J. Microbiol. Biotechnol. (2013), **23**(5), 724–730
http://dx.doi.org/10.4014/jmb.1211.11079
First published online March 13, 2013
pISSN 1017-7825 eISSN 1738-8872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Immunomodulatory and Anti-Allergic Effects of Orally Administered *Lactobacillus* Species in Ovalbumin-Sensitized Mice

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Received: November 28, 2012 / Revised: January 2, 2013 / Accepted: January 8, 2013

We investigated the effects of orally administered probiotic bacteria (*Lactobacillus* species) as allergic immune modulators in ovalbumin (OVA)-sensitized mice. BALB/c mice were intraperitoneally injected with OVA twice at a 2-week interval for allergy sensitization. The mice were then orally administered *Lactobacillus casei* YIT9029 (L1), *L. casei* HY7201 (L2), *L. brevis* HY7401 (L3), or *L. plantarum* HY20301 (L4) every 2 days for 3 weeks. Total IgE levels significantly decreased in sera of L3-administered mice but increased in the other groups. OVA-specific IgE levels decreased slightly in sera of mice administered L1, L3, and L4 but increased significantly in L2-administered mice. In passive cutaneous anaphylaxis (PCA) using sera from administered mice, only the L3-administered group showed reaction inhibition. High expression of TLR-2 with interferon (IFN)-γ stimulation on peripheral blood mononuclear cells occurred in L3- or L4-administered mice. Th1 cytokines, including IFN-γ and interleukin (IL)-12, increased in splenocytes of L3-administered mice; however, IL-4 decreased in L1- and L4-administered groups; IL-5 decreased in all experimental groups. IL-6 decreased in the L3-administered group; and IL-10 decreased in L1-, L2-, and L3-administered groups. L3 induced anti-allergic effects by increasing Th1 cytokines, decreasing Th2 cytokines, and inhibiting the PCA reaction, whereas L2 administration increased allergic effects.

Key words: Allergy, oral administration, probiotics, *Lactobacillus*

Allergies are adverse immune responses to foreign substance such as pollen, drugs, insect products, or certain food ingredients. When an allergen enters the body, it activates T-helper type 2 (Th2) cells, which stimulate B cells to differentiate into immunoglobulin E (IgE)-secreting plasma cells. Secreted allergen-specific IgE binds the IgE-specific Fc receptor on blood basophils or mast cells throughout the body. Second exposure to the allergen leads to cross-linking of bound IgE, triggering degranulation and cytokine releases, resulting in typical manifestations, including systemic anaphylaxis and localized anaphylaxis such as hay fever, asthma, hives, and eczema [1, 7, 12]. The prevalence of food allergy has increased worldwide and is recognized as one of the most predominant causative factors for atopic dermatitis, gastrointestinal distress, respiratory distress, and life-threatening anaphylactic responses [7, 29].

Infancy or childhood is a critical period for establishing the T-helper type 1 (Th1)/Th2 balance; a distorted balance in the immune response is related to reduced microbial exposure [28]. Therefore, early and frequent exposures to microbial stimuli can help maintain the Th1/Th2 balance, which may be an appropriate approach for preventing or treating allergic immune responses.

Probiotics are live microorganisms that can improve the balance of beneficial intestinal microflora in the host organism. Lactic acid bacteria (LAB) and bifidobacteria are the most common microorganisms used as probiotics; certain yeasts and bacilli can also be used to relieve symptoms of allergic responses [4]. The functions of probiotics, particularly LAB, have been studied in a range of human diseases, including cancer, infectious diseases, gastrointestinal disorders, and allergies [1, 7]. Among the many benefits associated with the consumption of probiotics, modulation of immune activity and enhancement of intestinal barrier function have received the most attention [6].

