



# *Lactobacillus reuteri* induces intestinal immune tolerance against food allergy in mice



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## ABSTRACT

The effect of *Lactobacillus reuteri* against food allergy was investigated in ovalbumin (OVA)-sensitized BALB/c mice. Oral administration with *L. reuteri* restored the deteriorated profile of enteric flora, and attenuated allergic diarrhoea, mast cell activation, and serum IgE production in allergic mice. The production of signature T helper (Th)1 and 2 cytokines, namely IFN- $\gamma$  and IL-4, by splenocytes was suppressed by *L. reuteri*. Concordantly, the intestinal expression of IFN- $\gamma$ , IL-4, T-bet and GATA3 was down-regulated. However, *L. reuteri* augmented the expression of IL-10, TGF- $\beta$  and Foxp3, and the number of IL-10-secreting CD11c<sup>+</sup>CD103<sup>+</sup> mesenteric lymph node (MLN) cells. Furthermore, direct exposure to heat-killed *L. reuteri* attenuated OVA-induced cell proliferation and IL-2 secretion by MLN cells. These results demonstrate that *L. reuteri* possesses anti-allergic activities via modulating enteric flora and promoting tolerogenic immune responses, and suggest *L. reuteri* as a functional probiotic against food allergy.

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## 1. Introduction

Lactobacilli with probiotic activities have been considered functional foods due to various beneficial health effects to the host, such as modulation of immune function (Lin, 2003). Over the past decade, *Lactobacillus reuteri* is one of the most commonly used and studied probiotics based on its safety and several biological activities, such as anti-inflammation, antimicrobial effects, protection against pathogens, and relief of gastrointestinal discomfort (Cleusix, Lacroix, Vollenweider, & Le Blay, 2008; Juarez, Villena, Salva, de Valdez, & Rodriguez, 2013; Kabuki, Saito, Kawai, Uemura, & Itoh, 1997; Liu, Fatherree, Dingle, Tran, & Rhoads, 2013; Shornikova, Casas, Mykkanen, Salo, & Vesikari, 1997; Urbanska & Szajewska, 2014; Wolf, Garleb, Ataya, & Casas, 1995). *L. reuteri* can be found in many foods, as well as in the gut (Dellaglio, Arrizza, & Ledda, 1981). We previously reported that *L. reuteri* was one of the major enteric strains sensitive to the prebiotic effect of diosgenin, a plant-derived steroidal sapogenin exhibiting anti-allergic and immunomodulatory properties (Huang, Cheng, Deng, Chou, & Jan, 2012; Huang, Ku, & Jan, 2009; Huang, Liu, & Jan, 2010; Huang, Wang, Lin, Hori, & Jan, 2017). As

enteric bacteria are known to play an important role in the maintenance of intestinal homeostasis, oral consumption with *L. reuteri* has been suggested as a potential strategy for the management of intestinal immune disorders (Koboziev, Reinoso Webb, Furr, & Grisham, 2014).

Food allergy is an aberrant immune response to dietary components, usually proteins (Sampson, 2004). The clinical signs of food allergy may range from minor gastrointestinal discomfort to severe anaphylactic shock and even death (Sampson, 2004). The prevalence of food allergy is approximately 7–10% in preschoolers and 3–4% in adults, which appears to be continuously increasing in the past 10–15 years (Gupta et al., 2011; Prescott et al., 2013). The current medical strategy for managing food allergy relies on total avoidance of relevant allergens. Pharmacotherapy is primarily used for the relief of the hypersensitivity symptoms. Hence, the development of safe and effective measures to prevent or treat food allergy is a relevant issue.

Food protein-induced allergic responses are generally primed at intestinal mucosa, and the hypersensitivity reactions are triggered by the degranulation of mast cells armed with allergen-specific immunoglobulin (Ig)E (Untersmayr & Jensen-Jarolim, 2006). IgE-mediated food allergy is a T helper (Th)2-dependent disease. The major Th2-type cytokine IL-4 is pivotal to trigger IgE class switching (Coffman, Savelkoul, & Leberman, 1989; Untersmayr & Jensen-Jarolim, 2006). Hence, shifting immune balance from the Th2 to Th1 direction has been proposed as a strategy to correct the aber-

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rant T cell immunity associated with food allergy (Coffman et al., 1989). In addition to modulation of T cell immunobalance, enhancement of functional regulatory T (Treg) cells is considered a potential approach to induce immune tolerance against allergic reactions (Chehade & Mayer, 2005). To date, Foxp3 has been well recognized as a lineage-determining transcription factor, as well as a relevant marker for the development of Treg cells (Sakaguchi, 2004; Zheng & Rudensky, 2007). The immunosuppressive mechanisms of Treg cells include cytokine-dependent and -independent pathways. Among cytokine-dependent pathways, IL-10 and TGF- $\beta$  are the major cytokines to suppress exaggerated immune responses. Compelling evidence indicates that IL-10 and TGF- $\beta$  are crucial for maintaining immune tolerance and attenuating allergic responses (Beyer et al., 2002; Hansen et al., 2000; Joetham et al., 2007; Meiler et al., 2008; Oh et al., 2002). Dendritic cells (DCs) play a pivotal role in the priming and differentiation of naive T cells (Kapsenberg, 2003; Tan & O'Neill, 2005). A functionally distinct subset of CD103<sup>+</sup> DCs has been identified in mesenteric lymph nodes (MLNs) that induces Treg cell differentiation and gut-homing receptor expression in responding T cells (Coombes et al., 2007; Johansson-Lindbom et al., 2005; Ruane & Lavelle, 2011; Sun et al., 2007). Therefore, it is suggested that CD103<sup>+</sup> DCs are required for the induction of immune tolerance against enteric antigens.

