

Untargeted Metabolomic Profiling Reveals Changes in Gut Microbiota and Mechanisms of Its Regulation of Allergy in OVA-Sensitive BALB/c Mice

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ABSTRACT: Gut microbiota plays an important role in the regulation of food allergy. However, the interactions between the gut flora and immune system are not well studied. Here, we obtained ovalbumin (OVA)-sensitive BALB/c mice, combined with serum untargeted metabolomics to investigate the mechanisms of the interactions. The serum metabolomics results showed that 17 serum metabolites were downregulated, enriched in the aminoacyl-tRNA biosynthesis pathway, whereas indole-3-propionic acid (IPA) was increased. Six operational taxonomic units (OTUs) at the family level were altered and correlated with immune endpoints. Combined metabolomic and microbiomic analyses revealed that IPA levels were correlated with differential bacterial OTUs and a positive correlation with Treg in splenic lymphocytes. These results suggest that the regulatory effects of intestinal flora on allergic responses may be achieved by metabolizing tryptophan to produce indole derivatives and the aminoacyl-tRNA biosynthesis pathway. The formation of OVA tolerance in mice may be related to the enrichment of *Peptostreptococcaceae*, *Ruminococcaceae*, and *Lactobacillaceae*.

KEYWORDS: food allergy, ovalbumin, gut microbiota, metabolomics, metabolites, *Lactobacillaceae*, *Clostridium*, aminoacyl-tRNA biosynthesis, indole-3-propionic acid

INTRODUCTION

Food allergy has gradually become one of the major problems threatening human health. Patients with food allergies may develop hives, diarrhea, and other symptoms with only minimal exposure to the allergen and may die in severe cases.¹ There has been a significant increase in the incidence of food allergies worldwide in recent years.^{2,3} Food allergy is a complex disease caused by the interactions of genetic, epigenetic, and environmental influences, and the mechanisms of which have not been well studied.⁴ Eggs are recognized as one of the eight most common allergy-causing foods, and OVA is a major allergen of egg whites, often causing allergic reactions in infants and children that persist into adolescence.^{5–7} Existing studies indicate that food allergies share a common type 2 immune response mechanism,⁸ but the diversity of the clinical symptoms presented by food allergies suggests that additional pathogenic processes are engaged in the formation of their heterogeneous phenotypes. Unfortunately, we know very little about the immune mechanisms behind specific food allergic reactions. Several studies have demonstrated a correlation between the development of food allergies and the changes in the intestinal flora,^{9,10} which in turn affects the body's tolerance to food allergies.^{11–13} For example, *Clostridium*-containing microbiota can significantly increase interleukin-22 (IL-22) production by innate lymphocytes, which enhances the epithelial barrier, reduces intestinal permeability to dietary antigens, and limits the entry of allergens into the circulatory system.¹⁴ *Lactobacillus plantarum* IM76 and *Bifidobacterium longum* IM55 can attenuate OVA-induced allergic rhinitis by restoring intestinal microbial

disorders and regulating type helper 2 cell/Treg (Th2/Treg) imbalance in mice.¹⁵ The effect of gut microbiota on the immune system may be related to its ability to produce specific metabolites.¹⁶

The ability of untargeted metabolomics to detect alterations induced by environmental and genetic factors makes it particularly useful for unraveling disease mechanisms in patients with complex atopic diseases, thereby helping to study the profound immune responses that occur when the intestinal flora affects the immune system. Some studies have used metabolomics in the study of inhalant allergic diseases and have identified some of the metabolic signatures associated with asthma severity and endotype.^{17,18} By contrast, only a few studies have used untargeted metabolomics for the study of food allergies. Crestani *et al.*¹⁹ found that, by using serum untargeted metabolomics, children with food allergies exhibited specific metabolomic features, including lower levels of ceramide and sphingomyelin, which may reflect the interactions between the intestinal immune system and the altered gut flora.

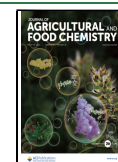
The integration of gut microbiomics with metabolomics has promoted the study of disease mechanisms, and it has been

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applied in the study of some diseases.^{20–22} For example, Olson *et al.*²³ used multiomics techniques, including gut microbiomics and serum metabolomics, to uncover that the gut flora mediates the antiepileptic effects of a ketogenic diet through the inhibition of γ -glutamylolation and to identify several strains associated with epilepsy protection, providing evidence for the development of gut microbial-based epilepsy therapies. Furthermore, urine metabolomics combined with the analysis of the intestinal flora revealed that *Panax ginseng* ameliorated the symptoms associated with androgen deficiency in rats by modulating steroid hormone biosynthesis, bile acid biosynthesis, and tryptophan metabolism.²⁴ Multiomics techniques can also help to explore the mechanisms by which the intestinal flora affects the physiological state of the organism. Lai *et al.*²⁵ utilized high-coverage metabolomics of blood specimens, brain tissues, and fecal specimens to present a comprehensive biochemical landscape of intestinal flora-driven inter-organ material transport and brain-gut axis communication in mice, thereby laying the groundwork for the future investigation of microbial effects on host metabolism and gut-brain communication. However, only a few studies have used multiomics to study the immune system. Wu *et al.*²⁶ evaluated the mitigating effects of lauric acid on LPS-challenged broilers in combination with the gut microbiome and the serum metabolome and found significant correlations between the serum metabolome, gut flora, and immune system. There are few studies combining the gut microbiome with the serum metabolome in the investigation of food allergies.

The aim of this study was to investigate the relationship between the development of food allergy and the altered composition of the gut flora in OVA-allergic mice in conjunction with serum untargeted metabolomics. The OVA-sensitive mice were obtained by multiple gavage administrations and intraperitoneal injections of OVA. Spleens were collected for *in vitro* immune analysis. Blood was used to detect the immune indicators and untargeted metabolome. Feces were collected to detect the gut flora by 16S rDNA sequencing. We conducted univariate and multifactorial analyses of the experimental results, analyzed the correlations among the indicators, and discussed them comprehensively with the results reported in previous studies to enhance the understanding of the relationship between the intestinal flora and food allergy. Our results provide new insights on the potential mechanisms by which the gut flora regulates the immune system in allergic individuals and help to establish a gut flora-based approach to food allergy intervention.

MATERIALS AND METHODS

Reagents. OVA and bull serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The HRP-labeled goat anti-mouse immunoglobulin G (IgG) antibody was purchased from Beyotime Institute of Biotechnology (Jiangsu, China), and a mouse OVA specific immunoglobulin E (OVA sIgE) ELISA kit was purchased from Cusabio Biotech Co., Ltd. (Wuhan, China). A mouse histamine (HIS) ELISA kit and mouse monocyte chemoattractant protein-1 (mMCP-1) ELISA kit were procured from Elabscience Biotechnology Co., Ltd. (Hubei, China). Mouse IL-4, IL-5, and interferon- γ (IFN- γ) high sensitivity ELISA kits were obtained from MultiSciences (Lianke) Biotech Co., Ltd. (Hangzhou, China). The RPMI 1640 medium and fetal bovine serum were purchased from Gibco (Gaithersburg, MD, USA). FITC rat anti-mouse CD4, APC rat anti-mouse CD25, PE rat anti-mouse Foxp3, PE rat anti-mouse IFN- γ , APC rat anti-mouse IL-4, and purified rat anti-mouse CD16/CD32 were acquired from BD Biosciences (Franklin Lakes,

NJ, USA). The transcription factor buffer set, staining buffer, anti-rat Ig, and κ /negative control compensation particles were also purchased from BD Biosciences. All reagents used in this study were in analytical grade.

Animals. BALB/c mice (SPF grade, female, aged 4–6 weeks, weight of 18–22 g) were obtained from Beijing VitalRiver Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were housed in a controlled environment (humidity of $55 \pm 5\%$ and temperature of $25 \pm 1^\circ\text{C}$) on 12 h light/12 h dark cycles and allowed to drink and eat *ad libitum*. All experimental procedures were approved by the Animal Care and Use Committee of the Zhejiang Academy of the Center of Safety Evaluation, the Zhejiang Academy of Medical Sciences (No. KY-2020-009).

