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Original Article

Yogurt-sourced probiotic bacteria alleviate shrimp tropomyosin-induced allergic mucosal disorders, potentially through microbiota and metabolism modifications

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ABSTRACT

Background: Shrimp tropomyosin (TM) is a major food allergen that may cause serious allergic responses, lactic acid-producing bacteria (LAPB) are believed to alleviate food allergy, but the mechanisms have not been fully clarified. The aim of this work is to investigate the mechanisms of LAPB in ameliorating food allergy-induced intestinal mucosal disorders and to investigate whether or not these disorders occur by the regulation of gut microbiota and metabolism.

Methods: A TM allergy BALB/c mouse model was established, and two LAPB strains, *Bifidobacterium longum* (Bi) and *Bacillus coagulans* (Bc), were used for oral treatment in sensitized mice. The allergic mucosal disorders were assessed by histological analysis and ELISAs. Additionally, microbiota and metabolic modifications were determined by 16S rRNA gene amplicon sequencing and GC-TOF-MS, respectively.

Results: YSPB administration suppressed TM-induced intestinal mucosal disorders, restored allergenic Th2 cell over-polarization and dysbiosis, and regulated gut arginine and proline metabolism pathways. Statistical analysis suggested the metabolites aspartate and arginine, as well as several commensal flora groups, to be the critical mediators in the process.

Conclusions: These data demonstrated the correlation between allergic mucosal disorder, T cell subtype differentiation, gut microbiota composition and intestinal metabolism especially the arginine and proline metabolism pathways. We also revealed the significant effects of LAPB in ameliorating food allergy and maintaining the mucosal ecosystem. This study confirmed the efficiency of LAPB in relieving food allergy, provided Bi and Bc as the potential treatment approaches, and suggested amino acid metabolism pathways might be the novel targets for potential clinical applications.

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Introduction

Shellfish is worldwide favorite food with increasing consumption due to the good flavor and rich nutrition, but is also one of the most common allergic food groups, with a prevalence of up to 10.3% in specific areas.¹ Food allergies are a serious health problem that can damage the gastrointestinal tract and skin ecosystems, sometimes even resulting in life-threatening anaphylactic responses.² Tropomyosin (TM) is the major antigen of shellfish that provokes immunoglobulin (Ig)E-mediated hypersensitivity reactions.^{3,4} The

gut serves as the primary organ to interact with food allergens and is thus tightly associated with food allergy progression.⁵ Generally, food allergens can be ingested in the gastrointestinal tract, transported by gut epithelial cells and sensed by gut-associated lymphoid tissues, resulting in immunological responses, such as T cell differentiation and cytokine production, as well as mast cell, eosinophil, and other leukocyte infiltration, which further induce subsequent immunologic intestinal tissue lesions and mucosal disorders.^{5,6}

In addition to host gastrointestinal cells, the microbiota of the gut has also been proven to play a critical role in regulating food allergies.⁷ Environmentally induced alterations in the commensal microbiome are implicated in the prevalence of food allergies. For instance, germ-free or antibiotic-treated mice show much stronger allergic responses than mice with intact microbiota,⁸ and people susceptible to food allergies have more distinctive microbiota than

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others.^{7,9,10} In contrast, food allergies also impair the gut microbiota and induce dysbiosis.^{11,12} The potential reason was suggested to be the oral tolerance programming ability of the microbiota,¹³ but the exact mechanisms have not yet been fully revealed.

Previous studies have also demonstrated the strong link between gut bacterial metabolites and host immunity. Bacterial metabolites, such as short-chain fatty acids (SCFAs),¹⁴ bile acid¹⁵ and amino acids,^{16,17} can regulate intestinal homeostasis in a variety of ways. Previous studies have demonstrated the critical role of amino acids, such as arginine, in intestinal epithelial cells, dendritic cells, macrophages and T cells. Their role is at least partially dependent on the mTOR signaling pathway.^{18,19} Arginine metabolism in macrophages can balance the activities of arginase and nitric oxide, influencing the tissue repair response.^{20,21} Additionally, the arginine metabolism pathway has also been proven to be associated with Th2 cell-mediated airway inflammation.^{22–24}

The involvement of gut microbiota and metabolism made it possible to regulate these physiology activities by specific control of food and nutrition intake, though the application will not be mature before the fully elucidation of the underlying mechanisms. Nowadays, although significant research has been conducted regarding the complex and systematic physiological process, the detailed mechanisms of food allergies have not been fully elucidated; as a result, there is no safe and efficient therapeutic approach that is clinically available.²⁵ Fortunately, in recent years, it has been reported that probiotics have strong effects on alleviating food allergies.²⁶

Yogurt is world famous fermented food that can serve as a plentiful source of probiotics. Previous studies have indicated that yogurt-sourced probiotic bacteria (YSPB) inhibit inflammation and alleviate food allergies by reconstructing gut microbiota and regulating intestinal metabolism and mucosal immune responses.^{27–29} Our results also showed that the YSPB strains *Bifidobacterium longum* (Bi) and *Bacillus coagulans* (Bc) ameliorated TM-induced allergy by the dendritic cell-dependent induction of the regulatory T cell (Treg) and the suppression of mTOR signaling.^{4,11} However, the direct targets for YSPB as well as the cellular and molecular anti-allergic mechanisms have not been clarified, limiting further clinical applications.

To intensively investigate food allergies, several mouse models have been established to date,³⁰ and a TM-sensitization model has been established in our laboratory.^{4,11} However, the durations of these models are too long,^{31,32} which severely limited the efficiency of the scientific research. In addition, only few studies have focused on the allergen-induced imbalance of the mucosal ecosystem. Therefore, in the present study, a short-time TM-induced food allergy mouse model was established, and YSPB were introduced. Using this model, we focused on the effects and mechanisms of allergy alleviation by YSPB and investigated the participation and interconnection of intestinal mucosal disorders, microbiota changes and metabolism alterations throughout the process. These results provided a favorable theoretical basis for the mechanism of food allergies. We also assessed the relevant therapeutic effect of probiotics and predicted potential targets for the clinically prevention and treatment of food allergies.

Methods

Ethics approval and consent to participate

This study was carried out in strict accordance with the recommendations of the National Guidelines for the Care and Use of Laboratory Animals of China. All the animal procedures were approved by the Zhejiang Gongshang University Laboratory Animal Welfare Ethics Review Committee.

In vivo immunization protocols

The mice were randomly divided into 10 groups ($n = 6$) for TM sensitization and YSPB administration, with 5 groups for each sex. For the groups with TM treatment, the mice were sensitized by intraperitoneal (IP) injection of 100 μ L fully emulsified TM (Supplementary Fig. 1) together with equal volumes of incomplete Freund's adjuvant (Sigma–Aldrich, St. Louis, USA). The total amounts of TM were 200 μ g on days 7, 14 and 21, followed by a booster challenge with 1200 μ g TM on day 28 and 35. The blank control (Ctrl) received 200 μ L sterile PBS, and the negative control group (Ad) received an equal amount of emulsified incomplete Freund's adjuvant with sterile PBS at each sensitization and challenge point. The therapeutic groups were orally administered with YSPB (Bi or Bc, 2×10^{10} CFU, 300 μ L per mouse) for 22 consecutive days from day 14–35, while the other groups received the same volume of sterile PBS. The mice were bled from the retro-orbital plexus on days 6, 22 and 36, and the serum samples were stored at -20°C until analysis. The symptoms of clinical anaphylaxis were monitored 30–60 min after the last challenge using a scoring system as described by Tan *et al.*¹⁴ and fecal samples were also collected at that time and immediately stored at -80°C . The mice were fasted overnight after the last challenge and sacrificed for immunological analysis. The detailed dosages of TM and YSPB are listed in Supplementary Table 1, and the protocol is illustrated in Figure 1.

Bacterial strains and culture conditions

The commercial YSPB strain *B. longum* subsp 1.2202 (ATCC 15697) (Bi) was purchased from China General Microbiological Culture Collection Center (CGMCC) and anaerobically cultured in MRS Broth (Haibo Biotech, Shandong, China) at 37°C for 24 h. The potential probiotic strain *B. coagulans* 09.712 (Bc) was originally obtained from fermented milk curd and kept in our own lab. Bc was anaerobically cultured in Trypticase Soy Broth (TSB) (Haibo Biotech, Shandong, China) at 37°C for 48 h. The indicated bacteria were collected by centrifugation and re-suspended in PBS for oral administration in the mouse models.

Animals

Six-week-old BALB/c mice (SPF) were purchased and housed in the Laboratory Animal Research Center of Zhejiang Chinese Medical University. The mice were housed in a room with a 12 h light–dark cycle at $22\text{--}24^{\circ}\text{C}$ under specific pathogen-free conditions.