Several strains of enteric bacteria with probiotic activities are effective to ameliorate allergic inflammation via various immunological mechanisms, such as the enhancement of Th1 or Treg cell functions depending on different bacterial strains (Ashraf, Vasiljevic, Day, Smith, & Donkor, 2014; Frossard, Steidler, & Eigenmann, 2007; Lyons et al., 2010; Shida et al., 1998; Yasuda, Serata, & Sako, 2008). We previously reported that *L. murinus* and *L. reuteri* were the major enteric strains sensitive to the prebiotic effect of diosgenin in mice with food allergy (Huang et al., 2012). We also found that diosgenin-mediated anti-allergic effects were associated with an up-regulation of the functionality of both Th1 and Treg cells (Huang et al., 2009, 2010, 2017). Recently, we further demonstrated that *L. murinus* was effective to attenuate allergic reactions, and shift the T cell immunobalance from the Th2 to Th1 direction, but did not affect regulatory immunity (Huang, Shen, Liang, & Jan, 2016). Interestingly, *L. reuteri* has been shown to attenuate airway allergic responses via the induction of Treg cells (Forsythe, Inman, & Bienenstock, 2007; Karimi, Inman, Bienenstock, & Forsythe, 2009). To date, it remains unclear if *L. reuteri* is a functional probiotic against intestinal allergic responses. In addition, the underlying mechanisms of *L. reuteri*-mediated immunomodulatory effects has yet to be fully elucidated. Hence, the objective of the present study is to test our hypothesis that *L. reuteri* may be a functional probiotic capable of inducing intestinal tolerogenic DCs and Treg cells against food allergy.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich Chemical (Saint Louis, MO, USA), unless otherwise stated. Anaerobe container system and supplies for bacterial incubation were purchased from BD Biosciences (San Jose, CA, USA). Antibodies, reagents and standards used for ELISA were purchased from BD Biosciences. Enzymes and reagents used for RT-PCR were purchased from Promega (Madison, WI, USA).

### 2.2. Preparation of *L. reuteri*

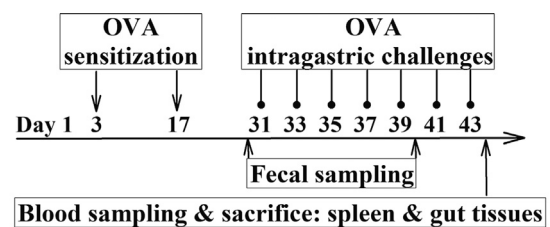
*L. reuteri* (NCBI accession number: AB425917) was isolated from the faeces of mice treated with diosgenin as previously described

(Huang et al., 2012). *L. reuteri* was cultured in fresh de Man, Rogosa and Sharpe (MRS) broth anaerobically at 37 °C for 48 h. After centrifugation, the bacteria were harvested and then resuspended in fresh MRS broth to adjust a final concentration of 10<sup>10</sup> colony forming units (CFU)/mL for oral gavage to mice.

### 2.3. Animals and protocol of food allergy model

Male BALB/c mice, 4–5 weeks old, were obtained from the Animal Breeding Center of the National Taiwan University Hospital (Taipei, Taiwan). Mice were kept in plastic cages containing a saw-dust bedding and acclimated to the new environment for at least 1 week prior to experimentation. The housing conditions are maintained at 23 ± 2 °C with a relative humidity of 60 ± 20%. Except for the days of allergen challenge, the mice have free access to food and water.

We employed a murine model of food allergy previously described (Brandt et al., 2003; Huang et al., 2009). The mice were randomly divided into the following groups (Fig. 1): nonsensitized (NS), ovalbumin (OVA)-sensitized and challenged (OVA), vehicle-treated and OVA-sensitized and challenged (VH), and *L. reuteri*-treated and OVA-sensitized and challenged (*L. reuteri*). *L. reuteri* (1 × 10<sup>9</sup> CFU/0.1 mL MRS broth per mouse) and the vehicle (VH; 0.1 mL MRS broth per mouse) was administered daily by gavage throughout the experiment. The dose of *L. reuteri* was chosen on the basis of previous results showing the effectiveness to suppress allergic airway responses in mice (Forsythe et al., 2007). Except for the NS group, each mouse was sensitized with OVA by intraperitoneal injection using 0.1 mL sensitization solution containing 50 µg OVA and 1 mg alum on day 3, and boosted with a double dose on day 17. To induce allergic reactions, mice were challenged repeatedly with OVA (50 mg in 0.3 mL saline) by gavage every other day from day 31 to day 43. Mice were deprived of food 3 h before administration with *L. reuteri* on the days of challenge and were challenged with OVA 1 h after the administration. Allergic diarrhea characterized as profuse liquid stool was checked visually for 3 h after each OVA challenge. Fresh faecal samples from each group were collected before the 1st challenge (day 30) and after



NS: Untreated, and without OVA sensitization but challenge

OVA: Untreated, and with OVA sensitization & challenge

VH: 0.1 mL MRS broth/mouse, daily

*L. reuteri*: 10<sup>9</sup> CFU live *L. reuteri* in 0.1 mL MRS broth/mouse, daily

**Fig. 1.** Protocols of *L. reuteri* administration and OVA sensitization and challenge. BALB/c mice were randomly divided into the following groups: nonsensitized (NS), OVA-sensitized and challenged (OVA), vehicle-treated and OVA-sensitized and challenged (VH), and treated with *L. reuteri* and OVA-sensitized and challenged (*L. reuteri*). Each mouse in *L. reuteri*-treated group was administered with 1 × 10<sup>9</sup> CFU *L. reuteri* in 0.1 mL MRS broth (vehicle; VH) by oral gavage daily throughout the experiment. Except for the mice of the NS group, each mouse was intraperitoneally sensitized with 50 µg OVA and 1 mg aluminum potassium sulfate on day 3, and later boosted with a double dose on day 17 followed by repeatedly challenged with OVA by gavage every other day from day 31 to day 43. Fresh faecal samples from each group were collected before 1<sup>st</sup> challenge (day 30) and after 5<sup>th</sup> challenge (day 40). 3 h post the last OVA challenge, serum sample from each mouse was collected, and the mice were sacrificed to isolate spleen and duodenal tissues for further experiments.

the 5th challenge (day 40). The mice were sacrificed 3 h post the last OVA challenge, serum samples, the spleen and duodenal tissues that were previously reported to exhibit marked allergic responses (Huang et al., 2009, 2010, 2016), were collected for further experiments. The animal experiments were approved by the Institutional Animal Care and Use Committee of the National Taiwan University.

