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2′-Fucosyllactose alleviates OVA-induced food allergy in mice by ameliorating intestinal microecology and regulating the imbalance of Th2/Th1 proportion†

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Food allergy (FA) has become a prominent problem in public health. 2′-Fucosyllactose (2′-FL) was reported to alleviate FA symptoms; however, the regulatory mechanism is still unclear. This study evaluated the 2′-FL antiallergic potential in an ovalbumin (OVA)-sensitized mouse model and explored the systemic effects of 2′-FL on gut microecology and the intestinal immune barrier. The results showed that 2′-FL alleviated allergy symptoms, decreased serum allergic indicator levels, enhanced the intestinal barrier, and attenuated low-grade inflammation. The up-regulation of G protein-coupled receptors (GPRs) was associated with higher levels of short-chain fatty acids (SCFAs) in 2′-FL intervention mice. 2′-FL also improved the intestinal microbiota diversity and increased the abundance of *Akkermansia*, *Lachnospiraceae UCG-006*, and *Ruminococcaceae* while suppressing *Muribaculaceae*, *Desulfovibrionaceae*, and *Erysipelotrichaceae*. Additionally, 2′-FL ameliorated the imbalance of Th2/Th1, mainly by decreasing Th2-type immune response and enhanced CD4 + Foxp3 + Treg immunoreaction. These results suggest that 2′-FL restores intestinal barrier defects, gut microbiota disorder, and immune impairment while alleviating ovalbumin-induced allergic symptoms in FA mice.

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1. Introduction

Allergic diseases are systemic disorders caused by the excessive immune response of the immune system to harmless substances in the environment.[1] Nowadays, the prevalence of these diseases is rising steadily worldwide. About 250 million people have been affected by food allergy (FA) in recent decades, according to the World Allergy Organization (WAO), and it is estimated that an increasing number of patients will endure the symptoms of allergic diseases for a long time. Allergy has been listed by the WHO as one of the top three major diseases in the 21st century. Many types of indigestible proteins, such as ovalbumin in eggs, casein in milk, and gluten in wheat, can cause food anaphylactic reactions and trigger acute clinical symptoms as food antigens, especially in children.[2–5] Nevertheless, the therapeutic effects of strict allergen avoidance and drug treatment are limited in cases of frequent exposure.[6–8] To date, there is an urgent need to explore more efficacious alternative therapeutic approaches consequently.

Several studies demonstrated the relationship between the gut microbiota and FA in which an imbalanced microbial structure led to a higher susceptibility to allergens.[8,9] Gut microbial composition determined the characteristics of the mucous layer. Fewer but well-adapted bacteria, including *Akkermansia muciniphila*, could promote mucus production and then form a physical barrier against external infection.[10] The colonization of microbiota is necessary for the maturation of the immune system in early life, and their housing conditions are also capable of altering the specific immune activity to antigens and regulating adaptive and effector immunoreactions.[11] Therefore, the positive interaction among gut microbiota, the intestinal barrier, and immune cells is of great significance for maintaining body health, and a balanced intestinal homeostasis environment may play a beneficial role in FA treatment in various aspects.

Human milk oligosaccharides (HMOs) are the third major solid component in human milk, next to lactose and fat. Among the class of nondigestible carbohydrates, 2′-fucosyllactose (2′-FL), a type of neutral fucosylated HMO, has the highest relative abundance. The reducing end of 2′-FL, which is the

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same as the other HMOs, is composed of lactose (Galβ1-4Glc), and fucose is then attached to the Gal terminal of lactose *via* an α1–2 bond.[12,13] In addition, as a latest prebiotic, 2′-FL has been identified as food generally recognized as safe (GRAS) by the United States and a novel food raw material by the European Union.[14,15] Only about 1% of the structurally intact 2′-FL is absorbed in the digestive tract, and most of this oligosaccharide will reach the distal small intestine and colon, where it is utilized as a fermentation substrate by specific intestinal microorganisms (*e.g.*, *bifidobacterium* and *lactobacillus*) so as to promote the construction of advantageous intestinal flora and the production of beneficial metabolites.[16–18] Previous studies found that 2′-FL modulated the gut microbiota in a structure-dependent manner.[19] 2′-FL binds with histo-blood group antigens (HBGAs) or soluble receptors on the intestinal epithelium to recognize or prevent pathogenic bacteria and parasites as an antiadhesive antimicrobial and pathogen decoy receptor. On the other hand, probiotics dominate the infant gut microbiota during breastfeeding *via* producing transport proteins and intracellular/extracellular glycosidase, which directly participate in the utilization of 2′-FL. Prebiotic 2′-FL plays positive roles in multi-aspects, which can be summed up as reducing the virus and pathogenic bacteria infection,[13,20] enhancing the intestinal barrier function,[21–23] relieving irritable bowel syndrome (IBS) and intestinal inflammation,[24–26] promoting brain development and increasing cognitive ability,[27–29] directly or indirectly regulating innate and adaptive immune responses,[30] and alleviating allergic diseases.

Several observational studies have shown that breastfeeding is significantly associated with lower rates of hypersensitivity diseases (such as atopic dermatitis, anaphylactic rhinitis, and FA) in early and even later stages life.[31,32] Further, the 2′-FL supplement has been proven to reduce allergy symptoms and partially improve the gut microbial dysbiosis in infants with a cow's milk protein allergic disease (CMPA).[33] Additionally, a few animal experiments showed that 2′-FL can alleviate allergic diseases. For instance, L. Castillo-Courtade *et al.* first demonstrated that 2′-FL relieved FA symptoms in mice by inducing IL-10 + T regulatory cells and indirectly stabilizing mast cells in 2015.[34] Besides, 2′-FL has been confirmed to reduce inflammation and improve the intestinal flora structure in a β-lactoglobulin induced-allergic mouse model.[35] Aili Li *et al.* found that 2′-FL attenuated β-lactoglobulin induced FA through the toll-like receptor 4/nuclear factor-κB signaling pathway mediated by miR-146a.[36] An *in vitro* experiment found that when exposed to intestinal epithelial cells, 2′-FL enabled dendritic cells (DCs) to drive type Th1 and regulatory immune development.[37] Nevertheless, most of the current studies are limited to clinical experiments to verify the hypoallergenicity of 2′-FL,[33,38,39] and there is still a lack of systematic studies expounding the impacts of 2′-FL on the gut microbiota, intestinal barrier function, and immunity in the allergic constitution. Meanwhile, the question of how 2′-FL restores the immune tolerance to food allergens and which pathways or mechanisms it takes has not been fully explored and solved.

In our study, mice were sensitized and challenged with ovalbumin (OVA), the most abundant protein in egg white, *via* multiple intraperitoneal injections to induce FA. The antiallergenic potential of 2′-FL to alleviate food allergy was also evaluated. The results demonstrated that 2′-FL relieved allergic symptoms by improving the mucosal barrier function and ameliorating intestinal microecology. Meanwhile, 2′-FL regulated the abnormal immune response in sensitized mice by reversing the imbalance of Th2/Th1 proportion and increasing the number of Treg cells. This study aimed to reveal the vital effects of 2′-FL on the gut microenvironment and immune system of food-allergic individuals and provide a valuable theoretical basis for the application of 2′-FL in the prevention and control of FA as a novel prebiotic.

2. Materials and methods

2.1. 2′-FL and experimental animals

2′-FL (chemically synthesized, purity >90.8%), used in this study, was obtained from Glycarbo Co. Ltd (Takamatsu, Japan).

The specific-pathogen-free Balb/c mice (6 weeks old, female) in this study were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd (Beijing, China) and housed in the Nankai University Laboratory Animal Center (permission number: SYKX 2019-0001) at a temperature of 22 ± 2 °C, humidity 50 ± 15% environment with 12 h light/dark cycles. All the animal protocols were ratified by the Institutional Animal Care and Use Committee of Nankai University (approval number: 2022-SYDWLL-000163) and implemented in accordance with the national guidelines for experimental animal welfare and ethics. One week before the start of the experiment, the mice were randomly divided into 3 groups (control group (CON), OVA-sensitized group (OVA), and 2′-FL intervention group (2′FL); n = 8 per group) and numbered, with 4 mice in each cage for acclimatization. The mice were fed a standard diet and allowed access to water *ad libitum*.

2.2. Construction of Balb/c mice OVA sensitization and 2′-FL intervention model

The construction of the food allergy model consisted of sensitization and challenge. **Sensitization:** mice in the OVA group and 2′FL group were sensitized by OVA intraperitoneal injection (i.p.) on days 0, 7, and 14, respectively. OVA (Sigma Aldrich, America) emulsification mixed with Freund's complete adjuvant (Sigma Aldrich, America) in a final volume of 200 μL was used for the first sensitization, and Friedrich's incomplete adjuvant (Sigma Aldrich, America) was used in the second sensitization and the third boosting immunization. The final concentration of OVA was 0.25 mg mL^{-1} all three times. Mice in the CON group were sensitized three times with 0.9% sterile normal saline instead. After sensitization, mice in 2′FL group were intragastric administered 2′-FL (500 mg per kg body weight) for 3 weeks continuously, and the CON and OVA

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group mice were treated and replaced with 0.9% sterile saline. **Challenge:** mice in group OVA and 2′FL were first challenged (i.p.) on day 32 (the concentration of OVA was 0.25 mg mL^{-1}), 4 days before the final challenge (the concentration of OVA was 2.5 mg mL^{-1}), to enhance immune response. Mice in the CON group were given 0.9% sterile normal saline equally. At the end of the experiment, the mice were anesthetized with ether after a 12 h fasting and sacrificed by posterior orbital venous plexus blood sampling and cervical dislocation with samples collected (serum, spleen, colon). The spleen index was calculated as the ratio of spleen weight against the body weight of mice.

