 2d). There was no significant difference in IFN- γ levels between the groups, but the values in the PA+HG group were closer to those in the CK group (Figure 2e). Mouse-specific IgE assay revealed that peanut

sensitization induced a significant elevation in serum sIgE levels. However, GOS administration at different doses resulted in varying degrees of reduction in sIgE levels across all treatment groups, with the most pronounced decrease observed in the PA+HG group ($P < 0.001$) (Figure 3d). IL-4 and IL-13 act on B cells, increasing IgE levels and causing allergic reactions. IFN- γ is an antagonist of IL-4, and its increased secretion in allergic organisms can negatively regulate allergic reactions in the body. GOS can downregulate the secretion of IL-13 cytokines and the expression of IL-13 mRNA in colitis, and strongly regulate the levels of IL-4, IL-5, and IFN - γ in serum of patients with atopic dermatitis^[18, 23]. Supplementing GOS/FOS (9:1 ratio) in the diet can induce systemic immune regulation of Th1 dependent adaptive response in mice. Therefore, GOS has the potential to enhance the immunity of peanut allergic mice.

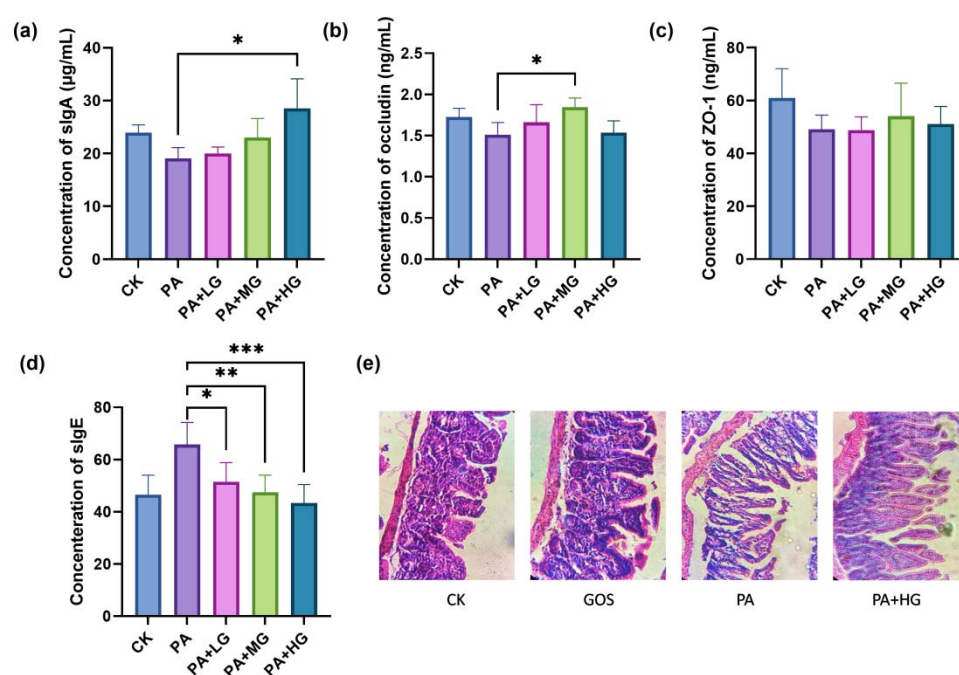


Fig. 3 Effects of GOS on the intestinal in peanut allergic mice. One hour after high-dose stimulation, the intestinal barrier was lysed to measure occluding (a) and ZO-1 (b), 5cm of small intestine was removed and the mucosa was scraped to measure sIgA (c), and ileum was used for tissue section (d). Data and picture were obtained from 3 independent experiments with 8 mice in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the indicated control.

