Effect of 3 lactobacilli on immunoregulation and intestinal microbiota in a β -lactoglobulin-induced allergic mouse model

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ABSTRACT

Milk is one of the earliest and most common allergen sources in the world, with β -lactoglobulin representing a major allergen protein. Numerous studies have reported that probiotics exert antiallergic and anti-inflammatory effects. Here, we examined the effects of 3 strains of *Lactobacillus* on immunomodulatory functions, intestinal barrier functions, and intestinal microbiota through a β -lactoglobulin-induced allergic mouse model. We found that the oral administration of *Lactobacillus plantarum* ZDY2013 and *Lactobacillus rhamnosus* GG suppressed allergic response, attenuating serum IgE and relieving anaphylaxis symptoms. The 3 strains of *Lactobacillus* could induce T helper (Th) 1 or T regulatory cells to differentiate to inhibit the Th2-biased response for regulating Th1/Th2 immune balance. Furthermore, *L. plantarum* ZDY2013 and *L. rhamnosus* GG enhanced intestinal barrier function through the regulation of tight junction. We also found that *L. plantarum* ZDY2013 and *L. plantarum* WLPL04 could regulate alterations in intestinal microbiota caused by allergies. In particular, *Rikenella*, *Ruminiclostridium*, and *Lachnospiraceae* UCG-006 were considerably reduced after treatment with *L. plantarum* ZDY2013 and *L. plantarum* WLPL04. These results suggested that 3 *Lactobacillus* strains may serve as an effective tool for the treatment of food allergies by regulating immune and gut microbiota.

Key words: *Lactobacillus*, food allergy, immunoregulation, intestinal microbiota

INTRODUCTION

Food allergy is a vital public health problem that affects adults and children, and its incidence has in-

creased considerably in recent decades (Neau et al., 2016). In Western countries, approximately 2 to 5% of children are affected by cow milk allergy (Shandilya et al., 2016), in which β -LG is the major allergen (Braun-Fahrlander and Von Mutius, 2011; Jo et al., 2014). To date, treatments for food allergies have been ineffective due to a lack of a clear understanding of the correlative molecular mechanisms. Therefore, the best remedy for the disease is to avoid exposure to food allergens (Hyung et al., 2017); however, accidental intake is difficult to completely avoid (Zhang et al., 2017).

Food allergies are mainly caused by allergens stimulating CD4⁺T cells to differentiate into T helper (Th) 2 cells, which produce IL-4, IL-5, and IL-13. These cytokines induce B cells to produce antigen-specific IgE antibodies, which combine with mast cells to stimulate mast cell degranulation and the release of histamine, prostaglandins, and leukotrienes, resulting in food allergy symptoms (Kaminogawa et al., 1999). However, the mechanism by which individuals respond to allergens remains uncertain.

The hygiene hypothesis is the first to link allergies to microbes, arguing that environmental changes are a major factor for the development of allergies (Strachan, 1989; Neau et al., 2016) and that improvement of hygiene conditions delays the establishment of microbes in infants, reduces stimulation of the body's immune system, and results in immature development of the body's immune system and immune function disorder, thereby leading to an increase in the incidence of food allergies and other allergic diseases (Gordon, 2011). The hygiene hypothesis supposes that moderate microbial stimulation plays an important role in establishing immune system maturation and oral tolerance (Nieuwenhuizen and Lopata, 2005). Therefore, intestinal commensal bacteria play a major role in promoting the maturation of the intestinal immune system and the delivery of mediators that trigger host immune and defense responses, regulating Th cell balance and acquisition of oral tolerance, and maintaining the integrity of intestinal epithelial cells (Slack et al., 2009; Chistiakov et al.,

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2015; Fábrega et al., 2016). This intestinal microbiota disorder has been linked to increase the permeability of epithelial cells (Perrier and Corthesy, 2011; van Ree et al., 2014) and development of chronic inflammatory diseases, such as allergies and inflammatory bowel diseases (Artis, 2008; Tlaskalová-Hogenová et al., 2011; Penders et al., 2013).

In the study of allergic mechanisms, numerous probiotics have exhibited potential immunomodulatory effects. Despite cell limitations, the cell model performs an important position in the initial screening of different bacterial cells and bacterial metabolic components of immune response (Kobayashi et al., 2017). Numerous clinical reports have shown that probiotics can promote the differentiation of T regulatory (**Treg**) cell development and rebalancing Th1/Th2 responses to a Th1-dominant state (Forsythe, 2011; Kim et al., 2014). For example, *Bifidobacterium infantis* 14.518 screened from infant feces can potentially alleviate shrimp tropomyosin-induced allergy by tolerogenic dendritic cell-dependent induction of Treg cells and alterations in gut microbiota (Fu et al., 2017). *Lactobacillus murinus* NBRC14221 can modulate gut microbiota, intestinal CD11c⁺ cell functionality, and the Th1/Th2 immune balance against food allergy (Huang et al., 2016a). Furthermore, a mixture of *Lactobacillus* (Lmix) colonization improves the gut epithelial barrier and reduces allergic sensitization to Bet v 1 through the increased production of circulating and secretory IgA and the regulatory cytokine transforming growth factor (**TGF**)- β (Kozakova et al., 2016).

