Preventive Effects of a Probiotic Mixture in an Ovalbumin-Induced Food Allergy Model

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Although there has been a steady increase in the prevalence of food allergies worldwide in recent decades, no effective therapeutic strategies have been developed. Modulation of the gut microbiota composition and/or function through probiotics has been highlighted as a promising target for protection against food allergies. In this study, we aimed to investigate the allergy-reducing effects of a probiotic mixture (P5: Lactococcus lactis KF140, Pediococcus pentosaceus KF159, Lactobacillus pentosus KF340, Lactobacillus puracasei 698, and Bacillus amyloliquefaciens 26N) in mice with ovalbumin (OVA)-induced food allergy. Administration of P5 significantly suppressed the oral OVA challenge-induced anaphylactic response and rectal temperature decline, and reduced diarrhea symptoms. Moreover, P5 also significantly inhibited the secretion of IgE, Th2 cytokines (interleukin (IL)-4, IL-5, IL-10, and IL-13), and Th17 cytokines (IL-17), which were increased in mice with OVA-induced food allergy, and induced generation of CD4+Foxp3+ regulatory T cells. These results revealed that P5 may have applications as a preventive agent against food allergy.

Keywords: Food allergy, probiotics, Th1/Th2 balance, IgE suppression, Treg induction

Introduction

Allergic diseases are caused by hypersensitivity of the immune system to foreign substances (allergens), such as drugs, insect products, pollen, or food ingredients. These diseases include hay fever, food allergies, atopic dermatitis, allergic asthma, and anaphylaxis. Food allergies, which are caused by food allergens, such as ovalbumin (OVA) and ovomucoid from eggs, casein and beta-lactoglobulin from milk, tropomysinos from shrimp, peanut agglutinin from peanuts, and gliadin from wheat [1], occur when the immune system reacts to food allergens after ingestion. Ingested allergens permeate into the gastrointestinal tract via the paracellular diffusion pathway [2] and are then taken up by antigen-presenting cells, which present selected peptides via the major histocompatibility complex class II to activate naïve T cell differentiation into Th cells, resulting in the activation of Th2 cells. Th2 cells produce cytokines such as interleukin (IL)-4, IL-5, and IL-13 and induce the generation of antigen-specific immunoglobulin E (IgE) from B cells. IgE then binds to a high-affinity IgE receptor (FceRI) on mast cells [3]. A second exposure to the allergen leads to crosslinking between IgE and the allergen [4]. Allergen-induced mast cells exhibit degranulation, releasing a variety of inflammatory mediators that can elevate allergic symptoms, such as itchiness, tongue swelling, hives, trouble breathing, pruritus, abdominal pain, vomiting, diarrhea, urticaria, hypotension, and lethal anaphylactic shock [5, 6]. IgE-mediated food allergy has been estimated to affect 1–3% of the adult population and up to 3–7% of children [7–9]. Although there has been a steady increase in the prevalence of food allergy worldwide in recent decades [10], no effective therapeutic strategies have been developed, and the primary management of
food allergies consists of strictly avoiding relevant allergens [11]. Recently, probiotics have been highlighted as a promising target of protection against allergic disease. Probiotics are defined as live microorganisms that, when administered at adequate amounts, confer health benefits to their human hosts [12]. During the last decade, a growing body of evidence has accumulated to show that probiotics alleviate allergic disease through the induction of immune tolerance [13], the modulation of intestinal immunity [14], the enhancement of the gut mucosal barrier [15], and the production of substances such as short-chain fatty acids [16]. In food allergy, a number of previous studies have also suggested that specific probiotic strains have the ability to alleviate food allergies. For example, Lactobacillus casei Shirori prevents food allergic symptoms (rectal temperature, IgE, IL-4, and IL-5) and inhibits dendritic cell (DC) maturation and T cell activation [17].