#### 2.4. Culture of faecal bacteria

The collected faecal samples were dispersed in saline and inoculated on tryptic soy agar (TSA) and MRS agar plates to culture non-specific bacteria and lactic acid bacteria (LAB), respectively, as previously described (Huang et al., 2012, 2016). After incubation for 48 h, the number of CFU was counted and the density of microbes was expressed as CFU/g of faeces.

#### 2.5. Necropsy and intestinal tissue preparation

The duodenum (1.5–4.5 cm toward the anal side from the pylorus) were isolated, fixed, embedded in paraffin, and sectioned at a thickness of 4–5  $\mu$ m. Tissue sections were subjected to toluidine blue staining to detect mast cells.

#### 2.6. Mesenteric lymph node (MLN) cell preparation and flow cytometric analysis

MLNs from the same group of mice were excised, pooled and kept in cold RPMI 1640 medium containing 10% fetal bovine serum. Single-cell suspensions were prepared by gently teasing the tissues and passing the cells through a nylon mesh cell strainer (BD Pharmingen, San Jose, CA, USA). After washing, the cells were stained with FITC-conjugated anti-IL-10, PE-conjugated anti-CD11c and PerCP/Cy5.5-conjugated anti-CD103 (Biolegend, San Diego, CA, USA), and then analyzed using a flow cytometer (BD FACSCalibur, San Jose, CA, USA).

#### 2.7. Preparation of heat-killed *L. reuteri* and treatment of MLN cells

Mice were sensitized with OVA by intraperitoneal injection using 0.1 mL sensitization solution containing 50  $\mu$ g OVA and 1 mg alum, and boosted with a double dose 2 weeks later. The mice were sacrificed 2 weeks after the boost. MLNs were collected and made into single cell suspensions. After washing twice with phosphate-buffered saline (PBS), *L. reuteri* was heated at 100 °C for 30 min. The MLN cells ( $5 \times 10^6$  cells/mL) were seeded into 96-well culture plates (0.1 mL/well), treated with heat-killed *L. reuteri* (1–100  $\mu$ g/mL), and then re-stimulated with OVA (100  $\mu$ g/mL) for 68 h. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) stock solution (5 mg/mL in PBS) was added to each well (10  $\mu$ L/well) and incubated for 4 h. At the end of incubation, the formed formazan was dissolved with 10% sodium dodecyl sulfate in 0.01 M HCl, and then the optical density (O.D.) was read at 570 nm, and at 630 nm as background. Stimulation index was calculated by [O.D. of cells re-stimulated with OVA/O.D. of cells without OVA-re-stimulation]. The supernatants of cultured MLN cells were collected for IL-2 measurement.

#### 2.8. Enzyme-linked immunosorbent assay (ELISA)

The levels of total and OVA-specific IgE in serum were quantified by ELISA as previously described (Huang et al., 2009). The splenocytes ( $5 \times 10^6$  cells/mL) prepared from each group were cultured in the presence of OVA (50  $\mu$ g/mL) for 72 h to induce antigen-specific IL-4 and IFN- $\gamma$  production. The levels of cytokines

in supernatants from cultured splenocytes and MLN cells were measured by ELISA as previously described (Huang et al., 2016).

#### 2.9. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from the duodenal tissues was extracted and the steady state mRNA expression of target genes was measured by RT-PCR. The extracted RNA were tested for DNA contamination by a side-by-side reaction without RT, and no PCR products were confirmed for all samples. The used PCR primers are summarized in Table S1. The procedures of total RNA extraction, RT-PCR and quantification of PCR products were performed as previously described (Huang et al., 2010). The results are expressed as the density ratio between the gene of interest and the reference standard ( $\beta$ -actin).

#### 2.10. Statistical analysis

Data are expressed as the mean  $\pm$  standard error of mean (SEM) for each treatment group. Dunnett's two-tailed *t*-test was used to assess the statistical difference between the treatment groups and the VH control, except for diarrhoea occurrence. To analyze differences of diarrhoea occurrence between the treatment groups and the VH control, Chi-square tests were employed. *p*-Values of less than 0.05 were defined as statistically significant.

### 3. Results

#### 3.1. Effects of *L. reuteri* on the density of enteric bacterial flora

We firstly examined the influence of *L. reuteri* administration on the density of faecal lactic acid bacteria (LAB) and non-specific bacteria. Before OVA challenge, the density of LAB and non-specific bacteria was comparable between non-sensitized and OVA-sensitized mice (Fig. 2; NS vs. OVA). Administration with *L. reuteri* markedly increased the density of LAB, but not non-specific bacteria (Fig. 2; *L. reuteri* vs. VH). After the 5th OVA challenge, allergic mice exhibited a diminished density of faecal LAB and an increased density of non-specific microbes (Fig. 2; NS vs. OVA). These results were consistent with our previous reports showing a deteriorated profile of the enteric flora in mice with food allergy (Huang et al., 2012, 2016). The alterations of the enteric bacteria observed in allergic mice were restored by administration with *L. reuteri*, in which the density of LAB and non-specific microbes was significantly increased and decreased, respectively (Fig. 2; *L. reuteri* vs. VH).

#### 3.2. Effects of *L. reuteri* on allergic responses

A successful induction of allergic diarrhoea was observed in OVA-sensitized mice from the 3rd OVA challenge (Fig. 3A; NS vs. OVA). Administration with *L. reuteri* significantly reduced the occurrence of diarrhoea in allergic mice from the 3rd OVA challenge (Fig. 3A; *L. reuteri* vs. VH). Concordantly, *L. reuteri* attenuated mast cell infiltration and degranulation (Fig. 3B and C), and the serum production of total and OVA-specific IgE in allergic mice (Fig. 3D).