3.3 Impacts on the Intestinal Barrier Function

Besides imbalances in immune cell differentiation and activation, injuries in the intestinal epithelium and barrier function often accompany food allergy. If the intestinal barrier function is damaged, allergens will enter the body and trigger an allergic reaction. The expression levels of the tight junction (TJ) proteins, such as ZO-1, occluding, and sIgA, IgE were examined by ELISA (Fig. 3a-d). The integrity of the intestinal barrier was further analyzed by hematoxylin and eosin (H&E) staining of the jejunum tissue (Fig. 3e). The mice exposed to peanut extract regularly had decreased the level of sIgA ($P > 0.05$), ZO-1 ($P < 0.05$) and occluding ($P > 0.05$) compared with CK group. Administration of GOS inhibited the reduction of sIgA ($P < 0.05$) and two TJ proteins caused by stimulation with peanut extract. It has been suggested sIgA helps reduce entry of food allergens into the gut^[24]. GOS could enhanced expression of mucin synthesis genes in goblet cell, which may protect the mucosa from toxins. The in vitro Caco-2 cell experiment showed that disrupting the integrity of

the Caco-2 barrier by disrupting the tight junction may allow peanut proteins to across the intestinal epithelium^[25]. Studies have shown that in ovalbumin (OVA)-induced food allergy models, supplementation with β -carotene significantly upregulates the expression of the tight junction proteins occludin (+24.39%) and ZO-1 (+26.23%), and promotes intestinal mucosal sIgA secretion, thereby enhancing both physical and immunological intestinal barrier functions and alleviating allergic symptoms^[26]. Similarly, in OVA-sensitized BALB/c mouse models of food allergy, β -sitosterol has been found to upregulate the expression of occludin and ZO-1, and to promote intestinal sIgA secretion, thus reinforcing the physical and biochemical barrier functions of the intestinal epithelium and preventing food allergy^[27]. Furthermore, in rat models of food allergy, OVA sensitization led to reduced expression and altered distribution of occludin and ZO-1, accompanied by increased intestinal permeability and mucosal inflammation; notably, supplementation with probiotics (*Clostridium butyricum* and *Lactobacillus reuteri*) reversed these changes by upregulating occludin and ZO-1 expression, thereby restoring intestinal barrier integrity^[28].

The state of the villi and crypts in the small intestine reflects the functional status of the small intestine. Through the analysis of GOS in cytokine secretion and intestinal immune regulation, it can be seen that both medium-dose and high-dose GOS have immunomodulatory effects on PA mice. In this experiment, the PA+HG group was selected to explore the effect of GOS on the internal structure of intestinal tissue, and the results are shown in Fig. 3(e). No pathological changes were found in the small intestines of the CK group and the GOS group. Compared with the CK group, the PA group had more ruptured villi and increased cell debris, while the degree of villi rupture in the PA+HG group was significantly lower than that in the PA group, indicating that GOS can effectively alleviate peanut protein-induced intestinal injury in mice. Studies have shown that GOS has the ability to restore the integrity of atrophied and ruptured (jejunum and ileum) intestinal villi in inflammatory mice. Yang^[29] pointed out that GOS can reduce mucosal damage in mice with alcohol withdrawal syndrome, restore intestinal mucosal integrity, and restore the morphology of intestinal villi and mucosa to normal.

3.4 Analysis of intestinal microbial composition

To understand the mechanisms underlying the action of GOS in this model, we collected fecal samples longitudinally and analyzed the α -diversity of the gut microbiota by calculating the Chao1, Observed species, Shannon, and Simpson indexes in the different groups of mice, as shown in Fig. 4(a). Compared to the CK group, the diversity and richness of the gut microbiota in the PA group mice decreased, indicating intestinal microbiota dysbiosis in PA mice. After GOS intervention, the Chao1 index, Observed species, Shannon index, and Simpson index all increased, suggesting that GOS can enhance the diversity and richness of the gut microbiota in mice, restoring the disrupted microbiota to a state close to the initial condition. Although the changes in each alpha index across the groups did not reach statistical significance in this experiment, overall, GOS played a positive role in modulating the gut microbiota dysbiosis induced by peanut protein. The abundance rank curve is shown in Fig. 4(b). A higher evenness in community composition is indicated by a flatter curve. The length of the curve along the horizontal axis reflects the number of ASVs/OTUs. The figure

shows that the PA+HG group had the flattest curve, while the CK group had the greatest span and the PA group the smallest, indicating the order of gut microbiota abundance: CK group > PA+HG group > PA group. After GOS intervention, both the abundance and evenness of the gut microbiota in mice were improved.