When administered orally before or concomitant with sensitization with ovalbumin (OVA), numerous LAB strains, including *Lactobacillus* or *Bifidobacterium*, can reduce total and OVA-specific IgE production; measurements of

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associated Th1 and Th2 cytokines such as interferon (IFN)-γ, interleukin (IL)-2, IL-4, IL-5, and IL-6 in OVA-stimulated splenocytes from LAB-fed mice also indicate that highly strain-dependent and different mechanisms are involved [9–11, 21, 25]. These investigations revealed that specific probiotic bacteria are a promising tool for promoting potentially anti-allergic processes through immune regulation.

In this study, we investigated the effect of orally administered *Lactobacillus* species as allergic immune modulators in OVA-sensitized mice. Previously, we examined 10 *Lactobacillus* species and screened for immunomodulation effects on murine splenocytes by IFN-γ and IL-4 detection. Four *Lactobacilli* were selected and tested to examine whether oral administration of these *Lactobacilli* had immunomodulatory and/or anti-allergic effects in OVA-sensitized mice. Cytokine profiles, Toll-like receptor (TLR)-2 expression, and total IgE and OVA-specific IgE detection using both the enzyme-linked immunosorbent assay (ELISA) and passive cutaneous anaphylaxis (PCA) were also examined in this study.

Materials and Methods

Preparation of Microorganisms

Lactobacillus casei YIT9029 (L1), *L. casei* HY7201 (L2), *L. brevis* HY7401 (L3), and *L. plantarum* HY20301 (L4) were provided by Korea Yakult Co. Ltd. (Seoul, Korea) (Table 1). Bacteria were cultured in Lactobacilli-MRS broth (Difco Laboratories, Franklin Lakes, NJ, USA) at 37°C for 18 h. Cells were collected by centrifugation at 5,000 ×*g* for 30 min at 4°C and then washed twice with phosphate-buffered saline (PBS). Pelleted cells were lyophilized and weighed for further analysis.

Animal Experiments

Animal experiments were performed under the approval of the Seoul National University Institutional Animal Care and Use Committee in accordance with the laboratory's animal ethics guidelines.

After 7-day acclimatization, forty-eight 6-week-old female BALB/*c* mice (Charles River Laboratories, USA) were randomly divided into 6 groups: negative control (PBS-treated); positive control; and L1, L2, L3, or L4 administration. Mice in the positive control and experimental groups were intraperitoneally injected with 20 μg of OVA mixed with 2 mg of $Al(OH)_3$ twice at a 2-week interval for allergy sensitization. Mice in the normal control group were injected with PBS. For oral administration of bacteria, lyophilized cells were suspended in distilled water and administered orally to OVA-sensitized mice (2 mg per mouse) every 2 days for 3 weeks. Next, the mice were euthanized, and blood and splenocytes were collected for further study (Fig. 1).

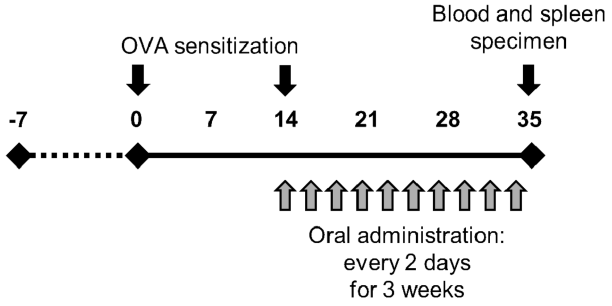

Fig. 1. Timeline of mouse OVA sensitization.
Mice were acclimatized for a week before the first sensitization on day 0. Sensitizations were performed on days 0 and 14 by intraperitoneal injection with 20 μg of OVA mixed with 2 mg of $Al(OH)_3$. The lyophilized probiotics were suspended and then orally administered to the OVA-sensitized mice (2 mg per mouse) every 2 days for 3 weeks. Following oral administration, the mice were euthanized, and blood and splenocytes were collected for further study.

For the PCA test, twenty-four 6-week-old male Wistar rats (Charles River Laboratories, USA) were used.