2.3. Mice food allergy symptom score

Food allergy symptoms in mice were scored within 30 minutes of the last challenge. The observation methods and scoring criteria were referenced from the literature with a slight modification.[40] Briefly, the scoring criteria are shown in Table 1.

2.4. Determination of serum biochemical indicators

The concentrations of OVA-specific IgE (OVA-sIgE), histamine (HIS), IgG2a, IgG1, and mast cell protease-1 (Mcpt-1) in the serum of mice were determined according to the description of Nanjing Jiancheng Institute of Bioengineering ELISA kit (Nanjing, China).

2.5. Histological analysis and morphological observation

After sacrificing the mice, the distal colon tissues were taken for histopathological sections. The tissue samples were immersed in 4% paraformaldehyde for 24–48 h for fixation. After dehydration with gradient ethanol, the tissue samples were embedded in paraffin, trimmed, and sliced. After dewaxing treatment, the tissues were dyed with hematoxylin–eosin (H&E) and periodic acid-Schiff (PAS) stain, and then the colonic morphology was observed under the microscope to complete the pathological evaluation. Eight colon sections in each group were randomly numbered and interpreted in a blinded manner. Sections were scored individually by an independent investigator blinded to the type of treatment based on the given scoring criteria. Scores from individual mice were subsequently decoded, and regrouped numbers were analyzed statistically. The histological scoring criteria for the colon tissue are shown in Table S1.†

Table 1 Mice food allergy symptom score

Scores	Allergic symptom
0	Without any food allergic symptoms, normal, dry, and solid feces
1	Piloerection, scratching mouth and nose repeatedly, swollen, and moist feces
2	Less activity, swelling around the eyes and mouth, higher respiratory rate, soft, and mucous feces
3	Prolonged immobility, shortness of breath, rash around the mouth or tail, wet, and shapeless feces
4	No response after the challenge, muscle twitching, and watery feces with severe perianal staining

2.6. Immunohistochemistry (IHC)

Tissue samples were paraffin-embedded and cut into 4 μm thick slices. The antigen was repaired through a water bath of repair solution. Then, slices were routinely dewaxed and rehydrated, washed in PBS, and incubated in 0.03% H_2O_2 in methanol at room temperature for 30 min to neutralize endogenous peroxidase. After nonspecific proteins were blocked, slices were incubated with a primary monoclonal antibody against anti-ZO-1 (21773-1-AP, Proteintech Group, Wuhan, China) and anti-Occludin (27260-1-AP, Proteintech Group, Wuhan, China) at 4 °C for 8–10 h. Slices were then washed and non-specific stained. DAB reagents were used for the chromogenic reaction, and hematoxylin was added for nuclei staining.

2.7. Gas chromatographic analysis of short-chain fatty acids (SCFAs) in mice feces

Standard curves were constructed with standard mixtures (including acetic acid, propionic acid, butyric acid, and valerate) of gradient concentrations. Fresh fecal samples were weighed about 0.2 g (the weighing mass was accurately recorded) before 1 mL ultra-pure water and two steel balls were added. A tissue homogenizer was utilized for homogeneity sufficiently, followed by centrifugation (12 000*g*, 5 min), and the supernatant was collected. Then, 100 μL 10% sulfuric acid was added to acidize the supernatant, and 0.5 mL ethyl ether was added to extract SCFAs. The samples were vortex-mixed for 2 min and then centrifuged (12 000*g*, 5 min) after static extraction. The upper layer of the organic phase was filtered using 0.22 μm nylon membranes and collected in 2 mL brown autosampler vials. The samples were detected on the same day after treatment.

Gas chromatography adopted high-purity nitrogen as carrier gas and helium as the protective gas. The heating conditions were 80 °C for 2 min, 80–180 °C for 10 min, and 180 °C for 5 min. 1 μL samples were injected at a time, and the single run time was 15 minutes. A DB-FFAP capillary column (30 m × 0.25 mm i.d., 0.25 μm, Agilent) was used for gas chromatography to quantify SCFAs in our study. The single standard solution was injected first, with the relative peak time recorded. Then, the standard mixtures of different concentrations were injected, and the relative peak area was recorded for the construction of standard curves. Different groups of samples were injected finally, and the concentration of each sample was calculated.

2.8. Real-time quantitative polymerase chain reaction (qRT-PCR) analysis

According to the method used in previous studies,[41] mRNA from the spleen and colon of mice used as a template for reverse transcription into cDNA was extracted with TriQuick reagent. The real-time quantitative PCR (RT-qPCR) technique was utilized to determine the expression level of related gene levels. The specific primer sequences are shown in Table S2.† The results were normalized with *β-actin* as the internal para-

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meter and calculated by the $2^{-\Delta\Delta Ct}$ method, and the results of the CON group were adopted as the standard for other groups.

2.9. Gut microbiota profiling (DNA extraction and amplicon generation) and bioinformatic analysis

The microbial total genomic DNA of the colon contents of mice in different groups was extracted, purified, and identified. Universal primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify the V3–V4 hypervariable region of bacterial 16S rRNA gene. Species taxonomic analysis, alpha diversity analysis, principal coordinate analysis (PCoA), and linear discriminant analysis (LEfSe) were performed on the BMKCloud platform. Spearman correlation analysis was used to find the correlations between intestinal microbiota and allergic parameters.

2.10. Flow cytometry

After anesthetized and euthanized, the spleen, mesenteric lymph nodes (MLNs), and Peyer's patches (PPs) of mice were separated to prepare single-cell suspension in a clean bench. Spleen cells were treated with erythrocyte lysis buffer. Cells were re-suspended in RPMI 1640 complete medium for cell count. Cell samples were first stimulated for 6 h with Leukocyte Activation Cocktail, with BD GolgiPlug™ (BD Biosciences, CA, USA) according to the description. The cells were then surface-stained with anti-CD3e-FITC, anti-CD4-BB700, and anti-CD25-BV421 (BD Biosciences). After treating cells with Transcription Factor Fixation/Permeablization Buffer Set (BD Biosciences), anti-IL-4-BV605, anti-IFN-gamma-BV785 and anti-FoxP3-AF647 (Biolegend, CA, USA) were utilized to label intracellular/intracellular factors. Fluorescence minus one (FMO) internal staining controls were set up by pooling cells from different experimental groups (CD3e+ CD4+ IL-4+ for Th2 cells and CD3e+ CD4+ IFN-gamma+ for Th1 cells). The BD FACSArica III system (BD Biosciences) and FlowJo software (Tree Star, Inc., CA, USA) were used to acquire and analyze the flow cytometric data.

2.11. Statistical analysis

Prism 8.0.1 (GraphPad, La Jolla, CA) was used for experimental data analysis, and all the results were presented as the mean ± SD. The statistical analysis of different groups was performed using the one-way analysis of variance (ANOVA), Dunnett's multiple comparison tests, and Kruskal–Wallis test. Statistical significance was set at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, or $^{****}p < 0.0001$ *versus* the OVA group.

3. Results

3.1. 2′-FL alleviates OVA-induced food allergy symptoms

The effect of 2′-FL on the allergic symptoms was evaluated based on OVA-sensitized mouse model (Fig. 1A). Body weight was recorded one day before and after each time of allergen sensitization (i.p.) during the study and was measured weekly during the intervention (i.a.) period thereafter (Fig. 1B). The first sensitization resulted in a loss of body weight, which was not repeated in the two subsequent sensitizations. However, as shown in Fig. 1C, the weight gain of mice calculated at the end of the study was significantly higher in the OVA group exposed to allergens than in the CON group (3.59 ± 0.402 g *vs.* 1.94 ± 0.427 g). After the intervention of 2′-FL, the excessive weight gain of mice was alleviated (3.092 ± 0.509 g).

On the last day of animal experiment, mice were challenged with a high dose of allergen, and then scored for FA symptoms within 30 min according to specific criteria (Fig. 1D). The three groups of challenged mice displayed different degrees of allergy symptoms. Compared with the CON group, the mice in OVA group showed evident food allergy symptoms, such as swelling, bluish mouth, higher respiratory rate, prolonged immobility, and diarrhea. In contrast, the allergic symptoms were attenuated in most mice in 2′-FL group, showing occasional muzzle scratching, formed feces, and higher levels of activity.

The intuitional comparison of spleens in different groups of mice in Fig. 1E exhibited that OVA stimulation significantly increased the volume of spleens in the OVA group, while the spleens in 2′-FL-treated mice were more similar to those in the CON group. Moreover, the spleen weight and index were evidently lower in mice intervened with 2′-FL than in OVA-sensitized mice (Fig. 1F and G). These results indicated that 2′-FL possessed the potential to alleviate food allergy induced by OVA.

