



Oral intervention of *Lactobacillus pentosus* S-PT84 attenuates the allergenic responses in a BALB/C mouse model of egg allergy

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ABSTRACT

Food protein-induced allergies are primarily aggravated due to imbalance immune responses. Earlier studies by different research groups have reported that the intervention of *Lactobacillus pentosus* (*L. pentosus*) S-PT84 can modulate T-helper (Th)1/Th2 balance through regulatory T cells and can effectively promote type 1 immunity by activating dendritic cells and natural killer cells, such biological activity makes *L. pentosus* S-PT84 a potential mediation in controlling food allergy. Thus, this study aimed to evaluate the effects of *L. pentosus* S-PT84 against egg ovalbumin (OVA)-induced allergic response in mice. BALB/c mice (n = 12/group) were sensitized with OVA (50 µg/mice) via intraperitoneal injection (IP) for four weeks and subsequently administered with three different doses of *L. pentosus* S-PT84 via pelleted diet. The allergenic status was assessed by clinical signs, serum histamine, mouse mast cell protease (MMCP) level, and antibody activity, cytokines level in splenocytes, and expression of T regulatory cells (T-regs) in blood. The intervention of *L. pentosus* S-PT84, precisely at the high dose (0.6 % *L. pentosus* S-PT84 in pelleted diet) group, significantly reduced the clinical allergenic symptoms and reduced the histamine and MMCP levels in serum. However, the intervention of *L. pentosus* S-PT84 did not affect the OVA-specific IgE, IgG concentration, but led to lower the total IgE and IgG titers, suggesting that the therapeutic effect of *L. pentosus* S-PT84 may be due to development of immune tolerance. Moreover, differences in the immune response were observed after *L. pentosus* S-PT84 intervention, as it significantly reduced the production of IL-4, IL-17, and increased the population of CD25⁺ Foxp3⁺ cells. Thus, it can be concluded that the intervention of *L. pentosus* S-PT84 provides a potential therapeutic strategy to reduce the chicken egg OVA-induced allergic symptoms.

1. Introduction

Food protein-induced allergy is an increasing problem and a global health concern of the modern era. Food allergy affects nearly 3–4 % of the adult population and 10 % of the infants in North-America (Sampson, 2016; Loh et al., 2018). Food allergy is attributed to dysregulation of immunological responses (IR) (Sampson, 2004), and predisposition of type-1 IR-associated allergic reactions are increasing at an alarming rate. Over the past few decades, the increases in the prevalence of allergic response are primarily affecting the industrialized regions of the world. It was earlier reported that IgE mediated type-1 hypersensitivity induced by food-proteins can be severe even sometimes life-threatening (Macdougall et al., 2002; Sicherer and Sampson, 2010). Egg, peanut, and milk are the top three of the eight major food

allergens in human (Sampson, 2004). In young children, the egg is considered as one of the major allergens, although it outgrows at a later stage in life (Savage et al., 2007; Caubet and Wang, 2011). Mainly glycoproteins from egg white have identified as food allergens. Among the major egg white proteins, ovalbumin (OVA) and ovomucoid (OVM) are the most prominent allergens (Honma et al., 1991). OVM is heat resistant and also resistant to digestive enzymes (Caubet and Wang, 2011). Whereas OVA is not heat resistant, can be digested by gastrointestinal digestive enzymes, and can be absorbed by the gastrointestinal epithelial cells (Martos et al., 2010; Caubet and Wang, 2011).

Transport mechanism of allergic epitopes of food proteins across the gastrointestinal epithelial cells is not completely explored yet. However, it is highly likely that gut permeability, allergen transport, and tolerance induction are intrinsically linked with each other and

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develops the onset of the food allergy (Price et al., 2013; Reitsma et al., 2014). Thus, intact tight-junctions of gastrointestinal epithelial cells resist the allergens to absorb or permeate to the bloodstream, whereas an inflamed and permeable gut cannot stop the invasion of the food allergens. An apparent defect in the gastrointestinal epithelial barrier was noted in the patients with food allergy, and an *in-vitro* experiment also showed that food allergens could decrease the expression of tight junction proteins, including occluding, claudin-1, and ZO-1 (Pizzuti et al., 2011). Although some of the food allergens can disrupt the tight junctions and increases gut para-cellular permeability, recent studies suggested that gastrointestinal microbiome also plays a vital role in modulating gut permeability (Kelly et al., 2015; Prince et al., 2015). Therefore, maintaining gut health is one of the critical aspects of preventing or managing the food-induced allergenic response.

Modulating the gut microbial population could be an effective strategy to control gut permeability and the invasion of food allergens into the bloodstream. Numerous studies have indicated that lactic acid bacteria (LAB) can alter the gut microbial population and affect bacterial immunogenicity (Greene and Klaenhammer, 1994; Ouwehand et al., 1999; Schiffrin et al., 1997; Perdigon et al., 2001). LAB can modulate both mucosal and systemic immune responses and are widely used as a health beneficial food ingredient. Earlier studies have shown that oral administration of *Lactobacillus casei* (LcS) increased Natural killer (NK) cell activity in aged mice (Hori et al., 2003), fermented milk containing LcS enhances NK activity in humans by stimulation of interleukin-12 (IL-12) production (Shida et al., 2006; Takeda et al., 2006; Takeda and Okumura, 2018). A specific strain, *Lactobacillus pentosus* (*L. pentosus*) S-PT84, isolated from fermented Japanese pickle known as “Shibazuke” has shown to exert immune-regulating activities, recognized as probiotic and well known for its health beneficial activity. *In-vitro* and *in-vivo* functions of *L. pentosus* S-PT84 are well documented in the literature (Koizumi et al., 2008). Supplementation of *L. pentosus* S-PT84 can modulate T helper type 1 (Th-1)/ T-helper type2 (Th-2) balances toward a Th1-dominant state (Nonaka et al., 2008), also induces immunomodulatory properties and effectively promote type-1 immunity by activating the productions of IL-12 and interferon gamma (IFN- γ) from the macrophages and enhances the NK cell activity (Koizumi et al., 2008). *L. pentosus* S-PT84 also exhibits anti-allergic effects by promoting the production of IgA in the Peyer’s patch to improve intestinal immunity (Izumo et al., 2010). Such biological functions make *L. pentosus* S-PT84 as a potential intervention in controlling allergy and infectious diseases, (Koizumi et al., 2008; Nonaka et al., 2008; Izumo et al., 2010). However, the therapeutic potential of *L. pentosus* S-PT84 in modulating allergic responses have not been completely elucidated yet. In this study, we used heat killed bacteria as it is easy handling for formulating it into custom diet and actual application as health supplement. In such circumstances, the present study aims to evaluate the anti-allergic effect of heat-killed *L. pentosus* S-PT84 through a mouse model of egg allergy.