Total IgE and OVA-Specific IgE in Serum

Tail-vein blood was obtained from each mouse, and serum total IgE and OVA-specific IgE were analyzed by ELISA. For total IgE detection, a plate was coated with 200 ng of anti-mouse IgE monoclonal antibody (BD Biosciences, USA) for 2 h at room temperature (RT). After 3 washes with PBS containing 0.05% Tween-20 (PBST), the plate was blocked with 200 μl of blocking solution containing 3% bovine serum albumin (BSA) in PBST for 2 h at RT. The plate was washed 3 times with PBST, 100 μl of diluted serum was applied, and the plate was incubated overnight at 4°C. After 3 washes with PBST, biotinylated anti-mouse IgE antibody (BD Biosciences, USA) was added and incubated for 1 h at RT. After 3 washes with PBST, 100 μl of avidin-horseradish peroxidase (HRP) (1:1,000) was added and incubated for 1 h at RT. After 3 washes with PBST, 50 μl of color development solution (3,3',5,5'-tetramethylbenzidine dissolved in dimethyl sulfoxide) was added and incubated for 20 min. Stopping solution (50 μl H_2SO_4) was added, and the absorbance was measured at 450 nm using a microplate reader. For OVA-specific IgE detection, the plate was coated with 500 ng of OVA in 0.1 M $NaHCO_3$; the procedure was conducted as described above. All samples were tested in triplicate. Serum titers for OVA-specific IgE were expressed as relative ELISA units, referring to a laboratory standard sera pool [16].

Table 1. Experimental strains of *Lactobacillus* species in this study.

Designation	Strain	Reference
L1	*Lactobacillus casei* YIT9029	[19]
L2	*Lactobacillus casei* HY7201	[14]
L3	*Lactobacillus brevis* HY7401	[15]
L4	*Lactobacillus plantarum* HY20301	New strain

PCA

Serum IgE antibodies specific to OVA were determined using PCA tests [22]. Four rats were used from each group. The serum of experimental mice was diluted with PBS (1:25, 1:50, 1:100, and 1:200), and the rats were injected intradermally with 100 μl of each diluted serum into shaved dorsal skin sites. The next day, 1 mg of OVA with 0.5% Evans blue in saline was injected intravenously into the tail vein. After 1 h, rats were euthanized, and the dorsal skin of the rats was removed to measure the pigment area. Diameters of blue spots on the internal surface of the skin were measured [13].

TLR-2 Expression in Peripheral Blood Mononuclear Cells (PBMCs)

Blood from sensitized mice was collected with anticoagulant in PBS. Cells were pelleted by centrifugation at 1,000 ×*g* for 10 min at 4°C and resuspended with red blood cell lysis buffer. Five minutes later, cells were re-collected and seeded at 1×10^6 cells per well with or without 10 ng/ml of IFN-γ in a 24-well plate. After 2-day incubation, cells were subjected to flow cytometry analysis with carboxyfluorescein-conjugated rat anti-mouse TLR-2 antibody (BD Biosciences) using FACSCalibur according to the manufacturer's instructions. Quantitation was performed using the Cell Quest software (BD Biosciences).

Cytokine Production from Splenocytes

Spleens were collected, and single-cell suspensions were prepared and resuspended in RPMI-1640 medium (Gibco BRL, Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL), 50 μg/ml of streptomycin, and 100 U/ml of penicillin. Cells were then seeded at 1×10^5 cells per well in a 96-well plate. After 3-h incubation, 10 μg of OVA was added for re-stimulation. The culture supernatant was harvested 48 h later, and the concentrations of IFN-γ, IL-4, IL-5, IL-6, IL-10, and IL-12 (BD Biosciences) were determined by sandwich ELISA using rat anti-mouse antibody for each cytokine. All samples were tested in triplicate.

Statistical Analysis

All laboratory assays were repeated at least 3 times, independently. The results are expressed as mean ± standard deviation. The statistical significance of variation among different groups was analyzed using the one-way analysis of variation and least significant difference tests using the SPSS software (SPSS Inc., IL, USA). Differences were considered statistically significant at $p < 0.05$.