In recent reports, probiotics can regulate immune balance, improve intestinal barrier function (Vanderpool et al., 2008), and change the structure of intestinal microbiota, resulting in an effective alleviation of allergic responses (Delcenserie et al., 2008; Kwon et al., 2010; Diesner et al., 2016). For example, *Lactobacillus fermentum* CECT5716 can prevent intestinal barrier dysfunction in newborn rats (Vanhaecke et al., 2017). *Bifidobacterium infantis* can relieve food allergies by modulating the microbial population, and specific gut bacterial changes can play a substantial role in disease progression (Yang et al., 2017). *Lactobacillus johnsonii* BS15 can exert beneficial effects on intestinal microbiota and enhance intestinal immunity in healthy broilers as well as those afflicted with subclinical necrotic enteritis (Wang et al., 2018).

The effects of probiotics on the prevention or treatment of allergic diseases, such as atopic dermatitis, allergic rhinitis (**AR**), and food allergy (Ouweland, 2007), have been studied. However, the underlying mechanisms are not fully understood, and different probiotics exhibit different capacities of immunomodulatory and regulating intestinal microbiota. The purpose

of our study was to explore the effects of *L. plantarum* ZDY2013, *L. plantarum* WLPL04, and *L. rhamnosus* GG on immune regulation, intestinal barrier function, and intestinal microbiota through a β -LG-induced mice model and consequently provide theoretical support for the function of probiotics in preventing and alleviating allergic diseases.

MATERIALS AND METHODS

Lactobacillus Strains and Culture Conditions

Lactobacillus plantarum ZDY2013 isolated from homemade fermented soybeans (Huang et al., 2015), *L. plantarum* WLPL04 isolated from human breast milk (Jiang et al., 2016), and *L. rhamnosus* GG were cultured under anaerobic conditions at 37°C in sterile de Man, Rogosa, Sharpe broth (Beijing Solarbio Science & Technology Co. Ltd., Beijing, China). Subsequently, lactobacilli were harvested, centrifuged at $5,000 \times g$ for 5 min at 25°C, and washed in sterile PBS. The concentration of each suspension was adjusted to 10^9 cfu/mL.

Animals and Housing Conditions

Female BALB/c mice aged 6 to 8 wk were purchased from Jinan Peng Yue Experimental Animal Breeding Co. Ltd. (Jinan, China). All mice used in our study were cared for in accordance with the *Guidelines for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (1996), and all experimental procedures were approved by the Animal Care Review Committee of Nanchang University. Animals were housed in an air-conditioned room ($23 \pm 2^\circ\text{C}$) with a 12-h light/12-h dark cycle. The mice were allowed free intake of food and purified water. All animal experiments were started after 1 wk of accumulation.

Experimental Design

Mice were randomly divided into a control group (**C**), sensitized group (**M**), and probiotics group (3 LAB). To induce an allergic response, mice were sensitized with 0.2 mL of 2 mg/mL allergen (1 mL of Freund's adjuvant and 1 mL of 2 mg/mL β -LG) by i.p. injection on d 7, 14, 21, and 28 and challenged twice, which was stimulated once every 30 min with β -LG (5 mg/mouse) on d 35. From d 7 to 35, mice in the 3 probiotic groups were administered 200 μL /mouse of lactobacilli [10^9 cfu/mL of *L. plantarum* ZDY2013 (**ZDY2013 group**), *L. plantarum* WLPL04 (**WLPL04 group**), or *L. rhamnosus* GG (**LGG group**)] in PBS by gavage, 4 times per week. Animals from the control and sensitized groups received 200 μL of sterile PBS (Figure 1).

Evaluation of Anaphylaxis Reaction

Anaphylaxis symptoms were scored by visually monitoring mice for 1 h after challenge. Anaphylactic symptoms were rated as 0 = no symptoms; 1 = hair up, scratching head and ear; 2 = reduced activity, swelling around the eyes and mouth; 3 = rest for more than a minute; 4 = loss of consciousness, no activity upon prodding; and 5 = convulsion, death (Li et al., 1999).

Detection of IgE and Cytokines by ELISA

The blood was taken from retro-orbital plexus on d 35 and then centrifuged at $3,000 \times g$ for 20 min at 4°C; the serum was then stored at -80°C until used. Levels of serum total IgE, IFN- γ , IL-4, IL-17A, and TGF- β were measured by ELISA according to the manufacturer's recommendations (NeoBioscience Co. Ltd., Shanghai, China).

Histological Analysis

Colon tissues of experimental mice were removed on d 35 and fixed in 10% phosphate-buffered formalin and embedded in paraffin. The colon sections were stained with hematoxylin and eosin for observation of intestinal mucosal integrity and lymphocytic infiltration. All analyses were performed under a microscope at 200 $\times$ magnification.