2. Materials and methods

2.1. Materials

L. pentosus S-PT84 was provided by Suntory Wellness Ltd (Kyoto, Japan). Briefly, *L. pentosus* S-PT84 was cultivated in a medium containing glucose and yeast extract (Aromild TM, Kohjin Life Science Ltd, Tokyo, Japan) and SK yeast extract Hi-K (Nippon Paper Ltd, Fukushima, Japan) at 37 °C for 24 h. Cultured bacteria were collected by centrifugation at 9190 g for 5 min, washed twice with sterile saline and once with distilled water, and heat-killed (HK) at 95 °C for 5 min. *L. pentosus* S-PT84 was lyophilized for further experiments. The customized pelleted mice diet incorporating *L. pentosus* S-PT84 were prepared in three different concentrations (0.006, 0.06, and 0.6 %, w/w) in this study (Table S1). The customized diets were prepared by Envigo (Teklad diets # 2014:14 % protein rodent diet) (Haslett, MI, USA). All antibodies used in the experiments were purchased from BD Biosciences (Pharmingen, San Diego, CA, USA), Sigma-Aldrich (Oakville, ON, Canada), or eBioscience (San Diego, CA, USA).

2.2. Animal study

Six-eight weeks old female BALB/C mice were obtained from Charles River (Senneville, QC, Canada). The mice were kept in the University of Guelph Central Animal Care facility at least 1 week for acclimatization. After 1 week of acclimatization, the mice were randomly divided into 5 different groups (n = 12/group). During the acclimatization and experimental period the mice were exposed to a 12 h (light:dark) cycle, in a humidity and temperature-controlled (23 °C) quiet room. Standard mice chow or the experimental diet and water *ad libitum* were provided to all the mice. The formulation of treatment diets is illustrated in Supplementary Table-S1. All experimental protocols were carried out in accordance with the Canadian Council for Animal Care guidelines, and all animal use protocols were approved by the University of Guelph Animal Care Committee (AUP1567).

2.3. Experimental design, allergen sensitization, and treatment

Fig. 1 summarizes the detailed experimental design; the entire study is 14 weeks long including 4 weeks of sensitization and 10 weeks of *L. pentosus* S-PT84 intervention. At the beginning of the experiment OVA (50 μ g/mice) was given by intraperitoneal (IP) injection (dissolved in 50 μ L of saline and 50 μ L of alumini adjuvant) once a week for four weeks to all the animals except the Negative-Control (Neg-Con) group. On the 5th week, animals were randomly selected from each treatment group, and OVA-specific IgE levels were checked in blood serum to ensure the allergenic sensitization. Following sensitization, HK *L. pentosus* S-PT84 was orally administered via pelleted diet to 3 different treatment groups; treatment-low dose (Trt-Low: 0.006 %); treatment-medium dose (Trt-Med: 0.06 %); and treatment-high dose (Trt-High:0.6

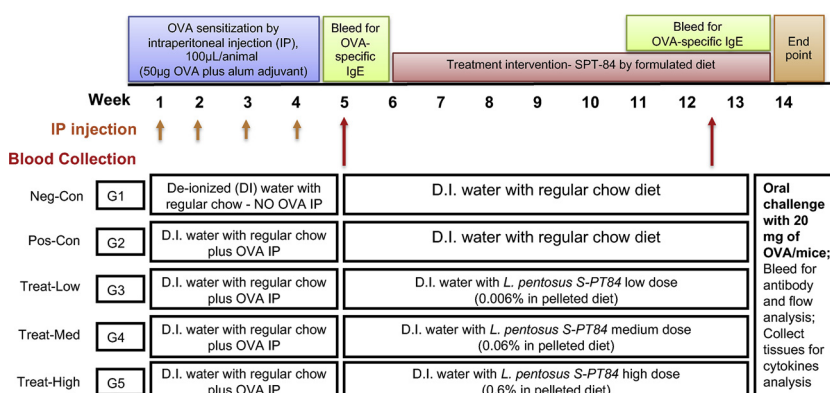


Fig. 1. Experimental study design. Five groups (n = 12/group) of female BALB/C mice, 6–8 weeks old were used in this study. During the first 4 weeks, mice were sensitized with ovalbumin [OVA: 50 μ g/mice dissolved in 100 μ L of saline:aluminum adjuvant (1:1)] given intraperitoneally (IP) once a week. The animals in the negative control group did not receive an injection only de-ionized (DI) water with regular chow. From 5th week onwards *L. pentosus* S-PT84 were administered as pelleted diet with three dosages (Low: 0.006 %; Medium: 0.06 %; and High:0.6 %) up to 13th week. On the 14th week, mice were orally challenged with OVA (20 mg/mouse), scored for clinical allergic reactions and then humanely euthanized by cervical dislocation. Blood and other tissues samples were then collected for further analysis.

%), whereas the Positive-Control (Pos-Con) and Negative-Control (Neg-Con) groups received regular chow diet. Blood samples were collected again after 12th week to measure the OVA-specific IgE levels as an indicator of allergic sensitization. On the final day of the 14th week, mice were fasted overnight for 8 h prior to oral challenge with 20 mg/mouse of OVA. All mice were humanely euthanized after 30 min of monitoring for clinical signs of allergic reactions. Blood was collected by cardiac puncture for various biomarker assays. The serum samples were obtained after centrifugation (10,000x g for 20 min at 4 °C) and stored at –80 °C for further biomarkers (histamine, mast cell protease) and antibody titers analysis. Some whole blood samples were collected separately for flow-cytometric analysis. Spleen was collected for *ex vivo* re-stimulation assays.

2.4. Oral OVA-challenge and assessment of clinical signs

Clinical scores were assigned as described earlier (Rupa and Mine, 2012; Rupa et al., 2014) and the total scores for each animal were obtained by adding scores for the individual symptoms. Briefly, mice were challenged with oral gavage of 20 mg of OVA/mice on the final day after 8 h of fasting. After the oral challenge, the mice were observed for 30 min, and clinical scores were assigned in a blinded manner by three independent and experienced observers. The scale for the clinical scores is as follows; 0 = no sign of discomfort; 1 = repeated scratching and/or rubbing around the snout and head; 2 = puffiness around eyes and nose, gastrointestinal signs-diarrhea, and respiratory difficulty, 3 = wheezing, labored respiration, cyanosis around the mouth and tail; 4 = tremor and convulsions; and 5 = death. Thus, the clinical scores were indicative of the severity of the allergic reaction.