RESULTS

Total IgE and OVA-Specific IgE in Serum

Four *Lactobacillus* species (L1, L2, L3, and L4) having immunomodulatory effects on murine splenocytes were selected and tested in this study. To monitor the effects of administration of these *Lactobacillus* species, serum was collected from each mouse group following OVA sensitization. Total serum IgE and OVA-specific IgE levels are presented in Fig. 2. When mice were sensitized with OVA twice, total IgE levels were significantly increased in positive controls (10.01 ± 1.43 ng/ml) (Fig. 2A). When

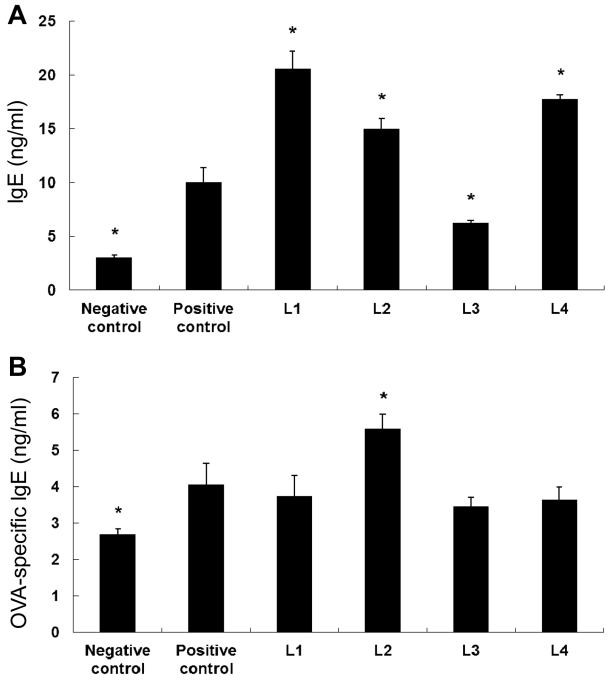

Fig. 2. Effect of oral administration of *Lactobacillus* species on production of total IgE (**A**) and OVA-specific IgE (**B**) in serum from OVA-sensitized mice.

Total IgE and OVA-specific IgE were determined by ELISA. Serum titers for OVA-specific IgE were expressed as relative ELISA units; negative control (PBS-treated mice); positive control (OVA-sensitized mice); and L1, L2, L3, and L4 (oral administration with L1, L2, L3, and L4 in OVA-sensitized mice). Statistical significance between the positive control and each group is indicated with an asterisk ($p < 0.05$).

bacteria were administered to each group, total IgE levels were significantly decreased in the L3-administered group only (6.27 ± 0.26 ng/ml) but significantly increased in the other groups (L1, 20.61 ± 1.63 ng/ml; L2, 14.99 ± 0.97 ng/ml; L4, 17.74 ± 0.41 ng/ml) compared with positive controls ($p < 0.05$).

OVA-specific IgE levels showed different results (Fig. 2B). The level of OVA-specific IgE decreased nonsignificantly in the L1-, L3-, or L4-administered group (L1, 3.74 ± 0.57 ug/ml; L3, 3.47 ± 0.24 ug/ml; L4, 3.64 ± 0.36 ug/ml) compared with the positive control (4.06 ± 0.58 ug/ml), whereas a significant increase was observed in the L2-administered group (5.60 ± 0.40 ug/ml).