Additionally, oral administration of Lactococcus lactis NCC 2287 for 7 weeks in sensitized mice reduced allergic manifestations and production of IL-12, C-C motif chemokine ligand (CCL) 11, and CCL17 [18]. Lactobacillus plantarum NRIC0380 alleviates allergic symptoms by inducing Tregs and controlling the Th1/Th2 balance [19]. Schiavi et al. [20] reported that oral administration of a probiotic mixture containing eight different strains (Lactobacillus acidophilus, Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus casei, Lactobacillus plantarum, Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium breve, and Streptococcus salivarius subsp. thermophilus) could have beneficial effects in OVA-sensitized mice, with a reduction in symptom severity and downregulation of Th2 cytokine mRNA expression. Moreover, Yang et al. [21] reported that Lactobacillus paracasei L9 reduces β-lactoglobulin allergic sensitization by inducing regulatory DCs and manipulating the balance of Th1/Th2 responses. These studies suggested that the main inhibitory effects of probiotics in the food allergic response involve restoration of the Th1/Th2 immune balance and acquisition of oral tolerance by inducing regulatory cells, such as Tregs, regulatory B cells, and tolerogenic DCs.

In a preliminary study searching for anti-allergic probiotics, we found five probiotics (Lactobacillus pentosus KS 340, Lactococcus lactis KF140, Bacillus amyloliquefaciens 26N, Lactobacillus paracasei 698, and Pediococcus pentosaceus KF159 isolated from Korea traditional foods) that possess the IL-4 suppressive effect and Treg inducing effect via in vitro test. Therefore, in this study, we aimed to investigate the effects of a mixture of these five probiotics on alleviation of allergic symptoms, including diarrhea and anaphylactic response, in an OVA-induced food allergy model.

### Materials and Methods

**Materials**

De Man, Rogosa, and Sharpe (MRS) and tryptic soy broth (TSB) media were purchased from Difco (USA). RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, and Dulbecco’s phosphate-buffered saline (D-PBS) were purchased from Gibco (USA). OVA (grade VI) and red blood cell lysis buffer were purchased from Sigma-Aldrich (USA).

**Isolation and Preparation of Probiotics**

Lactobacillus pentosus KF340 (KCCM11675P), Lactobacillus paracasei 698 (KFCC11569P), Lactococcus lactis KF140 (KCCM11673P), Pediococcus pentosaceus KF159 (KCCM11674P), and Bacillus subtilis 26N (KCCM11287P) were isolated from Korean traditional fermented foods and identified by 16S rRNA gene sequence analysis. Lactobacillus pentosus KF340, Lactobacillus paracasei 698, Lactococcus lactis KF140, and Pediococcus pentosaceus KF159, isolated from kimchi (Korean traditional fermented cabbage), were cultured in MRS medium at 37°C for 24 h. Bacillus amyloliquefaciens 26N, isolated from deonjang (Korean traditional fermented soybean paste), was cultured in TSB medium at 37°C for 24 h. For preparation of live probiotics, cultured cells were collected by centrifugation at 5,000 × g for 15 min and then washed twice with PBS. The pellet cells were lyophilized and stored at −80°C for further study. The probiotic mixture (P5) contained 1 × 10^7 CFU of each of the five strains.

**Immunoregulatory Effects of P5 Ex Vivo**

To evaluate the modulation of the Th1/Th2 balance by P5, BALB/c mice (5 weeks old, female, 18–20 g) were sensitized with 10 μg of OVA adsorbed in 1 mg/ml Imject Alum (Pierce, USA) by intraperitoneal (i.p.) injection on days 0 and 14. After the second OVA injection (day 28), the mesenteric lymph nodes (mLNs) were collected from OVA-sensitized mice and placed in ice-cold RPMI containing 10% FBS, antibiotics, and 2-mercaptoethanol. The mLNs were ground with the sterile flat bottom of a syringe piston to homogenize the sample, and homogenized single cells were then washed twice with RPMI medium and centrifuged (415 × g, 5 min). For cytokine analysis, the cells were adjusted to 5 × 10^6 cells/ml in RPMI medium using the trypsin blue exclusion method. In 24-well plates, the cells were co-cultured with 1 × 10^7 CFU of the probiotic mixture (P5) in the presence or absence of OVA (100 μg/ml/well). The plates were incubated at 37°C in a humidified incubator with 5% CO₂ for 72 h. After incubation, the supernatants were collected to analyze IL-4 and IFN-γ. To evaluate the anti-inflammatory and CD4+Foxp3-induction effects of P5, mLNs were obtained from normal BALB/c mice as described above. For analysis of cytokines (IL-10 and IL-12), the cells were adjusted to 5 × 10^6 cells/ml in RPMI medium using the trypsin blue exclusion method. The cells were then co-cultured with 1 × 10^7 CFU of the probiotic mixture (P5) in the presence of gentamicin (Gibco, USA) at 37°C in a humidified incubator with...
5% CO\textsubscript{2} for 72 h. For CD4+Foxp3+ analysis, mLN-derived lymphocytes (1 × 10\textsuperscript{6} cells/well) from BALB/c mice were cultured in 96-well plates in the presence of antigen (100 μg/ml OVA) and T-cell receptor stimuli (10 μg/ml plate-bound anti-CD3 monoclonal antibodies (17A2) and 2 μg/ml soluble anti-CD28 monoclonal antibodies (37.51); Biolegend, USA). At that time, the cells were treated with 1 × 10\textsuperscript{7} CFU of the probiotic mixture (P5) and incubated at 37°C in a humidified incubator with 5% CO\textsubscript{2} for 72 h.