Quantification of Cytokines and Tight Junction Protein Gene Expression in the Colon

Quantitative analysis of *Il12a*, *Ifng*, *Il4*, *Il10*, *Tnf*, *Tbx21*, *Gata3*, *Rorc*, *Foxp3*, *Ocln*, *Cldn1*, and *Tjp1* in the mouse ileum tissues was performed by reverse

transcription quantitative PCR. Total RNA was isolated from the tissue samples of the mouse colon using Takara MiniBEST Universal RNA Extraction Kit (Takara, Shiga, Japan) according to the manufacturer's protocol (He et al., 2017). We reverse transcribed RNA (500 ng) into cDNA using the Takara PrimeScript™ RT reagent kit with gDNA Erase. Reverse transcription quantitative PCR was performed on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Waltham, MA) using the following program: 30 s at 95°C, followed by 40 cycles of 10 s at 95°C, and 1 min at 58 to 62°C. The sequences are listed in Table 1. The housekeeping gene *Gapdh* was included for normalization.

High-Throughput Sequencing and Bioinformatic Analysis

The 16S rRNA gene amplicon sequencing was performed on the Illumina MiSeq platform (Novogene Bioinformatics Technology Co. Ltd., Beijing, China) according to the previous report (Caporaso et al., 2011), which was used to determine sequences of the primers targeting the V4 hypervariable region of the bacterial 16S rRNA genes (515F = 5'-GTGCCAGCMGCCGCGGTAA-3', 806R = 5'-GGACTACNNGGTTATCTAAT-3'). Bioinformatic analysis of sequencing data were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso et al., 2011). Briefly, raw data from all samples were filtered and spliced to obtain high-quality clean reads. The effective tags were then clustered to operational taxonomic units (OTU) at an identity threshold of 97% similarity. Taxonomic ranks were assigned to OTU representative sequence using Ribosomal Database Project (RDP) Classifier v 2.2 (Cole et al., 2005). Finally, an OTU

Figure 1. Experimental design of the mice model of food allergy to β -LG. Female BALB/c mice aged 6 to 8 wk were sensitized 4 times by intraperitoneal injection with β -LG (400 μ g/mouse) plus Freund's adjuvant and challenged twice with β -LG (5 mg/mouse). In parallel, mice were orally supplemented with a probiotic strain or PBS for 4 wk.

Table 1. Information of gene primers in the study

Gene	Direction	Sequence (5'-3')
<i>Gapdh</i>	Forward	CTGAACGGGAAGCTCACTGG
	Reverse	ATGCCTGCTTACCACCTTC
<i>Tbx21</i>	Forward	CCACCTGTTGTGGTCCAAGT
	Reverse	CATTCGCCGTCCTTGCTTAG
<i>Gata3</i>	Forward	AAGCTCAGTATCCGCTGACG
	Reverse	GTTTCCGTAGTAGGACGGGAC
<i>Il12a</i>	Forward	AGTGACATGTGGAATGGCGT
	Reverse	GGCGGGTCTGGTTTGATGAT
<i>Il10</i>	Forward	TTTGAATTCCCTGGGTGAGAA
	Reverse	GCTCCACTGCCTTGCTCTTATT
<i>Ifng</i>	Forward	GATCCTTTGGACCCCTGACTT
	Reverse	TGACTGTGCCGTGGCAGTAA
<i>Il4</i>	Forward	TCAACCCCCAGCTAGTTGTC
	Reverse	TGTTCTTCGTTGCTGTGAGG
<i>Tnf</i>	Forward	AGCCACGTCGTAGCAAACC
	Reverse	TACAACCCATCGGCTGGCAC
<i>Rorc</i>	Forward	CCGCTGAGAGGGCTTCAC
	Reverse	TGCAGGAGTAGGCCACATTACA
<i>Foxp3</i>	Forward	CAGCTGCCTACAGTGCCCCCTAG
	Reverse	CATTTGCCAGCAGTGGGTAG
<i>Ocln</i>	Forward	GCTGTGATGTGTGTTGACTG
	Reverse	CAAGGAGGTCCATCTGGCAG
<i>Cldn1</i>	Forward	GGGGACAACATCGTGACCG
	Reverse	AGGAGTCGAAGACTTTGCACT
<i>Tjp1</i>	Forward	GATCCCTGTAAGTCACCCAGA
	Reverse	CTCCCTGCTTGCACTCCTATC

table and a phylogenetic tree were produced according to α diversity (within a sample) and the β diversity (between samples) analysis.

Statistical Analysis

Statistical significance was determined by GraphPad Prism5 (GraphPad Software, La Jolla, CA). All data are expressed as the mean $\pm$ standard error of the means; P -values < 0.05 were considered statistically significant.

RESULTS

Lactobacillus Reduces β -LG-Induced Symptoms in a Mouse Model

β -Lactoglobulin-induced food allergy symptoms were evaluated and scored for anaphylactic response. Severe symptoms of β -LG-induced food allergy were observed in the sensitized group (anaphylactic response, 2.67 ± 0.82 points). By contrast, the ZDY2013 and LGG groups showed considerable suppression of anaphylactic response (1.33 ± 0.82 and 1.25 ± 0.71 points, respectively, Figure 2A). In addition, the level of total IgE in the sensitized group increased by 15.95 ng/mL compared with that of the control group, but decreased by 4.43 and 3.01 ng/mL in the ZDY2013 and LGG groups, respectively (Figure 2B). The administration of ZDY2013 and LGG obviously inhibited β -LG-induced

food allergy, suggesting a protective effect of *Lactobacillus* on food allergy in mice.