PCA

OVA-specific IgE in the serum of sensitized mice was specifically determined using the PCA test. The skin of rats was injected subcutaneously with serially diluted serum from sensitized mice, and then antigen was injected intravenously with Evans blue; positive reactions were exhibited as blue spots (Fig. 3). No blue spot was observed in any serum dilution in the negative control (Fig. 3Aa). Blue spots were observed in the 1:25, 1:50, and 1:100

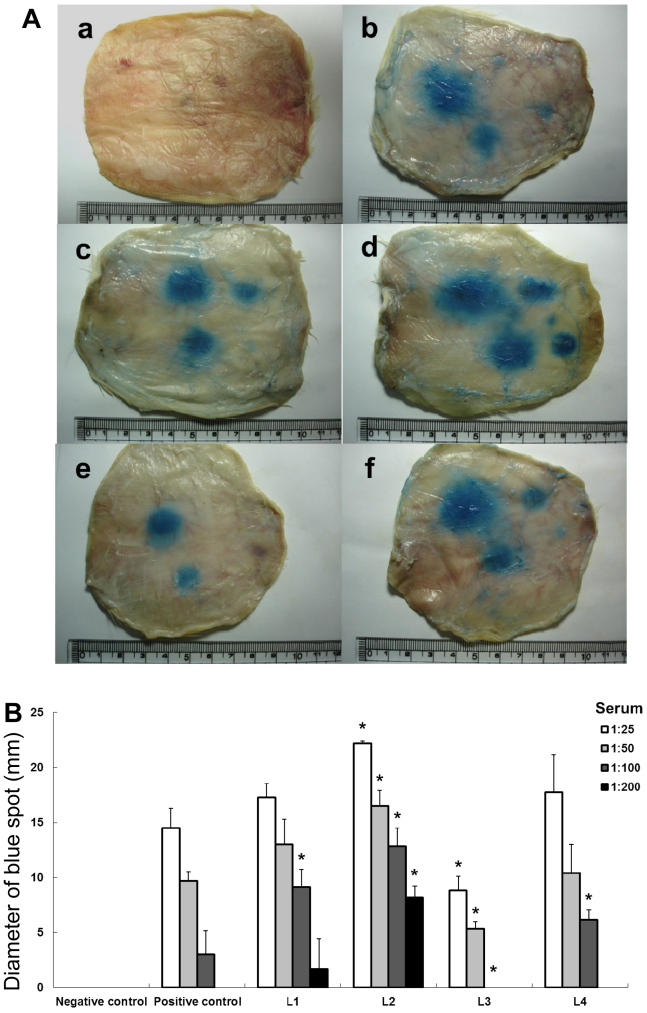

Fig. 3. PCA reaction of Wistar rat treated with mouse serum.
The serum with OVA-specific IgE from the mice was diluted with PBS (1:25, 1:50, 1:100, and 1:200) and then injected intradermally into the rat. OVA was injected with Evans blue into the tail vein and the positive reactions were shown as blue spots (**A**); negative control (a), positive control (b), L1- (c), L2- (d), L3- (e), and L4- (f) administered group. The diameters of blue spots on the internal surface of the skin were measured (**B**). Statistical significance between the positive control and each group in the same serum dilution is indicated with an asterisk ($p < 0.05$).

serum dilutions in the positive control (Fig. 3Ab). In both the 1:25 and 1:50 serum dilutions, the average size of blue spots was significantly decreased in the L3-administered group (Fig. 3Ae), whereas a significant increase was observed in the L2-administered group (Fig. 3Ad) compared with the positive control. In the 1:100 serum dilution, no blue spot was observed in the L3-administered group, whereas a significant increase in the average size of blue spots was observed in the other groups. In the 1:200 serum dilution, only the L2-administered group showed significant blue spots (Fig. 3B).