2.5. Measurement of serum histamine and mouse mast cell protease in serum

Histamine concentrations in mouse serum were determined by Enzyme-linked Immunosorbent Assay (ELISA) using a commercial kit (Histamine EIA, LDN Labor Diagnostika Nord, Nordhon, Germany) following the manufacturer's guidelines. ELISA was also used to quantify the serum concentrations of the mouse mast cell protease enzyme (MMCP) as per the manufacturer's instruction (eBio-sciences, CA, USA). Serum samples were pooled within each group (n = 6) due to the limitations in the volume for conducting different biomarker assays and antibody titers analysis.

2.6. Measurement of total and OVA-specific immunoglobulin Isotypes-IgG, IgE, IgG1, and IgG2a in serum

Mice serum samples obtained on the 14th week were used to measure and quantify the total and OVA-specific IgE, IgG1, and IgG2a by commercial ELISA kits (eBiosciences) as per manufacturer's recommendation. In brief, 96-well high binding microtiter plates (Costar Corning Inc., NY, USA) were coated with 2 µg/well of OVA dissolved in 50 mM carbonate buffer; NaHCO₃/Na₂CO₃, pH 9.6 (100 µL/well) for 24 h at 4 °C with gentle shaking over an Orbital Shaker. Rat monoclonal anti-mouse IgE was used for IgE followed by streptavidin-HRP conjugate, rabbit anti-mouse IgG conjugated to alkaline phosphatase was used for the detection of IgG, and rat monoclonal anti-mouse IgG1 and IgG2a were used followed by streptavidin-HRP conjugate. After coating the plates with antigen, on the following day, the plates were washed with 3 times with 200 µL/well of wash buffer (0.05 % Tween PBS 0.01 M, pH 7.4) and then blocked by the blocking buffer (200 µL/well, 37 °C for 1 h) provided by the respective ELISA kits. Washing steps were then repeated and then the diluted serum samples (100 µL/well) were added in duplicates and incubated overnight at room temperature with gentle shaking. The serum samples were diluted in 0.05 % PBST at 1:10 (v/v) for IgE measurement and 1:100 (v/v) for IgG, IgG1, or IgG2a. Next day, the plates were again washed, and the bound antibodies were detected

by adding respective detection antibodies, provided by the manufacturer, at room temperature for 2 h with gentle shaking. Specific binding activity was detected by the addition of 100 µL/well of 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich). The reaction was terminated after 30 min. with the addition of 1 N H₂SO₄ and absorbance at 450 nm was measured using a microplate reader (Bio-Rad 550, Hercules-CA, USA).

2.7. Flow cytometry

Flow cytometry was performed as described before (Rupa et al., 2014). Briefly, whole blood was collected after OVA challenge from individual mice and pooled in duplicates n = 6/group. Staining procedure for each target was carried out as per manufacturer's guideline BD Bioscience, 50 µL of pooled blood samples were first stained with 100 µL of Fluorescein isothiocyanate FITC-conjugated anti-mouse CD25 antibody 558642; BD Bioscience and then phycoerythrin PE-conjugated anti-mouse Foxp3 clone FJKS-16; eBioscience. The stained cells were acquired and measured via fluorescence-activated cell sorter (FACSscan Flow cytometer; BD Bioscience, USA). Data analysis was done using FCS Express 4 Plus software (Denovo software, Los Angeles, CA, USA), and the results were presented as the percentage of double-stained CD25⁺ Foxp3⁺ cells.

2.8. Quantitation of specific cytokines from mouse cultured splenocytes

The spleen samples from individual mouse were collected, and the splenocytes were isolated and plated (2.5 × 10⁶ cells/mL) on a 24-well flat bottom tissue culture plate (Corning Costar) in triplicates. Two hours after plating, the cells were either left unstimulated or stimulated with 50 µg/well of OVA. Culture supernatants were collected after 72 h of incubation at 37 °C and then stored in –20 °C for further analysis. Concentrations of IL-4, IL-17, IL-12, IL-10, IFN-γ, and Transforming growth factor-beta (TGF-β) were measured in culture supernatants using ELISA cytokine kits following the manufacturer's guidelines (eBiosciences).

2.9. Statistical analysis

The statistical analysis of the experimental data was analyzed using Graphpad Prism 7.0 (GraphPad Software, San Diego, CA, USA). All the data were first analyzed to check the Normal distribution by D'Agostino & Pearson test. If they data were not normally distributed then, logarithmic transformation was performed and checked again for normal distribution. All the normally distributed data were then analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. The datasets which were failed the normality test, were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparison test. All data were expressed as means ± standard error of mean (SEM).

3. Results

3.1. Reduction in clinical scores and allergic responses

Clinical scores post oral OVA challenge indicate a significant increase in the frequency of mice showing clinical signs in the Pos-Con group compared to the Neg-Con group (Fig. 2; *P* < 0.001). However, a significant reduction in clinical signs and allergic responses after *L. pentosus* S-PT84 intervention, especially in the Trt-Med and Trt-High groups compared to the Pos-Con group (Fig. 2; *P* < 0.01). However, the animals in the Trt-Low group did not exhibit any significant reduction in the frequency of the allergic symptoms compare to the Pos-Con group.

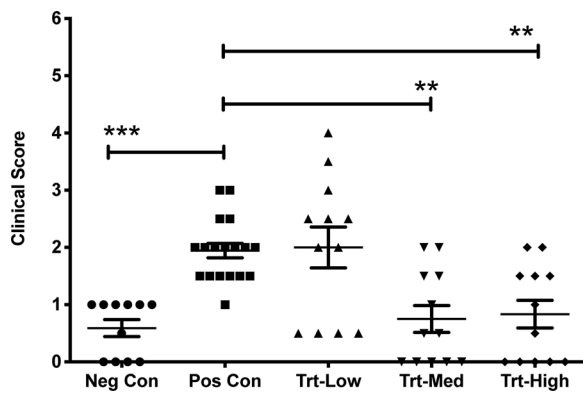


Fig. 2. Clinical scoring of allergic symptoms. On the 14th week of experimental period, clinical scores for each mouse post-OVA challenge were calculated. Average scores assigned by three independent observers in a blinded fashion are represented. The statistical differences between groups were determined, ** and *** indicate $P < 0.01$ and $P < 0.001$ respectively as compared to the positive-control (Pos-Con) group.