3.2. 2′-FL regulates immune markers of food allergy in the serum of mice

Acute food allergy is strongly mediated by IgE.[6] We measured OVA-sIgE levels in the mouse serum and found that OVA-sIgE content was significantly reduced by 2′-FL intervention in OVA-allergic mice (Fig. 2A). Besides, on account that mast cell degranulation is a major inducement of food allergy symptoms, levels of Mcpt-1 and HIS in the mouse serum were measured as well. As expected, the 2′-FL treatment effectively alleviated elevated levels of Mcpt-1 and HIS in the serum of allergic mice (Fig. 2B and C). We also evaluated the expression levels of immunoglobulin IgG1/IgG2a, which were associated with the Th2/Th1 type immune response. As shown in Fig. 2D and E, OVA-induced food allergy significantly increased the content of IgG1 and decreased the content of IgG2a in mice. Nevertheless, the IgG1/IgG2a imbalance in the 2′FL group was relieved by intragastric administration of 2′-FL to sensitized mice (Fig. 2F).

3.3. 2′-FL relieves low-grade impairment of the intestinal barrier

The integrity of the intestinal mucus barrier is a non-negligible part of food allergies. For the reason that 2′-FL is mainly utilized by specific microorganisms in the colon as fermentation substrate, the gut tight junction (TJ) proteins in the colon were determined by IHC staining experiments and qPCR. Tight junctions, such as ZO-1 and members of the occluding family,

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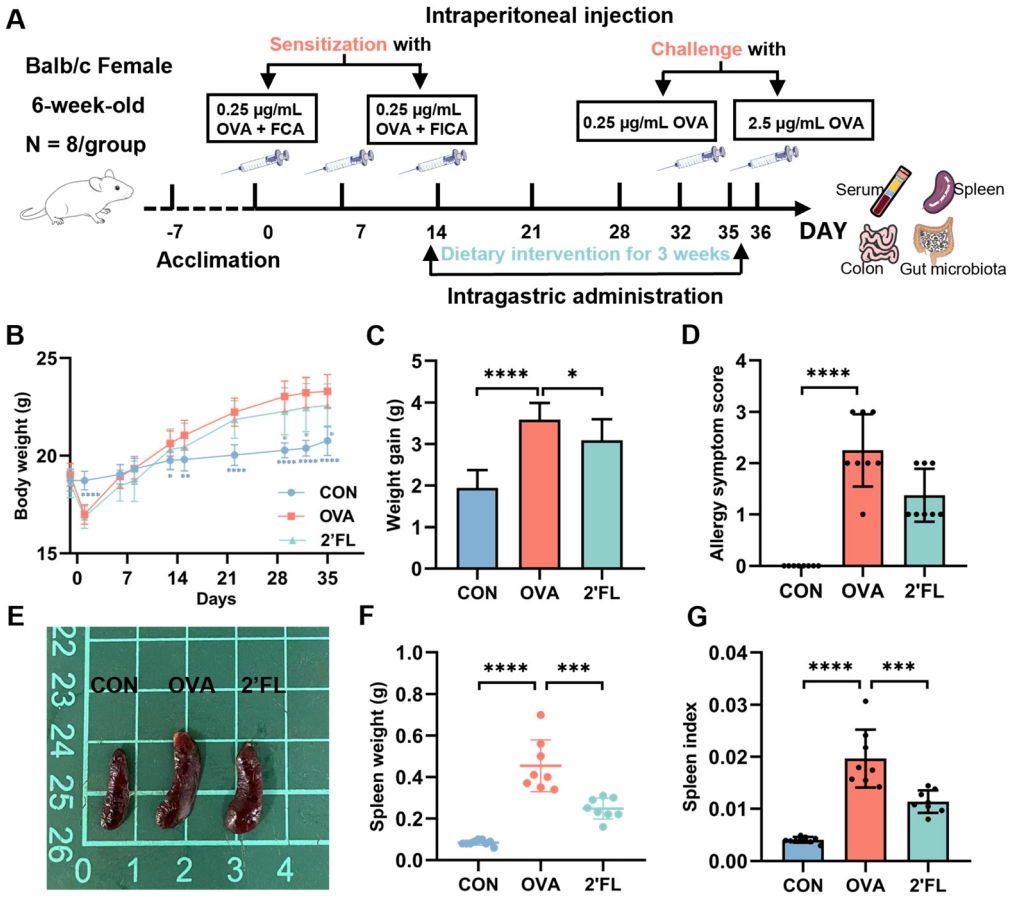

Fig. 1 2′-FL alleviates OVA-induced food allergy symptoms in mice. (A) Schematic diagram of Balb/c mice OVA-sensitization and 2′-FL intervention model. (B) Body weight. (C) Weight gain during the entire experimental period. (D) Allergy symptom score measured within 30 min after the last challenge. (E) Representative images of the spleen of mice. (F) Spleen weight. (G) Spleen index. Data are shown as the mean ± SD (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001, or ****p < 0.0001 *versus* the OVA group. CON: control; OVA: OVA-sensitized mice; 2′FL: OVA-sensitized mice treated with 2′-FL.

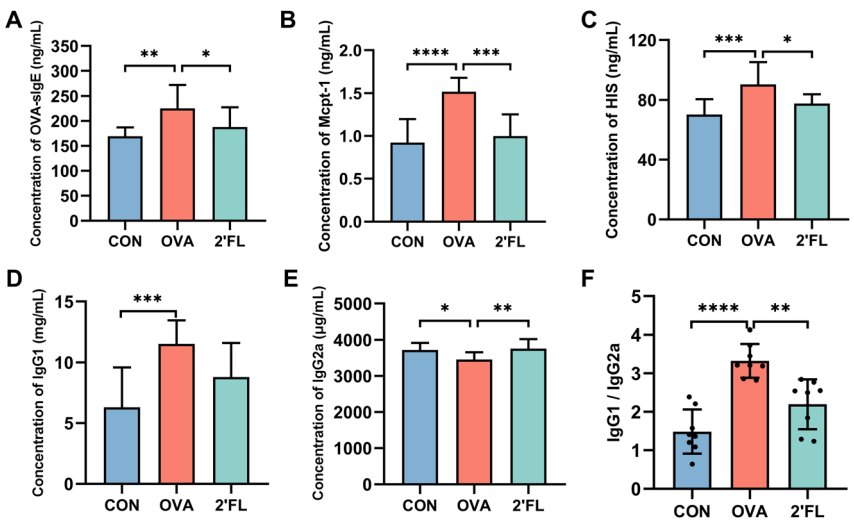

Fig. 2 2′-FL regulates immune markers of food allergy in the serum of mice. Concentration of immune markers (A) OVA-specific immunoglobulin E (OVA-sIgE), (B) mast cell protease-1 (Mcpt-1), (C) histamine (HIS), (D) immunoglobulin G1 (IgG1), (E) immunoglobulin G2a (IgG2a), and (F) IgG1/IgG2a. Data are shown as the mean ± SD (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001, or ****p < 0.0001 *versus* the OVA group. CON: control; OVA: OVA-sensitized mice; 2′FL: OVA-sensitized mice treated with 2′-FL.

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are major proteins that make up the mucosal barrier of the intestine. The IHC staining results confirmed that exposure to OVA resulted in the decline of TJs proteins in spinous and granular layers of mucosa, while the relative mRNA expressions of ZO-1 and Occludin were remarkably improved in mice with 2′-FL treatment (Fig. 3A and B).