Availability of data and materials

The datasets generated during the current study are available at the NCBI's Sequence Read Archive (SRA) accession numbers: SRX3990703 to SRX3990732 (<https://www.ncbi.nlm.nih.gov/sra/SRX142507>). More details are shown in Supplementary Methods.

Results

Oral administration of YSPB attenuates allergic responses in shrimp TM-sensitized mice

To intensively study the mechanisms of food allergies, we first established a food allergy mouse model. In the female groups, compared with the control (Ctrl) and adjuvant (Ad) groups, the TM-sensitized (TM) group showed significantly reduced body weight gain (Fig. 2A) and higher anaphylaxis scores (Fig. 2B), as well as more severe clinical symptoms, such as diarrhea, greasy hair (Fig. 2D), and high levels of serum histamine (Fig. 2C) and IgE

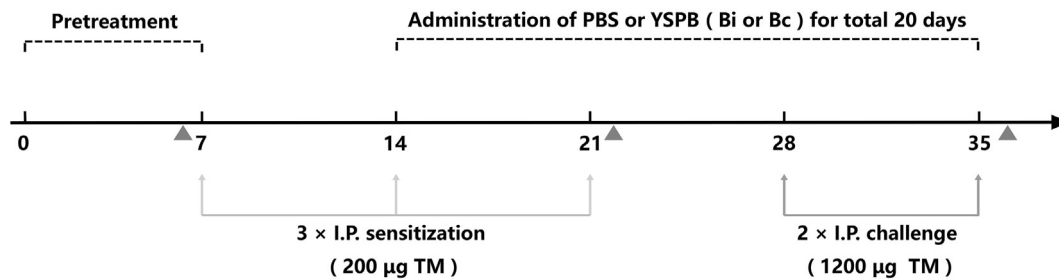


Fig. 1. Experimental design of *in vivo* immunization. Mice ($n = 6$) were sensitized by intraperitoneal (IP) injection of 200 µg fully emulsified TM together with equal volumes of Freund's adjuvant in 200 µL on days 7, 14 and 21, followed by a booster challenge with 1200 µg TM on day 28, 35. The blank control group (Ctrl) received 200 µL sterile PBS, and the negative control group (Ad) received an equal amount of emulsified Freund's adjuvant with sterile PBS at each sensitization and challenge point. Bi or Bc treated groups were orally administered with the indicated dose (2×10^{10} CFU, 300 µL per mouse) for 22 consecutive days from day 14–35, and the rest of the groups received the same volume of sterile PBS. The mice were bled from the retro-orbital plexus on days 6, 22 and 36, and the serum samples were stored at -20°C until analysis. Fecal samples were collected individually 30–60 min after the last challenge and immediately stored at -80°C . Mice were fasted overnight after the last challenge and sacrificed on day 36.

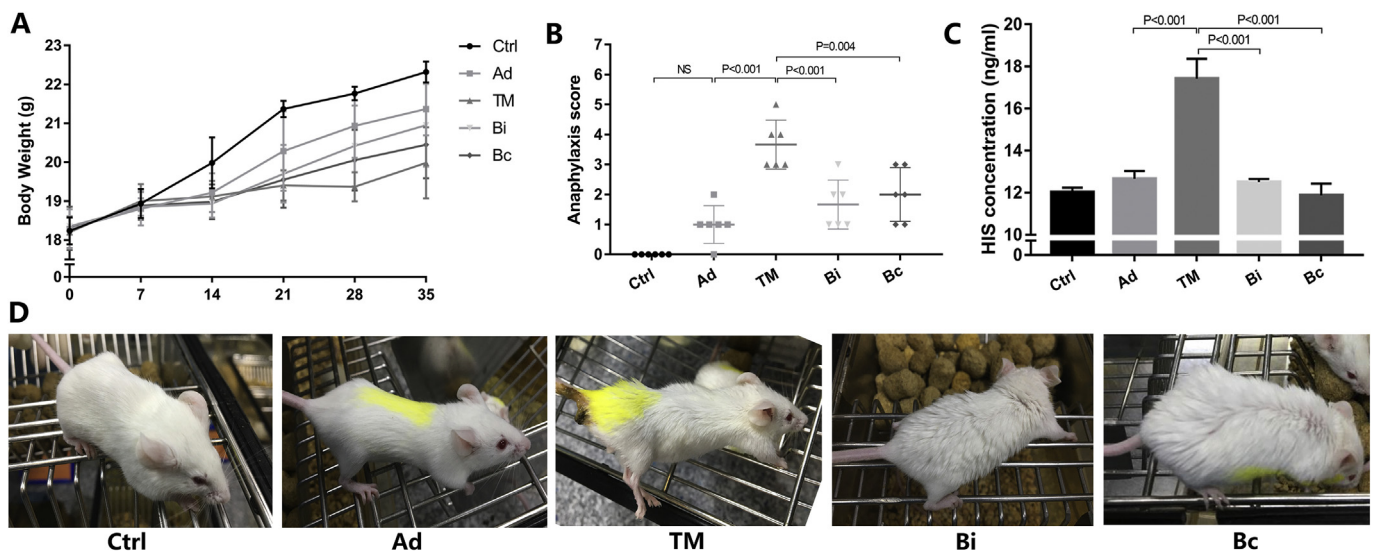


Fig. 2. YSPB suppressed TM-sensitization in female Balb/c mice. (A) Body weight changes of female Balb/c mice in each group. (B) Symptoms of clinical anaphylaxis were monitored 30–60 min after the last challenge. (C) Serum histamine concentrations were measured after the last challenge. (D) The appearance of female Balb/c mice in each group after the last challenge. Data are presented as the mean $\pm$ S.D; P values are determined by two-tailed Student's t test. NS, not significant.

(Fig. 3A). Interestingly, compared to the female mice, the male mice showed weaker or even no allergic symptoms, including body weight gain rate (Supplementary Fig. 2A), anaphylaxis score (Supplementary Fig. 2B) and histamine concentration, throughout the sensitization process (Supplementary Fig. 2C,D). Moreover, TM-specific IgE was not detected in the male mice sera (data not shown). This observation was consistent with the corresponding data for humans, although the underlying mechanisms remain unknown.³³ Consequently, only the female groups were chosen for the subsequent intensive investigations.

To investigate the effect and mechanism of the previously identified YSPB strains Bi and Bc in modulating food allergies, we introduced these strains into the established allergy mouse model. Bi or Bc treatment alleviated the body weight gain reduction (Fig. 2A), anaphylaxis symptoms (Fig. 2B) and serum histamine (Fig. 2C) and IgE (Fig. 3A) production induced by TM-sensitization. The results demonstrated that the oral administration of YSPB suppressed the development of TM-induced food allergies.

YSPB supplementation alleviated TM-induced small intestine allergic mucosal disorders

Serious food allergies usually cause immunologic gastrointestinal manifestations⁵; thus, we investigated allergic mucosal

disorders during TM sensitization in the mouse model by histological analysis in a blinded manner. Compared with those in the Ad group, the number of intestinal mast cells and goblet cells in the TM group was significantly increased, which could be associated with abnormal bacterial adhesion and intestinal permeability.³⁴ On the other hand, these cells were restored to normal levels as in the Ad group after either Bi or Bc treatment (Fig. 4A, B). Similarly, CD4⁺ T cells and eosinophils were also over-accumulated in the intestine after TM sensitization, abrogating the accumulation in the presence of YSPB (Fig. 4C, D). In addition, we investigated the apoptosis of intestinal epithelial cells and observed a significant increase in the number of apoptotic TUNEL-positive cells in the crypts of the TM group when compared to the Ad group, which was also relieved by YSPB administration (Fig. 4E).