3.2. Attenuation of histamine and mast cell protease-1 concentration

The serum histamine and MMCP-1 concentrations were significantly higher in the Pos-Con group compared to the Neg-Con group (Fig. 3A; $P < 0.001$ and Fig. 3B; $P < 0.001$). But the *L. pentosus* S-PT84 treatment significantly decreased the serum histamine and MMCP-1 levels in the Trt-Med (Fig. 3A; $P < 0.01$ and Fig. 3B; $P < 0.05$) and Trt-High (Fig. 3A; $P < 0.001$ and Fig. 3B; $P < 0.01$) groups compared to the Pos-Con group. However, the animals in the Trt-Low group did not exhibit any significant reduction in either in the histamine and MMCP-1 levels compare to the Pos-Con group.

3.3. Immunoglobulin isotype-related antibody activity in serum samples

Total IgE, IgG, and IgG1 were significantly increased in the Pos-Con group compare to the Neg-Con (Fig. 4A; $P < 0.001$; Fig. 4C; $P < 0.001$; and Fig. 5A; $P < 0.001$). *L. pentosus* S-PT84 intervention significantly reduced the total IgE levels only in the Trt-High (Fig. 4A; $P < 0.01$) group. The total IgG levels were significantly reduced after the *L. pentosus* S-PT84 intervention in all treatment groups, Trt-Low, Trt-Med, and Trt-High (Fig. 4C; $P < 0.01$). However, the total IgG1 levels remain unchanged (Fig. 5A) after *L. pentosus* S-PT84 intervention compared to the Pos-Con group. The total IgG2a levels did not significantly increase in the Pos-Con group compare to the Neg-Con (Fig. 5C). However, a significant increase of total IgG2a was observed in the Trt-Low ($P < 0.05$), Trt-Med ($P < 0.001$) groups compare to the Pos-Con group, no significant difference was observed in the Trt-high group compare to the Pos-Con group (Fig. 5C). The OVA-specific IgE (OVA-sp-IgE) and OVA-sp-IgG levels did not change after *L. pentosus* S-PT84 intervention (Fig. 4B and 4D). Interestingly, both the OVA-sp-IgG1 and OVA-sp-

IgG2a levels were significantly decreased in all the *L. pentosus* S-PT84 treatment groups, Trt-Low, Trt-Med, and Trt-High (Fig. 5B; $P < 0.01$ and Fig. 5D; $P < 0.01$).

3.4. Proportion of T-regulatory cells in blood indicates immune regulation

The number of T-regulatory cells was measured from whole blood by flow cytometry. The percentage of CD25⁺ Foxp3⁺ cells were significantly decreased in the Pos-Con (Fig. 6A; $P < 0.001$) group compared to the Neg-Con group. A significant increasing trend proportional to the increased dosage of *L. pentosus* S-PT84 intervention was observed in the percentage of CD25⁺ Foxp3⁺ cells as compared to the Pos-Con group (Fig. 6A; Trt-Med $P < 0.01$ and Trt-High $P < 0.01$). A representative flow-cytometric image of cell count for each treatment group is shown in Fig. 6B.

3.5. Cytokine concentration

Total of six different kinds of cytokine productions was measured on stimulated and unstimulated spleen cells. Concentrations of IL-4, IL-17a, and IFN- γ levels were significantly increased in the Pos-Con group compare to Neg-Con group, but the levels of these cytokines were significantly decreased in all the *L. pentosus* S-PT84 treatment groups (Trt-Low, Trt-Med, and Trt-High) (Fig. 7A, 7B, and 7C) compared to the Pos-Con group, with only exception of Trt-High group in IL-4 expression (Fig. 7A), here the IL-4 expression was showing a reducing trend but did not reach statistical significance. Surprisingly, IL-10 and IL-12 levels were increased in Pos-Con group compare to the Neg-Con group, and the levels of IL-10 in the Pos-Con group was significantly increased (Fig. 7D). The intervention of *L. pentosus* S-PT84 significantly decreased the IL-10 levels in the Trt-Med and Trt-High (Fig. 7D) compare to the positive controls. *L. pentosus* S-PT84 treatment Trt-Low group significantly increased the IL-12 levels compare to the controls (Fig. 7E). However, neither the Pos-Con nor the treatments significantly affected the TGF- β levels in the splenocytes.

4. Discussion

The predominance of food allergy or food-protein induced allergic conditions such as atopic dermatitis, allergic rhinitis (hay fever), and asthma are increasingly common disorders, especially in the industrialized countries, and are a severe social health concern. Previous reports suggested that the intervention of LAB, *L. pentosus* S-PT84 could exhibit immunomodulatory properties by modulating the Th-1/Th-2 balances (Nonaka et al., 2008) and promote type-1 immunity by activating the NK and dendritic cells activity (Koizumi et al., 2008). The development of food allergy depends on the Th-2-derived immune response characterized by the production of cytokines that eventually promote the production of IgE and other associated chemical mediators (Robinson and Kay, 1996; Romagnani, 2000). Thus, *L. pentosus* S-PT84 could serve a potential candidate for modulating food allergy.

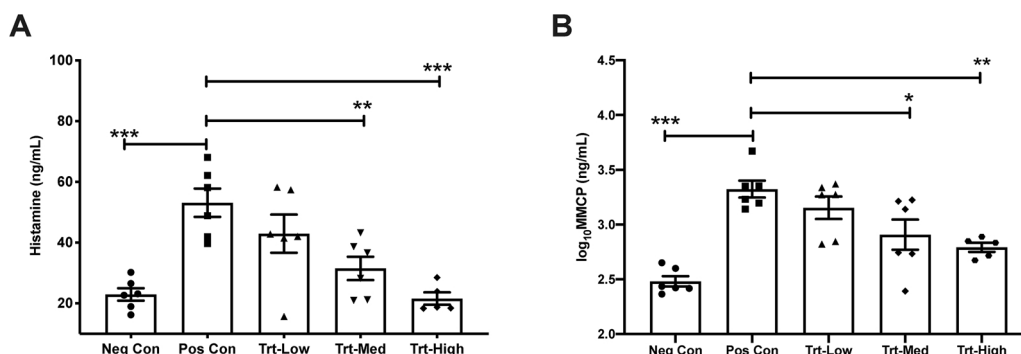


Fig. 3. Serum histamine and mast cell protease levels. (A) Serum histamine concentration and (B) Mouse mast cell protease (MMCP). The histamine and MMCP concentrations were measured by ELISA. Data represented as mean $\pm$ standard error of mean (SEM) from $n = 6$ per treatment group, *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ respectively, as compared to the positive-control (Pos-Con) group.