Experimental Animals and Feeds
Twenty-eight BALB/c mice (5 weeks old, female, 18–20 g) were purchased from Orient Bio Inc. (Korea). After acclimating for 1 week, the mice were randomly divided into normal (naïve), untreated (sham), dexamethasone-treated (positive), and P5-treated groups. All mice were maintained in the same room under conventional conditions with a regular 12-h light/dark cycle and temperature and relative humidity maintained at 23 ± 2°C and 50% ± 5%, respectively. Mice were allowed free access to food and water. All animal experimental procedures were reviewed and approved by the Ethics Committee of the Experimental Animal Research Laboratory, Korea Food Research Institute (Approval Code KFRI-224).

Induction of Experimental Food Allergies by Oral Administration of OVA
To evaluate the effects of P5 on the OVA-induced allergic response in vivo, mice were sensitized with 20 μg of OVA adsorbed on 2 mg/ml Imject Alum (Pierce) by i.p. injection on days 0 and 14. From day 28, mice were orally challenged with 50 mg of OVA in saline every 3 days, for a total of five times. To investigate the preventive effects of P5, lyophilized P5 was suspended in saline and orally administered (5 × 10\textsuperscript{8} CFU/head) every day from days 28 to 40 (Fig. 1). Following the final challenge, diarrhea, anaphylaxis, and rectal temperature were measured as indexes of food allergy symptoms. Diarrhea and anaphylaxis were observed by visually monitoring the mice for 60 min after challenge. The following criteria were used for scoring diarrhea: 0, normal stools; 1, a few wet and unformed stools; 2, a number of wet and unformed stools with moderate perianal staining of the coat; and 3, severe and watery stools with severe perianal staining of the coat. The following criteria were used for anaphylactic responses: 0, no symptoms; 1, reduced activity, trembling of limbs; 2, loss of consciousness, no activity upon prodding; 3, convulsions; and 4, death. Rectal temperature was measured using a Thermalert TH5 monitoring thermometer (Physitemp, USA). Body weight and water intake were carefully monitored and measured every week. On day 40, the mice were sacrificed, and sera, spleens, and mLNs were collected.

Preparation of Primary Cells and Cell Cultures
Spleens and mLNs were obtained from sacrificed mice on day 40 and ground with the sterile flat bottom of a syringe piston to homogenize the sample. Homogenized single cells were then collected and treated with red blood cell lysing buffer (Sigma-Aldrich) for spleens. The cells were then washed twice with RPMI medium and centrifuged (415 × g, 5 min). Subsequently, the cells were adjusted to 5 × 10\textsuperscript{6} cells/ml in RPMI medium using the trypan blue exclusion method. In 24-well plates, the cells were cultured in the presence of OVA (100 μg/ml/well). The plates were incubated at 37°C in a humidified incubator with 5% CO\textsubscript{2} for