To get more precise observations of the TM-mediated mucosal disorders and the effect of YSPB, we quantified the histological results above with the symptom scores. As presented in Figure 4F–J, it is clear that the TM group exhibited the most severe allergic symptoms, including inflammatory cell (such as mast cells and eosinophils) infiltration, goblet cell over-proliferation and crypt cell apoptosis. All these symptoms were significantly improved by YSPB administration ($p < 0.05$, by Student's t test). The quantified results also showed that the duodenum is the major position for inflammatory cell infiltration, followed by the jejunum

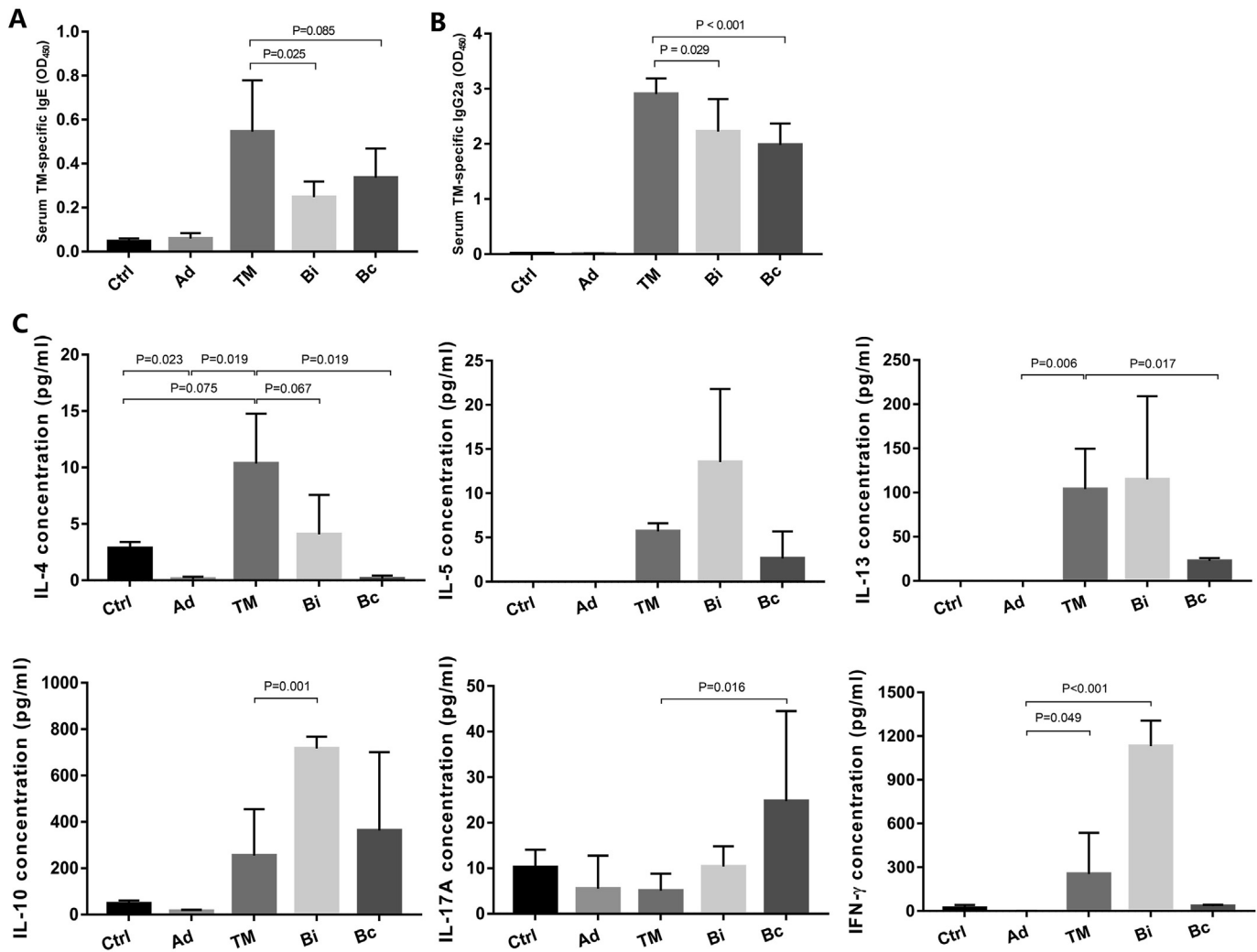


Fig. 3. YSPB relieved TM-induced allergy by polarizing T cell differentiation. (A) Serum TM-specific IgE concentrations were measured after the last challenge by ELISA. (B) Serum TM-specific IgG2a concentrations were measured after the last challenge by ELISA. (C) Splenic cells isolated from the mice of each group were cultured *in vitro*. After stimulation by purified TM, produced cytokines (IL-4/5/10/13/17A, IFN-γ) in the supernatants were measured by ELISA. Data are presented as the mean ± S.D; P values are determined by two-tailed Student's t test.

and ileum, leaving the colon almost intact. Similarly, the changes in mast cell, goblet cell, eosinophil, and apoptotic cell numbers were also slight in the colon, suggesting that the small intestine, rather than the large intestine, was the major location of allergic mucosal disorders.

YSPB switched T cell subtypes into an anti-allergic pattern

After confirming the activity of YSPB in alleviating allergic mucosal disorders, we then made efforts to investigate the possible T cell immune response regulated by YSPB after TM sensitization. We found that TM-sensitized mice produced higher levels of both TM-specific IgE and IgG2a, which were reduced by the oral administration of YSPB (Fig. 3A, B). To characterize the systemic T cell responses induced by TM sensitization, splenic cells were isolated from the mice of different groups and cultured *in vitro*. After being stimulated by purified TM proteins, the culture supernatants were collected, and their cytokine profiles were analyzed by ELISA (Fig. 3C). Th1- (IFN-γ), Th2- (IL-4/5/13) and Treg- (IL-10) but not Th17- (IL-17A) type cytokines were increased in the TM group, indicating the triggering of inflammation, allergy and immune

balancing at the same time. Splenic cells from the Bi group showed significantly higher production of IL-10 and IFN-γ and lower production of IL-4, suggesting that Bi utilized multiple methods, including inducing Treg-mediated oral tolerance and skewing the immune system towards inflammatory Th1 polarization and away from allergenic Th2 polarization to relieve allergic responses. On the other hand, splenic cells from the Bc group showed a significantly higher production of IL-17A and a lower production of IL-13, demonstrating that Bc promoted inflammatory Th17 differentiation and directly inhibited allergenic Th2 differentiation. These results suggested that Bi and Bc rely on different mechanisms but share Th2 as a common target to alleviate TM-induced allergy.

Moreover, to further investigate the molecular mechanisms *in vivo*, we measured the mRNA expression levels of allergy-related genes (including cytokines and transcription factors) in the spleen, small intestine and large intestine from each group (Supplementary Fig. 3). The expression changes of these genes showed significant tissue specificity: IL-13 was upregulated by TM only in the small intestine, while IL-4 and IL-10 upregulation was only observed in the spleen, indicating the major production organs of these cytokines. On the other hand, the expression of all the