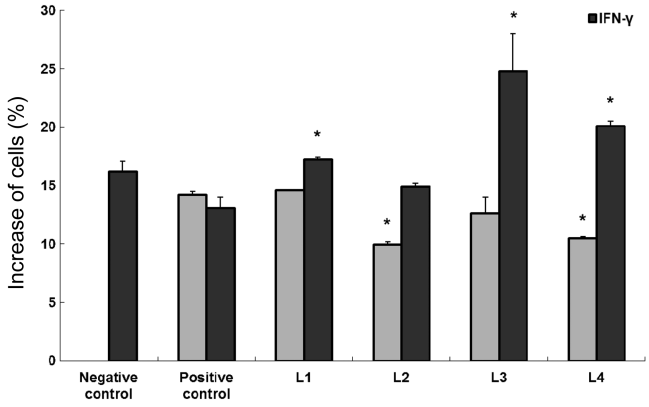

Fig. 4. Effect of oral administration of *Lactobacillus* species on TLR-2 expression of PBMCs from OVA-sensitized mice.
Mouse PBMCs were isolated and cultured with (dark grey) or without (light grey) IFN-γ stimulation for 48 h, and then subjected to flow cytometry analysis with carboxyfluorescein-conjugated rat anti-mouse TLR-2 antibody. Statistical significance between the positive control and each group in the same IFN-γ treatment is indicated with an asterisk ($p < 0.05$).

TLR-2 Expression on PBMCs

Changes in TLR-2 expression in PBMCs were determined by flow cytometry using carboxyfluorescein-conjugated rat anti-mouse TLR-2 antibody (Fig. 4). TLR-2 expression was decreased significantly in both the L2 and L4 groups compared with the positive control in the condition of non-IFN-γ treatment (light grey). However, TLR-2 expression increased significantly in all experimental groups, except the L2-administered group following IFN-γ treatment (dark grey). The L3-administered group showed the highest elevation of TLR-2 expression.

Cytokine Production from Splenocytes

Splenocytes were isolated from OVA-sensitized mice following oral administration of *Lactobacillus* species and then cultured with OVA re-stimulation. Th1 and Th2 cytokines, including IFN-γ, IL-4, IL-5, IL-6, IL-10, and IL-12, were determined in the culture supernatant. IFN-γ, which is produced in Th1 cells and inhibits allergic responses [26], was significantly increased in L1- and L3-administered groups (Fig. 5A). IL-12, which is secreted from antigen-presenting cells (APCs) and activates Th1 cytokine secretion [32], was significantly increased in L3- and L4-administered groups (Fig. 5B).

Among Th2 cytokines, IL-4, which induces B-cell class switching to produce IgE [30], was significantly decreased in L1- and L4-administered groups (Fig. 5C), and IL-5, which is produced by Th2 cells and enhances IL-4-dependent IgE production [24], was significantly decreased in all experimental groups (Fig. 5D).

IL-6, secreted from T cells and monocytes, has a role in IL-4-dependent IgE synthesis [5]. IL-6 was significantly