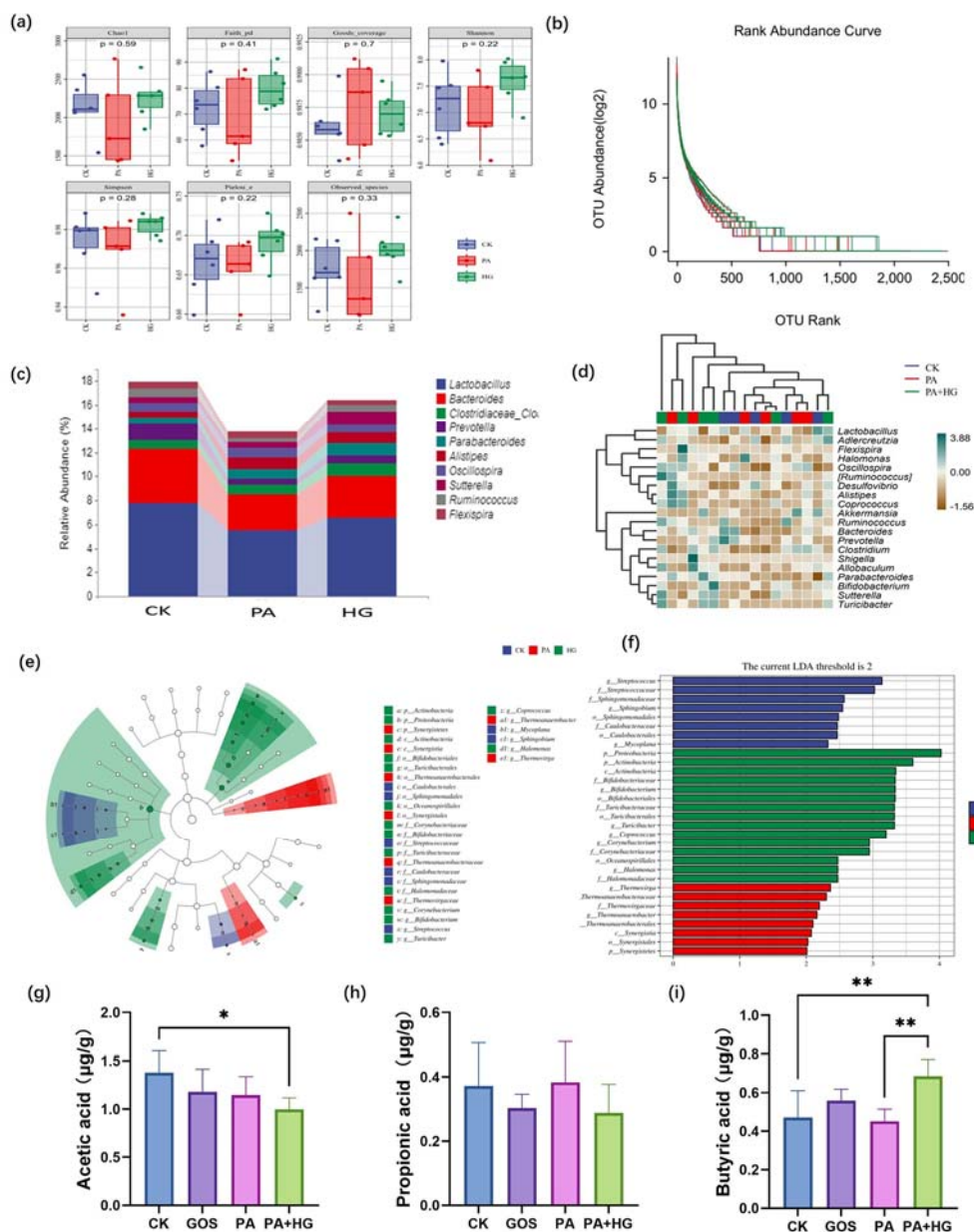


Fig.4 Effects of GOS on intestinal flora in mice. GOS effects on Gut Microbiota α -diversity in PA Mice(a), The rank-abundance curves for samples from each group of mice(b), Elative abundance of bacteria presented at genus level(c), Clustering heatmap of the effects of GOS on the top 20 bacterial genera(d), LEfSe analysis of the mice gut microbiome data(e)(f), Changes in Short-Chain Fatty Acid Concentrations Across Groups. Acetate acid(g), Propionate acid(h), Butyrate acid(i). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the indicated control.

Fig.5 The mechanism of GOS in alleviating the allergic reaction to peanuts.

Further analysis of the species composition of the gut microbiota in different groups revealed that at the phylum level, *Bacteroidetes* and *Firmicutes* were the dominant phyla in all groups, and their abundances were downregulated after peanut sensitization. At the genus level, the top ten bacterial genera in the gut microbiota are shown in Fig. 4(c). The dominant genera were *Lactobacillus* and *Bacteroides*. Compared to the CK group, the abundances of *Lactobacillus*, *Bacteroides*, *Prevotella*, and *Ruminococcus* were downregulated in the PA

group, and these were restored after GOS intervention. *Alistipes*, considered inflammation-related, was upregulated in the PA group compared to the CK group and was restored after GOS intervention. Compared to the CK group, the abundances of *Clostridiaceae_Clostridium*, *Parabacteroides*, and *Sutterella* were significantly upregulated, while the abundance of *Flexispira* decreased.

To further compare the species composition differences between samples, a cluster heatmap was used to analyze the species composition of each sample. Fig.4(d) shows the abundance data of the top 20 genera by average abundance. The CK, PA, and PA+HG groups exhibited distinct microbial markers. The CK group exhibited a high abundance of *Lactobacillus*. After PA