Establishment of the OVA-Sensitive Mice. Mouse sensitization was conducted according to the method by Sun *et al.*²⁷ with minor modifications. The mice were divided into two groups randomly ($n = 9$) and designated as the Tris-HCl group (control) and the OVA group (OVA). Sensitization was achieved in the OVA group by an intragastric administration of 250 mg/kg-bw OVA dissolved in 0.1 mol/L Tris-HCl (pH 7.4) on day 0, 7, 14, 21, and 28. Mice of the control group received an equal volume of 0.1 mol/L Tris-HCl (pH 7.4) by gavage at the same times. On day 35, the mice in the OVA group were challenged with a 4-fold protein dose of OVA by intraperitoneal injection, and the mice of the control group were injected intraperitoneally with an equivalent volume of Tris-HCl (Figure 1). The body weight of the mice during sensitization was

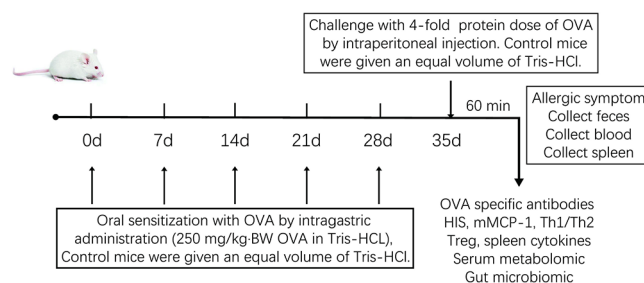


Figure 1. Experimental scheme of BALB/c mice sensitized weekly by intragastric gavage.

obtained. The mice were observed for 60 min after challenge, and the allergic symptoms were scored, according to Table 1.²⁸ Blood was

Table 1. Allergic Symptom Scoring

score	symptoms
0	no symptom
1	scratching mouth and nose
2	piloerection; reduced activity; swelling around the eyes and mouth; higher breathing rate
3	shortness of breath; blue rash around the mouth and tail
4	no activity after stimulation, shivering, and muscle contractions
5	death

gathered in serum separator tubes, and the serum was stored at -80°C until use. Feces was collected in sterile tubes. Mice were asphyxiated by CO_2 inhalation, and spleens were collected.

Detection of OVA sIgG, sIgE, HIS, and mMCP-1 in Serum. According to reported procedures,^{29,30} the sIgG level in sera was measured by indirect ELISA. Briefly, a 96-well plate was coated with OVA. BSA solution (1% BSA/PBS) was used as the blocking reagent. After washing with PBST (0.05% Tween 20/PBS), diluted serum (1:500 in PBST) was added and incubated for 90 min at 37°C . Mouse IgG was recognized by the HRP-labeled anti-IgG antibody, and TMB solution was used for color development. After the addition of 50 μL of sulfuric acid (2 mol/L), the absorbance of each well was detected at 450 nm. Details of the method for detecting OVA sIgG

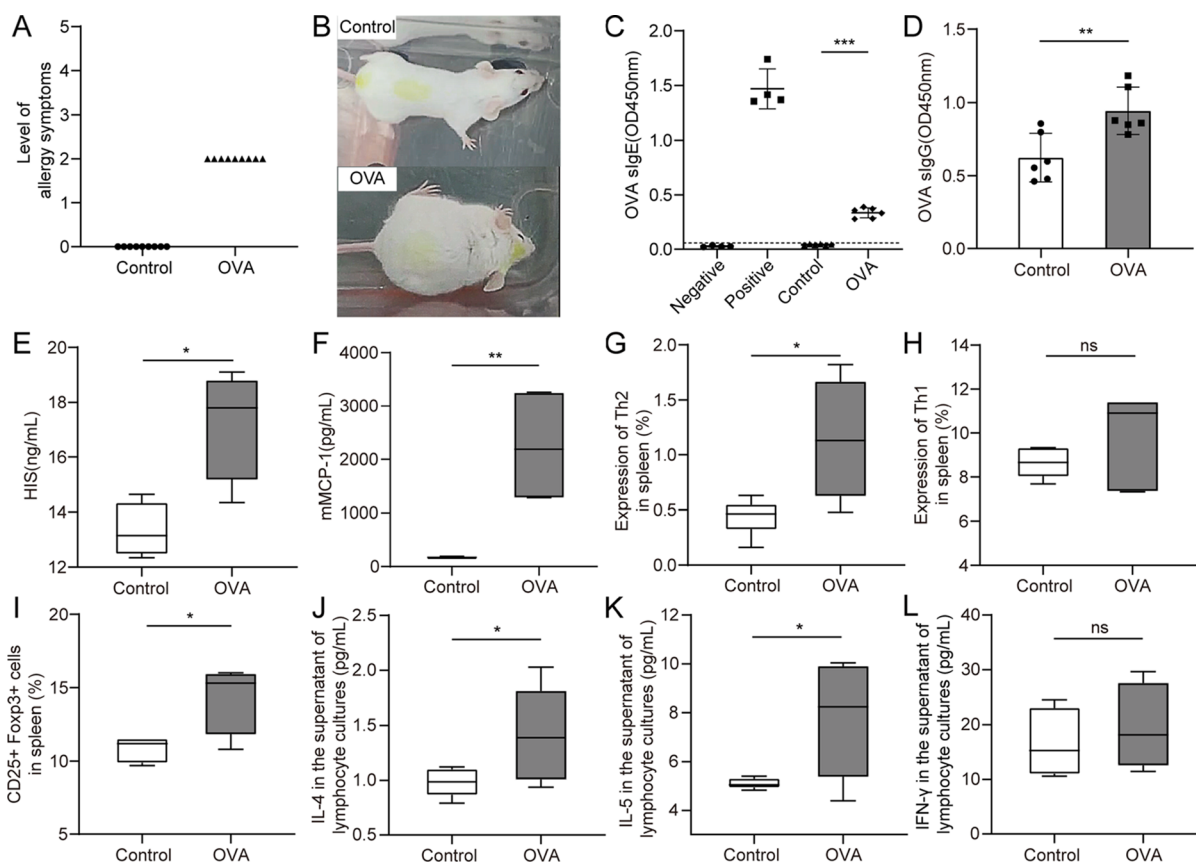


Figure 2. Allergic symptoms of mice and specific immunoglobulin levels, HIS levels, and MCP-1 levels in serum of mice. (A) Allergic symptom score ($n = 9$). (B) Piloerection occurred in OVA-sensitive mice. (C) OVA sIgE was detected by a commercial kit ($n = 6$). Negative and positive are the standard samples provided by the kit with a positive judgment criterion of $2.1 \times \text{OD}_{\text{Negative}}$ (dashed line). (D) OVA sIgG was detected by indirect ELISA ($n = 6$). (E) HIS and (F) mMCP-1 were detected by an enzyme immunoassay kit ($n = 9$). The percentage of Th1/Th2, Treg in mouse spleen lymphocytes, and levels of cytokines secreted by mouse spleen cells ($n = 9$). (G) Percentage of Th2. (H) Percentage of Th1. (I) Percentage of Treg. IL-4 (J), IL-5 (K), and IFN- γ (L) were quantified on supernatants using commercial kits ($n = 9$). ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$. ***: $p < 0.001$.

are provided in the [Supporting Information](#). The levels of OVA sIgE, HIS, and mMCP-1 in sera were determined by commercial ELISA kits.

Splenic Lymphocyte Analysis. *Splenic Lymphocyte Isolation and Cytokine Analysis.* Mouse spleens were aseptically isolated, ground in RPMI 1640, and filtered through a cell strainer. The supernatant was discarded by centrifugation, and the precipitate was resuspended by erythrocyte lysate and maintained on ice for 3 min. The supernatant was discarded by centrifugation, and the cell precipitate was washed two times with pre-chilled Hank's buffer. Cells were resuspended in RPMI 1640 (10% fetal bovine serum), and the cell concentration was adjusted to $1 \times 10^7/\text{mL}$. Splenocytes were seeded into 24-well culture plates so that each well contained 4×10^6 cells and subsequently stimulated with OVA and incubated for 24 h (37°C , 5% CO_2). The supernatant was collected, and the contents of IL-5, IL-4, and IFN- γ were measured using commercial ELISA kits.