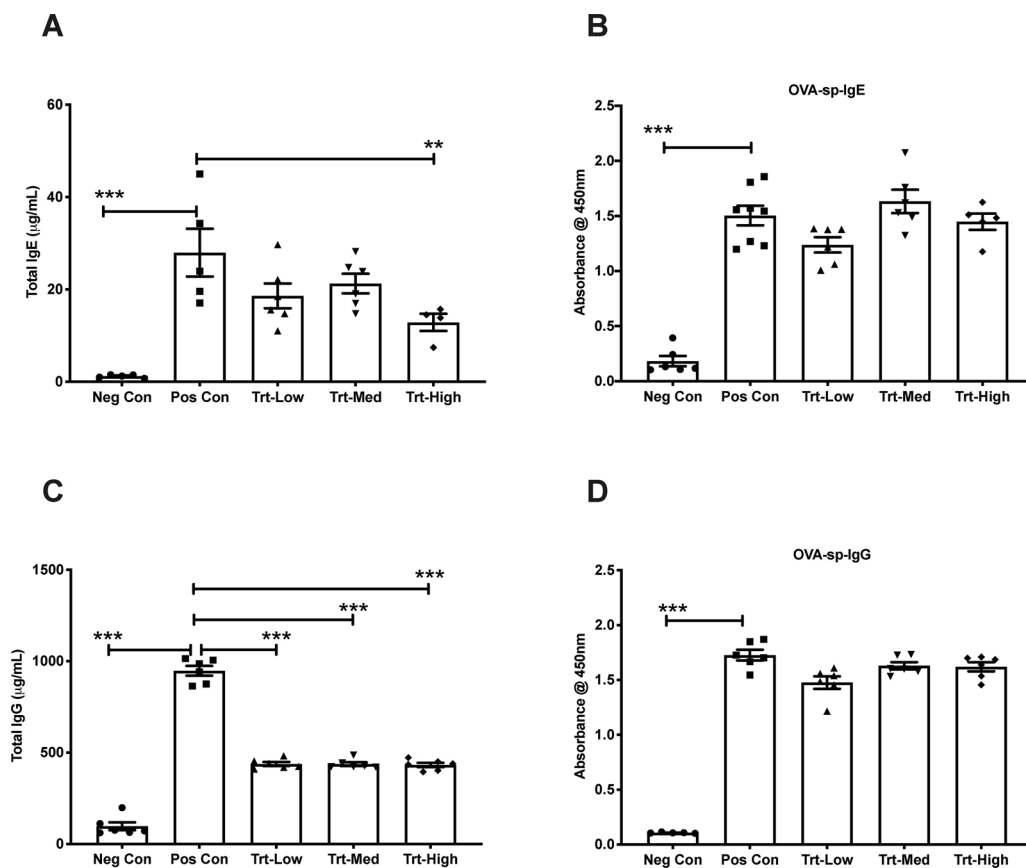


Fig. 4. Serum total and OVA-specific IgE and IgG levels. (A) Total IgE and (B) OVA-Specific IgE (OVA-sp-IgE), (C) Total IgG, and (D) OVA-specific IgG (OVA-sp-IgG) concentration in serum. Total and OVA-sp-IgE and IgG concentrations were measured by ELISA. Data represented as mean $\pm$ standard error of mean (SEM) from $n = 6$ per treatment group, ** and *** indicate $P < 0.01$ and $P < 0.001$ respectively, as compared to the positive-control (Pos-Con) group.

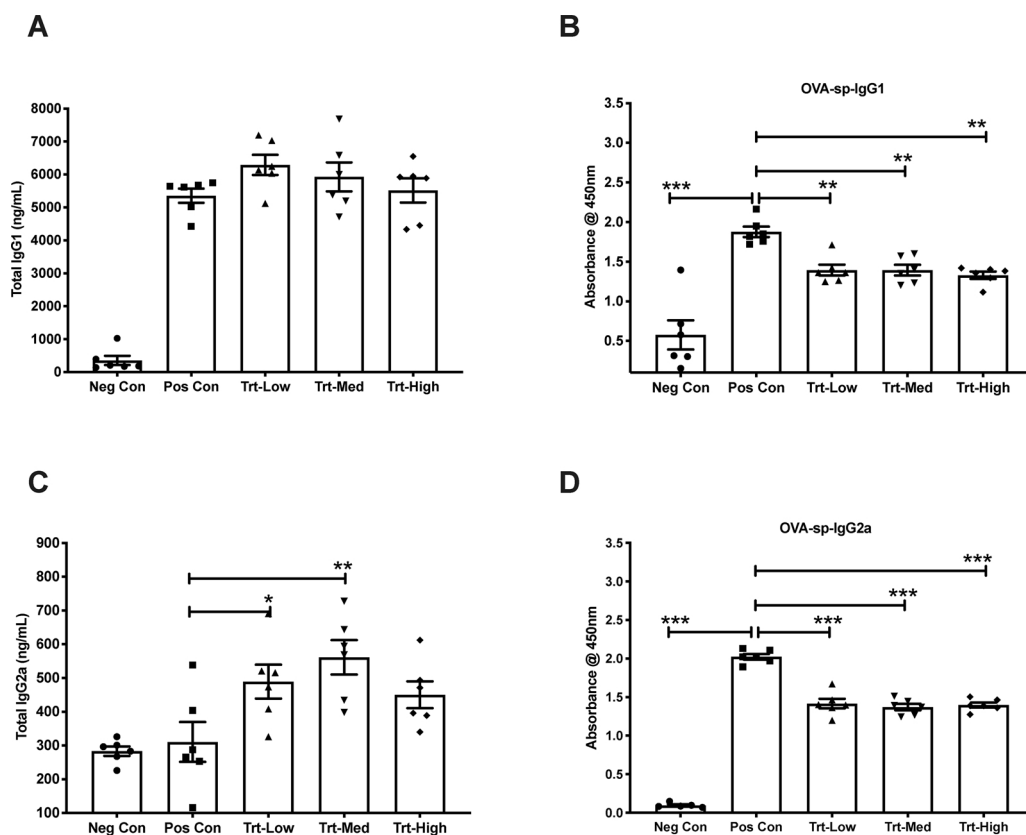


Fig. 5. Serum total and OVA-specific IgG1 and IgG2a levels. (A) Total IgG1, (B) OVA-specific IgG1 (OVA-sp-IgG1), (C) Total IgG2a, and (D) OVA-specific IgG2a (OVA-sp-IgG2a) concentration in serum. Total and OVA-sp-IgG1 and IgG2a concentrations were measured by ELISA. Data represented as mean $\pm$ standard error of mean (SEM) from $n = 6$ per treatment group, ** and *** indicate $P < 0.01$ and $P < 0.001$ respectively, as compared to the positive-control (Pos-Con) group.