Effect of *Lactobacillus* Supplementation on T Cell Balance

We measured the cytokine in serum from mice with β -LG-induced food allergy. Compared with the sensitized group, groups administered WLPL04 and LGG showed increased secretion of IFN- γ in serum ($P < 0.05$; Figure 3A). Interleukin-4, which is the major inducer of B-cell switching to IgE production (Akdis et al., 2011), was significantly decreased in groups treated with LGG ($P < 0.05$; Figure 3B). Transforming growth factor- β , which is expressed by regulated dendritic cells, can directly mediate the conversion of T cells into Treg cells and induce immune tolerance (Kwon et al., 2010), and is significantly increased with ZDY2013 ($P < 0.01$) and LGG ($P < 0.05$; Figure 3D). However, no evident changes of IL-17A (Figure 3C) were observed in the 3 *Lactobacillus* groups.

Effect of *Lactobacillus* Administration on Th1/Th2/Th17/Treg Balance in the Colon

The intestinal profile of the immune response induced by the administration of the 3 *Lactobacillus* strains was evaluated by analyzing the mRNA expression of *Ifng*, *Il12*, *Il4*, and *Il10* (Figure 4). To further explore the

effects of different *Lactobacillus* strains on Th responses in the colon, we measured the mRNA expression of Th-specific transcription factors (Figure 5). The expression of *Tbx21* (Th1; $P < 0.001$), *Ifng*, *Il12*, and *Foxp3* (Treg; $P < 0.001$) was significantly decreased in the food allergy group compared with the control group, whereas the expression of *Gata3* (Th2; $P < 0.001$) and *Il4* increased considerably. Supplementation with ZDY2013 induced a downregulation of the allergen-induced Th2 (*Gata3*; $P < 0.001$) and Th17 (*Rorc*; $P < 0.001$) responses but increased T-bet (*Tbx21*; $P < 0.01$) and IFN- γ (*Ifng*) at the colon. The expression levels of *Gata3* ($P < 0.001$) and *Il4* were significantly reduced with the administration of WLPL04, whereas the expression of *Foxp3* ($P < 0.001$) remarkably increased. With LGG treatment, the expression levels of *Tbx21* (T-bet; $P < 0.001$), *Foxp3* ($P < 0.05$), *Ifng*, *Il12*, and *Il10* were significantly increased. However, the expression of *Gata3* ($P < 0.001$) and *Il4* substantially decreased. To determine the extent of Th response skewing in the colon, we calculated the ratios for *Gata3/Tbx21* (Th2/Th1) and *Foxp3/Rorc* (Treg/Th17) mRNA expression. We observed a Th2-skewed immune response in the sensitive groups as represented by a significant increase in *Gata3/Tbx21* ratio compared with the control group; ZDY2013, WLPL04, and LGG reduced this ratio.

Effects of the Colonization of 3 *Lactobacillus* Strains on Intestinal Barrier Function

Intestinal barrier function is crucial in maintaining homeostasis; the destruction of barrier function can lead to intestinal disorders, such as leaky gut syndrome, inflammatory bowel disease, and food allergy. Although the change in intestinal permeability by the destruction of barrier function is considered a risk factor in the development of food allergy, this hypothesis has yet to be substantiated with experimental data (Chahine and Bahna, 2010). Therefore, we explored whether *Lactobacillus* can regulate intestinal barrier function in our mouse model of food allergy by measuring the tight junction proteins claudin-1 (*Cldn1*), occludin-1 (*Ocln*), and ZO-1 (*Tjp1*) in the colon (Figure 6). We found decreased transcriptional expression of claudin-1 and occludin-1 in β -LG-induced mice, whereas the expression levels of claudin-1 and occludin-1 were restored after administration of ZDY2013 and the expression level of occludin-1 increased after supplementation with LGG. However, no changes in the relative levels of ZO-1 mRNA expression were observed with probiotic strain treatment. These results suggest that enhancement of tight junction protein mRNA expression might contribute to the attenuation of food allergy.

Figure 2. *Lactobacillus* reduced β -LG-induced symptoms in a mouse model of food allergy: (A) anaphylactic response and (B) level of total IgE. Mice were treated from d 7 to 35 with 200 μ L of *L. plantarum* ZDY2013 (ZDY2013 group), *L. plantarum* WLPL04 (WLPL04 group), or *L. rhamnosus* GG (LGG group). The results are expressed as the mean $\pm$ SD; P -values determined by one-way ANOVA followed by Tukey's post hoc test. Significant differences of the sensitized (M) group versus the control (C) group are indicated as ### $P < 0.001$. Significant differences of ZDY2013, WLPL04, and LGG group versus M group are indicated as * $P < 0.05$, ** $P < 0.01$.