#### 3.3. Effects of *L. reuteri* on the immune profile of Th cell subsets

As T helper (Th) cells play a pivotal role in the immunopathology of food allergy, we characterize the profile of T cell immune balance. The expression of T cell-derived key cytokines (IFN- $\gamma$ , IL-4, IL-10 and TGF- $\beta$ ) and transcription factors (Foxp3, GATA-3 and T-bet) was markedly increased in the OVA group compared

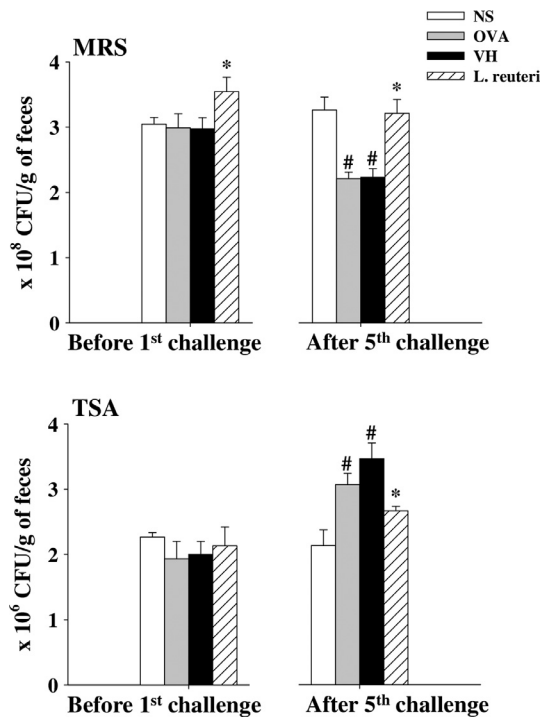


**Fig. 2.** Effects of oral administration with *L. reuteri* on the density of faecal bacteria. The murine faeces were isolated from each group as described in Materials and methods. The faecal samples were incubated in triplicate on MRS and TSA agar plates under anaerobic conditions. The number of colonies in the range of 25–250 CFU per plate was counted manually. Results are a representative of three independent experiments each in triplicate. Data are expressed as mean  $\pm$  SEM from triplicate experiments. # $p < 0.05$  compared to the NS group. \* $p < 0.05$  compared to the VH group.

to the non-sensitized control (Fig. 4; OVA vs. NS), demonstrating an enhanced T cell activation in OVA-sensitized and challenged mice. In line with the suppressive effect on IgE production, *L. reuteri* significantly down-regulated the production of IL-4, a signature Th2 cytokine (Fig. 4). Interestingly, the expression of the Th1 cytokine IFN- $\gamma$  by splenocytes was also attenuated in *L. reuteri*-treated mice (Fig. 4). We further measured the duodenal expression of key cytokines of Th1, Th2 and Treg cells, including IFN- $\gamma$ , IL-4, IL-10 and TGF- $\beta$ . Consistent with findings on splenocytes, the expression of IFN- $\gamma$  and IL-4 mRNA was down-regulated by *L. reuteri* administration (Figs. 4 and S1). However, the expression of IL-10 and TGF- $\beta$  was up-regulated (Figs. 4 and S1). We also examined the mRNA expression of lineage-determining transcription factors for Th1, Th2 and Treg cells, namely T-bet, GATA3 and Foxp3, respectively. Administration with *L. reuteri* markedly suppressed the expression of T-bet and GATA3, but enhanced that of Foxp3 (Figs. 4 and S1).

#### 3.4. Effects of *L. reuteri* on CD11c<sup>+</sup>CD103<sup>+</sup> MLN cells

We next investigated the effect of *L. reuteri* on the expression of CD103 on CD11c<sup>+</sup> MLN cells. The percentage of CD11c<sup>+</sup>CD103<sup>+</sup> cell population was significantly increased in MLNs of mice treated with *L. reuteri* (Fig. 5A and B). Moreover, the production of IL-10 by CD11c<sup>+</sup>CD103<sup>+</sup> cells was markedly higher than that of CD11c<sup>+</sup>CD103<sup>-</sup> cells (Fig. 5C).

#### 3.5. Supplement with heat-killed *L. reuteri* prevented the proliferation and activation of OVA-primed MLN T cells re-stimulated with OVA

We further investigated whether direct exposure to *L. reuteri* affected the proliferation and activation of intestinal immune cells.

MLN cells isolated from allergic mice were directly exposed to heat-killed *L. reuteri* (1–100  $\mu$ g/mL) in culture, and then re-stimulated with OVA (100  $\mu$ g/mL) to induce cell proliferation and cytokine expression. The stimulation index and production of IL-2, a key cytokine expressed by activated T cells (Liao, Lin, & Leonard, 2011), were markedly attenuated by heat-killed *L. reuteri* at concentrations  $\geq 50$   $\mu$ g/mL (Fig. 6). These results showed that direct exposure to killed *L. reuteri* attenuated the proliferation and activation of MLN T cells.

## 4. Discussion

Results from the present study confirm our hypothesis that *L. reuteri* is a functional probiotic capable of inducing intestinal immune tolerance against food allergy. Several lines of evidence substantiates this conclusion. First, oral administration with *L. reuteri* restored the deteriorated profile of intestinal bacterial flora in allergic mice. Second, *L. reuteri* administration attenuated allergic responses, including the occurrence of allergic diarrhoea, mast cell activation, and serum IgE production. Third, both Th1 and Th2 responses were down-regulated, whereas the levels of Treg cell-associated transcription factor and cytokines were up-regulated. Lastly, *L. reuteri* administration significantly increased the population of IL-10-secreting CD11c<sup>+</sup>CD103<sup>+</sup> tolerogenic DCs. On the basis of these findings, intake of *L. reuteri* may be beneficial for improving both microbial balance and aberrant immune responses in the intestine associated with food allergy.