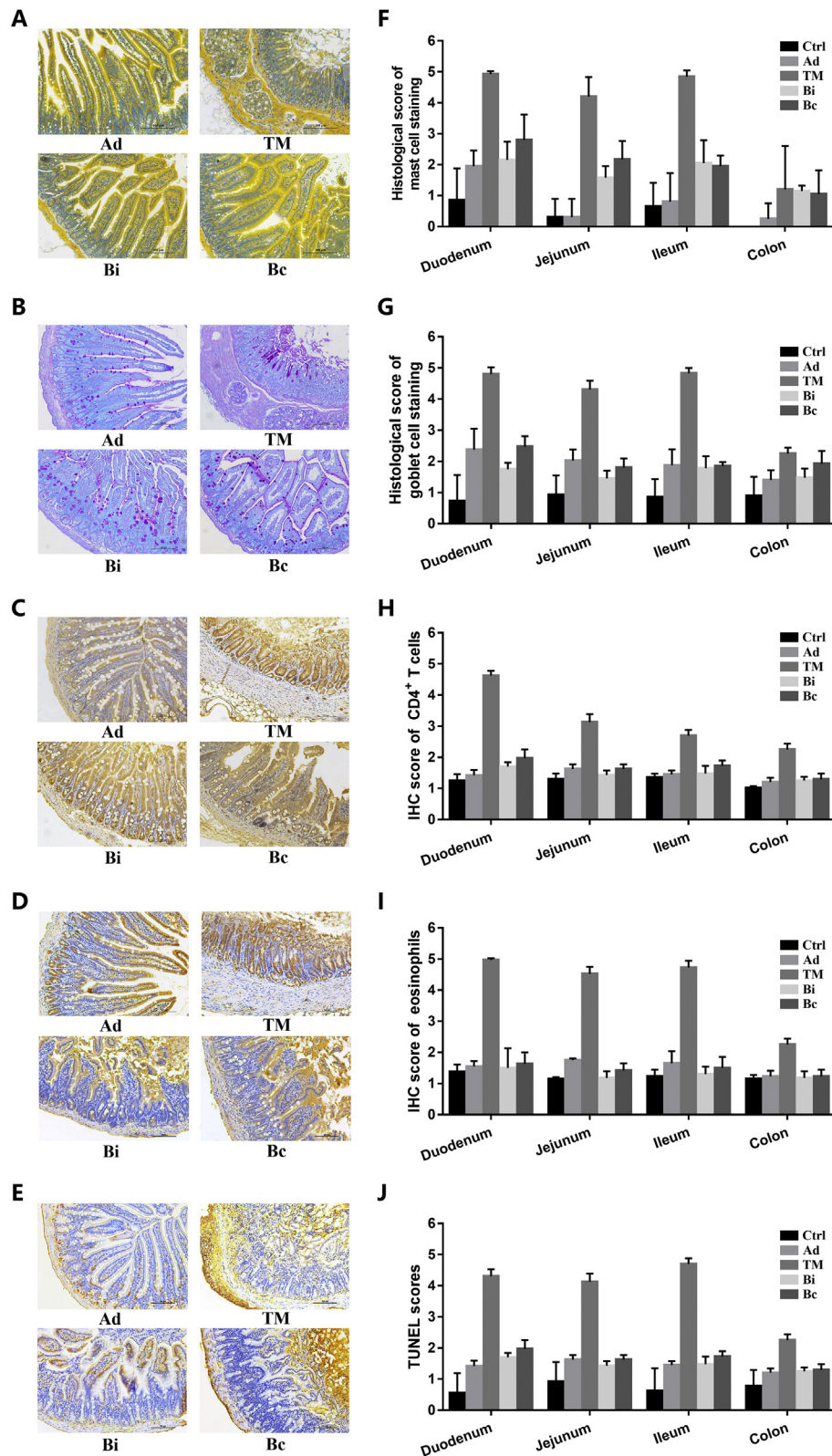


Fig. 4. YSPB supplementation alleviated TM-induced allergic mucosal disorders. Histological analysis was performed with gut sections from the duodenum, jejunum, ileum and colon of each mouse group. (A, F) Mast cells were detected with an AS-D chloroacetate esterase staining kit. (B, G) Goblet cells were detected by a periodic acid-Schiff kit. (C, H) CD4⁺ T cells were detected by immunohistochemistry. (D, I) Eosinophils were detected by immunohistochemistry. (E, J) Apoptotic cells were detected by TUNEL assay. Stained gut sections were photographed with a Leica microscope at $\times 200$ magnification.

tested genes showed marginal changes in the large intestine, suggesting that the large intestine is a less important organ for allergic responses, which is consistent with the histological results. Notably, Bc but not Bi further upregulated IL-10 gene expression in the spleen, which is inconsistent with the ELISA results and could be due to the alternative way of production and functional organs of IL-10.

Overall, we suggest that the spleen is the predominant organ for T cell differentiation during food allergies and that the small intestine is a major effector organ of cytokine production.

Administration of YSPB maintained gut microbiota homeostasis during TM-sensitization

Many studies in recent years have shown that food allergies are accompanied by dysbiosis.^{7,35–37} To test if this is also the case in this study, we assessed the microbiota changes during TM-sensitization and YSPB administration by 16S rRNA gene amplicon sequencing (Supplementary Fig. 4). As shown in Supplementary Figure 4C, the preliminary analysis of the sequencing results demonstrated that TM-sensitization markedly alters the composition of the gut microbiota, reducing the population of the *Bacteroidetes* but augmenting *Firmicutes* ($p < 0.05$, analyzed by Student's *t* test), which was consistent with a previous study on airway allergic disease.³⁸ On the other hand, YSPB treatment reconstructed the microbiota in terms of the increment of *Bacteroidetes* as well as the decrement of *Firmicutes* ($p < 0.05$).

Moreover, *Lachnospiraceae*, the most abundant family in the mouse gut microbiota, was significantly increased in the TM group and restored after YSPB treatment (Fig. 5A). It has been reported that *Lachnospiraceae* is associated with the production of butyrate necessary for oral tolerance.³⁹ Likewise, a principal component analysis (PCA) (Fig. 5B) of the high-throughput data also revealed a consistent correlation between TM-sensitization, YSPB administration and microbiota composition; a clear separation between the Ad and TM group was observed. In contrast, in the Bi and Bc groups, the separation with the Ad group decreased, and the three groups were partially overlapped.

These results indicate a potential relationship between microbiota homeostasis and YSPB-mediated food allergy alleviation, but the detailed underlying mechanisms remain to be elucidated by further investigation.

YSPB restored TM-induced metabolic disorders by regulating the arginine and proline metabolism pathways

In addition to the microbiota, gut metabolism has also been proven to be a critical target for treating food allergies by probiotics.^{14,15,40,41} To verify whether gut metabolism participated in YSPB function, we determined the metabolome of each group by GC-TOF-MS. PCA of the GC-TOF-MS data showed clearly that both TM-sensitization and YSPB administration significantly modified the gut metabolome (Fig. 6A). A heat map of the hierarchical clustering analysis further revealed differential metabolites between different groups (Fig. 6B). An intensive pathway analysis revealed that these differential metabolites were enriched in the arginine and proline metabolism pathways (Fig. 6C), which was further confirmed by KEGG analysis (Fig. 6D). In detail, precursor metabolisms, such as alanine, aspartate, and glutamine pathways significantly decreased in the TM group compared to the Ad group. In contrast, after the oral administration of Bc, the precursor metabolic pathways were restored (Fig. 6D). However, though statistically significant (Fig. 6C), the impact of Bi on the pathway was marginal (data not shown).

The metabolic analysis demonstrated that amino acid (especially arginine and proline) metabolism pathways are tightly correlated with TM and YSPB treatment, indicating that Bc regulates metabolic homeostasis during TM-induced food allergy.

Identification of allergy-related gut microbiota groups and metabolites

To further analyze the correlation between allergic mucosal disorders and the modifications of microbiota and metabolism, we performed a correlation analysis (Spearman's correlation test) using the ELISA, microbiome and metabolome data (Table 1). The results showed that the concentration of gut aspartate is positively correlated with the Th2-type cytokines IL-5 and IL-13, while arginine is negatively correlated with the Th2-type cytokine IL-4, which is consistent with the metabolome analysis results. On the other hand, the commensal phylum *Firmicutes* is negatively correlated with Treg-type cytokine IL-10 production, while *Bacteroidetes* is positively correlated, indicating that these phyla can regulate Treg-mediated immune tolerance. In addition, at the family level, *Lactobacillaceae* is negatively correlated with IL-4, while the *Bacteroidales* S24-7 group is positively correlated with the Treg-type cytokine IL-10. All these statistical analysis results revealed the critical allergy-related gut microbiota groups and metabolites, demonstrating the strong correlation between

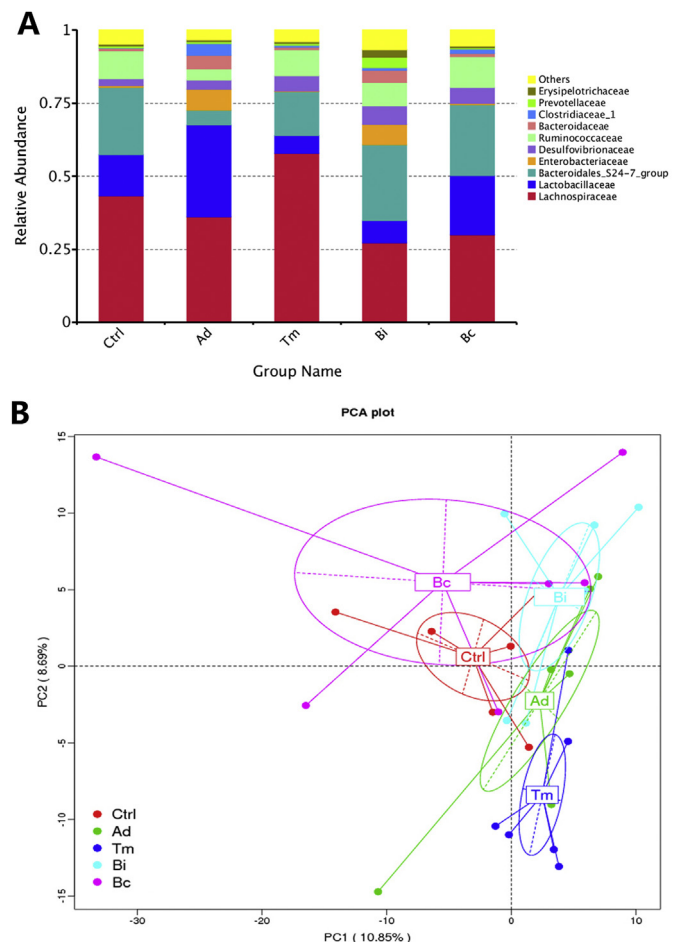


Fig. 5. YSPB suppressed TM-induced gut dysbiosis. (A) Mouse gut microbiota were determined by 16S rRNA gene amplicon sequencing, and the relative abundance of each bacterial family was calculated and displayed. (B) The microbiota data in each group were assessed by PCA.