sensitization, the microbial structure became more complex, with significant enrichment of *Oscillospira*, *Halomonas*, and potentially pathogenic bacteria such as *Flexispira* and *Shigella*. In contrast, the PA+HG intervention specifically enriched a series of beneficial bacteria, including *Coprococcus*, *Bifidobacterium*, *Akkermansia*, *Ruminococcus*, and *Turicibacter*.

To display the taxonomic hierarchy distribution of marker species in each group and the significantly enriched species and their importance within each group, LEfSe analysis was performed on the samples, as shown in Fig. 4(e) and 4(f). In the CK group, *g_Streptococcus*, *g_Sphingobium*, *f_Caulobacteraceae* and *g_Mycoplana* showed significant differences compared to the other two groups. The PA group showed significant enrichment of *g_Thermovirga*, *f_Thermoanaerobacteraceae*, *o_Synergistales* and *p_Synergistetes* compared to the other two groups. The PA+HG group showed significant enrichment of *p_Proteobacteria*, *p_Actinobacteria*, *g_Bifidobacterium*, *f_Turicibacteraceae*, *g_Turicibacter*, *g_Coprococcus* and *g_Halomonas* compared to the other two groups. The enrichment of atypical gut bacteria such as *g_Thermovirga*, *f_Thermoanaerobacteraceae*, *o_Synergistales* and *p_Synergistetes* in the PA group indicated gut microbiota dysbiosis. In the PA+HG group, compared to the PA group, the GOS treatment not only reversed the pathological state but also actively shaped a more favorable microecology, enriching three core beneficial bacteria: *g_Bifidobacterium*, *g_Coprococcus* and *g_Turicibacter*. This clearly demonstrates that GOS can effectively correct dysbiosis and directionally promote the growth of bacteria with clear health benefits. Compared to the CK group, GOS intervention introduced a more positive and functionally comprehensive consortium of beneficial bacteria, showing greater potential, particularly in enriching *g_Coprococcus* and the immunomodulatory *g_Bifidobacterium*.