Three *Lactobacillus* Strains Reduce Histological Changes in Allergic Mice

Hematoxylin and eosin staining visualized histological changes, such as mucosal or crypt damage and lymphocyte infiltration. Here, the sensitive group showed that intestinal epithelium and goblet cells were severely damaged in comparison with the control group. By contrast, the 3 *Lactobacillus* treatment group showed similar results to those of control mice (Figure 7).

Three *Lactobacilli* Modulate Fecal Microbiota Composition in β -LG-Induced Mice

We further performed α and β analyses of fecal microbiota composition of the 5 groups. The rarefaction curve is shown in Figure 8A, whereas the intestinal bacterial composition of the 5 groups is shown in Figure 8B. Four phyla (in descending order: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*) were the most dominant; among 10 bacterial phyla,

Figure 3. Effect of probiotic treatment on ex vivo cytokine secretion of serum from mice with β -LG-induced food allergy. Serum was collected after oral challenge of β -LG twice. Cytokine secretions were determined by ELISA. Mice were treated from d 7 to 35 with 200 μ L of *L. plantarum* ZDY2013 (ZDY2013 group), *L. plantarum* WLPL04 (WLPL04 group), or *L. rhamnosus* GG (LGG group). Results are expressed as means $\pm$ SD for (A) IFN- γ , (B) IL-4, (C) IL-17A, and (D) transforming growth factor (TGF)- β . Significant differences of the sensitized (M) group versus the control (C) group are indicated as ### P < 0.001. Significant differences of ZDY2013, WLPL04 and LGG group versus M group are indicated as * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 4. Effect of probiotic treatment on the colon tissue of the immune response. Colon gene mRNA expression was quantified by reverse transcription quantitative PCR. Mice were treated from d 7 to 35 with 200 μ L of *L. plantarum* ZDY2013 (ZDY2013 group), *L. plantarum* WLPL04 (WLPL04 group), or *L. rhamnosus* GG (LGG group). Results are expressed as means $\pm$ SD for (A) *Ifng*, (B) *Il12*, (C) *Il4*, and (D) *Il10*. Significant differences of the sensitized (M) group versus the control (C) group are indicated as ### P < 0.001. Significant differences of ZDY2013, WLPL04 and LGG group versus M group are indicated as * P < 0.05, ** P < 0.01, *** P < 0.001.

Firmicutes and *Bacteroidetes* accounted for an average of 87.76% of each sample's OTU. The changes in fecal microbiota across groups were examined by using principal coordinates analysis (PCoA) and unweighted pair-group method with arithmetic means (UPGMA) cluster. Similar PCoA distances were observed among ZDY2013 group and control groups compared with sensitized group (Figure 8C); UPGMA cluster analysis showed that ZDY2013 significantly changed the intestinal microbiota compared with β -LG-induced sensitized mice (Figure 8D).

Taxonomic assignment revealed marked differences between the control group and sensitized group (Table 2). At the family level, 4 bacterial families (*Lactobacillaceae*, *Enterococcaceae*, *Erysipelotrichaceae*, and *Clostridiales* vadin BB60 group) were much more abundant in the sensitized group. We identified 6 bacterial genera, including *Lactobacillus*, *Enterococcus*, *Rikenella*, *Ruminiclostridium*, unidentified *Ruminococcaceae*, and unidentified *Lachnospiraceae*. We found that the abundance at the genus level of 4 taxa (*Rikenella*, *Ruminiclostridium*, unidentified *Ruminococcaceae*, and un-

Figure 5. Effect of probiotics on relative expression of transcriptional regulation factors in colonic tissue. Colon gene mRNA expression was quantified by quantitative PCR. Mice were treated from d 7 to 35 with 200 μ L of *L. plantarum* ZDY2013 (ZDY2013 group), *L. plantarum* WLPL04 (WLPL04 group), or *L. rhamnosus* GG (LGG group). Results are expressed as means $\pm$ SD for (A) *Tbx21*, (B) *Gata3*, (C) *Rorc*, (D) *Foxp3*, and (E) *Gata3/Tbx21* and *Foxp3/Rorc*. Significant differences of the sensitized (M) group versus the control (C) group are indicated as ### P < 0.001. Significant differences of ZDY2013, WLPL04, and LGG group versus M group are indicated as * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 6. Effect of probiotics on relative expression of intestinal barrier function in colonic tissue. Colon gene mRNA expression was quantified by quantitative PCR. Results are expressed as means $\pm$ SD for (A) claudin-1 (*Cldn1*), (B) occludin-1 (*Ocln*), and (C) zonula occludens-1 (*Tjp1*). Significant differences of the sensitized (M) group versus the control (C) group are indicated as ### $P < 0.001$. Significant differences of ZDY2013, WLPL04, and LGG group versus M group are indicated as ** $P < 0.01$, *** $P < 0.001$.

identified *Lachnospiraceae*) decreased in the ZDY2013 (Table 3) and WLPL04 groups (Table 4). Supplemental Figure S1 (<https://doi.org/10.3168/jds.2018-15683>) shows Shannon and Simpson indices of α -diversity.