**Fig. 1.** Timeline of experimental procedures.
Female BALB/c mice (5 weeks old) were divided into naïve (n = 7), Dexa (n = 5), sham (n = 8), and P5 groups (n = 8). To induce food allergy, mice were immunized with 20 μg of OVA and 2 mg of alum by intraperitoneal (i.p.) injection on day 0; from day 28, mice were orally challenged with 50 mg of OVA in saline every 3 days, for a total of five times. Simultaneously (from day 28), the naïve and sham groups were provided saline (0.9%), and the P5 group was provided 5 × 10\textsuperscript{6} CFU of the probiotic mixture. As a positive control, dexamethasone (Dexa; 1 mg/kg BW) was administered orally every day from days 28 to 40. Diarrhea score, anaphylaxis symptoms, and rectal temperature were measured for 1 h after challenge as an index of food allergy symptoms.
72 h. After incubation, the supernatants were collected for cytokine analysis.

Measurement of Serum Immunoglobulins

To measure immunoglobulin levels, serum samples from each group were obtained by collecting blood from the orbital venous plexus. An enzyme-linked immunosorbent assay (ELISA) protocol was used to determine total IgE and OVA-specific IgE antibody levels in the samples. Aliquots (200 μl/well) of IgE capture antibody for total IgE (BD Pharmingen, USA) or OVA (10 μg/ml dissolved in 0.1 mol/l NaHCO₃, pH 8.2) for OVA-specific IgE were coated on 96-microwell plates (Nunc; Thermo Fisher Scientific, Denmark). The plates were incubated overnight at 4°C and then carefully washed three times with washing buffer (0.5 g/l Tween 20 in PBS). Serum samples were diluted 1:5 for total IgE and OVA-specific IgE determination. One hundred microliters of diluted sample was added to each well. Total IgE and OVA-specific IgE levels were determined using biotin-conjugated rat anti-mouse (BD Pharmingen) according to the manufacturer’s protocols. The plates were read using an ELISA plate reader (Molecular Devices, Inc., USA) at 450 nm.

Measurement of Cytokines from Cultured Splenocytes and mLN-Derived Lymphocytes

A cytokine assay kit (BD Pharmingen) was used to measure cytokine levels (interferon-gamma (IFN-γ), IL-12, IL-4, IL-5, IL-13, IL-10, and IL-17) in the supernatant, according to the manufacturer’s protocols. Briefly, recovered supernatants and standard solutions were transferred to 96-well plates precoated with the appropriate monoclonal antibodies raised against each of the target cytokines and then incubated at room temperature for 2 h. After thorough washing with the wash buffer included in the kit, a horseradish peroxidase-conjugated secondary antibody was added to each well, and incubation was continued at room temperature for 2 h. After removal of the secondary antibody, the substrate solution for the enzymatic reaction was added, and the samples were incubated for another 30 min in the dark. The reaction was terminated by addition of stop solution, and the absorbance was measured at 450 nm using a microplate reader (Molecular Devices, Inc.)

Immunofluorescence Staining of CD4 and Foxp3

For CD4+Foxp3+ analysis, mLN-derived lymphocytes (1 × 10⁶ cells/well) from BALB/c mice were cultured in 96-well plates in the presence of antigen (100 μg/ml OVA) and T-cell receptor stimuli (10 μg/ml plate-bound anti-CD3 monoclonal antibodies (17A2) and 2 μg/ml soluble anti-CD28 monoclonal antibodies (37.51)). The cells were incubated at 37°C in a humidified incubator with 5% CO₂ for 72 h. To stain Foxp3, cells were washed in fluorescence-activated cell sorting (FACS) buffer (1% fetal calf serum and 0.1% NaN₃ in PBS) and incubated with anti-CD16/32 antibody (2.4G2) for 5 min at 4°C to block Fc receptors (clone 2.4G2) (BD Pharmingen). Surface CD4 molecules were stained with phycoerythrin (PE)-labeled anti-CD4 (clone H129.19) (BioLegend) for 30 min at 4°C. The cells were then fixed and permeabilized using Foxp3 fixation/permeabilization concentrate and diluent (BD Biosciences, USA) for 1 h at 4°C. After the Fc receptors were blocked for 15 min, the cells were stained using peridinin chlorophyll-a-protein-Cy5.5-labeled anti Foxp3 (eBioscience, USA) in FACs buffer at 4°C for 30 min. Data were acquired by flow cytometry using BD FACSCanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star, USA).