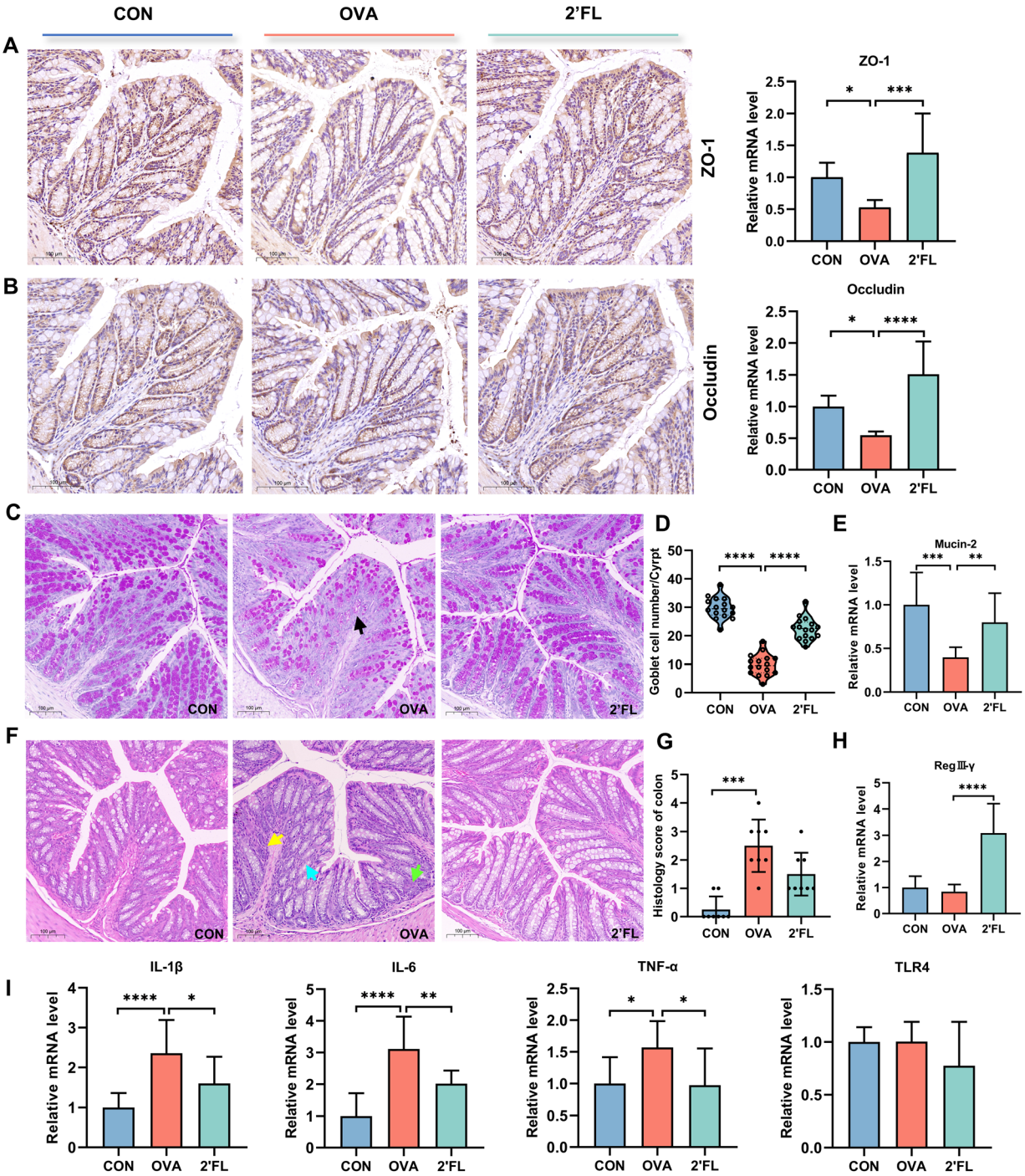

Fig. 3 2′-FL relieves low-grade impairment of the intestinal barrier. (A) Representative histologic images of immunohistochemistry (IHC) staining of colon sections and relative mRNA expression of ZO-1. (B) Representative histologic images of IHC staining of colon sections relative mRNA expression of Occludin. (C) Representative histologic images of periodic acid-Schiff (PAS) staining of colon sections. (D) Number of goblet cells. (E) Relative mRNA expression of Mucin-2. (F) Representative histologic images of hematoxylin and eosin (H&E) staining of colon sections. (G) Histology score of colons. (H) Relative mRNA expression of RegIII-γ. (I) Relative mRNA expression of IL-1, IL-6, TNF-α and TLR4. Data are shown as the mean ± SD (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001, or ****p < 0.0001 *versus* the OVA group. CON: control; OVA: OVA-sensitized mice; 2′FL: OVA-sensitized mice treated with 2′-FL.

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Additionally, PAS and H&E staining were used to observe the histopathology of the mouse distal colon. Goblet cells, the key producer of Mucin-2 protein, were observed with PAS staining, which can make mucoproteins in goblet cells appear purply red. Compared with mice in the CON group, OVA-induced food allergy caused damage to goblet cells in the colon of mice (as shown by the arrow in the figure) (Fig. 3C), and the relative mRNA expression level of Mucin-2 secreted by these cells was also significantly downregulated (Fig. 3E). While after the 2′-FL intragastric treatment, the number of goblet cells in the crypt lumen increased significantly (Fig. 3D), and the expression of Mucin-2 was obviously upregulated to maintain the formation of the mucin layer (Fig. 3E). In addition, histological scores were performed by observing the H&E staining of the colon. As shown in Fig. 3F and G, OVA induced the low-grade inflammation of the colon, aggregation of lymphocytes (yellow arrow), deformation in crypt structure (green arrow), and abnormal epithelial morphology (blue arrow). However, after 3 weeks of 2′-FL gavage in sensitized mice, those symptoms were relieved, and the morphological characteristics were more similar to the state of a normal mouse colon. The relative mRNA expression of RegIII-γ, as an antibacterial peptide secreted by epithelial cells, decreased in the colon of 2′-FL-treated mice, which was significantly distinguished from that in the other two groups (Fig. 3H). Subsequently, the levels of colonic inflammatory cytokines IL-1β, IL-6, and TNF-α, and the relative expression of TLR4 were measured, and the results illustrated that 2′-FL can attenuate low-grade colonic inflammation in food-allergic mice but had no significant effect on TLR4 expression (Fig. 3I).

3.4. 2′-FL affected the content of SCFAs and the expression of intestinal epithelial-related receptors

SCFAs play a positive role in alleviating anaphylactic disease. The levels of four SCFAs in colonic contents of mice in the 2′-FL group were evidently increased along with the intervention of prebiotics, showing significant differences from the other two groups (acetic acid, 1.83-fold increase; propionic acid, 2.76-fold increase; butyric acid, 2.98-fold increase; valeric acid, a 2.35-fold increase in mice fed 2′-FL compared to those in OVA group) (Fig. 4A).

Certain G-protein-coupled receptors (GPRs) can mediate SCFAs to regulate physiological processes and deliver signals to maintain cell homeostasis.[42] Several major SCFA receptors were determined in this study. The results indicated that the OVA-induced food allergy had no significant effect on GPR41 and GPR109A, except for GPR43, and the gene expression was lower than that of the CON group. However, the relative expression levels of these receptors were all evidently increased in OVA-sensitized mice treated with 2′-FL (Fig. 4B). We also measured the expression level of aromatic hydrocarbon receptors (AhR) in the colon and found that 2′-FL intervention also upregulated the AhR expression in our study (Fig. 4C).

3.5. Effects of 2′-FL on the intestinal microbiota in OVA-induced food allergy mice

It has been reported that the imbalance of the gut microbiome is related to food sensitivity.[43] Therefore, 16S rRNA sequencing was performed on the gut microbiota of mice in each group to investigate whether 2′-FL alleviated FA by reprogramming the intestinal microecology. The CON, OVA, and 2′FL groups

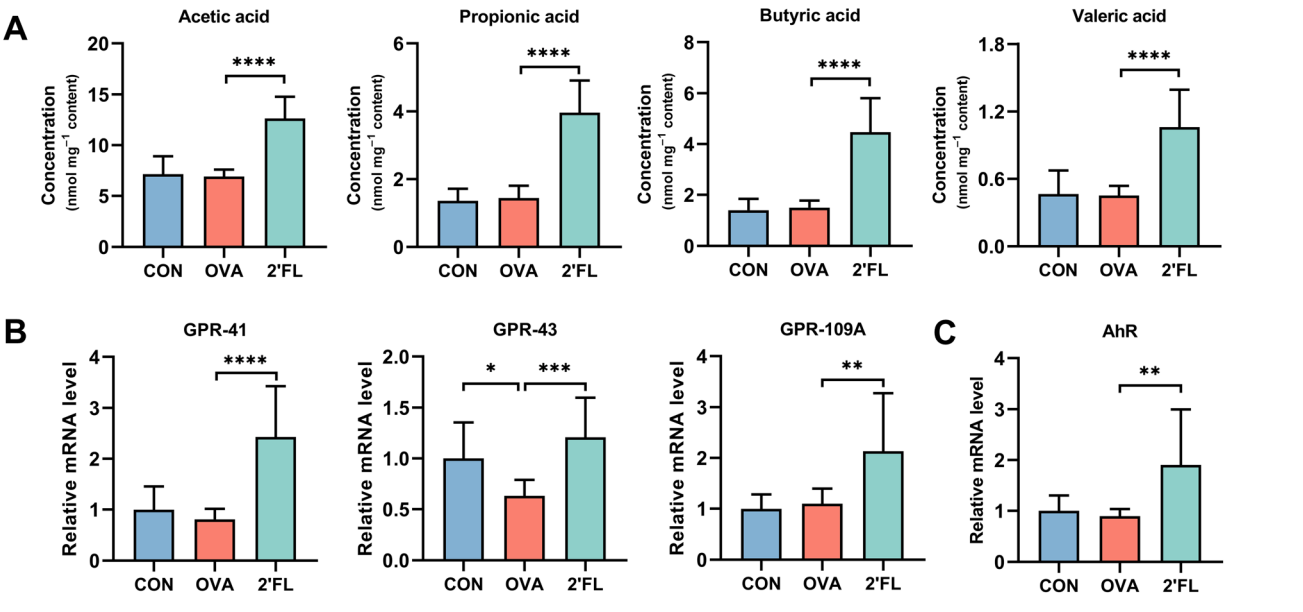

Fig. 4 2′-FL affects the content of short chain fatty acids and the expression of intestinal epithelial related receptors. (A) Concentration of acetic acid, propionic acid, butyric acid, and valeric acid in the colonic contents of mice. (B) Relative mRNA expression of GPR-41, GPR-43, GPR-109A and (C) AhR. Data are shown as the mean ± SD (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001, or ****p < 0.0001 *versus* the OVA group. CON: control; OVA: OVA-sensitized mice; 2′FL: OVA-sensitized mice treated with 2′-FL.