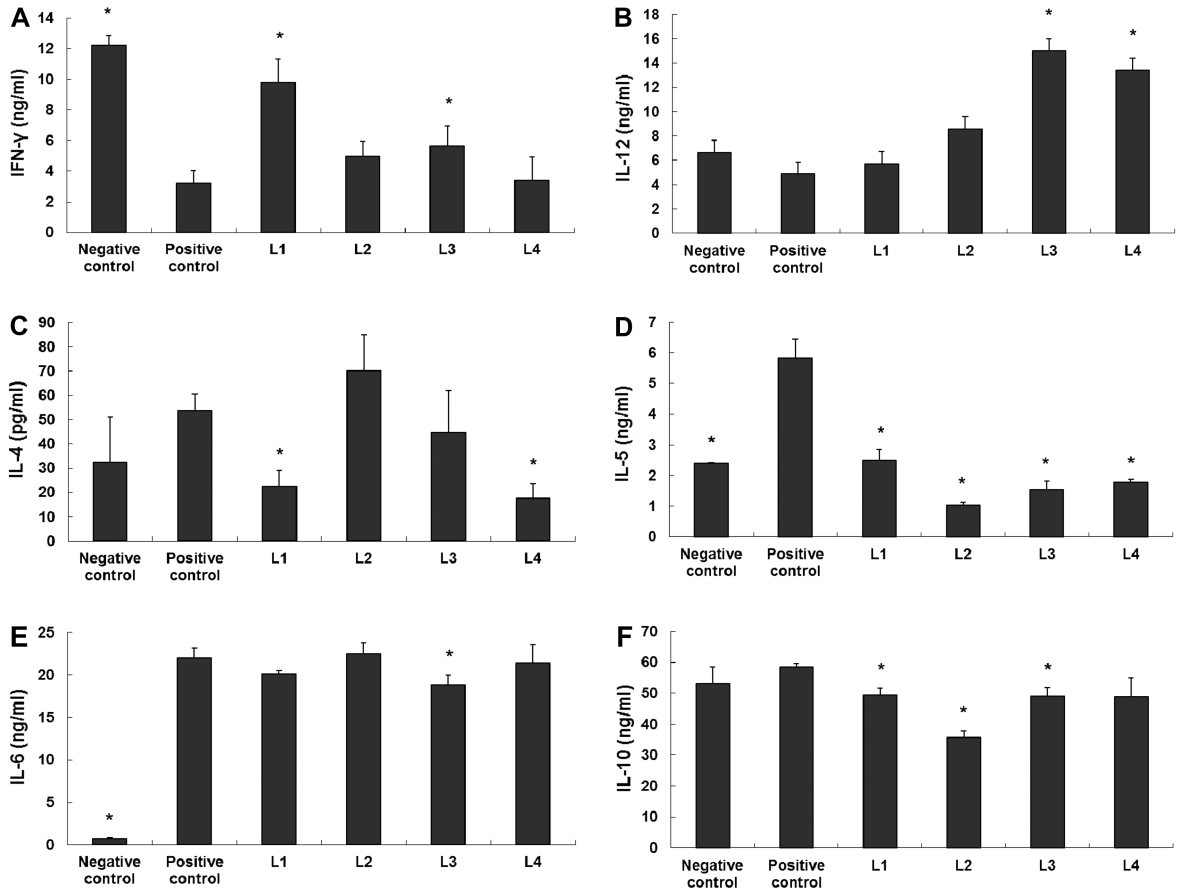

Fig. 5. Effect of oral administration of *Lactobacillus* species on cytokine releases of splenocytes isolated from OVA-sensitized mice. The isolated splenocytes were cultured with OVA for re-stimulation, and then the culture supernatants were used to determine the cytokines, including IFN-γ (**A**), IL-12 (**B**), IL-4 (**C**), IL-5 (**D**), IL-6 (**E**), and IL-10 (**F**), by sandwich ELISA. Statistical significance between the positive control and each group is indicated with an asterisk ($p < 0.05$).

decreased only in the L3-administered group compared with the positive control; no significant changes were observed in the other groups (Fig. 5E). IL-10, which inhibits Th1 responses [2], was significantly decreased in the L1-, L2-, and L3-administered group; there was no significant alteration in the L4-administered group (Fig. 5F).

DISCUSSION

Probiotic bacteria promote cellular immune responses through Th1 cell activation. Numerous strains of probiotic bacteria, including *Lactobacillus* and *Bifidobacterium*, can stimulate and regulate several aspects of natural and acquired immune responses [1, 6, 18]. Several strains, including *L. casei*, *L. acidophilus*, *L. bulgaricus*, *B. bifidum*, and *B. longum*, have been shown to reduce total or OVA-specific IgE production and to change associated cytokine patterns in a strain-dependent manner [9-11, 21, 25]. Some strains are effective even when heat-killed [3, 19, 20, 27, 33].

To investigate the immunomodulatory and anti-allergic effects of orally administered LAB in OVA-sensitized mice, 4 *Lactobacillus* species were used. Total IgE and OVA-specific IgE in serum, cytokine profiles in splenocytes, and TLR-2 expression in PBMCs were determined.