Statistical Analysis

Numerical data are expressed as the means ± standard deviations using SPSS software. The significance of differences between experimental conditions was analyzed using one-way analysis of variance (ANOVA) followed by Duncan’s multiple-range test. Results with p-values of less than 0.05 were considered significant.

Results

Effects of P5 on Cytokine Production Ex Vivo

To evaluate the modulatory effects of P5 on the Th1/Th2 balance, we examined the expression patterns of IFN-γ and IL-4 in mLN-derived lymphocytes (isolated from OVA-sensitized mice) co-cultured with P5 in the presence of OVA. As a result, co-culture with P5 increased IFN-γ (a Th1 cytokine) production and reduced IL-4 (a Th2 cytokine) production (Table 1). To evaluate the anti-inflammatory effects of P5, we measured the levels of the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokine IL-12 in mLN-derived lymphocytes (isolated from healthy mice) co-cultured with 1 × 10⁷ CFU of P5. Owing to the pleiotropic properties of IL-10 produced by many cell types, IL-10 secreted from DCs or Th2 cells in a Th2 skewing environment and a low pathogenic environment may act as a pro-inflammatory cytokine rather than an anti-inflammatory cytokine [22–26]. Thus, we used the mLN-derived lymphocytes isolated from healthy mice to evaluate the anti-inflammatory effect of P5. According to previous studies screening anti-inflammatory probiotics [27–29], there is a close correlation between the IL-10²/²/IL-12² phenotype ex vivo and in vivo immunomodulatory efficacy. Probiotics having the IL-10²/²/IL-12² phenotype (>10 for the IL-10/IL-12 ratio) ex vivo could suppress diverse immune disorders in vivo. In this study, co-culture with P5 induced the IL-10²/²/IL-12² phenotype (ratio of 23.94 for IL-10/IL-12; Table 1). These results suggested that P5 may alleviate allergic disease via modulation of the Th1/Th2 balance and anti-inflammatory properties.
Effects of Probiotics on Allergies

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Effects of P5 Administration on OVA-Induced Food Allergy Symptoms

Food allergy symptoms induced by OVA were evaluated and scored by the criteria for diarrhea, anaphylactic response, and rectal temperature (Fig. 2). Severe symptoms of OVA-induced food allergy, such as reduced mobility (score after 1 h, 2.29), increased explosive diarrhea (score after 1 h, 2.64), and decreased rectal temperature (Δtemp after 1 h, -9.74°C) were observed in the sham group. In contrast, the P5-treated group showed significant inhibition of OVA-induced anaphylactic response (score after 1 h, 0.53) and diarrhea (score after 1 h, 1.36). We also measured rectal temperature for 1 h after the fifth challenge with OVA. After 1 h, rectal temperature in the sham group was decrease to -9.74°C. However, P5 administration ameliorated the decrease in the rectal temperature by OVA to -3.5°C. These results demonstrated that P5 administration could alleviate food allergy symptoms through inhibition of

Table 1. Cytokine secretion in mLN-derived lymphocytes co-cultured with the probiotic mixture (P5) from OVA-sensitized mice and BALB/c mice.

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<th>OVA-sensitized mice</th>
<th>BALB/c mice</th>
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<tr>
<td></td>
<td>PBS</td>
<td>P5 (1 × 10⁷ CFU)</td>
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<tr>
<td></td>
<td>IFN-γ (pg/ml)</td>
<td>IL-10 (pg/ml)</td>
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<tr>
<td></td>
<td>2,648.76 ± 113.95</td>
<td>3,910.33 ± 13.81**</td>
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<td></td>
<td>IL-4 (pg/ml)</td>
<td>IL-12 (pg/ml)</td>
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<td>482.54 ± 14.54</td>
<td>235.26 ± 22.95**</td>
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<td>IL-12 (pg/ml)</td>
<td>IL-10/IL-12</td>
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<tr>
<td></td>
<td>ND</td>
<td>21.61 ± 4.26</td>
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<tr>
<td></td>
<td>Ratio (IFN-γ/IL-4)</td>
<td>Ratio (IL-10/IL-12)</td>
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<td>5.4</td>
<td>16.62</td>
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Each value is presented as the mean ± SD (n = 3). Bars represent significant differences from the PBS group at *p < 0.05 and **p < 0.01. Data were analyzed using ANOVA followed by Duncan’s multiple-range test.