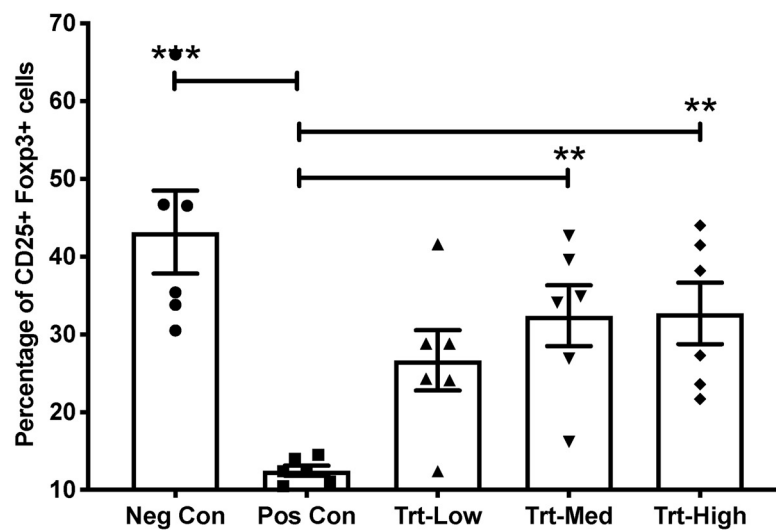
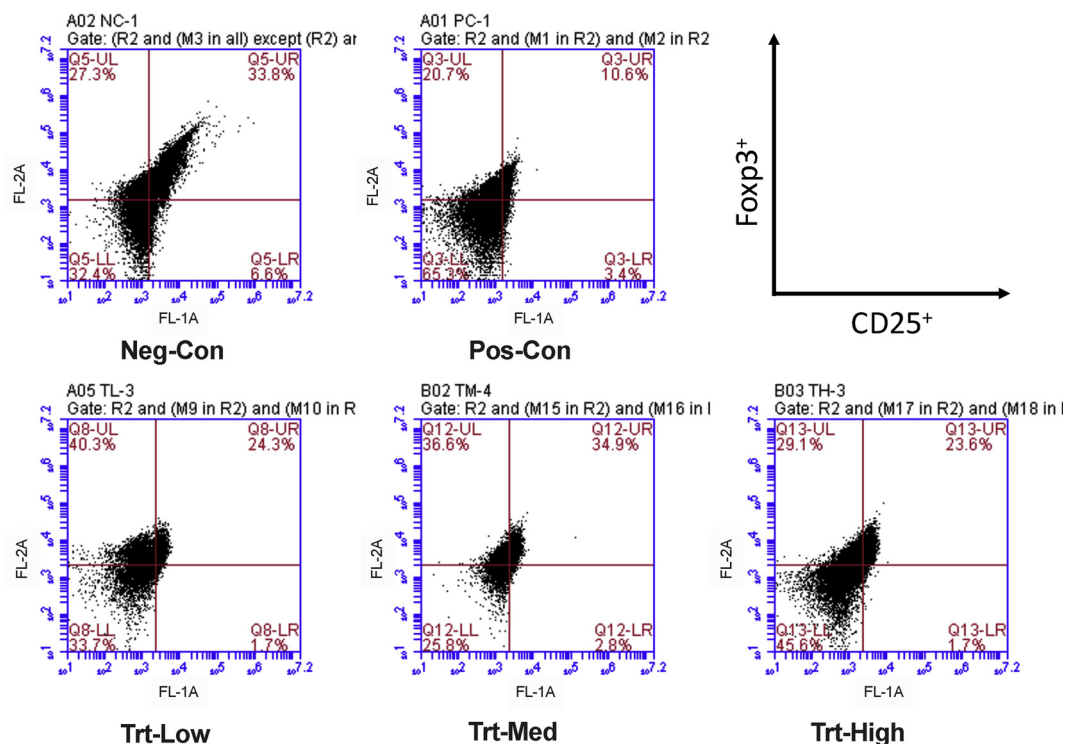
A**B**

Fig. 6. Percentage of CD25+ and Fopx3+ cells in serum. (A) Percentage of CD25+ Fopx3+ cells were determined by flow cytometry from whole blood of mice collected post oral challenge. Data represented as mean $\pm$ standard error of mean (SEM) from $n = 6$ animals per treatment group, ** and *** indicate $P < 0.01$ and $P < 0.001$ respectively, as compared to the positive-control (Pos-Con) group. (B) A representative image of CD25+ and Fopx3+ cells for each group and respective percentage of cells for each quadrant are shown.

Therefore, this study tested the hypothesis that oral intervention of *L. pentosus* S-PT84 could reduce the egg protein OVA-induced allergic symptoms in a mice model of egg allergy.

In this study, we have three different treatment groups of *L. pentosus* S-PT84 intervention, Trt-Low (0.006 %), Trt-Med (0.06 %), and Trt-High (0.6 %). The responses to the various treatments were assessed by allergic clinical symptoms, histamine, and mast cell protease concentration, serum total and anti-OVA anti-body as IgE, IgG, IgG1, and

IgG2a, as well as cytokine production levels in spleen, and percentage of T-reg cells in the blood. We demonstrated that 9 weeks of oral administration of *L. pentosus* S-PT84 through a pelleted diet (Trt-Med and Trt-High groups) could significantly reduce the clinical symptoms, suggesting the potential use of *L. pentosus* S-PT84 in the treatment of egg allergy induced disorders.

Increased in scratching frequency, allergic rhinitis, and asthma are the major clinical signs associated with egg allergy (Tariq et al., 2000).