Detection of Th1/Th2 Balance in Splenic Lymphocyte. Th1/Th2 cells were detected indirectly by measuring the expression levels of intracellular IFN- γ and IL-4. The FITC anti-mouse CD4 antibody was added to $100 \mu\text{L}$ of the splenic cell suspension ($1 \times 10^7/\text{mL}$), and the sample was maintained for 15 min in the dark at room temperature. The mixture was combined with fixative and rupture agents and maintained in the dark for 15 min. The supernatant was discarded by centrifugation. The PE anti-mouse IFN- γ antibody and APC anti-mouse IL-4 antibody were added and maintained for 15 min in the dark, and then, the supernatant was discarded by centrifugation. Resuspended in $500 \mu\text{L}$ of flow buffer, the cells were detected by flow cytometry (BD FACS Calibur, BD, USA). Th1 cells were identified

by $\text{CD4}^+\text{IL-4}^-\text{IFN-}\gamma^+$, while Th2 cells were identified by $\text{CD4}^+\text{IL-4}^+\text{IFN-}\gamma^-$.

Identification of Tregs. $\text{CD4}^+\text{Foxp3}^+\text{CD25}^+$ is often used as the specific marker to identify Tregs.^{31,32} The FITC anti-mouse CD4 antibody and APC anti-mouse CD25 antibody were added to $100 \mu\text{L}$ of the splenic cell suspension, and the sample was then kept in the dark for 15 min. The mixture was combined with fixative and rupture agents and incubated for 40 min in the dark. After adding permeation buffer, the sample was kept at room temperature for a few minutes, and the supernatant was dropped by centrifugation. Next, with the addition of the PE anti-mouse Foxp3 antibody, the sample was kept for 40 min at 4°C in the dark followed by washing two times. Resuspending in $100 \mu\text{L}$ of flow buffer, Tregs were analyzed by flow cytometry.

16S rDNA Amplicon Sequencing and Data Analysis. DNA was purified from fecal specimens using a ZymoBIOMICS DNA Microprep kit (Zymo Research, USA). The 16S rDNA (V4 region) of the DNA sample was amplified using specific primers (S15F, 5'-GTGYCAGCMGCCGCGGTAA-3'; 806R, 5'-GGAC-TACHVGGGTWTCTAAT-3'). The amplification products were mixed with 6-fold loading buffer followed by electrophoretic detection of the target fragments using a 2% agarose gel. The target bands were recovered using a Zymoclean Gel Recovery kit (Zymo Research), quantified by a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Foster City, CA, USA). Sequencing was implemented by the HiSeq Rapid SBS Kit v2 (500 cycles, Illumina, USA) with PE250 sequencing.

The double-ended sequences were stitched using FLASH 1.2.8 software. We separated the sequence of each sample from the raw

data based on the barcode and truncated the barcode sequence. Sequences were quality-filtered using QIIME 1.9.1 software to remove short and long sequences as well as sequences with ambiguous bases. OTU clustering was performed using Usearch software (<http://drive.com/uparse/>, version 10.0.240) based on the UPARSE algorithm at 97% consistency and annotated each sequence according to the SILVA database (<https://www.arb-silva.de/>) and UCLUST classification for analysis.

Serum Untargeted Metabolomics Analysis. *Metabolite Extraction and Analysis.* Metabolite extraction was implemented according to the method by Ji *et al.*³³ MSTFA (100 μ L, 1% trimethylchlorosilane, v/v) was added to each sample as the derivatization reagent, and the samples were incubated at 70 °C for 1 h. For the quality controls (QCs), 10 μ L of a fatty acid/methylester mixture (FAMES; C8-C16: 1 mg/mL; C18-C24: 0.5 mg/mL, chloroform) was added as the internal standards. Derivatized samples were used for gas chromatography–mass spectrometry (GC–MS) analysis. A Pegasus BT GC-TOF mass spectrometer (LECO, USA) was used to performed GC/time-of-flight MS (GC-TOF/MS) analysis, and a DB-5 MS column (internal diameter, 30 m $\times$ 0.25 mm $\times$ 0.25 μ m, Agilent, USA) was used. The injection volume was 1 μ L, and helium (purity, 99.995%) was utilized as the carrier gas. The specific parameters of the sample extraction method and GC–MS are described in the Supporting Information (Table S1).

Metabolite Profiling. The raw data were converted to “abf” format by Abf Converter software. MS DIAL with FiehnLib³⁴ was operated for coarse peak extraction, peak alignment, inverse fold analysis, and identification of the data.³⁵ The retention time index (RI) was applied for peak identification with a similarity threshold of 70% and a tolerance of 5000 for RI.

Statistical Analysis. The results are expressed as the mean $\pm$ standard deviation (SD). Statistical analysis, including the *t* test, Spearman's correlation analysis, and Pearson's correlation analysis, was implemented with GraphPad Prism 8.4.3 (GraphPad Software, California, USA). To correct for multiple comparisons, the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli post-hoc tests was used to control the false discovery rate (FDR). A *p*-value < 0.05 or FDR-corrected *p* (*q*) < 0.05 was considered statistically significant. Microbial analysis, including community composition analysis and diversity analysis, was operated by R 3.6.1 (the R Foundation for Statistical Computing, Vienna, Austria), and the linear discriminant analysis effect size (LefSe) analysis was implemented by the LefSe tool (<http://huttenhower.sph.harvard.edu/galaxy/>). The functional prediction results of the intestinal flora were obtained based on the microbial functional gene profile in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.genome.jp/kegg/>). Serum metabolomic data were analyzed after Pareto scaling and log transformation. Principal component analysis (PCA), latent structure orthogonal projection-discriminant analysis (OPLS-DA), and pathway enrichment analysis were implemented on the MetaboAnalyst 5.0 platform, a web-based metabolomics pathway analysis platform (<https://www.metaboanalyst.ca/>). The visualization was performed with R 3.6.1. The pathway map was analyzed with CytoScape and MetaMap (Boston, MA, USA).

■ RESULTS

OVA Gavage Induces Allergy in Mice. *Allergy Symptoms and Body Weight of Mice.* The sensitization process in Figure 1 shows the establishment of OVA-sensitive mice. Before and after sensitization, there was no significant difference in body weight between mice in the control group and OVA group (Figure S1). Within 60 min after intraperitoneal injection of OVA, the mice in the control group did not show any signs of sensitization, while the mice in the OVA group showed grade 2 allergic reactions (Figure 2A), specifically piloerection (Figure 2B) and a higher respiratory rate.

OVA *slgE* and *slgG* Levels in Sera. A specific antibody response is considered as one of the direct indicators for assessing allergen sensitization, and immunoglobulin levels in the serum are the most commonly used parameters to reflect the degree of a specific antibody response.³¹ The OVA IgE ELISA kit provides a negative control and a positive control and sets the criteria for a positive result as an OD_{450nm} value higher than 2.1 times of the OD_{Negative} value. According to the criteria, mice in the OVA group showed a significantly elevated level of OVA sIgE in the sera (*p* < 0.001) and were positive for OVA allergy (Figure 2C). The level of OVA sIgG of mice in the OVA group also appeared to be significantly elevated, compared to the control group (*p* < 0.01) (Figure 2D).

HIS and mMCP-1 Levels in Sera. HIS in the serum is mainly produced by mast cell or basophil degranulation, and it can reflect the severity of the allergic reaction.³⁶ When mice experienced allergic reactions, their serum HIS levels increased significantly. Compared with the control group (13.32 $\pm$ 0.97 ng/mL), the OVA group displayed a significant increase in the HIS content, reaching 17.25 $\pm$ 2.04 ng/mL (*p* < 0.05) (Figure 2E). mMCP-1 is a marker of increased intestinal permeability and mucosal mast cell proliferation and a serum marker of mast cell activation in mice with food allergies.^{28,37} The mMCP-1 content in allergic mice was consistent with the HIS level. Serum MCP-1 levels in the OVA group (2242.2 $\pm$ 881.3 pg/mL) were significantly anabolic, compared to those in the control group (164.7 $\pm$ 24.2 pg/mL, *p* < 0.01) (Figure 2F).