Fig. 2. Effects of the probiotic mixture (P5) on OVA-induced food allergy symptoms.

Food allergy symptoms induced by OVA were evaluated and scored by criteria for diarrhea symptoms, anaphylactic response, and rectal temperature for 1 h after challenge with OVA. Diarrhea score (A), anaphylactic response score (B), and rectal temperature (C) were evaluated on the fifth challenge. Each value is presented as the mean ± SD (naïve, n = 7; dexta, n = 5; sham and P5, n = 8). Bars represent significant differences from the sham group at *p < 0.05 and **p < 0.01. Data were analyzed using ANOVA followed by Duncan’s multiple-range test.
systemic and intestinal allergic reactions.

Effects of P5 Administration on Total and Specific IgE Levels in Serum

To investigation the effects of oral administration of P5 on total IgE and specific IgE levels in an OVA-induced food allergy model, sera were obtained from each group after the fifth OVA challenge. Total IgE and OVA-specific IgE were dramatically increased in the sham group. However, total IgE and OVA-specific IgE in the P5-treated group were significantly reduced by about 28% and 49%, respectively, compared with that in the sham group (Fig. 3).

Effects of P5 on Cytokine Patterns in Splenocytes and mLN-Derived Lymphocytes from Mice with OVA-Induced Food Allergy

In order to determine the immunomodulatory effects of P5, we investigated the cytokine patterns in spleens and mLNs isolated from mice with OVA-induced food allergy. In splenocytes restimulated with OVA, all Th2-related cytokines (IL-4, IL-5, IL-10, and IL-13) showed increased levels following induction of food allergy and were significantly inhibited by administration of P5 (Figs. 4A–4D). In contrast, Th1-related cytokines (IFN-γ, IL-12) were significantly increased in the P5-treated group compared with those in the sham group (Figs. 4E and 4F). We also investigated the role of IL-17, a Th17-related cytokine, in food allergy. IL-17 secretion was found to increase following induction of food allergy and was significantly suppressed by administration of P5 (Fig. 4G). These cytokine patterns were also observed in mLNs restimulated with OVA (Figs. 5A–5G).

Effects of P5 on the CD4+Foxp3+ T-Cell Population in Mice with OVA-Induced Food Allergy and Normal BALB/c Mice

In order to determine whether suppression of the Th2 and Th17 response was caused by Tregs, we measured the CD4+Foxp3+ T-cell population in mLNs from mice with OVA-induced food allergy. The CD4+Foxp3+ T-cell population was increased by about 1.6-fold in the P5-treated group compared with that in the sham group (Figs. 6A, 6B). Additionally, secretion of transforming growth factor (TGF)-β, a Treg-related cytokine, was increased in the P5-treated group compared with that in the sham group (Figs. 4H and 5H). In addition, in an ex vivo analysis using lymphocytes from mLNs of healthy BALB/c mice, co-culture with P5 induced a 3.1-fold increase in the CD4+Foxp3+ T-cell population (Figs. 6C and 6D) and a 3.7-fold increase in the expression of IL-10, an anti-inflammatory cytokine (Table 1), compared with those in PBS-treated cells. These data indicated that the suppressive effects of P5 on the Th2 and Th17 responses were related to induction of Tregs.

Discussion

In this study, we confirmed that the probiotic mixture P5, which was composed of five strains (i.e., Lactobacillus paracasei KF698, Lactobacillus pentosus KF340, Bacillus

![Fig. 3. Effects of the probiotic mixture (P5) on production of total IgE (A) and OVA-specific IgE (B) in serum from mice with OVA-induced food allergy.](image-url)
amyloliquefaciens KF26, Pediococcus pentosaceus KF159, and Lactococcus lactis KF140) inhibited Th2 and Th17 immune responses through regulation of the Th1/Th2 balance and Treg induction, thereby alleviating OVA-induced food allergy symptoms. This mechanism is similar to the findings from studies showing that oral administration of the probiotic mixture VSL#3 [20] and Lactobacillus plantarum NRIC0380 [19] alleviated symptoms of allergy through regulation of the Th1/Th2 balance and Treg induction.