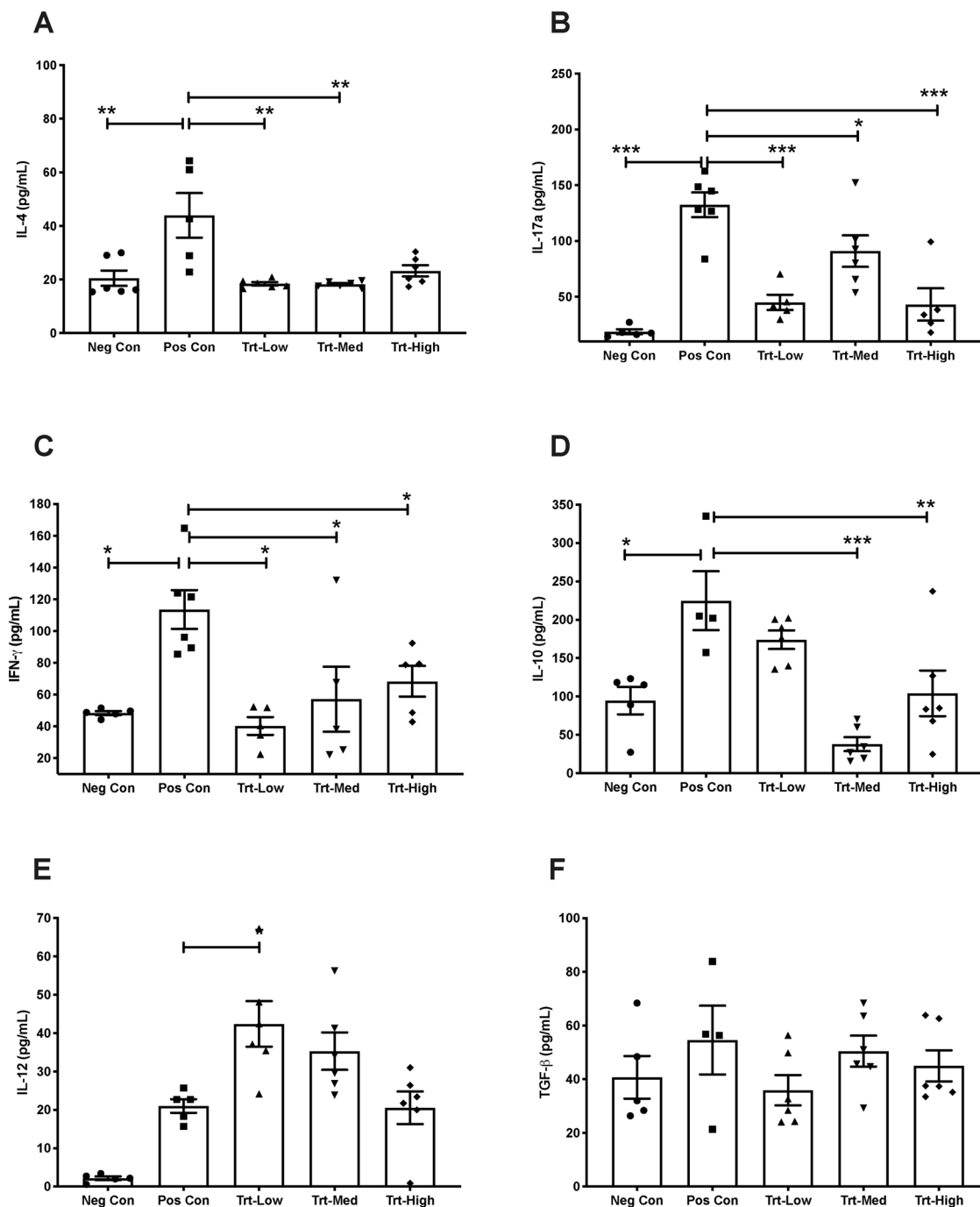


Fig. 7. Cytokine production cultured splenocytes. Spleens were collected from mice and pooled within each group ($n = 6$ per group). Single cell splenocyte cultures were prepared and plated at 2.5×10^6 cells/mL in triplicate wells in a 24-well plate and were either stimulated (Pos-Con, Trt-Low, Trt-Med, and Trt-High) with $50 \mu\text{g}/\text{well}$ of OVA for 72 h or unstimulated (Neg-Con). The cytokine concentration of cell culture supernatants for (A) IL-4, (B) IL-17a, (C) IFN- γ , (D) IL-10 (E) IL-12a and (F) TGF- β were determined by ELISA. Data represented as mean $\pm$ standard error of mean (SEM) from $n = 12$ per treatment group, *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ respectively, as compared to the positive-control (Pos-Con) group.

The present study demonstrated that oral intervention of *L. pentosus* S-PT84 significantly improves the clinical signs such as scratching, puffiness around the eyes, lethargy, gastrointestinal signs, diarrhea, tremor, and respiratory difficulty, supporting the therapeutic use of *L. pentosus* S-PT84 against food-induced allergic disorders. Histamine is a primary amine synthesized from histidine in the Golgi apparatus of mast cells, has been well recognized as a significant mediator of the allergic diseases (Xie and He, 2005). Histamine is released upon surface binding of antigen-specific IgE and is responsible for the adverse symptoms' characteristic of an allergic reaction (Cianferoni and

Muraro, 2012). Histamine levels were significantly reduced after *L. pentosus* S-PT84 intervention. Mast cells are an important mediator-secreting cell in allergic diseases, MMCP-1 predominantly expressed in the mucosal mast cells. The degranulation of mast cell leads to the increased levels of MMCP-1 indicating the increase in mucosal permeability in allergic reactions (Benedé and Berin, 2018; Galli and Tsai, 2012; Rupa et al., 2014). *L. pentosus* S-PT84 intervention significantly reduced the MMCP-1 levels in both Trt-Med and Trt-High dose groups. Our study demonstrated that *L. pentosus* S-PT84 intervention could potentially attenuate the development of allergic reactions. The

reduced levels of histamine and MMCP-1 directly correlate with the reduction of allergenic symptoms in the mice observed in the clinical evaluation study.