Because the beneficial effects of probiotics are strain-specific, previous studies have assessed the immunomodulatory effects of numerous probiotic strains, in which *L. reuteri* has been shown to increase the population of Treg cells and the production of two regulatory cytokines, namely IL-10 and TGF- $\beta$  (Juarez et al., 2013; Karimi et al., 2009; Liu et al., 2013; Özdemir, 2010). In addition, supplement with heat killed-*L. reuteri* augmented the development of Treg cells in MLNs (Livingston, Loach, Wilson, Tannock, & Baird, 2010). Results from animal studies further demonstrated the anti-inflammatory effects of *L. reuteri*, and suggested the promotion of Treg cell development as one of the major mechanisms contributing to its anti-inflammatory effects (Liu, Tran, Fatheree, & Rhoads, 2014; Liu et al., 2013). Although the immunomodulatory and anti-inflammatory activities of *L. reuteri* have been reported, direct evidence supporting the anti-allergic effects of *L. reuteri* remains limited. Forsythe et al. have shown that oral administration with *L. reuteri* ( $1 \times 10^9$  CFU/day for 9 consecutive days) inhibited allergic airway responses in mice (Forsythe et al., 2007). Simultaneously, the percentage and total number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells in spleens were significantly increased. Adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup> T cells from *L. reuteri*-treated mice to OVA-sensitized animals attenuated airway hyper-responsiveness and inflammation, indicating that the induction of functional Treg cells plays an important role in *L. reuteri*-mediated effects against allergic airway responses (Karimi et al., 2009). In the present study, we showed that *L. reuteri* at the same dose exhibited anti-food allergic effects, as evidenced by the suppression of allergic diarrhoea, mast cell activation, and serum IgE production. Notably, an enhanced development of Treg cells in the intestine was demonstrated, which may contribute to the observed anti-food allergic effects.

The enhanced development of Treg cells by *L. reuteri* was illustrated by an increased expression of Treg-related factors, including Foxp3, IL-10 and TGF- $\beta$ . Although IL-10 is a key regulatory cytokine expressed by Treg cells, other immunocytes such as Th2 cells may also produce it. However, the elevation of the other two factors Foxp3 and TGF- $\beta$  provide further evidence to substantiate the enhanced regulatory immunity by *L. reuteri*. It is noticed that the intestinal level of Foxp3, IL-10 and TGF- $\beta$  in the OVA group was

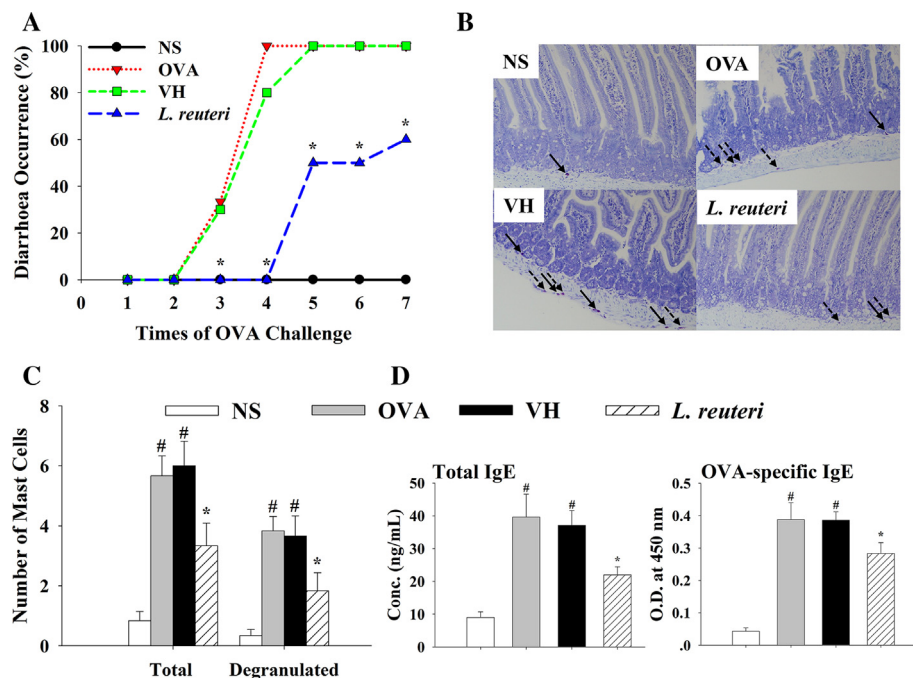


**Fig. 3.** Effects of oral administration with *L. reuteri* on allergic responses. BALB/c mice were sensitized and challenged with OVA to induced allergic diarrhoea as described in Materials and methods. (A) After each OVA challenge, diarrhoea was monitored visually for 3 h. Mice with profuse liquid stool were identified as diarrhoea-positive ones. ●, NS (n = 11); red ▽, OVA (n = 15); ■, VH (n = 20); ▲, *L. reuteri* (n = 20). Data were pooled from 3 independent experiments. After sacrifice, the duodenal tissue sections were prepared for toluidine blue staining. (B) Representative toluidine blue-stained sections are shown (original magnification, 200×). The solid and dashed arrows indicate non-degranulated and degranulated mast cells, respectively. (C) The number of total and degranulated mast cells was counted manually. Six measurements per tissue of each mouse were counted. (D) Serum samples were collected before sacrifice for measurement of total and OVA-specific IgE by ELISA. Data presented in Fig. C and D are pooled from 3 independent experiments and expressed as mean ± SEM. of 11–20 mice per group. #*p* < 0.05 compared to the NS group. \**p* < 0.05 compared to the VH group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