GOS have the ability to promote the proliferation of *Lactobacillus*, *Bifidobacterium*, etc. These probiotics have been proven to compete with pathogens for binding sites on the host cell membrane, preventing pathogenic bacteria such as *Escherichia coli*, *Salmonella*, *Clostridium perfringens*, and other strains from forming biofilms in the intestinal tract^[30, 31]. Moreover, *Clostridia* species have been found to suppress an inflammatory response in the intestine by promoting Treg^[32]. Zhai found that the intake of GOS increased the relative abundance of *Prevotella* in the fecal microbiota of mice, and further pointed out that the protective effect of GOS on mice depends on the regulation of the intestinal microbiota^[33]. This observation is in agreement with our data. Supplementation with GOS for 3 weeks, the abundances of *Lactobacillus*, *Marvinbryantia*, *Ruminococcaceae_UCG-008*, and *Blautia* were significantly increased, and the abundances

of *Lachnoclostridium*, *Gauvreauii_group*, and *Alistipes* were significantly decreased. Recent studies have shown that the composition of the intestinal microbiota affects the development of allergies, and exposure to specific bacteria, including *Bifidobacterium* and *Lactobacillus* strains, can attenuate allergic reactions^[34].

Early studies have shown that changes in the intestinal microbiota precede the occurrence of allergic diseases, and research on the structural changes of the intestinal microbiota is helpful in preventing the occurrence and development of allergic diseases^[35]. The regulation of allergic organisms by prebiotics or probiotics with anti-allergic effects has also been shown to be achieved by restoring the structure of the intestinal microbiota^[36]. Nasab^[37] found that the improvement and preventive effects of probiotics on allergic diseases can be achieved by inhibiting Th2 and shifting the response to Th1, inducing tolerance, increasing IL-10, Treg cells and their responses to controlling immune inflammation, increasing the IFN- γ /IL-4 ratio, reducing eosinophils, Th2 cytokines and IgE levels, and reducing the expression of chemokine and cytokine-signalling pathway genes. In our results, the mechanisms of GOS are related to not only immune cell response, but also the growth-promoting effects on several probiotics such as *g_Lactobacillus*, *g_Bifidobacterium*, *g_Ruminococcus* and *g_Coprococcus*.

Short-chain fatty acids (SCFA) are metabolites produced by the metabolism of dietary fiber and other carbohydrates by the intestinal microbiota. Studies have shown that low levels of SCFA can lead to allergic susceptibility, and increasing SCFA levels can improve the disease state^[38, 39]. Therefore, the structure of the intestinal microbiota and the content of short-chain fatty acids can affect the immune status of the body to a certain extent.

The concentrations of SCFAs in colonic contents were analyzed by HPLC, and the results are shown in Fig.4(g), 4(h) and 4(i). Compared to the CK group, the acetate acid concentration was lower in the PA, GOS, and PA+HG groups. The propionate acid concentration decreased in the GOS and PA+HG groups compared to the CK group. Butyrate acid content decreased in the PA group compared to the CK group but increased significantly in the GOS and PA+HG groups. Furthermore, the butyrate acid concentration in the PA+HG group was significantly different from that in the PA group.

We found that the changes in the gut microbiota were consistent with the changes in SCFA concentrations. Compared to the PA group, the abundances of *g_Coprococcus* and *g_Ruminococcus* were significantly upregulated in the PA+HG group. These bacteria are closely associated with butyrate production, leading to the increased butyrate concentration. The decrease in acetate concentration might be related to *Clostridiaceae* and *Ruminococcus*, which efficiently convert acetate to butyrate through the activity of acetate CoA-transferase, resulting in decreased acetate and increased butyrate concentrations^[40, 41].