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resulted in 1321, 1190, and 1792 operational taxonomic units (OTUs), respectively, for clustering all high-quality sequences with 97% identity. Venn diagram revealed the shared and specific microbial communities in the gut of the three groups of mice (Fig. 5A). Chao1 index and Shannon index are applied to estimate the richness and diversity of species in intestinal

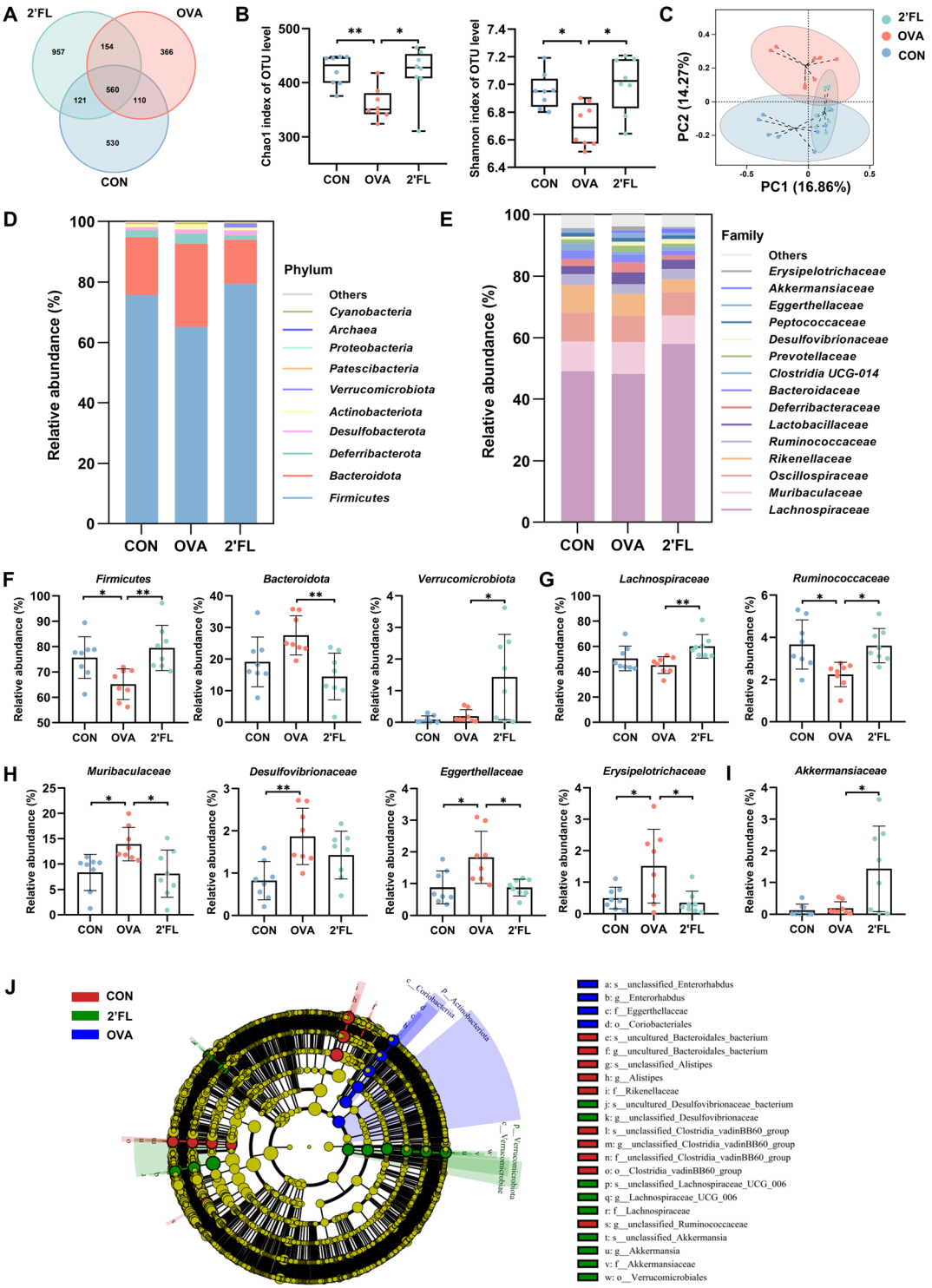

Fig. 5 Effects of 2′-FL on the intestinal microbiota in OVA-induced food allergy mice. (A) Venn diagram of the different groups. (B) Chao1 index and Shannon index. (C) PCoA. (D) Bacterial taxonomic composition at the phylum level. (E) Bacterial taxonomic composition at the family level. (F) Relative abundance of the significantly differential flora at the phylum level. (G–I) Relative abundance of the significantly differential flora at the family level. (J) Branch diagram depicting the output of the LEfSe analysis. Data are shown as the mean ± SD (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001, or ****p < 0.0001 *versus* the OVA group. CON: control; OVA: OVA-sensitized mice; 2′FL: OVA-sensitized mice treated with 2′-FL.

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microbiota. Fig. 5B showed that 2′-FL intervention significantly improved the decrease in the Chao1 index and Shannon index caused by OVA sensitization, suggesting that 2′-FL treatment inhibited the disturbance of intestinal flora diversity and evenness in allergic mice. In addition, PCoA demonstrated the beta diversity among the three groups, and the separation of OVA and CON groups showed shifts in microbial structure due to FA, whereas changes in microbial composition in the 2′FL group were restored (Fig. 5C).

To further explore the differences in intestinal flora between the 2′-FL intervention group and the OVA-sensitized group, Fig. 5D and E showed the relative richness histogram of the top 10/15 annotated species at the phylum/family level, respectively. Firmicutes and Bacteroidota, as the two dominant phyla, accounted for 75.68% and 19.12% of the microbial profiles of the CON group, respectively. The proportion of these two bacteria in the intestinal flora changed to 65.16% and 27.50% due to the stimulation of OVA, but the significant changes were restored to 79.49% and 14.49% in the 2′FL group (Fig. 5F). By analyzing the distinction in community composition at the family level between different groups of mice, 2′-FL was found to reverse the decrease in the relative abundance of beneficial bacteria families, such as *Lachnospiraceae* and *Ruminococcaceae*, in food allergic mice (Fig. 5G). Besides, the evident increase in *Muribaculaceae*, *Desulfovibrionaceae*, *Eggerthellaceae*, and *Erysipelotrichaceae* caused by OVA sensitization was partially or completely suppressed after 2′-FL treatment (Fig. 5H). It was worth noting that 2′-FL treatment significantly enriched the abundance of *verrucomicrobia* in the intestinal flora, and by combining the data at the family level, it was reasonable to speculate that this was mainly related to the obvious enhancement in the number of *Akkermanaceae* (Fig. 5F and I). The intestinal microorganism biomarkers among the three groups were found by LEfSe analysis. As illustrated in Fig. 5J, the bacteria from the genera of *Enterorhabdus* were enriched in the OVA group, while *Akkermansia* and *Lachnospiraceae* UCG-006 were observed at higher levels in the 2′FL group.

3.6. 2′-FL modulates and ameliorates the imbalance of Th2/Th1

The anaphylactic response is often accompanied by the imbalanced immune regulation of Th2/Th1. After sensitization with allergens, the body tends to have a stronger Th2-type immune response. Hence, we extracted single cells from the spleen, MLNs, and PPs of different groups of mice with or without 2′-FL intervention to detect Th2/Th1 cell differentiation by flow cytometry. Fig. 6A shows the gating strategy of the flow cytometry analysis in our study. Compared to the OVA group, Th2-type immunoreaction tendency obviously appeared in the spleens and PPs of the OVA group mice, demonstrating that the sensitized individuals produced more type Th2 immune responses internally, yet the imbalance of Th2/Th1 differentiation was reduced in the treated 2′FL group mice (Fig. 6B).

Furthermore, mRNA expression levels of key transcription factors of Th1 and Th2 cells (GATA-3 and T-bet) and their related cytokines were measured in the spleens and colons. The levels of Th2-related immune factors (IL-4, IL-5, and IL-6) in the OVA group mice sensitized were abnormally increased, causing more differentiation from Th0 cells to the direction of Th2 cells. However, with the treatment of 2′-FL, this imbalanced polarization in FA mice could be relieved (Fig. 6C). Additionally, transcription factor T-bet was found to be significantly reduced in the spleen in the OVA group mice. In contrast, 2′-FL can enhance the Th1 type immune response reflecting in upregulating the expression levels of Th1-related immune factors (IFN-γ, TNF-β) (Fig. 6D), thereby resisting the Th2/Th1 imbalance caused by the OVA-induced food allergy.