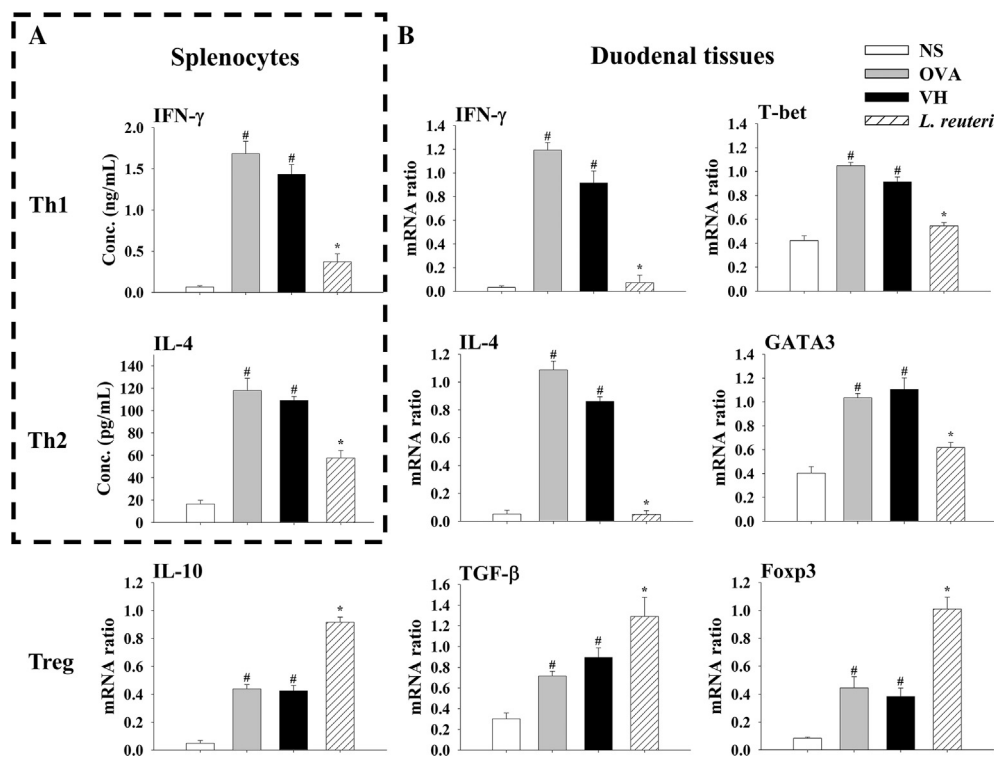


**Fig. 4.** Effects of oral administration with *L. reuteri* on the expression of T cell-associated cytokines and transcription factors. After the last OVA challenge, mice were sacrificed and their spleens and duodenal tissues were isolated as described in Materials and methods. (A) The splenocytes were prepared and re-stimulated with OVA for 72 h. The levels of IFN-γ and IL-4 in supernatants were quantified by ELISA. (B) Total RNA from the duodenal tissues was extracted and the steady state mRNA expression of target genes was measured by RT-PCR. Representative photos of RT-PCR products are shown in Fig. S1. The results were quantified as the density ratio between the gene of interest and the reference standard (β-actin). Results are a representative of three independent experiments each in triplicate. Data are expressed as mean ± SEM from triplicate experiments. #*p* < 0.05 compared to the NS group. \**p* < 0.05 compared to the VH group.

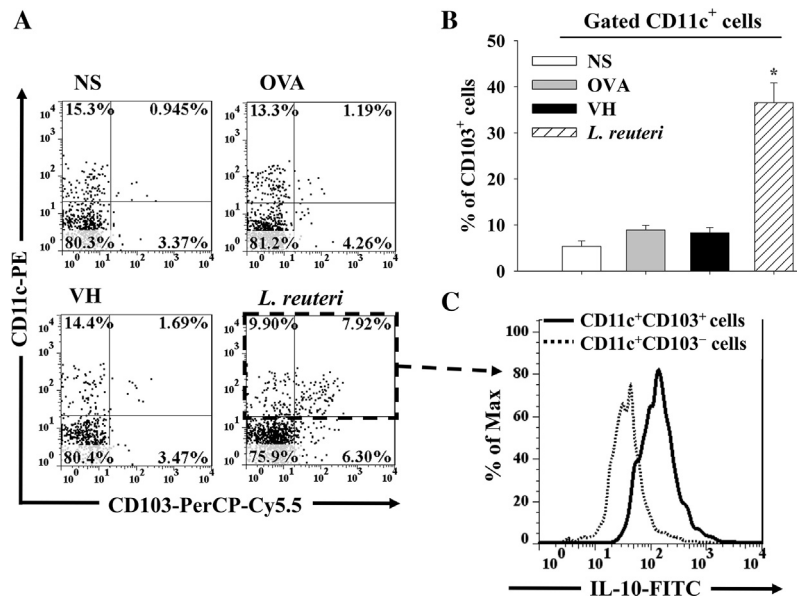


**Fig. 5.** Effects of oral administration with *L. reuteri* on IL-10 secreting CD11c<sup>+</sup>CD103<sup>+</sup> MLN cells. After the last OVA challenge, mice were sacrificed and their MLNs were isolated and the MLN cell suspensions were prepared as described in Materials and methods. The cells were stained with FITC-conjugated anti-IL-10, PE-conjugated anti-CD11c and PerCP/Cy5.5 anti-CD103. (A) Representative flow cytometry dot plots of CD11c/CD103 analysis are shown. (B) The percentage of CD103<sup>+</sup> cells in gated CD11c<sup>+</sup> cells is shown. (C) The gated CD11c<sup>+</sup>CD103<sup>+</sup> cells were analyzed for expression of IL-10, and the representative histogram and geometric mean fluorescence intensity are shown. Results are a representative of three independent experiments. Data are expressed as mean  $\pm$  SEM from triplicate experiments. \* $p < 0.05$  compared to the VH group.