Butyrate, as one of the primary short-chain fatty acids produced by gut microbial fermentation of dietary fiber, plays a central role in regulating immune homeostasis and suppressing allergic responses. First, butyrate significantly promotes the differentiation and function of colonic regulatory T cells by inhibiting histone deacetylases, thereby enhancing immune tolerance and directly suppressing excessive inflammatory responses^[42]. Second, butyrate enhances intestinal epithelial barrier function by activating the AMPK

signaling pathway, promoting the assembly of tight junction proteins, and reducing the translocation of food allergens and pathogenic microorganisms, thereby lowering the risk of sensitization at its source^[43]. Furthermore, butyrate can modulate dendritic cells toward a tolerant phenotype and indirectly suppress the production of allergen-specific IgE, further attenuating the intensity of Th2 immune responses^[44]. In experimental models, supplementation with butyrate or butyrate-producing bacteria has been shown to alleviate allergic airway inflammation^[45] and food allergy symptoms^[45]. Clinical studies have also observed a significant reduction in the abundance of butyrate-producing bacteria in the gut of allergic patients^[45].

GOS intervention significantly enriched butyrate-producing bacteria, including *Coprococcus* and *Ruminococcus*, along with the probiotic *Bifidobacterium*, leading to a marked increase in intestinal levels of butyrate and other short-chain fatty acids. We conducted Spearman correlation analysis among core butyrate-producing genera (*Coprococcus* and *Ruminococcus*), colonic butyrate, and IL-4, and presented the results as Supplementary Figure S3. The correlation data clearly show that the abundances of *Coprococcus* and *Ruminococcus* are significantly positively correlated with butyrate concentration, while both taxa exhibit significant negative correlations with serum IL-4 levels. These microbial metabolites directly suppressed Th2 immune responses, as evidenced by decreased serum levels of IL-4 and IL-13 and restored Th1/Th2 balance. Concurrently, butyrate enhanced intestinal barrier function by activating the AMPK signaling pathway, characterized by increased expression of tight junction proteins (ZO-1 and occludin) and elevated secretory sIgA production, thereby effectively preventing allergen penetration. Collectively, by modulating the gut microbiota structure and its metabolic output, GOS coordinately regulated both cellular and humoral immunity in the host, ultimately achieving effective alleviation of peanut allergy.

4. Conclusion

Overall, this study enhanced our understanding of GOS as a potential antiallergic supplement for preclinical immunotherapy or functional foods, and proposed the potential mechanism of how GOS suppressed allergic symptoms in mice with peanut-induced food allergy (Fig. 5). Dietary supplementation with GOS remodels the composition of the gut microbiota, marked by a significant enrichment of butyrate-producing bacteria including *Coprococcus* and *Ruminococcus*. The subsequent elevation in intestinal butyrate concentration contributes to enhanced intestinal barrier integrity via upregulating the expression of tight junction proteins ZO-1, Occludin, and sIgA, and simultaneously restores immune homeostasis by suppressing Th2-type cytokines such as IL-4 and IL-13 and promoting IFN- γ secretion, thereby ultimately attenuating allergic manifestations. Therefore, the regulatory effect of GOS on the disruption of gut microbiota caused by peanut protein can be achieved through cellular immunity, humoral immunity, and improving the composition of the gut microbiota. Additionally, this study has several limitations that should be further addressed in future research: (1) the differentiation of Tregs cells and the secretion of Th17 cells and IL-10 cytokines during the experiment are still unclear. (2) The degree of polymerization will affect the properties and functions of GOS. We have initially investigated the effect of the degree of polymerization on the degranulation of RBL cells, but how the degree of polymerization affects the anti-allergic effect remains to be

deeply studied. Therefore, the mechanism of GOS to reduce the incidence of peanut allergy needs further investigation. (3) The findings are based on a murine model of acute sensitization, which may not fully recapitulate the chronic and complex nature of human peanut allergy. Therefore, extrapolation of these results to humans should be done with caution.