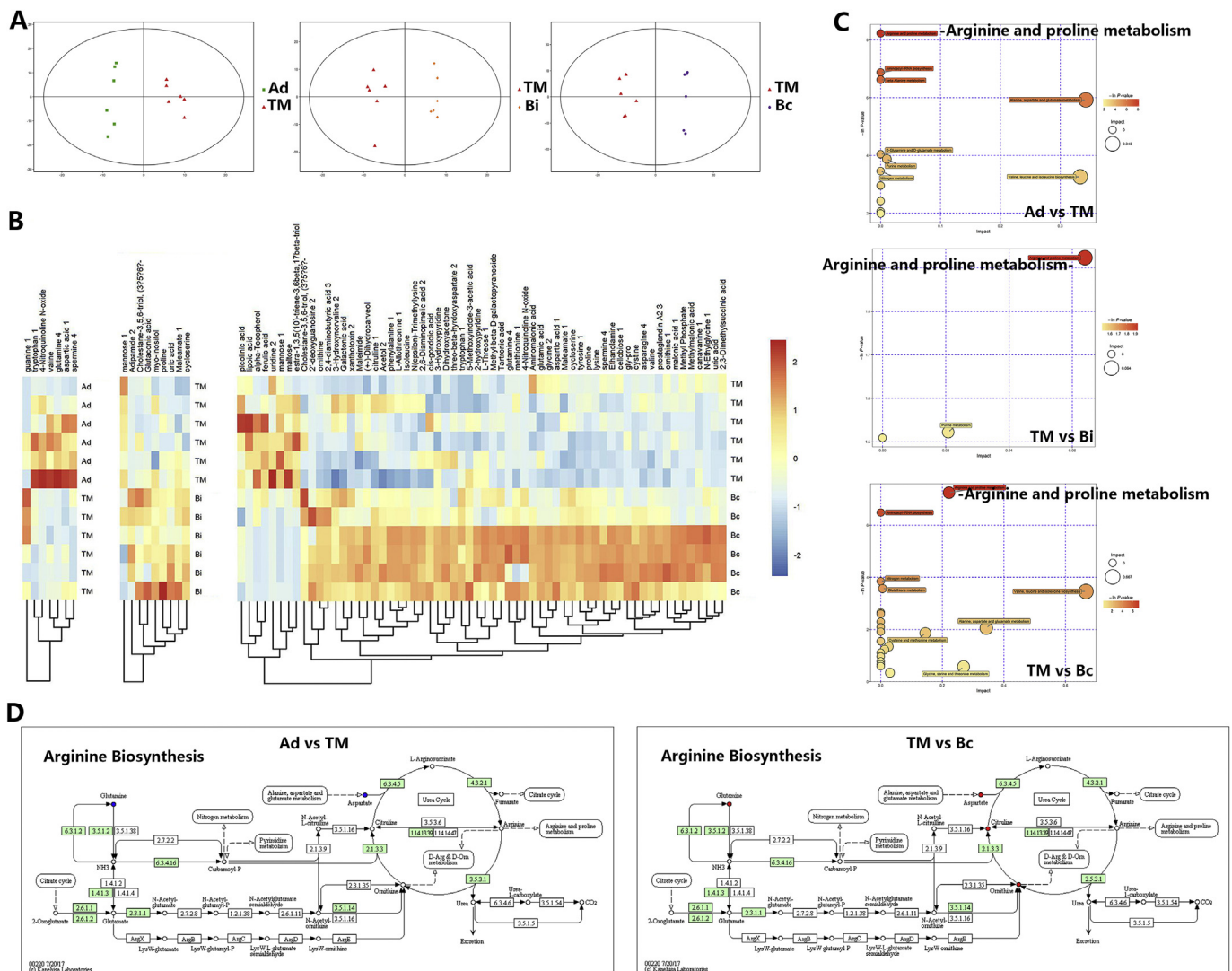


Fig. 6. YSPB regulated metabolic homeostasis during TM-sensitization. (A) Mouse gut metabolomes were determined by GC-TOF-MS, and the differences among groups were analyzed by PCA. (B) Differential metabolites among groups were revealed by a heat map of the hierarchical clustering analysis. (C) Critical metabolic pathways were revealed by pathway analysis. (D) Intensive analysis of arginine metabolism among different groups was performed by KEGG.

allergic mucosal disorders and the modifications of microbiota and metabolism as well as implying the underlying causal relationships.

Discussion

Yogurt is a natural source of probiotics presenting various beneficial effects on human bodies,⁴² as a traditional food, yogurt-sourced bacteria are generally safe and thus can be used more conveniently in clinical applications. Recently, experimental and clinical evidence has demonstrated that probiotics can inhibit inflammation and alleviate food allergies, which are involved in gut microbiota reconstruction, intestinal metabolism modification and mucosal immune response regulation.^{41,43} However, histological and immunological mediators involved in the process are not completely defined, and the mechanisms of ameliorating imbalance of mucosal ecosystems by YSPB have not been clarified, which greatly impaired the further development and clinical applications of probiotics in treating food allergy. In the present study, to investigate the anti-allergy and anti-inflammation effects, as well as the underlying mechanisms of YSPB activity, we established a

shrimp TM-sensitization mouse model, and focused on the changes in allergic mucosal disorders, microbiota and metabolism.

We first established a feasible allergic mouse model with relatively short time procedures. The results demonstrated that this model can reflect the physiological condition of food allergy. Compared with other models elsewhere, this model shortened the whole period from two months to one month and maintained equal allergic response levels. To further improve the sensitivity, we compared female and male mice in their response to TM sensitization and found that female mice showed much stronger responses than male mice. This finding may be due to the different hormonal profiles of different sexes, though the possible mechanism has not yet been revealed.^{30,33} Overall, our established allergic mouse model is efficient and sensitive for further studies, which could be a useful tool for investigations of other food allergens and developments of effective anti-allergic treatments.

Using this mouse model, we first explored the allergic mucosal disorders by histological analysis. Under TM sensitization conditions, CD4⁺ T cells, eosinophils and mast cells were observed to infiltrate into the intestine, and goblet cell proliferation and intestinal cell apoptosis were also observed, demonstrating allergic

Table 1
Spearman's correlation test with the ELISA, microbiome and metabolome data.

		IL-4	IL-5	IL-13	IL-10	IL-17A	IFN- γ
Aspartate	r [†]	0.564	0.821*	0.900*	0.500	-0.410	0.700
	P [‡]	0.161	0.044	0.019	0.196	0.246	0.094
Arginine	R	-0.975**	-0.564	-0.500	-0.200	0.667	-0.600
	P	0.002	0.161	0.196	0.374	0.109	0.142
<i>Lactobacillaceae</i>	R	-0.975**	-0.718	-0.600	-0.500	0.359	-0.800
	P	0.002	0.086	0.142	0.196	0.276	0.052
<i>Bacteroidales</i>	R	0.154	0.616	0.500	0.900*	0.718	0.700
	P	0.402	0.134	0.196	0.019	0.086	0.094
<i>Firmicutes</i>	R	-0.154	-0.616	-0.500	-0.900*	-0.718	-0.700
	P	0.402	0.134	0.196	0.019	0.086	0.094
<i>Bacteroidetes</i>	R	0.154	0.616	0.500	0.900*	0.718	0.700
	P	0.402	0.134	0.196	0.019	0.086	0.094

*P < 0.05, **P < 0.01.

[†] r, the calculated Spearman correlation coefficient.

[‡] P, P values calculated by Spearman correlation.

mucosal disorders during TM-induced food allergy.^{4,13,44} Interestingly, it should be noted that the histological changes were mainly present in the small intestine rather than the large intestine. Specifically, for CD4⁺ T cell infiltration, the severity decreased along the intestinal tract from the duodenum to the colon, implying that the proximal intestine is the primary location of allergic mucosal disorders, and additional investigations are needed to confirm this in clinical cases. Furthermore, all these disorders were relieved by YSPB, verifying the anti-allergic function of these strains.

The gut microbiota and its metabolism have been reported to be critical in modulating the host immune system and are potential therapeutic targets for food allergies. In the present study, we also found that the gut microbiota changed during TM-sensitization and

YSPB administration. Additionally, the arginine and proline metabolism pathways were identified to be associated with food allergies. Previous studies have demonstrated the critical role of amino acids, such as arginine, in regulating gut mucosal homeostasis and inflammation, which is at least partially dependent on the mTOR signaling pathway.^{19,22} Our previous studies also revealed that the mTOR pathway is involved in food allergies,⁴ assuming the potential role of the mTOR pathway in food allergen-mediated modifications on the arginine metabolism of the gut microbiota. As an intensively studied pathway, mTOR could be a good start point for applied researches on food allergy therapy.

Finally, by summarizing and analyzing all the data in our work, we identified several specific metabolites and commensal flora groups as critical factors in mediating the YSPB activity of alleviating TM-induced allergic mucosal disorders, providing the possibility of regulating immune system directly by influencing gut microbiota and metabolism, or indirectly by modifying food and nutrition intake.