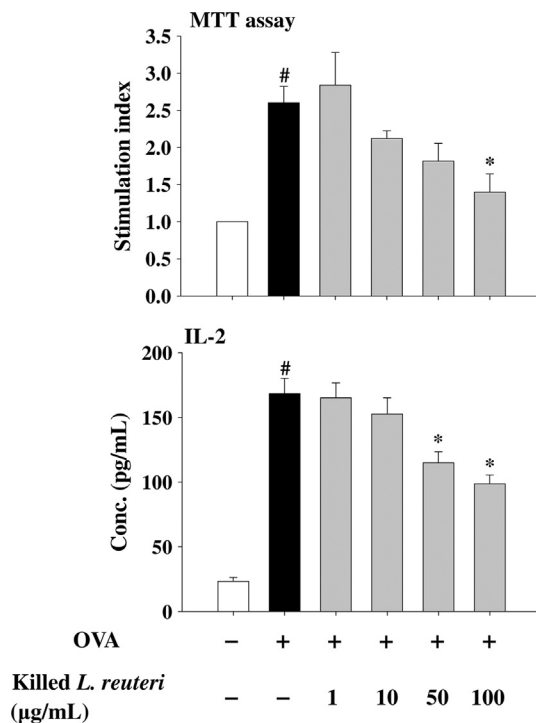


**Fig. 6.** Effects of heat-killed *L. reuteri* on the proliferation and activation of MLN T cells. Mice were sensitized with OVA by intraperitoneal injection using 0.1 mL sensitization solution containing 50  $\mu$ g OVA and 1 mg alum, and boosted with a double dose 2 weeks later. The mice were sacrificed 2 weeks after the boost and their MLN were harvested. The MLN cells ( $5 \times 10^6$  cells/mL) were pretreated with heat-killed *L. reuteri* (1–100  $\mu$ g/mL) for 30 min followed by re-stimulation with OVA (100  $\mu$ g/mL). After 72 h of culture, the metabolic activity was determined using an MTT assay, and the level of IL-2 in the supernatants was quantified by ELISA. Results are a representative of three independent experiments each in triplicate. Data are expressed as mean  $\pm$  SEM from triplicate experiments. \* $p < 0.05$  compared to the OVA group.

greater than that in non-sensitized mice, suggesting an up-regulation of the regulatory immunity in allergic mice. However, the up-regulation appears insufficient to counteract OVA challenge-induced allergic responses, as the OVA group completely developed diarrhoea following repeatedly OVA challenge. Nevertheless, the allergic diarrhoea was reduced by *L. reuteri*, and more importantly a concordant suppression of Th2 immunity was also observed. These results imply that *L. reuteri* more robustly enhances the regulatory immunity that is capable of counteracting the aberrant Th2-dominant allergic responses.

CD103<sup>+</sup> DCs in the MLNs have been identified as a subset of tolerogenic DCs that promote the generation of Treg cells (Coomes et al., 2007; Johansson-Lindbom et al., 2005; Ruane & Lavelle, 2011; Sun et al., 2007). Accordingly, the induction of CD103<sup>+</sup> DCs is considered a potential mechanism for probiotics to induce immune tolerance. For example, oral treatment with the probiotic mixture VSL#3, containing without *L. reuteri*, promoted the differentiation of CD103<sup>+</sup>CD11c<sup>+</sup> DCs and attenuated intestinal inflammation (Dolpady et al., 2016; Mariman, Tienen, Koning, & Nagelkerken, 2015). In addition, it has been reported that *Bifidobacterium breve* activated intestinal CD103<sup>+</sup> DCs to produce IL-10 and IL-27 thereby inducing functional Treg cells (Jeon et al., 2012). Notably, we showed that oral administration with *L. reuteri* increased the population of IL-10-secreting-CD11c<sup>+</sup>CD103<sup>+</sup> cells in the MLN cells. As IL-10 is one of the inhibitory cytokines produced by intestinal CD103<sup>+</sup> DCs, these results suggest that *L. reuteri* treatment may induce the development of functional tolerogenic DCs (Jeon et al., 2012). In parallel with the results of *in vivo* experiments, our *in vitro* experiments showed that exposure to heat-killed *L. reuteri* attenuated the proliferation and activation of MLN cells re-stimulated with the sensitized allergen OVA. These results suggest that the effect of *L. reuteri* against food allergy may be attributed to the induction of tolerogenic DCs, which generates gut-homing Treg cells to counteract the aberrant immune responses associated with food allergy. Previous reports have

demonstrated that *L. reuteri*-induced Treg cells played a crucial role in its anti-inflammatory effect (Juarez et al., 2013; Liu et al., 2013, 2014; Mao, Yu, Ljungh, Molin, & Jeppsson, 1996; Shornikova et al., 1997). Our results showing the induction of tolerogenic DCs provide further mechanistic insights on *L. reuteri*-mediated induction of Treg cells that may contribute to its anti-inflammatory and anti-allergic properties.

We have demonstrated that oral administration with *L. murinus* restored the deteriorated profile of enteric flora and modulated the functionality of intestinal DCs in mice with food allergy (Huang et al., 2016). *L. reuteri* is known to produce the antibiotic substance reuterin that suppresses the growth of some harmful microbes, hence we also investigated whether *L. reuteri* modulated the deteriorated profile of enteric flora in mice with food allergy (Talarico, Casas, Chung, & Dobrogosz, 1988). As expected, oral administration with *L. reuteri* significantly restored the deteriorated profile of enteric flora. These results link the anti-allergic effects of *L. reuteri* to its successful competition with other bacteria in the gut. It has been suggested that the intestinal environmental conditioning, such as the interaction between epithelial cells and luminal bacteria, may be essential for CD103<sup>+</sup> DC differentiation (Ruane & Lavelle, 2011). How the interaction between *L. reuteri* and other enteric probiotics may contribute to the induction of tolerogenic DCs remains elusive. This issue is intriguing and warrants further investigation.

We previously reported that both *L. murinus* and *L. reuteri* were the major enteric bacteria strains up-regulated by diosgenin in mice with food allergy (Huang et al., 2012). The lack of effects on enhancing Th1 immune responses by suggests that *L. reuteri* contributes partially to diosgenin-mediated anti-allergic effects. We recently reported that *L. murinus* is a functional probiotic against food allergy via shifting Th1/Th2 immune balance toward Th1 polarization (Huang et al., 2016). Interestingly, *L. reuteri* is also effective against food allergy possibly via the induction of tolerogenic DCs. Hence, the underlying immunological mechanisms contributing the anti-allergic effects between these two probiotics are completely different. It is suggested that both *L. reuteri* and *L. murinus* may contribute additively or synergistically to diosgenin-mediated anti-allergic effects. Further studies are required to address whether a combination of *L. reuteri* and *L. murinus* will produce the same profile of immunomodulatory effects as diosgenin does.

## 5. Conclusions

Our data confirm the hypothesis that *L. reuteri* is a functional probiotic capable of inducing intestinal immune tolerance against food allergy. Mechanistic investigation revealed that the intestinal IL-10-secreting CD103<sup>+</sup> DCs and functional Treg cells were induced by *L. reuteri*, which might maintain the immune tolerance and counteract the aberrant immune responses. These findings suggest that *L. reuteri* may be used as a functional probiotic for managing intestinal disorders associated with exaggerated immune responses, especially Th2-mediated allergic diseases.