3.7. 2′-FL enhances the immune response of Treg in OVA-sensitized mice

To further investigate the impacts of 2′-FL on regulatory T cell (Treg) immune responses, Treg cell counts were also measured. Results of flow cytometry showed that exposure to OVA significantly reduced the proportion of Treg cells in spleens, MLNs, and PPs of mice in the OVA group, but 2′-FL effectively alleviated the decrease of Treg cells in the OVA-induced food allergy model mice and restored them close to the normal level taking the CON group mice as the reference (Fig. 7A and B). By determining the levels of Treg cell key transcription factors and immune factors in the spleen and colon of mice, it was found that the mRNA relative expression levels of Foxp3 and TGF-β in 2′FL group mice were significantly increased compared with those in the OVA group mice (Fig. 7C and D). These results suggested that 2′-FL may further regulate the immune response in the FA mice by mediating CD4 + Foxp3 + Treg. Besides that, interestingly, the mice stimulated with the allergen OVA had higher levels of IL-10 (Fig. 7C and D), which is aligned with the previous studies of L. Castillo-Courtade *et al.* and Wróblewska *et al.*[34,44]

3.8. Correlation analysis between intestinal microbiota and allergic parameters

To identify the specific microbial strains that may mediate 2′-FL in alleviating FA, Spearman's correlation analysis was used to evaluate the correlations between the gut microbiota and allergy-related indicators, as shown in the heatmap (Fig. 8). Th2 cells key transcription factors and related cytokines were negatively correlated with *Akkermansia*, *Lachnospiraceae* UCG-006 and *Ruminococcaceae*, while positively correlated with *Anaerotruncus*, *Enterorhabdus*, and *Desulfovibrionaceae*. In addition, in terms of the intestinal barrier function, TJ protein expression and SCFA contents were negatively associated with the abundance of *Alistipes*, *Bacteroides*, *Erysipelotrichaceae*, and *Muribaculaceae* but were observed to be positively related to *Akkermansia* and *Lachnospiraceae* UCG-006.

4. Discussion

The development of FA, one of the prominent problems in food safety and public health emergencies, is proven to be closely related to intestinal barrier impairments and immune

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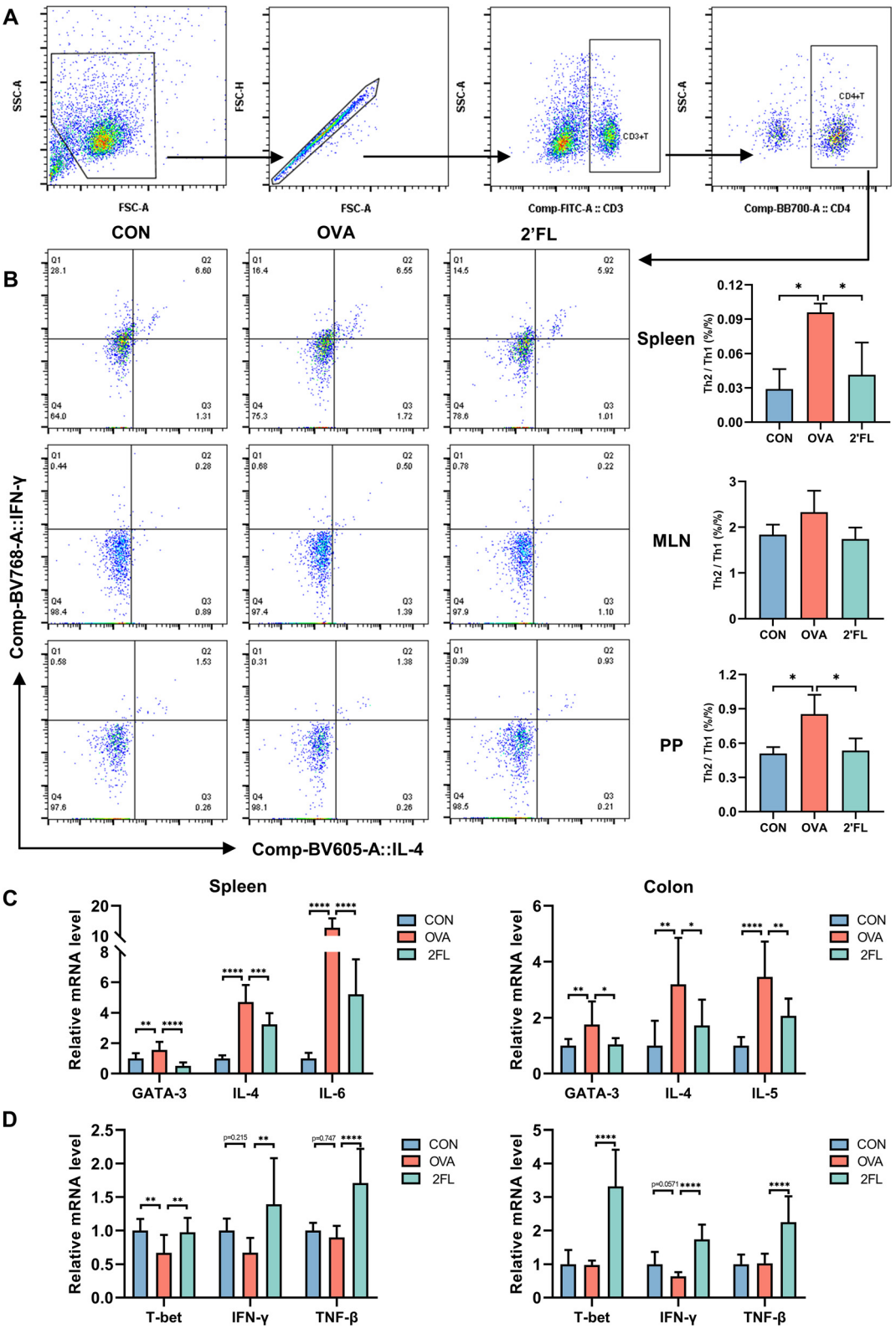

Fig. 6 2′-FL ameliorates the imbalance of Th2/Th1. (A) Gating strategy of the flow cytometry analysis. (B) Representative images of flow cytometry analysis and frequency of Th2/Th1 in spleens, MLNs, and PPs. (C) Relative mRNA expression of key transcription factors of Th2 cell and related cytokines in spleens and colons. (D) Relative mRNA expression of key transcription factors of Th1 cells and related cytokines in spleens and colons. Data are shown as the mean ± SD (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001, or ****p < 0.0001 *versus* the OVA group. CON: control; OVA: OVA-sensitized mice; 2′FL: OVA-sensitized mice treated with 2′-FL.

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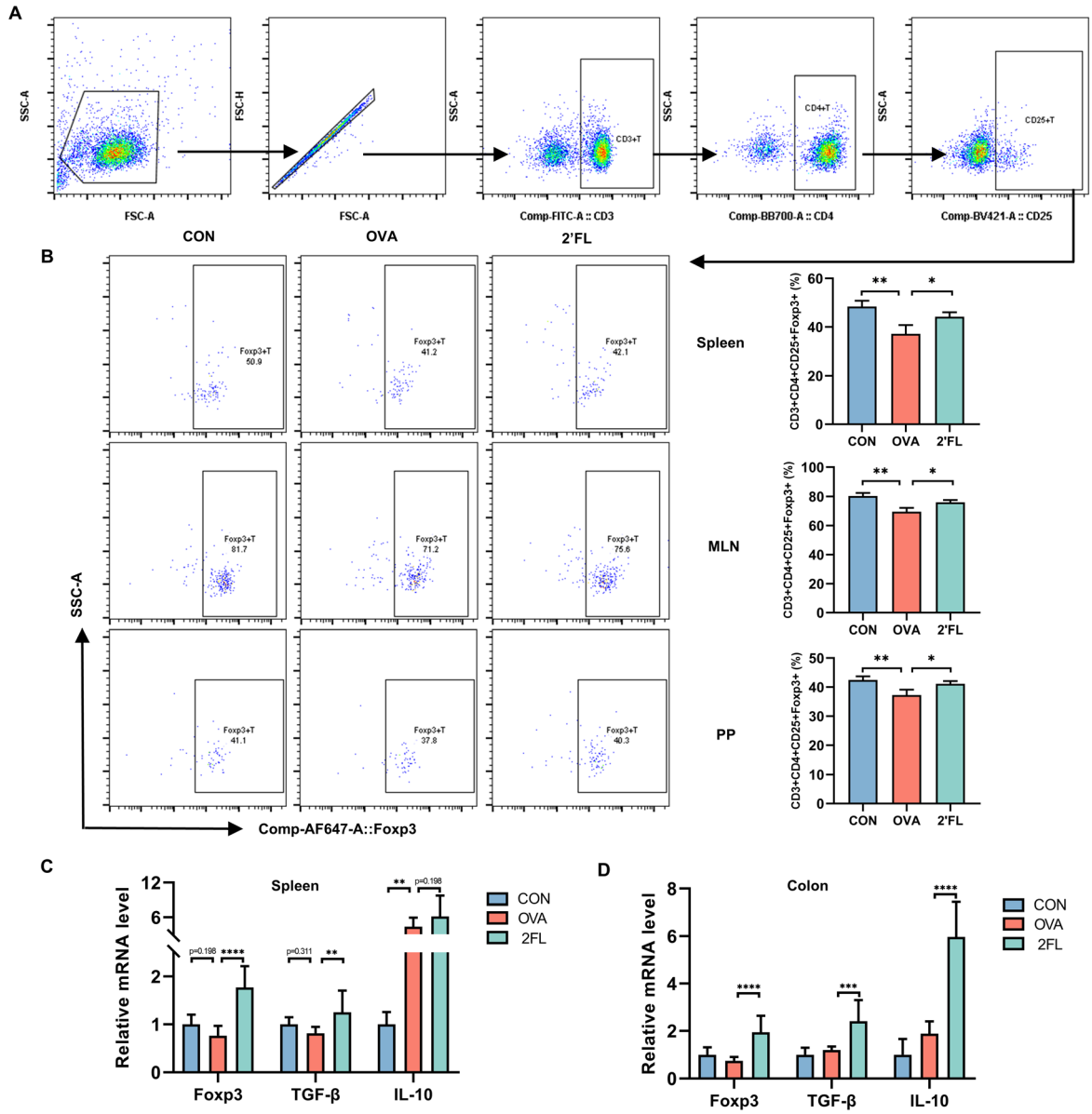