In mice sera, total IgE decreased only in the L3-administered group and increased in all the other groups. An increase in OVA-specific IgE was observed in the L2 group, whereas a nonsignificant decrease was observed in the others including the L3 group. Furthermore, in the PCA test, OVA-specific IgE was lower in the L3 group, whereas this value was higher in the L2 group. The PCA test has higher sensitivity and specificity in allergen-specific IgE detection [17]. Thus, in general, among the 4 *Lactobacillus* species used in this study, *L. brevis* HY7401L3 (L3) showed characteristic inhibition of allergen-specific IgE production.

Most food allergies are a type I hypersensitivity reaction; an allergen is presented to CD4+ Th2 cells specific to an antigen that stimulates B cell production of IgE antibodies. This allergen-specific IgE production is modulated by

various cytokines. For example, IL-4 and IL-5 enhance allergen-IgE production [24]; however, IFN-γ controls IgE production by suppressing Th2 cell proliferation, and IL-12 enhances NK cell activation and Th1 cell responses [26]. Therefore, modulating allergy-associated cytokine releases and controlling allergen-specific IgE production are most critical in developing effective anti-allergenic materials. However, interpreting the results on cytokine release studies is complicated, because these cytokines comprise the most pleiotropic group of immune response modulators.

We estimated levels of Th1 and Th2 cytokines in OVA-stimulated splenocytes. Among Th1 cytokines, IFN-γ inhibits IL-4-derived isotype switching [30]. This cytokine was strongly detected in L1- and L3-administered groups in this study. IL-12 is secreted from APCs, including macrophage and dendritic cells [32]; it was highly induced in the L3 and L4 groups. Among Th2 cytokines, the most important cytokine is IL-4, because it induces isotype switching to IgE [24, 30]. IL-4 was decreased in the L1 and L4 groups in this study. IL-5 also enhances IL-4-induced IgE production [24]. It was significantly lower in all groups compared with the positive control. IL-6 has a role in IL-4-dependent IgE synthesis and induces differentiation to antibody-secreting plasma cells [5]. Only the L3 group showed reduced IL-6 levels compared with the positive control. IL-10, known as human cytokine synthesis inhibitory factor, is an anti-inflammatory cytokine, which down-regulates Th1 cytokine expression [2]. In this study, IL-10 was decreased in the L1, L2, and L3 groups.

TLR can recognize pathogen patterns and produce pro-inflammatory antimicrobial mediators to protect against bacterial infections. It is highly expressed in APCs and involved in phagocytosis and cytokine release. Many probiotics are Gram-positive bacteria, and their cell wall components, such as lipoproteins and lipoteichoic acids, can induce Th1 cell responses *via* TLR-2 activation [1, 8]. TLR-2 activation induces inhibition of allergen-specific Th2 responses and IgE production [23, 31]. We measured changes in TLR-2 expression in PBMCs using flow cytometry in LAB-administered, OVA-sensitized mice. Generally, TLR-2 expression decreased (significantly in L2 and L4 groups). However, TLR-2 expression increased (significantly in L1, L3, and L4 groups) when IFN-γ was treated. Because IFN-γ can activate APCs, it may contribute to elevated TLR-2 expression.

In conclusion, *L. casei* HY7201 (L2) tended to induce allergic responses. *L. casei* YIT9029 (L1) induced higher Th1 cytokines and lower Th2 cytokines; however, no inhibitory effect on IgE production was observed. Following *L. plantarum* HY20301 (L4) administration, Th2 cytokines were effectively reduced, but IgE production was not decreased. In *L. brevis* HY7401 (L3) administration, Th1 cytokines increased, Th2 cytokines decreased, and IgE production was reduced. Therefore, among the 4 *Lactobacillus* strains used in this study, *L. brevis* HY7401 may be the most effective for oral administration to control allergic responses in OVA-sensitized animal experiments. Thus the feasibility for application of this strain as an anti-allergic substance can be considered. Further studies are needed to validate the anti-allergic effects and their detailed mechanisms for controlling or improving allergic hypersensitivity.

Acknowledgments

This work was funded by a research grant from Korea Yakult Co., Ltd to H-J. W.

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