Phenotyping of T-Cell Populations in Spleens. Th1 and Th2 cells inhibit each other functionally. When mice suffer from allergic reactions, Th2 cells overact, while Th1 cells are suppressed. In this study, the percentage of Th2 cells in spleen lymphocytes of mice in the OVA group (1.14 $\pm$ 0.55%) exhibited a significant increase compared to that in the control group (0.44 $\pm$ 0.16%, *p* < 0.05) (Figure 2G), but there was no significant disparity in the expression of Th1 cells (Figure 2H). Tregs are usually identified by Foxp3⁺CD25⁺ in CD4⁺ cells. In allergic individuals, Tregs play a vital role in preventing the development of autoimmune diseases and maintaining immune tolerance. They also have a role in maintaining the Th1/Th2 differentiation balance. In this study, the percentage of Treg differentiation was significantly higher in the OVA group (14.4 $\pm$ 2.4%) than in the control group (10.8 $\pm$ 0.8%, *p* < 0.05) (Figure 2I). The density plots obtained by flow cytometry are shown in Figure S2.

Splenocyte Cytokine Production in Response to OVA. The restimulation of mouse splenocytes with OVA and the analysis of cytokine production can confirm allergen specificity and verify Th1 or Th2 cell response types.³¹ IL-4 and IL-5 are mainly secreted by Th2 cells and promote allergic inflammation, whereas IFN- γ is mainly produced by Th1 cells and inhibits Th2 cells.³⁸ The levels of these cytokines are generally positively related to the abundance of the corresponding cells. The secretion of IL-4 and IL-5 in splenocytes of mice in the OVA group (1.42 $\pm$ 0.41 and 7.79 $\pm$ 2.39 pg/mL) showed a significant increase compared with that in the control group (0.97 $\pm$ 0.12 and 5.10 $\pm$ 0.21 pg/mL, *p* < 0.05) under the same OVA stimulation (Figure 2J,K). There was no significant disparity in the secretion levels of IFN- γ in all groups of cells (Figure 2L). The secretion of IL-5, IL-4, and IFN- γ was consistent with the Th1/Th2 differentiation balance.

Significant Alteration of Gut Microbiota in OVA Group Mice. *Changes in Diversity and Compositional Signatures after OVA Sensitization.* The flora in the feces of

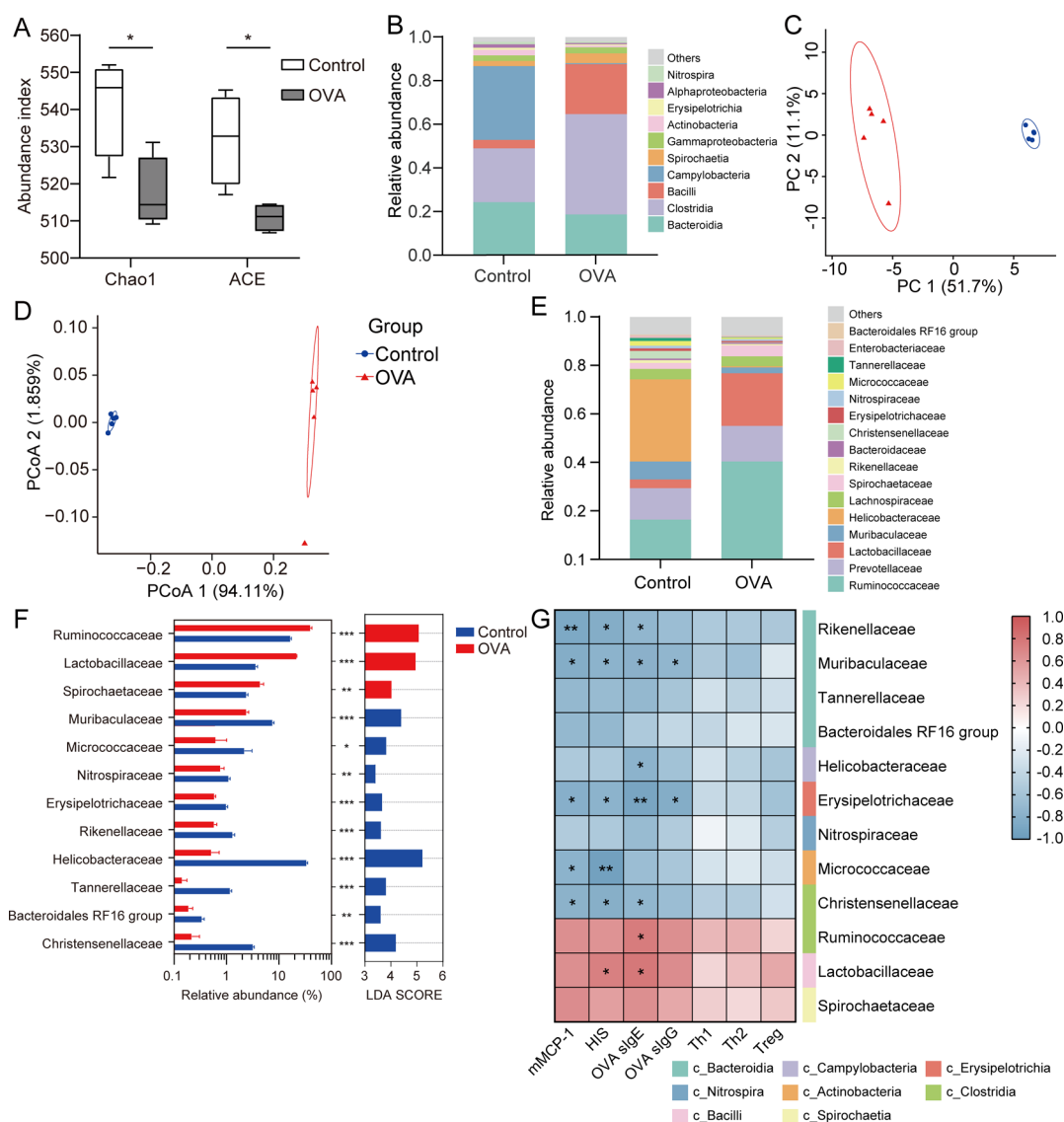


Figure 3. (A) Analysis of gut microbial α -diversity. (B) Changes and difference in microbiota composition at class levels. PCA (C) and PCoA (D) reflect the beta diversity between the intestinal flora of control mice and OVA-sensitive mice. Changes in the composition of the microbiota at the family level (E) and the relative abundance and LDA of significantly different OTUs at the family level (F). Spearman's correlation heatmap (G) between differentiated bacterial OTUs at the family level and immune endpoints. Red represents a positive correlation, and blue represents a negative correlation. The class of OTUs is labeled by side. $n = 5$ mice per group; *: $p < 0.05$, $q < 0.05$; **: $p < 0.01$, $q < 0.01$; ***: $p < 0.001$, $q < 0.001$.

the control and OVA groups of mice were analyzed. As shown in Figure 3, the intestinal flora of the two groups of mice differed significantly in strain diversity (α -diversity) and composition. The α -diversity of the intestinal flora in OVA-sensitive mice showed a significant decrease according to Chao1, ACE, and Shannon indices (Figure 3A and Figure S3A). The relative abundance of a variety of bacteria in the intestinal microbiota of the OVA group of mice differed at the class level (Figure 3B), with *Bacilli*, *Clostridia*, and *Campylobacteria* being the most pronounced ($p < 0.001$, $q < 0.001$). To verify that the amount of sequencing data was reasonable, the rarefaction curve was plotted, as shown in Figure S3B. The flattening of the curve indicated that our sequencing depth largely covered all species in the samples.

After further analyzing the composition at the family level, multivariate cluster analysis based on bacterial OTUs levels implied that OVA immunization strongly affected the

composition of the gut flora in mice (Figure 3C, PCA). PC1 and PC2 explained 51.7 and 11.1% of the total variation, respectively. The microbiomes in control and OVA-sensitive mice were significantly separated along PC1. PCoA was performed according to Bray–Curtis dissimilarity and calculated based on the abundance of OTUs, which showed significant disparities between the intestinal flora of the two groups (Figure 3D).