DISCUSSION

Probiotics play a significant role in regulating allergic reactions. A probiotic mixture can be effective in redirecting allergen-specific Th2-polarized immune responses toward Th1-Treg responses and in protection against anaphylactic reactions induced by the allergen of food allergy (Schiavi et al., 2011). *Lactobacillus rhamnosus* CGMCC 1.3724 and peanut oral immunotherapy effectively prevented peanut allergy in clinical trials (Tang et al., 2015). A *Bifidobacteria* mixture can notably improve AR symptoms and quality of life in children with pollen-induced AR and intermittent asthma (Miraglia Del Giudice et al., 2017). Although the administration of probiotics is proven an effective tool for alleviating allergies in experiments, different probiotics enact different mechanisms of action to allergic reactions. We explored the effect of 3 strains of *Lactobacillus* on β -LG-induced allergy; among these strains, *L. plantarum* ZDY2013 and *L. plantarum* WLPL04 performed numerous probiotic functions, such as acid resistance and antiadhesion of pathogens (Huang et al., 2016b; Jiang et al., 2016) through a mouse model. Supplementation with the ZDY2013 and LGG strains led to a considerable decrease in serum IgE, which is associated with Th2 and the important indicator in allergy reactions. Meanwhile, allergic reactions of mice, such as erection of hairs, edema around the eyes and mouth, and other symptoms, were substantially alleviated, as observed previously (Zhang et al., 2017).

Transcriptional regulatory factors such as T-bet, GATA-3, ROR- γ t, and Foxp-3 are responsible for helper T cell subsets differentiated from naïve T cells (Lin et al., 2015). To investigate the reactive mechanism of the 3 *Lactobacillus* strains, we explored their effects on immunoregulation in the colonic tissues of sensitized mice. Mice administered 3 strains of *Lactobacillus* exhibited Th2 resistance, and the expression of GATA-3 markedly decreased; GATA-3 is a key molecule that regulates the balance in the ratio Th1 cells and Th2 cells and is considered to be indicative of the pathogenesis of allergic diseases (Bae et al., 2011). Meanwhile, ZDY2013 and LGG induced colon expression of T-bet and regulated the differentiation of Th1. Both WLPL04 and LGG induced the expression of Foxp-3, which is a main regulator of functional differentiation of Treg cells (Pellerin et al., 2014) that can induce and maintain immune tolerance by inhibiting Th2 differentiation (Cottrez et al., 2000). Reports showed that

Foxp-3 might inhibit allergic reaction by controlling the degranulation of mast cell (Gri et al., 2008).

Transforming growth factor- β and IL-10 are Treg-related cytokines that are essential for maintaining immune tolerance and reducing allergic reactions (Huang et al., 2010). Moreover, IFN- γ and IL-12 secreted by Th1 cells can inhibit the differentiation of Th2 cells and relieve allergies (Kirjavainen et al., 1999). Studies have reported that the use of probiotic strains can promote the differentiation of Th1 and Treg, which perform important roles as adjunctive treatments for allergic diseases. Studies showed that the LGG strain can induce the production of IFN- γ and IL-10 and relieve skin symptoms in IgE-sensitized infants with atopic dermatitis (Pessi et al., 2000; Pohjavuori et al., 2004). In the present study, TGF- β increased after oral administration with ZDY2013 and LGG, whereas IFN- γ considerably increased after oral administration

of WLPL04 and LGG. Therefore, we speculate that *L. plantarum* ZDY2013 and *L. rhamnosus* GG can alleviate allergies by inducing the Th1 or Treg cell response.

Numerous studies reported that imbalances in the body's immune system result from food allergies and increased intestinal permeability (Bae et al., 2016; Kozakova et al., 2016; Neau et al., 2016). The increase in intestinal permeability and disruption of intestinal barrier function are known risk factors for developing food allergy (Chahine and Bahna, 2010). Studies have shown that food allergies increase intestinal permeability not only in children but also in asthma patients (Järvinen et al., 2013). In our study, the mRNA expression of tight junction proteins (e.g., claudin-1 and occludin-1) significantly increased after treatment with ZDY2013. These results were consistent with the findings of Nébot-Vivinus et al. (2014), which showed that multispecies probiotics can enhance occludin-1 expression to prevent

Figure 7. Effect of probiotics on colonic histopathology in mice model with β -LG-induced food allergy. Mice were treated from d 7 to 35 with 200 μ L of *L. plantarum* ZDY2013 (ZDY2013 group), *L. plantarum* WLPL04 (WLPL04 group), or *L. rhamnosus* GG (LGG group).

Figure 8. Probiotics modulates fecal microbiota composition in β -LG-sensitized mice. (A) Rarefaction curves of operational taxonomic units for each group. The center of the error bar is the arithmetic mean of the measured values, and the error bars represent the central value $\pm$ SD. (B) Principal coordinates analysis (PCoA) of the mice intestinal microflora of each group. (C) Microbial composition of mice feces of each group in phylum level. (D) Unweighted pair-group method with arithmetic means clustering of the mice intestinal microflora of each group. *L. plantarum* ZDY2013 (ZDY group), *L. rhamnosus* GG (LGG group), *L. plantarum* WLPL04 (WLPL group), control (C group), and mock (M group).