Fig. 7 2′-FL enhances the immune response of Treg. (A) Gating strategy of the flow cytometry analysis. (B) Representative images of flow cytometry analysis and frequency of Tregs in spleens, MLNs, and PPs. (C) Relative mRNA expression of key transcription factors of Treg and related cytokines in spleens. (D) Relative mRNA expression of key transcription factors of Treg and related cytokines in colons. Data are shown as the mean ± SD (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001, or ****p < 0.0001 *versus* the OVA group. CON: control; OVA: OVA-sensitized mice; 2′FL: OVA-sensitized mice treated with 2′-FL.

disorders. HMOs in human milk (HM), represented by 2′-FL, are not digested by human enzymes owing to their structural similarities to the O-chain structure presenting in human blood group antigens and mucus. They have evolved as natural prebiotics, acting through the intestinal microenvironment and selectively feeding certain species to guide healthier immunomicrobial relationships, and then benefit the development and maturation of the host immune system.[16,45] Therefore, 2′-FL, as the most abundant oligosaccharide content in HM, was hypothesized to be an anti-allergy nutritional supplement. It is vital to analyze the antianaphylaxis potential and probiotic effect of 2′-FL as well as its underlying mechanisms.

OVA-induced FA is an IgE-mediated allergic disease. Antigen-specific IgE is vital in the pathogenesis of FA because it mediates the activation and degranulation of mast cells through FcεR1 aggregation, releasing HIS and cysteine, leukotriene, and others, which will cause allergic symptoms, such as swelling of the surrounding tissues and low-grade inflammation.[6] Mast cell protease-1 is a marker of activation and proliferation of mast cells, which can promote intestinal permeability.[46] In mice, Th1 enhances the synthesis of IgG2a by B

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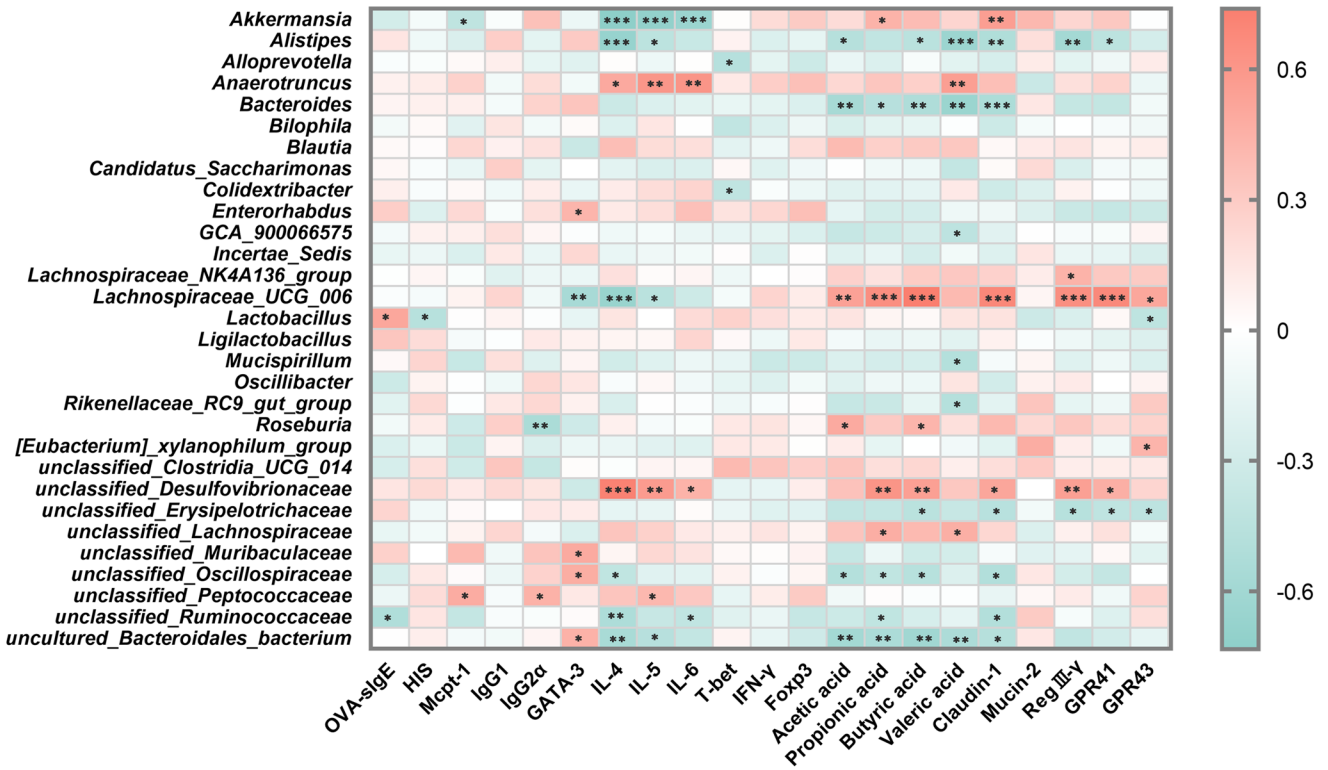

Fig. 8 Correlations between intestinal microbiota and allergic parameters. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cells through IFN-γ, while Th2 induces the secretion of IgE and IgG1 by B cells through IL-4.[47,48] The IgG1/IgG2a ratio has been recognized as an indicator of the Th2 or Th1 immune response in mice. In our study, we established a food allergy model by intraperitoneal injection of OVA in female Balb/c mice and subsequently found that the relief of allergic disease symptoms could be visually observed after continuous treatment with 2′-FL (500 mg per kg body weight) to sensitized mice for 3 weeks. Both stimulated by high concentrations of OVA, most of the OVA group mice showed evident shortness of breath, scratching the nose and mouth, and symptoms of prolonged immobility accompanied by diarrhea. In contrast, the 2′FL group mice had lower systemic swelling, and their body weight, spleen index, and behavioral status were more similar to the control group mice. We then measured concentrations of relevant immune biomarkers in the serum of different groups of mice. Compared with the OVA group, the contents of OVA-sIgE and HIS, especially Mcpt-1, in the 2′FL group mice were significantly decreased, and the ratio of IgG1/IgG2a was more consistent with that in the CON group, which may indicate an increased Th1 type response, and verify the ability of 2′-FL to inhibit IgE-mediated allergic diseases.

As a physiological functional unit, intestinal mucosa separates the intestinal cavity from the internal environment and plays important functions, such as protection, nutrition, and immunity.[49] Increased intestinal permeability is one of the causes of FA, along with immune abnormalities, expansion of inflammatory mediators, and dysregulation of intestinal flora.[40] While the intestinal mucosa is impaired, allergens are more likely to penetrate the intestinal barrier and stimulate the submucosal immune system.[50] The study of Tao Chen *et al.* concluded that allergens may generate intestinal mucosal permeability through intestinal TJ impairment, thus causing intestinal FA.[51] Bing Han *et al.* demonstrated that fucoxanthin could inhibit ovalbumin-induced food allergies by enhancing the intestinal epithelial barrier and regulating intestinal microbiota.[40] Therefore, we further investigated the effect of 2′-FL on the intestinal barrier function in sensitized mice. By staining the colon of mice, intestinal villi in the OVA group were found to be damaged to different degrees, and the number of goblet cells in the crypt was obviously reduced, but after the 2′-FL intervention, the above histological damages were significantly improved. Besides that, TJ proteins such as ZO-1 and Occludin-1 showed higher expression in the colon of 2′FL group mice. It is worth mentioning that the mucous layer, as a major driver of intestinal homeostasis, stores antimicrobial peptides and sIgA, and RegIII-γ is one of the antimicrobial peptides secreted by Paneth cells.[8] In our experiments, we found that FA did not cause a decrease in RegIII-γ levels, but supplementing 2′-FL significantly increased RegIII-γ content in the gut. Our results suggested that 2′-FL enhanced the intestinal epithelial barrier function, reduced the gut permeability to food allergens, and restricted the entry of antigens into the circulatory system in allergic mice. Moreover, the release of cytokines and inflammatory mediators is associated with the epithelial barrier degradation, leading to an inappropriate circula-

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tion that causes a further increase in intestinal permeability.[52] In our study, 2′-FL was found to inhibit low-grade inflammation in the colon of sensitized mice and the up-regulation of cytokines, such as IL-6, IL-1β, and TNF-α. Our results proved that 2′-FL firmed the integrity of the intestinal epithelial barrier and relieved inflammation in the intestine, which may be one of the pathways that 2′-FL alleviated FA symptoms.