Particular Bacterial OTUs Associated with OVA Sensitization. To investigate the OTUs significantly associated with OVA allergy in mice, we analyzed the compositional distribution of the intestinal flora in the two groups at the family level (Figure 3E) and screened 12 differential OTUs with relative abundance $>0.1\%$, LDA score >3.0 , and $q < 0.05$ (Figure 3F). An analysis of Spearman's correlation coefficient between the abundance of these 12 OTUs and immune endpoints was carried out (Figure 3G). *Muribaculaceae* and

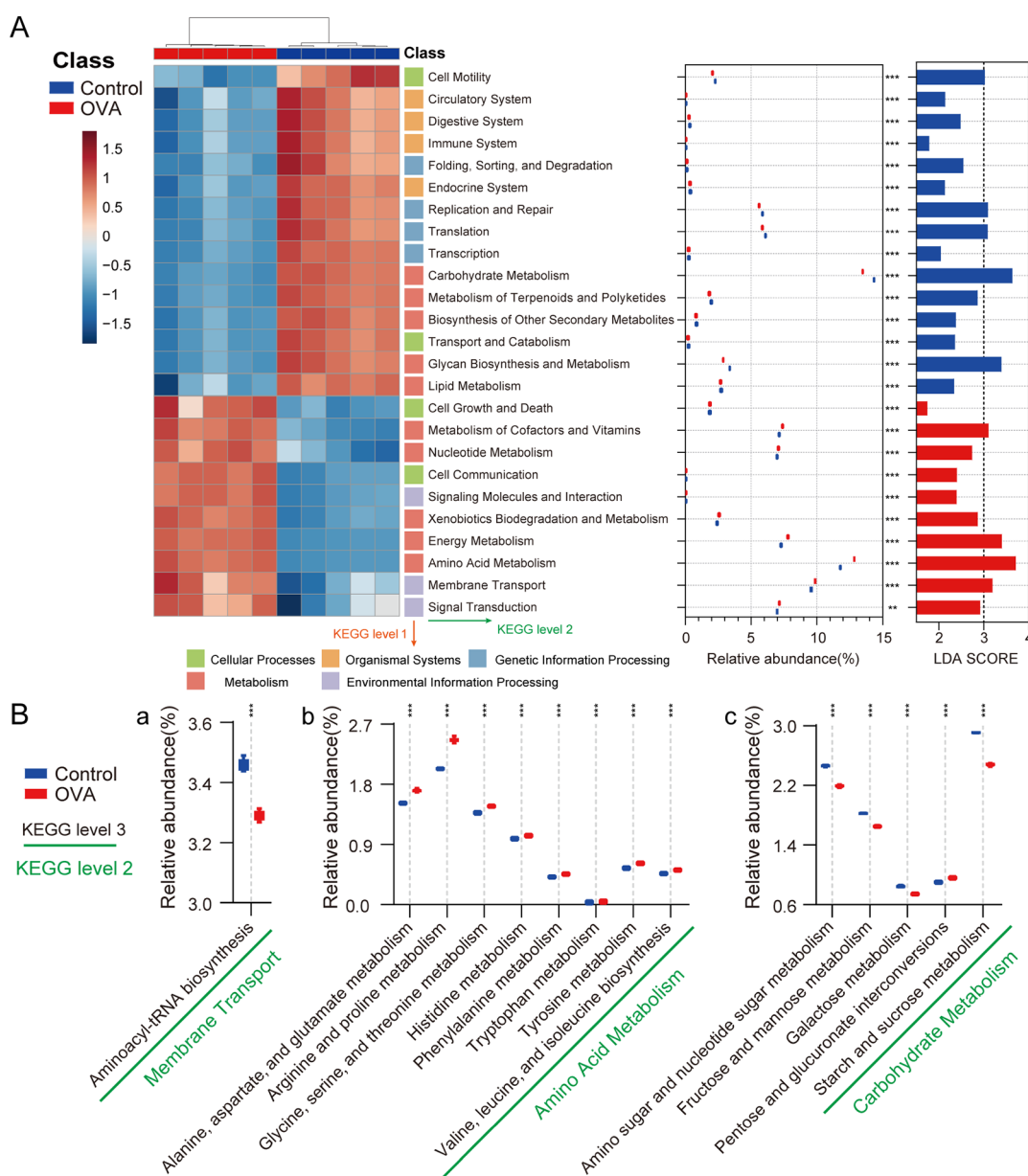


Figure 4. Changes in the predicted intestinal flora function in OVA-sensitive mice. (A) Heat map of Pearson's hierarchical clustering obtained from the selected pathways (level 2) based on the t test at the 95% level. The corresponding pathways at level 1 are labeled on the left. The relative abundance comparisons and LDA scores of these pathways between the control group (blue) and OVA group (red) are shown on the right. (B) The presence of differential functional pathways at level 3 screened with LDA score > 3.0 (level 2), LDA score > 2.5 (level 3), and $q < 0.05$, with the corresponding level 2 pathways labeled in green. $n = 5$ mice per group; **, $q < 0.01$; ***, $q < 0.001$.

Erysipelotrichaceae had a negative correlation with four immune endpoints (OVA sIgE, sIgG, mMCP-1, and HIS); *Rikenellaceae* and *Christensenellaceae* were significantly and negatively correlated with three major immune endpoints (OVA sIgE, mMCP-1, and HIS); *Micrococcaceae* was significantly and negatively correlated with mMCP-1 and HIS in serum, while *Lactobacillaceae* was significantly and positively correlated with HIS and OVA sIgE. *Helicobacteraceae* and *Ruminococcaceae* were significantly correlated with OVA sIgE levels. Among these, *Muribaculaceae* and *Rikenellaceae* belong to the *Bacteroidia* class; *Christensenellaceae* and *Ruminococcaceae* belong to the *Clostridia* class.

We further analyzed the differences in the intestinal flora between the two groups of mice at the genus level. Using LDA > 2.0 and $q < 0.05$ as criteria, 44 OTUs were screened for

significant differences at the genus level (Figure S3C), of which 19 belonged to the 12 groups mentioned above, including 10 belonging to *Ruminococcaceae*, 2 belonging to *Rikenellaceae*, and 2 belonging to *Erysipelotrichaceae*. In addition, four OTUs belong to *Lachnospiraceae*. Spearman's correlation analysis of the abundance of the 44 OTUs with immune indicators showed that eight OTUs belonging to *Rikenellaceae* were significantly correlated with several immune indicators (Figure S4, $p < 0.05$). At the class level, 21 of these 44 species belonged to the *Clostridia* class, and 18 of them were significantly correlated with immune indicators.

Predicted Functional Changes in Microbiota. To explore the effect of the altered gut flora on metabolism in OVA-sensitive mice, we compared the sequencing results with the KEGG database to analyze the key pathways. In OVA-sensitive

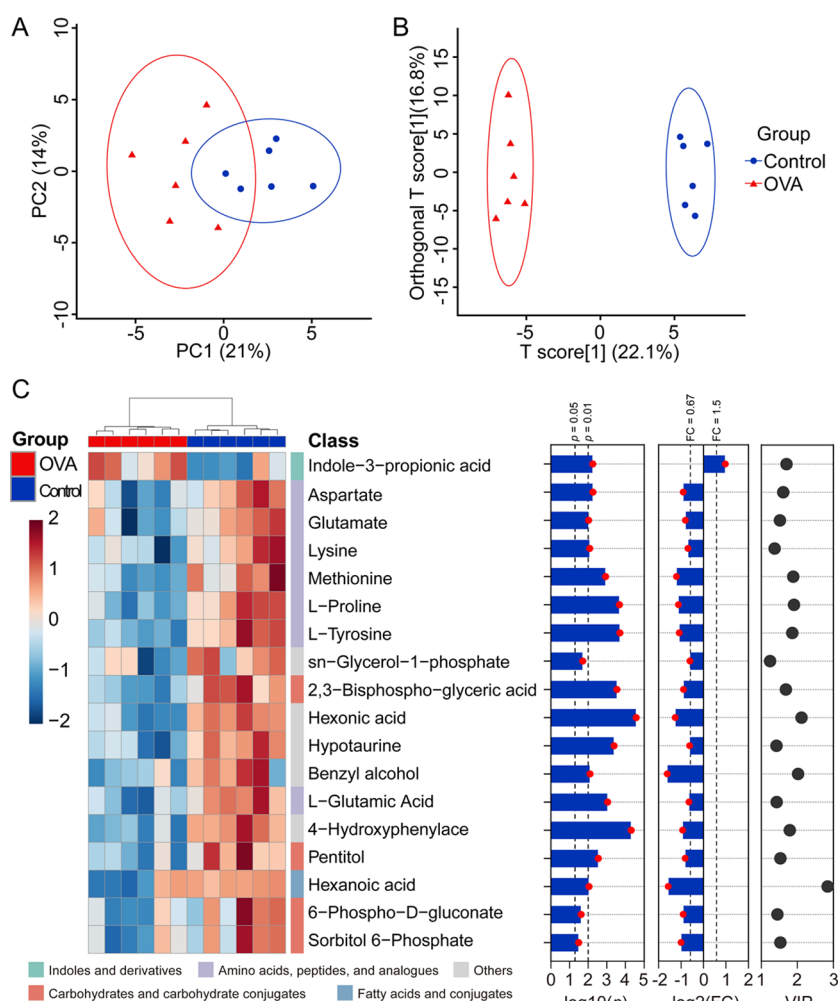


Figure 5. (A) Unsupervised PCA presents the primary source of metabolite variability to monitor alterations in serum metabolites ($n = 6$). (B) Supervised PCA analysis. OPLS-DA shows differences in serum metabolism groups between control mice and OVA-sensitive mice ($n = 6$). (C) Heat map obtained by Pearson's hierarchical clustering of metabolites selected based on fold change values >1.5 , VIP >1 , and t test at the 95% level. The type of metabolite is marked on the side.