Taken together, these data suggest a potential mechanism for food allergy-induced mucosal disorders being alleviated by YSPB, as shown in Figure 7. The intake of food allergens, such as shrimp TM, induced allergic T cell differentiation in the spleen and subsequent inflammatory cell infiltration in the small intestine, which finally resulted in allergic cytokine production, goblet cell over-proliferation and crypt cell apoptosis. On the other hand, intake of YSPB regulated the gut metabolism, especially the amino acid metabolism pathways, induced anti-allergic T cell differentiation and suppressed inflammatory cell infiltration, potentially through the mTOR signaling pathway. In addition, YSPB supplementation also relieved allergen-induced gut dysbiosis by modulating several critical microbiota groups. Finally, the overall effect of YSPB is the alleviation of allergic mucosal disorders. Though whether or not

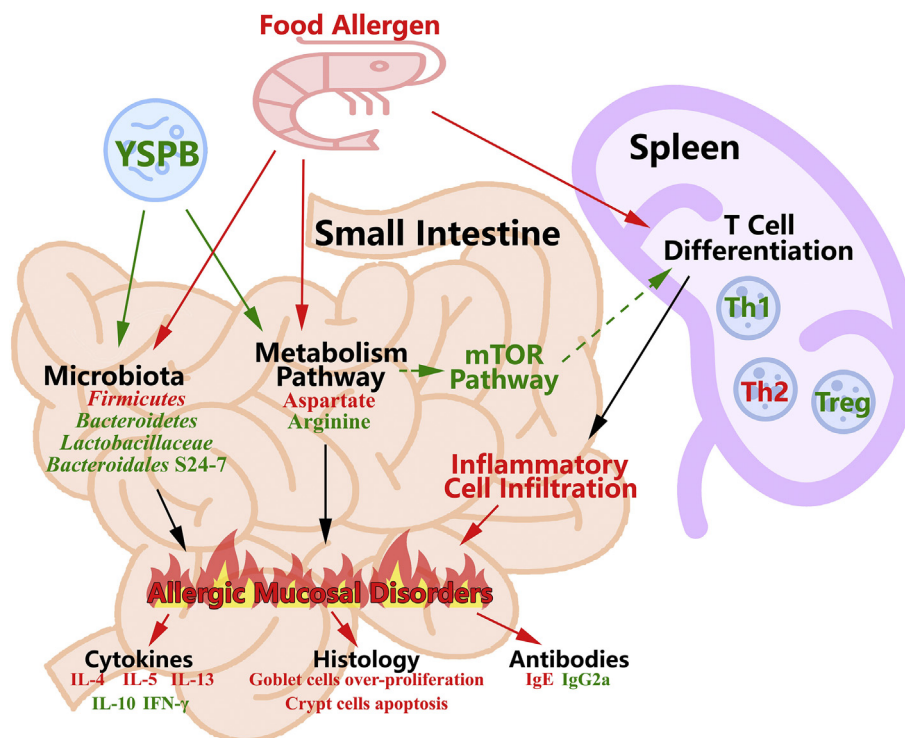


Fig. 7. An illustration of food allergy-induced mucosal disorders alleviated by YSPB. Shrimp TM induced allergic T cell differentiation in the spleen and inflammatory cell infiltration in the small intestine, which finally resulted in allergic mucosal disorders, including cytokine production, goblet cells over-proliferation and crypt cell apoptosis. On the other hand, administrating YSPB regulated gut arginine metabolism and subsequent mTOR pathway activation, induced anti-allergic T cell differentiation and suppressed inflammatory cell infiltration, thus alleviating allergic responses and mucosal disorders. Solid lines demonstrate the results found in the present work. Dotted lines denote the results reported previously. Allergic effects and factors are presented in red, and anti-allergic factors are presented in green.

these conclusions also apply to the human body requires further direct clinical studies, our results have already provided novel insights into the allergic mechanisms and proved specific strains of YSPB to be effective food allergy regulators, laying the theoretical foundation for further mechanism studies, providing potential therapeutic targets for food allergies, and shedding light on the application of probiotics in treating immune disorders.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2019.05.013>.

Conflict of interest

The authors have no conflict of interest to declare.

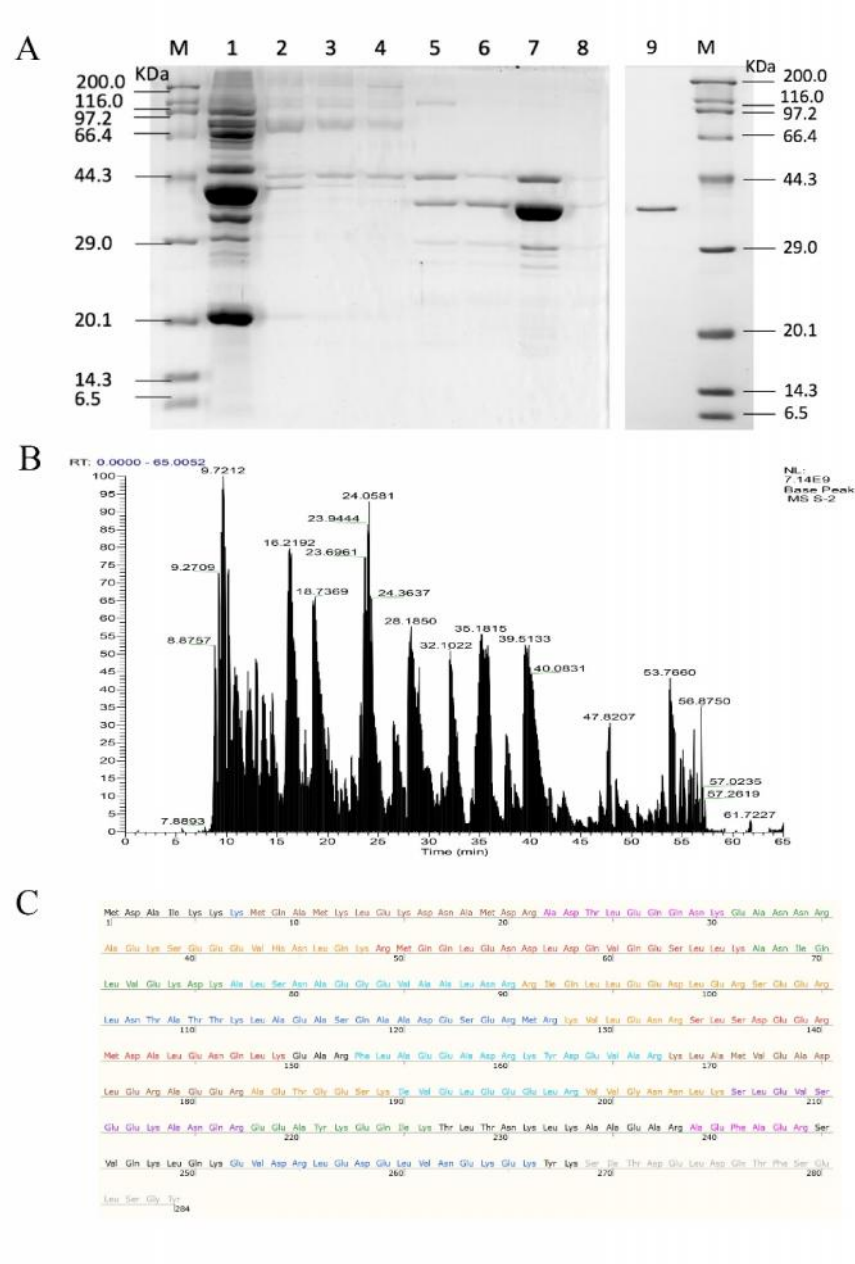
Authors' contributions

LF and YW designed research; SF and CW analyzed data; LF, SF, CW and MX performed research; LF, SF, CW and YW wrote the paper.