## Conflict of interest

The authors declare no conflicts of interest relevant to this work.

## Acknowledgments

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## Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jff.2017.01.034>.

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## **Supplementary Material**

### ***Lactobacillus reuteri* induces intestinal immune tolerance against food allergy in mice**

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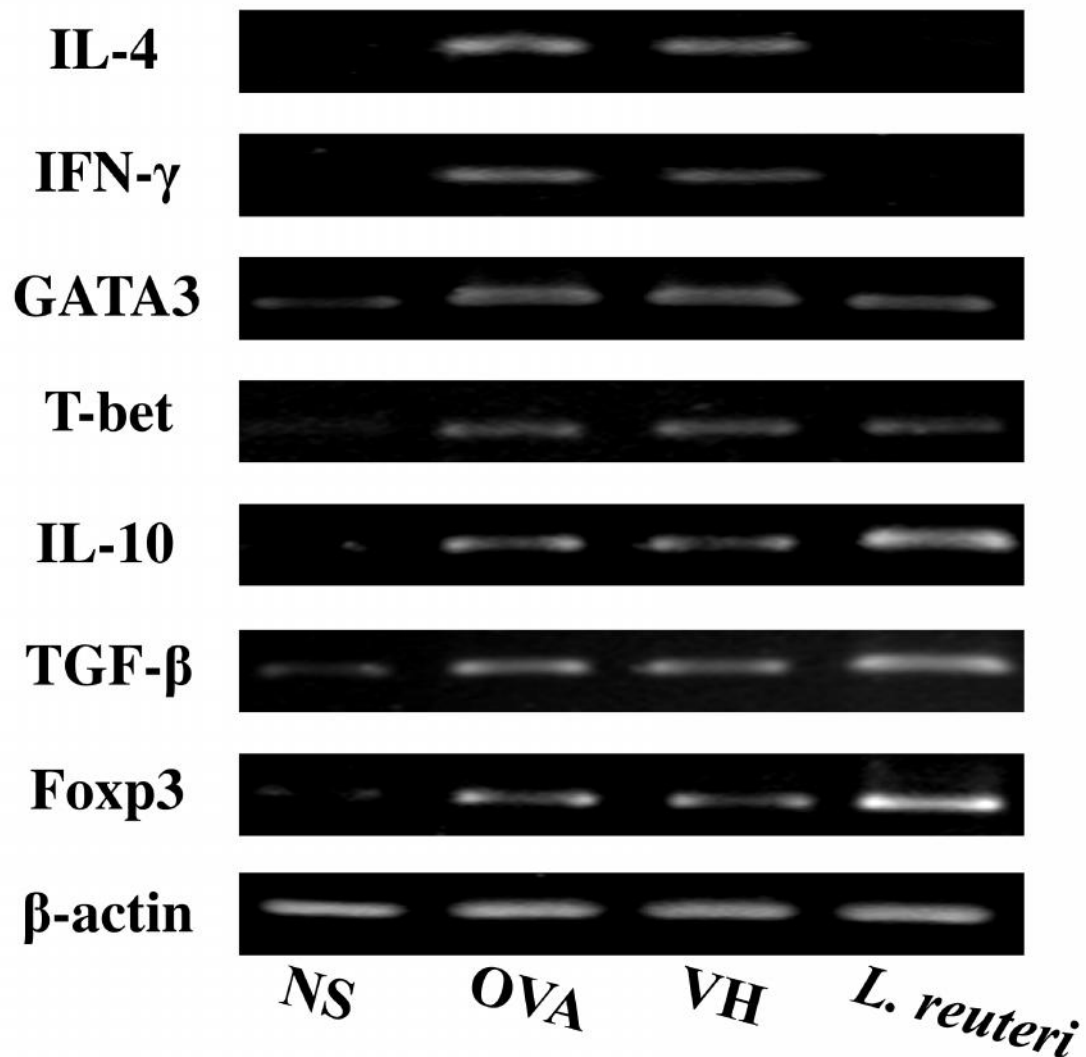


**Fig. S1** *L. reuteri* treatment attenuated IL-4, IFN-γ, GATA3 and T-bet mRNA expression, whereas enhanced IL-10, TGF-β and Foxp3 mRNA expression in the duodenum. After the last OVA challenge, mice were sacrificed and the duodenal tissues were isolated. Total RNA extracted from duodenal tissues of each group was used for RT-PCR performance as described in **Materials and methods**. Representative photos of RT-PCR products are shown. Quantitative data are shown in Fig. 4.

**Table S1** Primers used for RT-PCR analysis

| gene           | primer Sequence                                                   | length (bp) |
|----------------|-------------------------------------------------------------------|-------------|
| IL-4           | 5'-ACGGCACAGAGCTATTGATG-3'<br>5'-ATGGTGGCCAGTACTACGA-3'           | 454         |
| IFN- $\gamma$  | 5'-CATGAAAATCCTGCAGAGCC-3'<br>5'-GGACAATCTCTTCCCCAGCC-3'          | 314         |
| GATA3          | 5'-CTCCTTTTTTGCTCTCCTTTTC-3'<br>5'-AAGAGATGAG GACTGGAGTG-3'       | 254         |
| T-bet          | 5'-AACCAGTATCCTGTTCCCAGC-3'<br>5'-TGTCG CCACTGGAAGGATAG-3'        | 438         |
| IL-10          | 5'-GACACCTTGGTCTTGGAGCTTATT-3'<br>5'-TCCTTAATGCAGGACTTTAAGGGTT-3' | 258         |
| TGF- $\beta$   | 5'-GCAAAGATAACACACTGCAA-3'<br>5'-GAAGTCAATGTAGAGCTGCC-3'          | 525         |
| Foxp3          | 5'-CAGCTGCCTACAGTGCCCCTAG-3'<br>5'-CATTTGCCAGCAGTGGGTAG-3'        | 382         |
| $\beta$ -actin | 5'-ACTCATCGTACTCCTGCTTGCTGA -3'<br>5'-AGGGAAATCGTGCGTGACATCAAA-3' | 478         |