mice, the functional abundance of the three levels classified according to the KEGG database was significantly altered. Figure 4A shows that, compared to the control group, 25 pathways of level 2 were altered in the OVA group ($p < 0.05$, $q < 0.05$) and mainly concentrated in metabolism (level 1). The LDA score for each level 2 pathway showed that the major altered pathways were amino acid metabolism, membrane transport, energy metabolism, carbohydrate metabolism, translation, glycan biosynthesis and metabolism, cell motility, metabolism of cofactors and vitamins, and replication and repair (LDA score > 3.0). Using LDA >2.5 as the criterion, we further analyzed these level 2 pathways and screened out the significantly altered level 3 pathways among them ($p < 0.05$, $q < 0.05$), as shown in Figure 4B and Figure S5. In the gut flora of OVA-sensitive mice, the metabolic capacity of multiple amino acids was increased [Figure 4B-(b), $p < 0.001$], while the capacity of aminoacyl-tRNA biosynthesis was decreased [Figure 4B-(a), $p < 0.001$, $q < 0.001$, LDA score > 3.0]. In addition, the metabolic capacity of several carbohydrates was reduced [Figure 4B-(c), $p < 0.001$, $q < 0.001$], except for pentose and glucuronide ($p < 0.001$, $q < 0.001$).

Significant Alteration of Serum Metabolome in OVA Group Mice. Global Metabolomic Profiling.

A total of 269

metabolites were identified and quantified by GC-TOF/MS analysis. After removing duplicate metabolites and xenometabolites, 175 metabolites were used for subsequent statistical analysis (Table S2). PCA showed significant inconsistencies between the serum metabolic profiles of mice in the OVA group and the control group, and the score plots are shown in Figure 5A. The OPLS-DA model also showed significant clustering between the control and OVA groups (Figure 5B), with good ability to distinguish between the different serum samples ($R^2X = 0.221$, $R^2Y = 0.934$, $Q^2 = 0.756$). Permutations tests with 100 repetitions were implemented to avoid overfitting, demonstrating that the established OPLS-DA model could accurately distinguish between the two groups of mice (Figure S6). A total of 18 differential metabolites with a $p < 0.05$, fold change (FC) >1.5 , and variable importance of the projection values (VIP) >1 are presented in Figure 5C, which mainly included seven each of amino acids, peptides, and analogues, four each of carbohydrates and carbohydrate conjugates, and one each of indole and derivative. To better show the differences between the two groups of metabolites, the 18 differential metabolites were selected for hierarchical Pearson's clustering (Figure 5C). Among these serum metabolites, IPA was upregulated, and the levels of the

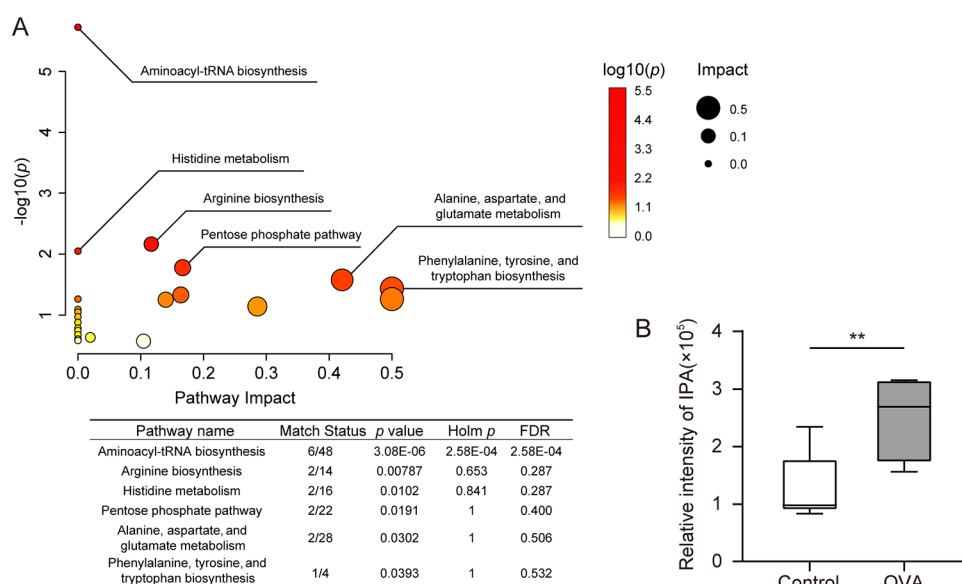


Figure 6. (A) Pathway analysis of serum metabolites in OVA-sensitive mice. The color (varying from yellow to red) means that the metabolites are in the data with different levels of significance. The dot size represents the pathway impact. (B) IPA levels in serum of mice. **: $p < 0.01$.

remaining 17 metabolites were decreased in the OVA group. Hierarchical Pearson's clustering analysis showed significant disparities between the control and OVA groups consistent with the results of PCA and OPLS-DA.

Pathway Analysis. The MetaMapp tool was applied to reflect the relationships between the metabolites. In this study, metabolic pathway analysis of OVA-sensitive mice was generated by MetaMapp and plotted by Cytoscape based on the functional groups and chemical structures in the PubChem classification (Figure S7A). Aminoacyl-tRNA biosynthesis was the prominent pathway affected in the OVA-sensitive mice (Figure 6A). Six metabolites in this pathway showed a decrease in OVA-sensitive mice, involving aspartate, methionine, lysine, L-tyrosine, L-proline, and glutamate (Figure S7B). In addition, the concentration of IPA in the sera of OVA-sensitive mice showed a significant increase (Figure 6B).

Correlation of Serum Metabolites with Particular Bacterial OTUs. To investigate the possible pathway of the reciprocity between the intestinal flora and immunity system, we analyzed Pearson's correlation coefficient between differential metabolites in the serum and differential OTUs at the family level and genus level, as shown in Figure 7A and Figure S8. A highly significant correlation was presented between the IPA level and the 11 differential OTUs ($p < 0.01$), and 4-hydroxyphenylacetic acid, hexonic acid, and benzyl alcohol were significantly associated with the 12 differential OTUs ($p < 0.05$). IPA was also significantly associated with all OTUs that differed at the genus level ($p < 0.05$). Pearson's correlation between the metabolome and immune indexes was also analyzed (Figure 7A). IPA and hexonic acid were significantly correlated with each immune index ($p < 0.05$). Among them, IPA levels had a significant positive correlation with Tregs ($R = 0.913$, $p < 0.01$). Some studies have reported that IPA affects Treg differentiation and is mainly produced by *Clostridium*.^{16,39} We further analyzed Pearson's correlation between the IPA level and the abundance of each family attributed to the *Clostridium* class. IPA levels were significantly and positively related to the abundance of the *Clostridium* class (Figure 7B), but differences emerged in the correlation with the abundance of each taxon at the family level (Figure 7C). Among them, the

more prominent ones were positively correlated with *Ruminococcaceae*, *Peptostreptococcaceae*, and *Clostridiaceae* 1 ($p < 0.01$) and negatively correlated with *Christensenellaceae*, *Family XIII* ($p < 0.01$).