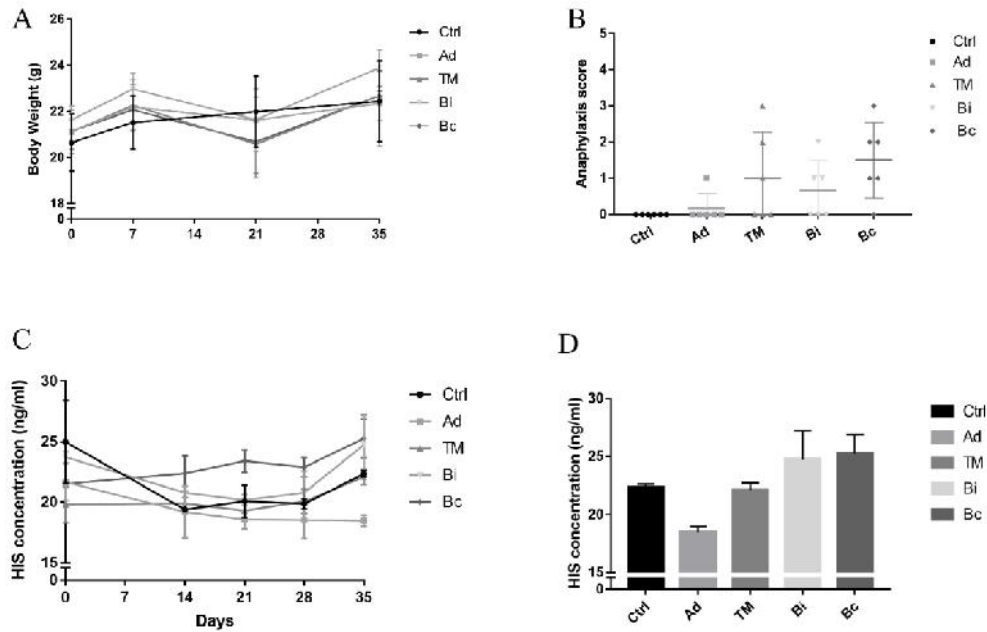
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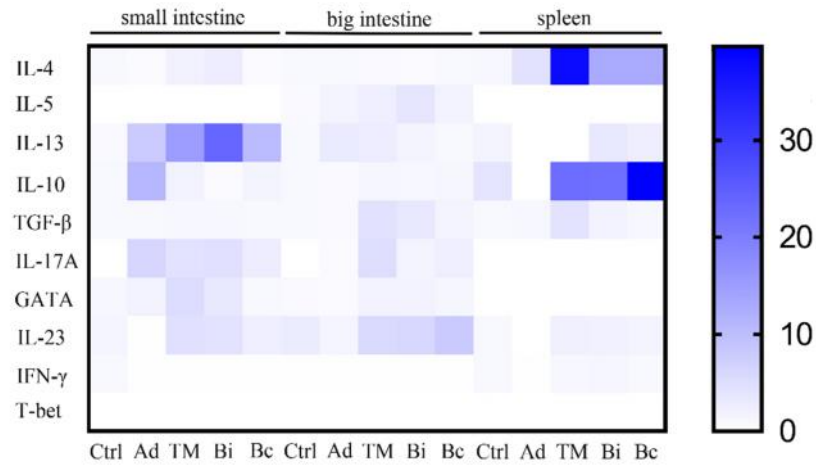
Supplementary Figures



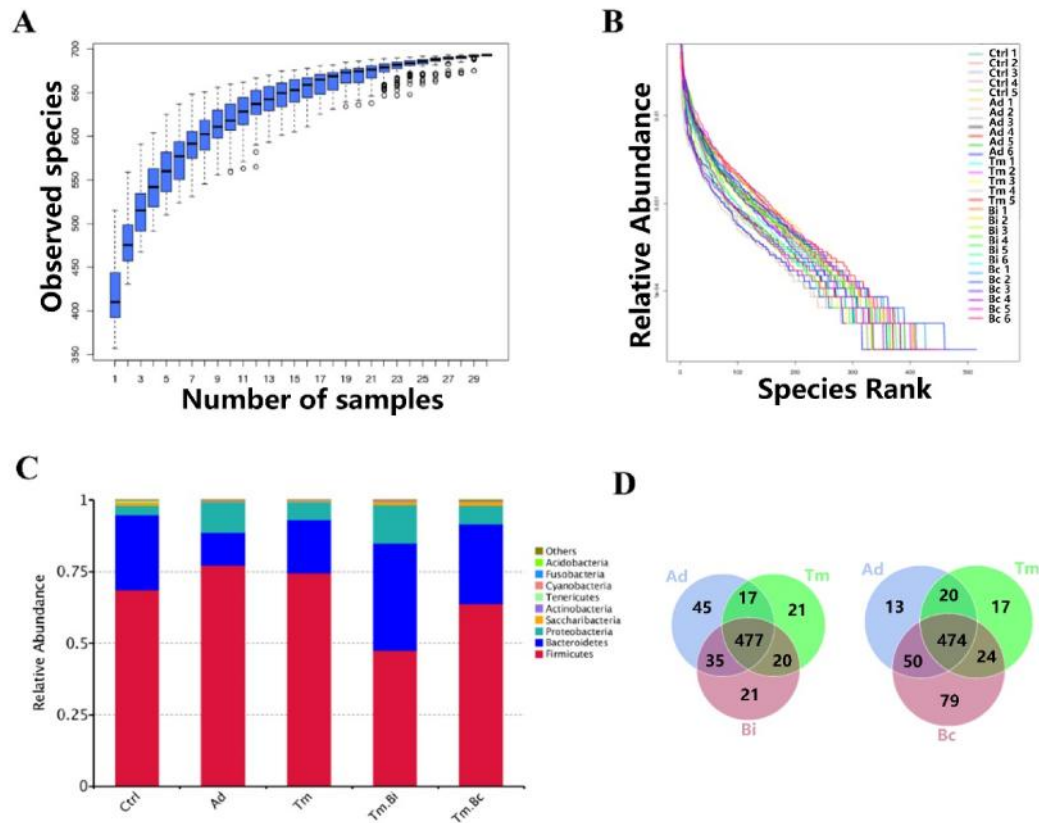
Supplementary Figure 1. Purification and Characterization of TM. (A) SDS-PAGE analysis of purified TM; **(B)** Mass spectrometric analysis of TM; **(C)** Amino acid sequence comparison of TM.



Supplementary Figure 2. Effects of YSPB on TM-sensitization in male BALB/c mice. Sera were collected on days 6, 22 and 36 (n=10) for all groups. Rate of weight change **(A)**, symptom score **(B)** and the level of histamine **(C, D)** were measured. Significance was determined by two-tailed Student's t test.



Supplementary Figure 3. Expression profiles of allergy-related genes. The expression levels of ten allergy-related genes (including genes of *IL-4/5/10/13/17A/23*, *TGF-β*, *GATA*, *IFN-γ* and *T-bet*) in different organs (spleen, small and large intestine) of all the groups were measured by qPCR and were expressed as a heat map.



Supplementary Figure 4. Bi and Bc maintained gut microbiota homeostasis during TM-induced food allergies. Mouse gut microbiota were determined by 16S rRNA sequencing, and the data were further analyzed. **(A)** Species accumulation boxplot. **(B)** Rank abundance curve. **(C)** Relative abundance of each phylum. **(D)** Venn graphs of the phyla in each group.

Supplementary Methods

Shrimp tropomyosin

The major shrimp allergen tropomyosin (TM) was isolated and purified from *Litopenaeus vannamei* as described by L. B. Smillie [1] with some modifications. Briefly, peeled and deveined frozen shrimp were homogenized with water, and the precipitate was collected and suspended in cold acetone. The homogenate was washed with cold acetone and dried. The dried powder was extracted in extraction buffer (20 mM Tris-HCl, 1 M KCl, pH 7.0) with continuous stirring, and the supernatant was collected and boiled. Insoluble proteins were removed by centrifugation, and the supernatant was adjusted to pH 4.6 for isoelectric precipitation. The precipitate was dissolved in PBS, further purified by ammonium sulfate fractionation (0 - 30% saturation), and re-suspended in PBS. The purified TM was verified by mass spectrometry, and the concentration was determined by a BCA Protein Assay kit (Pierce, Rockford, USA). The purity was analyzed by SDS-PAGE (Supplementary Figure 1).

Analysis of splenic cytokine profiles

The spleens of mice were excised from the other tissues and crushed through sterile 70 μm cell strainers in 10 mL PBS. After removing the erythrocytes with red blood cell lysis buffer (Sangon Biotech, Shanghai, China) for 5 min at room temperature, the cells were washed twice with PBS, counted and then re-suspended in complete culture medium of RPMI-1640 containing 10% fetal bovine serum and 1% penicillin/streptomycin (3×10^6 cells/mL). The cells were incubated at 37 °C in 5% CO₂ for 3 days, with the stimulation of TM (100 $\mu\text{g/mL}$). Then, the culture supernatants were harvested for cytokine profile (IL-4, IL-5, IL-13, IL-10, IL-17A, IFN- γ) determination using the Mouse ELISA Ready-SET-GO![®] kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. All the assays were performed in triplicate.

Determination of immunoglobulin and histamine levels in the serum

The shrimp TM-specific IgE and IgG2a in individual serum were determined by ELISA. Briefly, 96-well plates were coated with 100 μL TM (10 $\mu\text{g/mL}$) in carbonate coating buffer (0.05 M Na₂CO₃, 0.13 M NaHCO₃, pH 9.6) at 4 °C overnight. The plates

were washed with 250 μ L 0.05%-Tween 20/PBS and blocked with 200 μ L 5% BSA/PBS at 37 °C for 1 h. After washing, 100 μ L serum samples diluted with 5% BSA/PBS (1:10 for IgE and 1:100 for IgG2a) were added and incubated at room temperature for 2 hours. The plates were then washed and incubated with 100 μ L HRP-conjugated rat anti-mouse IgE or IgG2a secondary antibody (Abcam, Cambridge, MA, USA) at room temperature for 30 min. After incubating with substrate TMB for 15 min, the reaction was stopped by 1 M H₂SO₄, and the absorbency levels were measured at 450 nm. On the other hand, the concentration of histamine in the serum was measured by MOUSE HIS ELISA kits (Baomanbio, Shanghai, China) according to the manufacturer's instructions.