In IgE-dependent food allergies, food allergy symptoms are known to be caused by the predominant Th2 immune response. Th2 cytokines, such as IL-4, IL-5, IL-10, and IL-13, play an important role in the manifestation of allergic symptoms by inducing the production of antibodies, such as IgE or IgG1, and by promoting eosinophil proliferation, mast cell degranulation, and B-cell isotype switching [3]. Inhibition of the Th2 immune response can be controlled by enhancing the Th1 immune response. Many probiotics, such as Lactobacillus brevis HY7401 [30], Pediococcus pentosaceus Sn26 [31], and Bifidobacterium lactis [32], have been shown to have anti-allergic activities in various food allergy models through inhibition of the Th2 immune response by promoting the Th1 immune response. In the present study, analysis of the cytokine secretion pattern in splenocytes...
isolated from OVA-induced mice showed that in the P5-treated group, all Th2-related cytokines, including IL-4, IL-5, IL-10, and IL-13, were decreased compared with those in the sham group, and Th1-related cytokines, such as IL-12 and IFN-γ, were increased. This pattern was also observed in mLNs. Furthermore, both the anaphylactic response and the diarrhea score were decreased. Thus, the Th1/Th2 immunoregulatory activity of P5 appeared to affect both systemic and local immunity. The P5-mediated Th1/Th2 balance-regulating activity was also confirmed in an in vivo experiment; co-cultivation of lymphocytes from mLNs of OVA-sensitized mice and P5 at a concentration ratio of 1:10 resulted in increased IFN-γ and decreased IL-4.

Foxp3+ Tregs, which suppress the Th17, Th1, and Th2 immune responses, have been reported as immune cells capable of inhibiting the Th2 immune response. These cells are known to secrete the anti-inflammatory cytokines IL-10 and TGF-β and to play important roles in alleviating food allergies [33]. Tregs have been reported to act directly or indirectly on the sensitization and effector phases of food allergy development through various routes, such as inhibition of allergen-induced degranulation of effector T
cells [34], inhibition of the entry of eosinophils and other effector T cells into inflamed tissues [35], induction of tolerogenic DCs [36], inhibition of direct Th2 cell activation [33], and inhibition of IgE secretion by acting on B cells [37], thereby alleviating the symptoms of food allergy. Recent studies have shown that Foxp3+ Tregs induced by commensal intestinal bacteria play important roles in regulating allergic responses and balancing Th1/Th2 polarization [38–40]. It has been reported that these regulatory effects of Tregs in alleviating food allergy are by the stabilization of mast cells via balance of IL-4 (or IL-17) and TGF-β [41, 42] as well as the suppressive effect of IL-10 secreted from Treg. Thus, Treg induction is an important mechanism for the prevention and treatment of allergic diseases caused by probiotic ingestion. Treg induction in immune organs, such as mLN, Peyer’s patches, and the spleen, through ingestion of probiotics, such as Lactobacillus gasseri OLL2809 [43], probiotic mixture VSL#3 [20], and Lactobacillus plantarum NRIC0380 [19], has been reported to alleviate allergy in many animal models.

To confirm that the inhibitory effects of P5 on the Th2 and Th17 immune responses were caused by Treg induction, P5 and mLN were co-cultivated. The results showed that the CD4+Foxp3+ cell population and IL-10 secretion were increased by P5 treatment, suggesting that P5 inhibited Th2 and Th17 immune responses through Treg induction. In addition, in our OVA-induced food allergy model, the population of CD4+Foxp3+ cells and TGF-β secretion in mLN from the P5-administered group were increased compared with those in the sham group, and the levels of Th2-related cytokines and the Th17-related cytokine IL-17 were decreased compared with those in the sham group. Although the specific role of Th17 in the maintenance and development of food allergy symptoms needs to be studied further, IL-17 may be associated with food allergy development based on previous reports showing upregulation of IL-17 mRNA expression in food allergy-induced mice [44], increased IL-17 in antigen-restimulated lymphocytes [45], and decreased allergic symptoms and IL-17 expression in FceRI gene-knockout
mice [46]. In normal immune responses, Th17-dominant immune responses can be regulated by Treg induction because Th17 cells maintain a balance with Tregs [42]. Therefore, IL-17 inhibition by oral administration of P5 is also thought to be caused by Treg induction.