Table 2. Effects of β -LG-induced allergies on intestinal microbiota of mice at family and genus level¹

Item	M		C		P-value
	Mean	SD	Mean	SD	
Family					
<i>Lactobacillaceae</i>	0.32963	0.09257	0.09425	0.02302	0.012
<i>Enterococcaceae</i>	0.042508	0.01422	0.010466	0.00349	0.017
<i>Erysipelotrichaceae</i>	0.001977	0.00044	0.000906	0.0002	0.01
<i>Clostridiales</i> vadinBB60 group	0.003628	0.00053	0.001372	0.00119	0.024
Genus					
<i>Lactobacillus</i>	0.32963	0.09257	0.09425	0.02302	0.012
<i>Enterococcus</i>	0.042503	0.01422	0.010466	0.00349	0.017
<i>Rikenella</i>	0.007899	0.00282	0.000772	0.00037	0.014
<i>Ruminiclostridium</i>	0.014126	0.0066	0.003065	0.00489	0.039
Unidentified <i>Ruminococcaceae</i>	0.01052	0.005	0.001409	0.00116	0.033
Unidentified <i>Lachnospiraceae</i>	0.00925	0.00347	0.002535	0.00344	0.033
<i>Dorea</i>	0	0	0.000145	0.00018	0.206
<i>Ralstonia</i>	0.00023	0.0003	0.000032	0.00006	0.281

¹Mice were randomly divided into a control group (C) and sensitized group (M). To induce an allergic response, mice were sensitized with 0.2 mL of 2 mg/mL allergen (1 mL of Freund's adjuvant and 1 mL of 2 mg/mL β -LG) by i.p. injection on d 7, 14, 21, and 28, and challenged twice with β -LG (5 mg/mouse) on d 35. From d 7 to 35, mice from the control and sensitized groups received 200 μ L of sterile PBS.

the destruction of the gut barrier, and consequently prevented the penetration of harmful substances in the intestinal lumen and suppressed immune response.

The gastrointestinal microbiota of mammals exhibit 3 characteristics, namely high population density, wide diversity, and complexity of interactions (Zoetendal et al., 2004). The main functions of the microbiota include increasing the intestinal mucosal barrier, promoting immune system maturation, stimulating intestinal secretion of IgA, preventing the adhesion of pathogenic microorganisms, and participating in the formation

of immune tolerance (Chow et al., 2010). Numerous studies have illustrated that intestinal microbes are closely related to allergic diseases (Salzman et al., 2010; Diesner et al., 2016) and the increasing susceptibility to allergic and autoimmune diseases (Cho and Blaser, 2012). Kirjavainen and Gibson (1999) reported that intestinal microorganisms can downregulate allergic inflammation by counterbalancing type Th2 cell responses and enhancing antigen exclusion through IgA response. Furthermore, recent studies suggested that microbiota composition could influence immune-

Table 3. The effect of *Lactobacillus plantarum* ZDY2013 (ZDY2013) on intestinal microbiota of mice at family and genus level¹

Item	M		ZDY2013		P-value
	Mean	SD	Mean	SD	
Family					
<i>Rikenellaceae</i>	0.03702	0.0051	0.018001	0.01132	0.011
<i>Coriobacteriaceae</i>	0.010343	0.00271	0.004807	0.00187	0.018
<i>Desulfovibrionaceae</i>	0.014073	0.00606	0.004566	0.0016	0.047
Genus					
<i>Rikenella</i>	0.007899	0.00282	0.000804	0.00057	0.013
<i>Enterorhabdus</i>	0.008092	0.00196	0.004105	0.02642	0.026
<i>Desulfovibrio</i>	0.012781	0.00456	0.00396	0.00111	0.027
<i>Lachnospiraceae</i> UCG-006	0.003403	0.00065	0.000938	0.0006	0.001
<i>Ruminiclostridium</i>	0.014126	0.0066	0.001876	0.00119	0.032
Unidentified <i>Ruminococcaceae</i>	0.01052	0.00502	0.00224	0.00289	0.037
Unidentified <i>Lachnospiraceae</i>	0.00925	0.00347	0.003896	0.00152	0.046
<i>Acetatifactor</i>	0.002621	0.00109	0.000214	0.00016	0.020
<i>Dorea</i>	0	0	0.000557	0.00063	0.176
<i>Ralstonia</i>	0.00023	0.0003	0.000182	0.00019	0.309

¹Mice in the sensitized group (M) group were sensitized with 0.2 mL of 2 mg/mL allergen (1 mL of Freund's adjuvant and 1 mL of 2 mg/mL β -LG) by i.p. injection on d 7, 14, 21, and 28, and challenged twice with β -LG (5 mg/mouse) on d 35. From d 7 to 35, mice in the ZDY2013 group were administered 200 μ L/mouse of *L. plantarum* ZDY2013 by gavage, 4 times per week.