Histological analysis

Gut segments (duodenum, jejunum, ileum and colon) were collected after sacrifice, and the intestine luminal contents were removed by PBS using a blunt-ended lavage needle. The gut segments were fixed with 4% paraformaldehyde overnight at 4 °C and embedded in paraffin. Mucosal mast cells and goblet cells were detected using the Naphthol AS-D chloroacetate esterase staining kit (Sigma-Aldrich) and periodic

acid-Schiff staining kit (Sigma-Aldrich), respectively, following the manufacturer's instructions. Apoptotic cells were detected using the *In Situ* Death Detection Kit (Roche, Basel, Switzerland) following the manufacturer's instructions.

Immunohistochemistry

The dewaxed, rehydrated and retrieved gut tissues sections were incubated with 3% H₂O₂ to block endogenous peroxidase activity. Then, the sections were incubated with primary antibody (1:100 rabbit anti-mouse PRG2 polyclonal antibodies (GeneTAx) for eosinophils, 1:1000 rabbit anti-mouse CD4 monoclonal antibody (Abcam) for CD4⁺ T cells) at 4 °C overnight. Subsequently, the sections were incubated with HRP-labeled secondary antibodies (ZSGB Bio, Beijing, China) at 20 °C for 20 min. Then, the slides were developed with a DAB substrate solution (ZSGB Bio, Beijing, China) for 5 min and counterstained with hematoxylin for 2 min. The segments were finally dehydrated, cleared and mounted for further visualization by a microscope.

Quantitative real-time PCR

The total RNA extraction of the spleen and gut segments was performed using the

E.Z.N.A.[®] Total RNA Kit II (Omega, Norcross, GA, USA). The cDNA was generated with the HiScript II Q RT SuperMix synthesis kit (Vazyme Biotech, Nanjing, China) following the manufacturer's instructions. qPCR was conducted using the AceQ qPCR SYBR Green Master Mix (Vazyme Biotech, Nanjing, China) on a LightCycler[®] Nano Thermal cycler (Roche Applied Science, Penzberg, Germany). The housekeeping gene HRPT was included for normalization, and the results were calculated according to $\Delta\Delta CT$. The sequences of the used primers are presented in Supplementary Table 1.

Bacteria 16S rRNA gene amplicon sequencing and bioinformatics

The total genomic DNA from the samples was extracted using the CTAB/SDS method. 16S V3-V4 regions were amplified using specific primers with barcodes. All the PCR reactions were carried out with Phusion[®] High-Fidelity PCR Master Mix (New England Biolabs) and detected by agarose gel electrophoresis. The samples with a bright main strip between 400-450 bp were chosen for further experiments. The PCR products were mixed in equidensity ratios and purified with the Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using the TruSeq[®] DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's

recommendations, and the index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and the Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina HiSeq 2500 platform, and 250 bp paired-end reads were generated.

Paired-end reads was assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence, and merged using FLASH (V1.2.7, <http://ccb.jhu.edu/software/FLASH/>) [2]. Quality filtering on the raw tags were performed according to the QIIME (V1.7.0, <http://qiime.org/index.html>) quality controlled process [3]. Sequences analysis were performed by Uparse software (Uparse v7.0.1001, <http://drive5.com/uparse/>) [4]. Sequences with $\geq 97\%$ similarity were assigned to the same OTUs. For each representative sequence, the GreenGene Database (<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>) [5] was used based on RDP classifier (Version 2.2, <http://sourceforge.net/projects/rdp-classifier/>) [6] algorithm to annotate taxonomic information. Principal component analysis (PCA) was performed by WGCNA package, stat packages and ggplot2 package in R software (Version 2.15.3).

Metabolomic analysis

Fecal samples were collected sterilely after the last challenge. Metabolites were extracted and analyzed by gas chromatography and time-of-flight mass spectrometry (GC-TOF-MS). Briefly, the GC-TOF-MS analysis was performed using a gas chromatograph (Agilent 7890 GC system, Agilent Technologies, Santa Clara, CA, USA) coupled with a time-of-flight mass spectrometer (Pegasus HT, LECO UK, Stockport, UK). The system utilized a DB-5MS capillary column coated with 5% diphenyl cross-linked with 95% dimethylpolysiloxane (30 m×250 μ m inner diameter, 0.25 μ m film thickness; J&W Scientific, Folsom, CA, USA). A 1 μ L aliquot of the analyte was injected in the splitless mode. Helium was used as the carrier gas, the front inlet purge flow was 3 mL/min, and the gas flow rate through the column was 1 mL/min. The initial temperature was kept at 50 °C for 1 min, then elevated to 310 °C at a rate of 10 °C/min⁻¹, and then kept for 8 min at 310 °C. The injection, transfer line, and ion source temperatures were 280, 270, and 220 °C, respectively. The energy was -70 eV in the electron impact mode. The mass spectrometry data were acquired in full-scan mode with the m/z range of 50-500 at a rate of 20 spectra per second after a solvent delay of

6.17 min.

Chroma TOF 4.3X software and LECO-Fiehn Rtx5 database (LECO UK, Stockport, UK) were used to extract the raw peaks, to filter and calibrate the data baselines and to complete peak alignment, deconvolution analysis, peak identification and integration of the peak area. Both the mass spectra datasets and retention index matches were considered in the metabolite identification. The peaks were removed when they were detected in <50% of the QC samples or $RSD > 30\%$ in the QC samples.

Statistical analysis

All the quantitative results were based on three independent replications. A two-tailed Student's t test was used for analysis of the differences between the means of the groups.

All the results were expressed as the mean $\pm$ S.D. P values < 0.05 were considered statistically significant. A correlation analysis was performed using Spearman's correlation test, and the coefficient of correlation (r) was calculated to evaluate the correlation. The SPSS 18.0 statistical software package (SPSS Inc., Chicago, IL, USA) was used for the data analysis.

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Supplementary Tables

Supplementary Table 1 The dosage of TM and probiotics used in the mouse model

Groups [†]	Sensitization (µg)			Challenge (µg)		Probiotic (CFU/mL; µL)
	1	2	3	4	5	
Ctrl	0	0	0	0	0	/
Ad	0	0	0	0	0	/
TM	600	600	600	1200	1200	/
Bi	600	600	600	1200	1200	Bi: 2×10 ¹⁰ ; 300
Bc	600	600	600	1200	1200	Bc: 2×10 ¹⁰ ; 300

[†]Each group was further divided into male and female subgroups.

Supplementary Table 2 Primer sequences for qPCR

Genes	Forward primer (5' – 3')	Reverse primer (5' – 3')
<i>IL-4</i>	ACAGGAGAAGGGACGCCAT	GAAGCCCTACAGACGAGCTCA
<i>IL-5</i>	GAGGTTACAGACATGCACCATT	TCAGTTGGTAACATGCACAAAG
<i>IL-13</i>	AGACCAGACTCCCCTGTGCA	TGGGTCCTGTAGATGGCATTG
<i>IL-10</i>	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
<i>IL-17A</i>	AGGGAGAGCTTCATCTGTGG	AGATTCATGGACCCCAACAG
<i>IFN-γ</i>	TGGCATAGATGTGGAAGAAAAGAG	TGCAGGATTTTCATGTCACCA
<i>Foxp3</i>	AGAGTTCTTCCACAACATGGACTACTT	GATGGCCCATGGATAAGG

<i>TGF-β</i>	ACCGCAACAACGCCATCTAT	GCACTGCTTCCCGAATGTCT
<i>GATA-3</i>	CTTATCAAGCCCAAGCGAAG	CCCATTAGCGTTCCTCCTC
<i>IL-2</i>	CACATTTGAGTGCCAATTCGAT	GCGCTTACTTTGTGCTGTCCTA
<i>T-bet</i>	TCAACCAGCACCAGACAGAG	AACATCCTGTAATGGCTTGTG
<i>RORγt</i>	AGAAAGAAAAGGGGAACTGG	CTATTGTGGCTGCTGAGTTC
<i>IL-23</i>	TGCTGGATTGCAGAGCAGTAA	GCATGCAGAGATTCCGAGAGA
<i>HRPT</i>	CTGGTGAAAAGGACCTCTCG	TGAAGTACTCATTATAGTCAAGGGCA