DISCUSSION

We report that, in BALB/c female mice, excitation with OVA by multiple gavage administrations and intraperitoneal injections could obtain OVA-sensitive mice and significantly alter the serum metabolome and intestinal flora in the feces of the mice. Allergic symptoms and levels of OVA sIgE, OVA sIgG, HIS, and mMCP-1 can directly reflect the degree of the allergic response in mice. After intraperitoneal injection of OVA, the mice in the OVA group showed mild allergic symptoms such as piloerection and rapid breathing within 60 min, the serum OVA sIgE, sIgG, HIS, and mMCP-1 levels showed significant increases, and the percentage of Th2 cells that differentiated in the splenic lymphocytes increased, which showed that the mice of the OVA group had a Th2-type allergic reaction. However, the percentage of Tregs in splenic lymphocytes of mice in the OVA group appeared to be elevated. Tregs have a vital role in promoting immune tolerance.³² Wakabayashi *et al.*⁴⁰ reported that the repeated oral administration of OVA was necessary to induce OVA immune tolerance in mice, whereas intraperitoneal and intravenous injections of OVA exacerbated the allergic reactions. Therefore, we speculate that the OVA-sensitive BALB/c mouse could be obtained by gavage of OVA and single high-dose intraperitoneal injection of OVA, but repeated oral administration of OVA can induce OVA immune tolerance in mice.

The gavage of OVA may affect the sensitivity of mice to OVA by altering the intestinal flora. The results of 16S rDNA sequencing of the gut flora showed that OVA-allergic mice had a distinctive intestinal flora composition compared with healthy mice. The control mice had a higher α -diversity of gut flora than the OVA group. In the allergic population and individual mice, the occurrence of food allergy is often accompanied by a decrease in the intestinal flora α -diversity.⁴¹

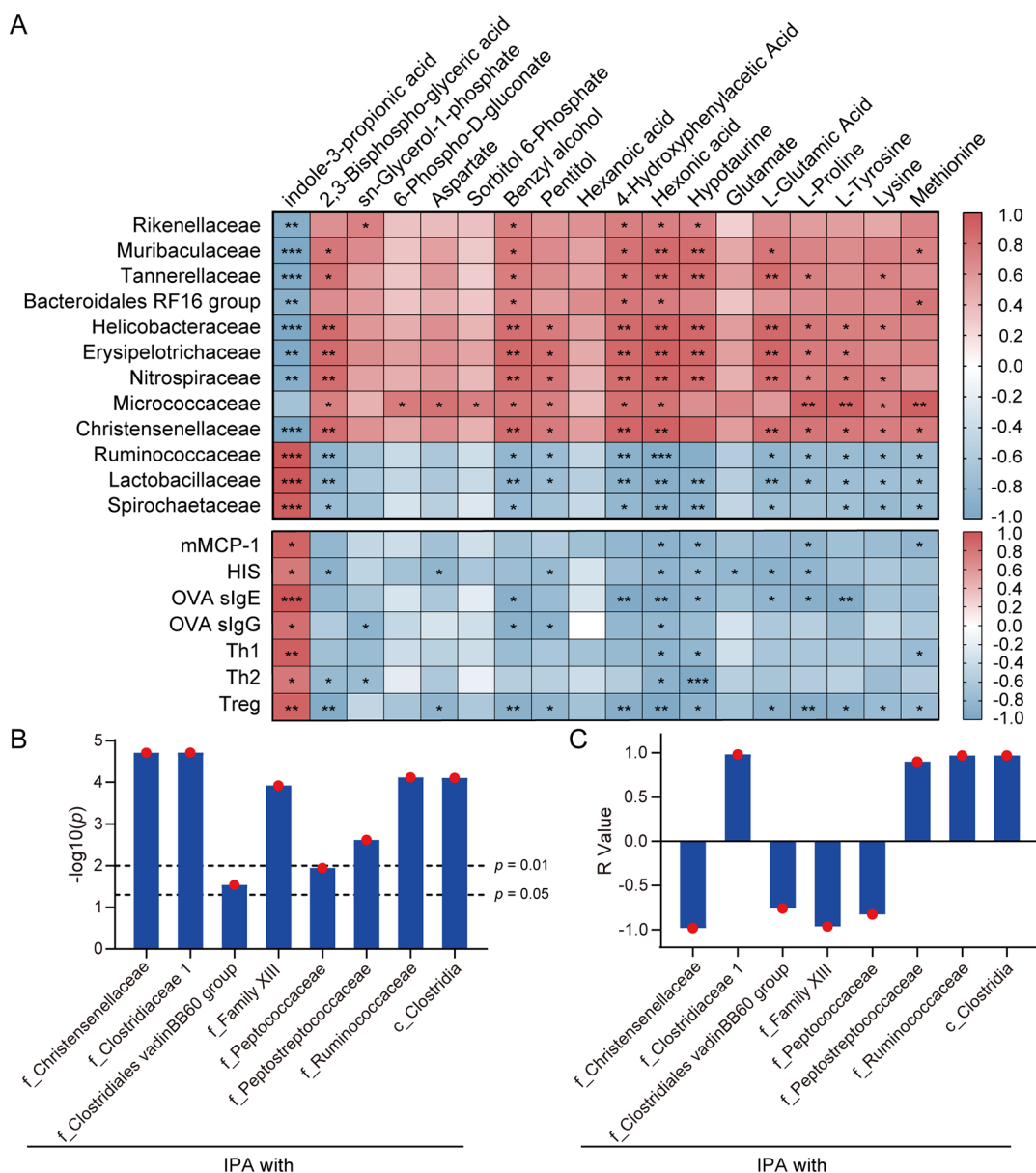


Figure 7. Heat map (A) of Pearson's correlation between differential serum metabolites and differentiated bacterial OTUs at the family level and Pearson's correlation between differential serum metabolites and immune endpoints. Red represents a positive correlation, and blue represents a negative correlation. Pearson's correlation analysis of IPA levels with the abundance of *Clostridium* and the abundance of OTUs at the family level belonging to the *Clostridium* class. (B) $-\log_{10}(\text{Pearson } p)$. (C) Pearson R . *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Allergic individuals showed significant changes in the abundance of several characteristic flora in their gut, and these flora have the potential to become therapeutic targets. For instance, Yao *et al.*⁴² demonstrated that the relative abundance of *Parabacteroides* and *Rikenellaceae_RC9_gut_group* in the intestinal flora of mice with OVA-induced allergic diarrhea was significantly reduced. The oral administration of alginate resulted in a trend of recovery in the abundance of these bacteria and alleviated the symptoms of allergic diarrhea in mice. Rivas *et al.*¹³ demonstrated that OVA-allergic mice have specific gut microbial characteristics, specifically the declined abundance of *Lachnospiraceae* and *Rikenellaceae* and the rising abundance of *Porphyromonadaceae* and *Lactobacillaceae*, but some exceptions, such as some *Lachnospiraceae* and *Rikenellaceae* strains. The transplantation of OVA-specific Tregs into allergic mice promoted the

formation of OVA immune tolerance and reversed the allergy-induced changes in the abundance of *Lachnospiraceae*, *Rikenellaceae*, and *Porphyromonadaceae*. In this study, we reported a significant downregulation in the abundance of *Rikenellaceae*, *Muribaculaceae*, *Erysipelotrichaceae*, and *Christensenellaceae* families in OVA-sensitive mice, a significant upregulation in the abundance of the *Lactobacillaceae* family, and a significant correlation with the main immunological indicators. Four genera belonging to *Lachnospiraceae* were significantly altered, and the abundance of *Parabacteroides* and *Rikenellaceae_RC9_gut_group* genera also decreased significantly (Figure S3C). The association of *Erysipelotrichaceae* with food allergies has not been confirmed, but *Erysipelotrichaceae* has been found to be associated with asthma triggered by air pollution in children with airway allergies. Zheng *et al.*⁴³ reported that hazy days alter the intestinal flora in the stool of

children with asthma, including the decrease in *Erysipelotrichaceae* and *Bifidobacteriaceae* and that short-term air pollution may trigger allergic asthma by altering the intestinal flora. *Muribaculaceae* was first identified by Lagkouvardos *et al.*⁴⁴ in 2019, and the related functional studies are not sufficient. A recent study has shown that 2-O- β -D-glucopyranosyl-L-ascorbic acid significantly alters the gut microbiota of mice during intervention in immunosuppressed BALB/c mice, with *Muribaculaceae* being one of the key taxa that may play a crucial role in modulating the immunity.⁴⁵ These findings imply that oral OVA may induce OVA allergy by altering the intestinal flora such as *Muribaculaceae* and *Erysipelotrichaceae*. *Christensenellaceae* is commonly found in the intestines of various animals, including humans and mice, and is becoming one of the significant factors influencing the health of organisms. Although it has been shown that *Christensenellaceae* is one of the major flora in the gut of infants with milk allergies and its abundance decreases with the intervention of *Bifidobacteria*,⁴¹ the mechanism by which *Christensenellaceae* interacts with the immune system is not clear. In addition, it is important to note that there is no uniform understanding of the trend of *Lactobacillaceae* in allergic individuals. Some studies have demonstrated that the abundance of *Lactobacillaceae* decreases in allergic individuals and that increasing the abundance of *Lactobacillaceae* has the effect of suppressing the allergic reaction.^{46,47}