Despite our results (increase of CD4+Foxp3+ cell and TGF-β, decrease of IL-17) supporting the Treg induction activity of P5, the decrease in IL-10 secretion in the in vivo model in contrast to that in vitro can be attributed to the pleiotropic properties of IL-10. IL-10 is a pleiotropic cytokine that is produced by many cell types, including macrophages, epithelial cells, mast cells, and Tregs, and has both pro- and anti-inflammatory effects during immune responses [22–24]. The function and activity of IL-10 are affected depending on the type of stimulus, the cell type producing IL-10, the immunological environment, and the genetic background of the cells or animal used. IL-10 secreted from immune regulatory cells, such as Tregs and tolerogenic DCs, inhibits the Th1, Th2, and Th17 immune responses by acting as an anti-inflammatory cytokine [47], whereas IL-10 secreted from DCs or Th2 cells promotes the development of the Th2 immune response and the mast cell response [25, 26, 48]. Although the role of IL-10 remains controversial in the allergic environment [47], many studies have demonstrated that IL-10 is associated with the development of allergic inflammation; Laouini and et al. [49] reported that DC-derived IL-10 plays an important role in the Th2 responses to antigen and in the development of skin eosinophilia in a murine model of allergic dermatitis; Polukort et al. [50] demonstrated that IL-10 plays an essential role in the progression of allergy in the OVA-induced murine food allergy model using BALB/c mice, suggesting that allergic symptoms, such as intestinal anaphylaxis, diarrhea, mast cell activation, and Th2 cytokine production, were alleviated in IL-10−/− mice compared with that in wild-type controls. In contrast to these reports, the research group of JJ Ryan reported that IL-10 prevents IgE-mediated activation of mast cells, and inhibits FcεRI expression in a model using C57BL/6 mice [51–53]. These discrepancies in the role of IL-10 during inflammatory conditions in vivo are thought to be due to the immune microenvironment and the genetic background of the animal used. Kolawole and Ryan [54] recently reported that the mast cell responsiveness to IL-10 may be altered by genetic background. Polukort et al. [50] reported that IL-10 can enhance mast cell expansion and promote allergic responses in BALB/c mice, which are known to have a strong Th2 bias.

In our experiment, we used BALB/c mice to establish the OVA-induced food allergy model, and administration of P5 started under a Th2 skewing environment (after 2nd immunization). In light of these, IL-10 detected in vivo may act a Th2-related cytokine secreted from DCs or Th2 cells, rather than as an anti-inflammatory cytokine secreted from Treg. Indeed, the increased secretion of IL-10 in the sham group, which showed severe symptoms of food allergy, was observed. Therefore, the decrease of IL-10 in vivo is assumed to result from the mitigation of a Th2-dominant immune response by P5. In contrast to that in vivo, the Treg population and IL-10 secretion by co-culture with P5 were increased ex vivo using lymphocytes from mLNs of healthy BALB/c mice, while IL-12 was decreased. Considering the decrease of IL-12, the IL-10 detected ex vivo might act as an anti-inflammatory cytokine. Thus, it is thought that such discrepancy between in vivo and ex vivo results in IL-10 secretion pattern may be due to differences in the immunological environment.

In summary, although further studies should be conducted to fully understand the regulatory mechanisms of P5 via Tregs induction in food allergy, our results indicated that oral supplementation with the probiotic mixture P5 attenuated OVA-induced allergic symptoms by regulation of the Th1/Th2 balance and suppression of Th2 and Th17 responses via induction of Tregs. Thus, our study provides evidence that P5 may be a potential candidate probiotic mixture for the prevention of food allergy disorders.

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References


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