SCFAs are metabolites produced by the fermentation of insoluble substrates by beneficial bacteria in the gut microbiome. They have been shown to improve the gut environment and regulate the intestinal immune system homeostasis by binding to GPRs, a signaling molecule.[42] As oligosaccharides, 2′-FL itself can be metabolized into dihydroxyacetone phosphate and lactate aldehyde, which are subsequently metabolized into acetic acid and propionic acid, respectively, thus increasing the total amount of SCFAs in the host intestine. The experiment conducted by Aurélien Trompette *et al.* concluded that intestinal microbiota increased the concentration of circulating SCFAs by metabolizing a high-fiber diet and then reduced allergic airway diseases.[53] The generation of macrophage and dendritic cell (DC) precursors were enhanced in mice treated with SCFA propionic acid in this study, with impaired ability to promote Th2 cell effector function, and the effect of propionic acid on allergic inflammation is dependent on GPR-41. In another experiment, Jian Tan *et al.* showed that more acetic acid and butyrate release improved oral tolerance and avoided FA, and mice lacking SCFA receptors showed exacerbated FA and lower levels of Treg cell differentiation.[54] Our results also displayed the increase of SCFA contents and the up-regulation of SCFA receptor expression. Moreover, SCFAs may influence Treg cells dependent on the activity of direct histone deacetylase (HDAC) inhibitors and regulate the mTOR pathway required for Th1 and IL-10+ T cell generation, thereby promoting immunity or immune tolerance.[55] After the 2′-FL intervention, the abundance of SCFAs and the differentiation tendency of Treg in OVA-induced food-allergic mice significantly increased, which may be one of the explanations for the mechanism of 2′-FL alleviating allergic diseases.

Intestinal microecology is composed of intestinal microflora and the environment in which they exist. The imbalance of the intestinal gut microflora and the reduction of bacterial diversity are the main causes of the high hyperreactivity of FA.[9] In this study, the improvement of intestinal microecology in OVA-sensitized mice by 2′-FL was reflected in alleviating damage to the intestinal mucosal barrier, reducing low-grade intestinal inflammation, stabilizing intestinal microflora, and other aspects. A decline in the relative abundance of *Lachnospiraceae*, which belongs to the *Firmicutes* and is found in the gut of most healthy individuals, has been shown to be associated with asthma in preschool children. The genus-level bacteria of *Lachnospiraceae* UCG-006 was proven to be positively correlated with cecal SCFAs but negatively related to serum IL-6 levels.[56] Hein M. Tun *et al.* reported that exposure to pets enriched *Ruminococcaceae* abundance in infants, which was negatively correlated with allergies in children.[57] Jianlong Yan *et al.* found in their studies that *Akkermansia* was positively correlated with SCFAs in mice, and then the increasing SCFA may improve the intestinal barrier function and relieve inflammation.[58] Our conclusion was consistent with the above phenomena that after the intervention of 2′-FL, the significant reduction of *Firmicutes*, *Lachnospiraceae*, *Ruminococcaceae*, and *Akkermansiaceae* caused by FA was effectively relieved. These characteristic strains with variation may be the target of treatment for FA induced by OVA. Additionally, the level of harmful bacteria *Desulfovibrionaceae* was also found upregulated by Bing Han *et al.* in the intestines of OVA-sensitized mice.[40] In a mother-infant pairs cohort pilot study, *Eggerthellaceae* was enriched in both infant feces and maternal milk who were diagnosed with cow milk protein allergy.[59] *Erysipelotrichaceae* had been reported to be more present in the intestines of organisms with higher inflammatory levels or metabolic disorders.[60] It was found in our study that 2′-FL inhibited the proliferation of *Muribaculaceae*, *Desulfovibrionaceae*, *Eggerthellaceae*, and *Erysipelotrichaceae* that were likely to induce overactive immune responses, which was aligned with Jiayuan Xu *et al.*[43] These findings emphasize the importance of homeostatic intestinal microecology in the positive regulation of alleviating FA. As described in the previous study, the gut microbiome-immune axis may serve as a target for nutrition-mediated modulation of FA.[8] Further, in our study, after 2′-FL entered the colon and reprogrammed the structure and composition of the intestinal microbes in allergic mice, the disrupted immune system was also found to be corrected.

Studies have shown that FA is related to the imbalance of differentiation of Th1 and Th2 cell subsets. Th2 exacerbates allergic reactions through IL-4, IL-5, and IgE[61] while Th1 alleviates allergic symptoms by activating target cells through IFN-γ, inhibiting IgE secretion and eosinophilic granulocyte production. Additionally, Treg cells are also involved in the development of FA, and the absence of Foxp3 + Treg may lead to immune tolerance disorders, autoimmunity, and allergic reactions. Yu Wang *et al.* found that L-arabinose alleviates gliadin-sensitized FA by balancing the ratio between Th1 and Th2 cells and upregulating Treg response in mice.[62] Shigeru Katayama *et al.* demonstrated that rutinosylated ferulic acid played an effective immunomodulatory role in allergy by upregulating regulatory T cells.[63] Kim WG *et al.* also illustrated that *Bifidobacterium longum* IM55 and *Lactobacillus plantarum* IM76 relieved allergic rhinitis in mice by reversing Th2/Treg imbalance.[64] Evidence has shown that 2′-FL acted through glycan receptors *in vitro* and *in vivo* to directly regulate immune cell function.[34,65] In this study, as expected, OVA sensitized mice showed obvious Th2/Th1 imbalances in PPs and spleens compared with the results of the CON group, corresponding with reference, but 2′-FL effectively alleviated these imbalances by promoting the Th1 type immune response. The key transcription factors and related immune factors were also determined in the spleen and colon of mice to validate the results. The levels of IL-4 and IL-5 in the 2′FL group were decreased, while the levels of IFN-γ and TNF-β were increased, and 2′-FL played a role in the regulation of the endogenous transcription factors GATA-3 and T-bet. 2′-FL also had a significant effect on

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Treg cells. The treatment of 2′-FL relieved the decrease of CD4 + CD25 + Foxp3 + Treg in FA mice, and up-regulated the expression of Foxp3 and TGF-β. Furthermore, immunosuppressed Tregs can maintain immune homeostasis in the body by regulating Th2- and Th1-type responses. The results acquired in the correlation analysis were consistent with the differential bacteria and T-cell differentiation observed in the three groups of mice. These specific strains improved the intestinal barrier function of food-allergic mice by significantly affecting TJ protein expression and metabolites, thereby inhibiting intestinal leakage of food allergens. We also noted that gut microbe biomarkers were mainly associated with key transcription factors and related cytokines of Th2 rather than Th1 cells. Nevertheless, the results of our study only proved the correlations between gut microbes and the allergic indicators mentioned above, while the causation is yet to be verified. These consequences suggested that 2′-FL may alleviate food allergy symptoms by reversing the Th2/Th1 imbalance, especially decreasing the Th2-type immunoreaction, and enhancing Treg immune response in OVA-sensitized mice.

5. Conclusions

In conclusion, our study suggested that 2′-FL effectively improved intestinal microecology and inhibited the abnormal immune response caused by allergen OVA by regulating the gut microbiota in FA mice. After 2′-FL intervention, the immune marker levels in the sensitized mouse serum, such as OVA-sIgE, HIS, and Mcpt-1, were recovered, and mast cell degranulation was effectively suppressed to release the anaphylactic symptoms. After 2′-FL reached the intestine, on the one hand, it improved the intestinal barrier function by up-regulating the expression of mucoproteins, reduced the low-grade inflammation caused by FA, produced more beneficial metabolites, such as SCFAs, and improved the intestinal microecology, as well as building a healthy and stable intestinal environment. On the other hand, 2′-FL significantly reversed the Th2/Th1 imbalance and the attenuation of Treg cell number caused by allergenic OVA, thus ameliorating the impaired immune system. Judging from the conclusions above, 2′-FL may play a profound role in the prevention and protection of allergic diseases as a novel prebiotic.

Abbreviations

FA	Food allergy
HMOs	Human milk oligosaccharides
2′-FL	2′-Fucosyllactose
GRAS	Generally recognized as safe
DCs	Dendritic cells
OVA	Ovalbumin
OVA-sIgE	OVA-specific immunoglobulin E
HIS	Histamine
Mcpt-1	Mast cell protease-1
H&E	Hematoxylin–eosin
PAS	Periodic acid-Schiff
SCFAs	Short-chain fatty acids
PCoA	Principal coordinate analysis
qRT-PCR	Real-time quantitative polymerase chain reaction
MLN	Mesenteric lymph node
PP	Peyer's patch
TJ	Tight junction
RegIII-γ	Regenerating islet-derived protein III-gamma
IL-4	Interleukin-4
IFN-γ	Interferon γ
TNF-α	Tumor necrosis factor-α
TGF-β	Transforming growth factor β
GPR-41	G protein-coupled receptor
TLR4	Toll-like receptor 4
Th	T helper
Treg	Regulatory T

Author contributions

Ruixin Kou: conducted experiments, collected, analyzed the data, and drafted the manuscript. Jin Wang: conceptualization, analyzed the data, and funding acquisition. Ang Li: conducted flow cytometry experiments. Yuanyifei Wang: methodology and conducted q-PCR experiments. Dancai Fan: review editing, supervision, and validation. Bowei Zhang: review editing, supervision, and validation. Wenhui Fu: Conceptualization and review editing. Jingmin Liu: software and review editing. Hanyue Fu: methodology and investigation. Shuo Wang: review editing, supervision, validation, and funding acquisition.

Conflicts of interest

There are no conflicts to declare.

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