Table 4. The effect of *Lactobacillus plantarum* WLPL04 on intestinal microbiota of mice at family and genus level¹

Item	M		WLPL04		P-value
	Mean	SD	Mean	SD	
Family					
<i>Ruminococcaceae</i>	0.083558	0.0188	0.031152	0.00807	0.007
Genus					
<i>Rikenella</i>	0.007899	0.00282	0.00306	0.00053	0.039
<i>Lachnospiraceae</i> UCG-006	0.003403	0.00065	0.000798	0.00079	0.003
<i>Ruminiclostridium</i>	0.014126	0.0066	0.002069	0.00146	0.032
Unidentified <i>Ruminococcaceae</i>	0.01052	0.00502	0.001447	0.00089	0.034
Unidentified <i>Lachnospiraceae</i>	0.00925	0.00347	0.002771	0.00228	0.025
<i>Parabacteroides</i>	0.000932	0.00047	0.006131	0.00307	0.041
<i>Acetatifactor</i>	0.002621	0.00109	0.000391	0.0004	0.020
<i>Anaerotruncus</i>	0.003537	0.00104	0.001645	0.00075	0.029
<i>Dorea</i>	0	0	0.000096	0.00012	0.219
<i>Ralstonia</i>	0.00023	0.0003	0.000343	0.00056	0.739

¹Mice in the sensitized group (M) group were sensitized with 0.2 mL of 2 mg/mL allergen (1 mL of Freund's adjuvant and 1 mL of 2 mg/mL β -LG) by i.p. injection on d 7, 14, 21, and 28, and challenged twice with β -LG (5 mg/mouse) on d 35. From d 7 to 35, mice in the WLPL04 group were administered 200 μ L/mouse of *L. plantarum* WLPL04 by gavage, 4 times per week.

mediated diseases, such as autoimmunity and allergy (Geuking et al., 2014).

Probiotics, which can regulate intestinal microbiota caused by allergies, are essential for preventing or alleviating allergic diseases. Kim et al. (2005) reported that *Bifidobacterium bifidum* BGN4 and *Lactobacillus casei* 911 may be useful probiotic bacteria for preventing allergy in mouse models. Harata et al. (2017) reported that *L. rhamnosus* GG (ATCC53103) and *Lactobacillus gasseri* TMC0356 can modulate gut microbiota to cure allergy caused by Japanese cedar pollinosis. Consistently, we found that lactobacilli can regulate the intestinal microbiota composition of β -LG-sensitized mice. A considerable increase in the Simpson index was observed in the sensitized group, and gut barrier function might have been impaired in sensitized mice. These phenomena possibly caused intestinal microecological imbalance, as identified through sequencing of 16S rRNA gene, which was used to determine the effect of 3 strains of *Lactobacillus* on β -LG-induced sensitized mice. Moreover, beneficial bacteria inherent in the intestinal tract may substantially decrease and some opportunistic bacteria may multiply. This result is similar to the study of Berni Canani et al. (2016), which showed that the microbiota of cow milk allergy infants is more diverse than that of healthy controls. The PCoA and UPGMA cluster analysis showed marked difference in microbiota between the ZDY2013 group and the sensitized group; these findings indicate that *Lactobacillus* markedly affects the intestinal microbiota structure of sensitized mice. *Ruminococcaceae* and *Lachnospiraceae* showed a significant increase in cow milk allergy guts (Berni Canani et al., 2016). *Ruminococcus* is positively correlated

with inflammation-related diseases and *Lachnospiraceae* is strongly correlated in obese patients (Meehan and Beiko, 2014); we consistently found that ZDY2013 and WLPL04 considerably decreased the abundance of these 2 species after oral gavage through *t*-test analysis. *Dorea* can be significantly reduced in the gut microbiome of food-allergic infants and is closely related to the balance of T cells, thus suggesting its potential in preventing allergies (Savage et al., 2018). In addition, some literature reported that *Dorea* could bring about major changes in the intestinal microbiota after adding lactic acid bacteria (Chen et al., 2014; Nishida et al., 2017). By contrast, *Ralstonia* exerts proinflammatory effects in patients with Parkinson's syndrome (Keshavarzian et al., 2015) and is enriched in chronic rhinitis (Ramakrishnan et al., 2015), indicating its destructive effect on the host immune system. In our research, we did not detect *Dorea* in the gut microbiota of sensitized mice; therefore, we speculated that probiotics may affect the production of intestinal metabolites, such as short-chain fatty acids, by regulating the structure of the intestinal microbiota and promoting the maturation of the immune system.

CONCLUSIONS

In conclusion, the possible mechanism of the 3 *Lactobacillus* strains that alleviated β -LG-induced allergy involved 3 main aspects. All 3 strains of *Lactobacillus* regulated intestinal immune balance; in particular, LGG and ZDY2013 inhibited Th2 responses by inducing differentiation of Treg cells or Th1 cells. However, LGG and ZDY2013 considerably increased the relative

expression of tight junction proteins, enhanced intestinal barrier function, and relieved allergies. In addition, ZDY2013 and WLPL04 regulated the structure of intestinal microbiota to affect allergies.

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