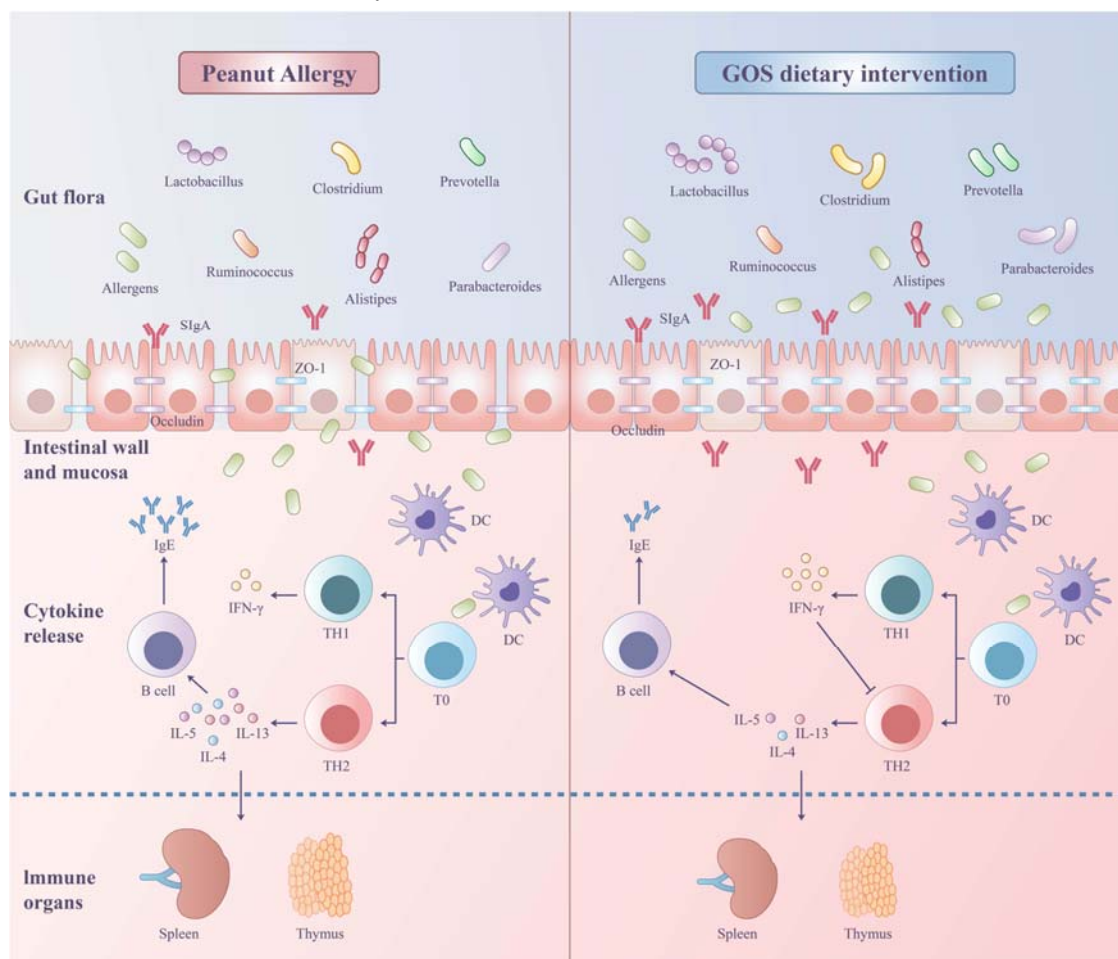


Fig.5 The mechanism of GOS in alleviating the allergic reaction to peanuts.

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