The altered intestinal flora in mice triggering OVA hypersensitivity may be associated with the downregulation of the aminoacyl-tRNA pathway. Compared to the control group, the serum metabolome of OVA mice showed a significant downregulation of the aminomethyl-tRNA synthesis metabolic pathway ($p < 0.001$). The functional prediction of the intestinal flora also revealed a decreased ability of aminoacyl-tRNA biosynthesis in OVA-sensitive mice. It has been demonstrated that aminoacyl-tRNA synthetase complex-interacting multicomplex protein 1 (AIMP1) negatively regulates Th2 responses in mice and that the transplantation of AIMP1-deficient CD4⁺ T cells into OVA-allergic mice exacerbates OVA-induced allergic airway inflammation.⁴⁸ The aminoacyl-tRNA synthetase complex (MACS) has a potential role in regulating Th1 immunity. Altering the structural components of MACS could regulate the production of CD8⁺CD25⁺ cells and the ability of these cells to secrete IFN- γ .⁴⁹ Furthermore, it has been suggested that the development of autoimmune illnesses, like idiopathic inflammatory myopathies, rheumatoid arthritis, and Reynaud's phenomenon, is associated with the production of antibodies against aminoacyl-tRNA synthetase.⁵⁰ Therefore, we infer that, in mice, the aminomethyl-tRNA synthesis pathway may play a vital role in the formation of food allergies and have the potential to be a therapeutic target.

Multiple oral administrations of OVA, which induced immune tolerance in mice, may be related to the production of IPA by the gut microbiota. The levels of IPA, one of the tryptophan metabolites produced by the intestinal flora and absorbed into the blood via the intestinal epithelium,¹⁶ showed a significant elevation in the OVA group in comparison with the control group and were positively correlated with the percentage of Tregs in splenic lymphocytes ($p < 0.01$). The IPA levels were also strongly correlated with most of the differential intestinal flora ($p < 0.01$). IPA can maintain mucosal homeostasis by reducing intestinal permeability, which is mediated by receptors including pregnane X receptor

(PXR) and aryl hydrocarbon receptor (AHR).^{51,52} IPA can also affect Treg differentiation. IPA and indoleacetic acid (IAA) produced by microbial metabolism can restore Treg responses by activating AHR but do not directly affect Th1/Th2 homeostasis.^{39,53} Recent studies showed that IPA is mainly produced by the metabolism of *Clostridium*, and its concentration is significantly related to the abundance of the intestinal flora.^{16,54} As shown in Figure 3B, the abundance of *Clostridium* in the feces of mice was significantly higher in the OVA group than that of control mice ($p < 0.01$), in which the abundance of *Peptostreptococcaceae* and *Ruminococcaceae* was significantly and positively correlated with IPA ($p < 0.01$, $r = 0.899$ and 0.968 , respectively). Tryptophan metabolism in the intestinal flora of mice in the OVA group was also significantly enhanced [Figure 4B-(b), $p < 0.001$]. A strain belonging to the *Peptostreptococcaceae* family was found to have the ability to produce IPA with indoleacrylic acid (IA), thereby having an inhibitory effect on inflammation.⁵⁵ The mechanism of the association between *Ruminococcaceae* and indole metabolites is also one of the key factors in maintaining the integrity of the intestinal barrier.⁵⁶ *Clostridium* may be one of the key intestinal flora regulating the immune system during the development of OVA immune tolerance in mice induced by multiple oral doses of OVA, and IPA may be one of the mediators of the adjustment of the immune system by the intestinal flora. We also found a significant positive correlation between *Lactobacillaceae* abundance and IPA levels (Figure 7A). Cervantes-Barragan *et al.*⁵⁷ found that *Lactobacillus reuteri* metabolizes tryptophan in the intestine of mice to produce indole derivatives, which activate the AHR on CD4⁺ T cells, prompting the conversion of CD4⁺ T cells to Treg, which may suggest that *Lactobacillaceae* affects tryptophan metabolism in the intestine and has a positive effect on the formation of oral immune tolerance. In addition, there was a significant inverse correlation between IPA and *Christensenellaceae* (Figure 7B,C), and we hypothesize that *Christensenellaceae* plays a role in both the formation of OVA allergy and the formation of tolerance, but further studies are needed to explore its function in food allergy.

In conclusion, oral sensitization and excitation by intraperitoneal injection of OVA induced OVA allergy in mice, but repeated oral administrations of OVA may cause mice to develop OVA tolerance, which was mainly reflected in the significant increases in OVA sIgE, sIgG, HIS, and mMCP-1 in the serum, the proportion of Th2-type cells, and IL-4 and IL-5 secretion in splenic lymphocytes after intraperitoneal injection of OVA in mice of the OVA group, but a significant increase in the proportion of Tregs in splenic lymphocytes was also observed. OVA-sensitive mice had significantly altered serum metabolic profiles with specific intestinal flora characteristics, including decreased α -diversity and reduced abundance of *Rikenellaceae*, *Muribaculaceae*, *Erysipelotrichaceae*, and *Christensenellaceae* families. The serum metabolome suggests that the development of OVA allergy is associated with a downregulation of the aminoacyl-tRNA synthesis pathway, while the effect of the intestinal flora on the immune system may be achieved by metabolizing indole derivatives produced from tryptophan such as IPA. The formation of OVA tolerance in mice may be related to the enrichment of *Peptostreptococcaceae*, *Ruminococcaceae*, and *Lactobacillaceae*. It should be noted that the relationship between the gut flora and food allergy and the mechanisms behind the effect are still not thoroughly investigated. In this study, the regulatory pathways of the

intestinal flora on OVA allergy were explored in conjunction with serum untargeted metabolomics, but the interactions between these flora and the deeper mechanisms of their effects on the immune system still need further investigation.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c07482>.

Detailed operation of serum metabolite extraction and parameters of GC–MS (Table S1), detailed procedure for the detection of OVA sIgG levels in serum, information on serum metabolites obtained by GC–MS after initial screening in all samples (Table S2), body weight changes of the two groups before and after the experiment (Figure S1), identification of Th1/Th2 and Treg in mouse spleen cells (Figure S2), complementary analysis of α -diversity of the intestinal flora in two groups (Shannon index and rarefaction curve) and comparison of differential OTUs at the genus level (Figure S3), Spearman's correlation between differentiated bacterial OTUs at the genus level and immune endpoints (Figure S4), comparison of differential functional pathways of the intestinal flora in two groups (Figure S5), permutation test for discriminating control and OVA-sensitive mice (Figure S6), pathway mapping of metabolites detected in OVA-sensitive mice and relative intensity comparison of metabolites contained in the aminoacyl-tRNA biosynthesis pathway in two groups (Figure S7), and Pearson's correlation between differential serum metabolites and differentiated bacterial OTUs at the genus level (Figure S8) (PDF)

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Notes

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■ ABBREVIATIONS USED

AHR, aryl hydrocarbon receptor; AIMP1, aminoacyl-tRNA synthetase complex-interacting multicompound protein 1; BSA, bull serum albumin; ELISA, enzyme-linked immunosorbent assay; HIS, histamine; HRP, horseradish peroxidase; IL-4/5/22, interleukin-4/5/22; IFN- γ , interferon- γ ; IPA, indole-3-propionic acid; mMCP-1, mouse monocyte chemoattractant protein-1; OTU, operational taxonomic unit; OVA, ovalbumin; PBS, phosphate buffer solution; PXR, pregnane X receptor; QC, quality control; RI, retention time index; sIgG, specific immunoglobulin G; sIgE, specific immunoglobulin E; Th1, type helper 1 cell; Th2, type helper 2 cell; TMB, tetramethylbenzidine; Treg, regulatory cell

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