The type-1 hypersensitivity reactions are characterized by increased in allergen-specific IgE as a critical biomarker (Platts-Mills, 2001; Galli and Tsai, 2012). However, our study demonstrated that *L. pentosus* S-PT84 intervention did not affect the OVA-sp-IgE concentration, suggesting that the therapeutic effect of *L. pentosus* S-PT84 may be due to the other local regulatory mechanisms inducing immune tolerance to the allergen OVA. Similarly, earlier studies have demonstrated that specific immunotherapy did not significantly reduce the levels of antigen-specific IgE, but still suppress the allergic responses (Jutel et al., 2003; Möbs et al., 2010). Previous studies from our laboratory also observed the same phenomena (Yang et al., 2013; Mine et al., 2019). Thus, the growing body of evidence suggests that the measurement of antigen-specific IgE levels in serum is not a reliable indicator of allergic reaction, as the allergen-specific serum IgE may not redirect the allergy-mediating activity of leucocyte FcεR1-bound antibody. These results also suggest that the reduction of allergic reactions or the increase in tolerance level may be modulated by several different kinds of mechanisms including clonal deletion and induction of tolerogenic T-regulatory cells that produce IL-10 and/or TGF-β (Akdis and Akdis, 2011; du Pré and Samsom, 2011). Additionally, significant decrease in the total IgE levels in the serum after the intervention of *L. pentosus* S-PT84 in the Trt-High dose group may also contribute to the reduction of allergic responses. However, the mechanism of decrease in total IgE levels and its correlation to the reduction of allergic symptoms needs to be further investigated. The measurement of serum OVA-specific IgG, and sub-isotopes IgG1 and IgG2a exhibit an interesting phenomenon. The OVA-sp IgG levels did not differ in the treatment groups with only exception to the Trt-Low group. On the contrary, the serum OVA-sp IgG1 and IgG2a levels were significantly reduced after the intervention of *L. pentosus* S-PT84 indicating the effect of treatment in reducing antibody isotypes. Additionally, the significant reduction of total IgG levels further suggests that it is most likely that allergic suppression induced by *L. pentosus* S-PT84 treatment protects against the development of allergic signs by an antibody isotype-mediated mechanism. However, it remains elusive at this moment that how *L. pentosus* S-PT84 may exert its beneficial effect on the reduction of allergic symptoms since the OVA-sp IgG and IgE antibody remain unaltered. IgA is also an essential component of mucosal immune homeostasis and plays a pivotal part in the maintenance of a vast community of commensal bacteria in the intestinal lumen while minimizing penetration of the gut epithelium (Mantis et al., 2011). In the present study, the total and OVA-sp IgA levels in the fecal extract remain largely unaltered (Data not presented).

The T-cells play an important role in sustaining immune tolerance and the maintenance of immune responses, such as the prevention of autoimmunity and modulation of allergic diseases (Nouri-Aria, 2009). The transcription factor Foxp3, a member of the forkhead-winged helix family, appears to be critical in the suppressive abilities of Treg cells. The decrease in Foxp3+ CD25+ Treg cells, resulting in an increase of Th-2-derived immune response by increasing the production of cytokines and eventually promote the production of IgE (Nouri-Aria, 2009). Our present study demonstrated that the intervention of *L. pentosus* S-PT84 significantly increases the percentage of Foxp3+ CD25+ Treg cells. IL-4 production by mast cells is critical in food allergic reaction (Chatila, 2005; Vignali et al., 2008; Noval Rivas and Chatila, 2016). IL-4 produced by the Th-2 cells promotes the production of antigen-specific IgE by B cells (Dourado et al., 2010; Mathias et al., 2011). IgE interaction with the FcεR1 expressed on mast cells act as an amplifier of the Th-2 cells and IgE responses during allergic sensitization (Burton et al., 2014). Importantly, dysregulated IgE mast cell activation and their subsequent IL-4 production profoundly inhibits allergen T-reg cells generation during allergic processes (NovalRivas et al., 2015). Following the increase in the percentage of Foxp3+ CD25+ T-Reg cells,

we also observed that *L. pentosus* S-PT84 intervention significantly reduced the IL-4 (i.e., a Th2-biased cytokine) levels in cultured splenocytes. These data suggest that *L. pentosus* S-PT84 intervention increase the T-reg cells generation and reduce the Th-2 cell-based IL-4 production. In contrast with the reduction in IL-4, *L. pentosus* S-PT84 intervention significantly upregulated the expression of IL-12 (only in the Trt-Low group) and significantly reduced the expression of IFN-γ indicating that the *L. pentosus* S-PT84 may be able to modulate both the Th-1/Th-2 response towards the alleviation of allergic reactions. Furthermore, in mice increase percentage of Foxp3+ Treg cells induce the production of IL-10 and TGF-β (Oboki et al., 2008). The reduction of IL-10 levels in cultured splenocytes after *L. pentosus* S-PT84 intervention (Trt-Med and Trt-High groups) also indicate the beneficial role of *L. pentosus* S-PT84 in the mitigation of allergic responses. However, in our study we could not able to detect any significant changes in TGF-β levels may be due to a Treg defect in cytokine secretion in this specific mouse model. Nevertheless, few previous studies suggested that the Foxp3 expression is more significant in food allergy tolerance induction as compared to IL-10 and TGF-β expression (Krogulska et al., 2011; Westerholm-Ormio et al., 2010). Few studies also indicate that IL-17 producing Th cells (Th 17 cells) may also contribute to the pathogenesis of classically recognized Th2-mediated allergic disorders (Oboki et al., 2008). Our data revealed that *L. pentosus* S-PT84 treatment significantly reduces the IL-17 levels in all treatment groups compared to the Pos-Con group, suggesting that the *L. pentosus* S-PT84 also contribute towards ameliorating the allergic reactions by controlling the Th-17 cells.

In conclusion, the present study provides evidence to support the therapeutic role of *L. pentosus* S-PT84 in the attenuation of OVA-induced allergic responses in a BALB/C mouse model of food allergy. Induction of immune tolerance through a shift from a Th2- to a Th1-skewed immune response, down-regulation of Th-17 cells, increase the generation of T-reg cells, suppression of histamine release, and modulation of mast cell functionality may be some of the contributing mechanisms responsible for the attenuation of the allergic responses. Overall, this study suggests that the oral intervention of *L. pentosus* S-PT84 may be a potent therapeutic strategy that can be used as a dietary supplement for the treatment of food allergy.

Authors' contributions

YM designed and supervised the study and YJ and KM performed the experiment. KM analysed the data and wrote the manuscript, YM and HS reviewed a final manuscript. All authors interpreted the data and agreed to publish.

Declaration of Competing Interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.molimm.2020.01.025>.

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Supplementary Table -1: Composition of experimental diet (g/kg)

	Control Diet	Treatment Diet		
		Low-dose (0.006%)	Medium-dose (0.06%)	High-dose (0.6%)
Corn starch	465	464.94	464.4	459
Casein	210	210	210	210
Sucrose	90	90	90	90
Maltodextrin	100	100	100	100
Lard	20	20	20	20
Soybean oil	20	20	20	20
Cellulose	37.2	37.2	37.2	37.2
L-Cystine	3	3	3	3
Choline bitartrate	2.8	2.8	2.8	2.8
Calcium phosphate	2	2	2	2
Mineral mix (AIN-93G)	35	35	35	35
Vitamin mix (AIN-93G)	15	15	15	15
Heat-killed S-PT84	-	0.06	0.6	6
Protein (% kcal)	20.5	20.5	20.5	20.6
Carbohydrate (% kcal)	69.1	69	69	68.9
Fat (% kcal)	10.5	10.5	10.5	10.5