International Archives of Allergy and Immunology

Experimental Allergy – Research Article

Int Arch Allergy Immunol 2021;182:277–291
DOI: 10.1159/000511146

Received: February 19, 2020
Accepted: August 25, 2020
Published online: November 4, 2020

Lactococcus lactis NZ9000 Prevents Asthmatic Airway Inflammation and Remodelling in Rats through the Improvement of Intestinal Barrier Function and Systemic TGF-β Production

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Keywords
Allergy · Intestine · Lactic acid bacteria · Lung inflammation · Probiotics

Abstract
Introduction: The use of probiotics has been broadly popularized due to positive effects in the attenuation of aberrant immune responses such as asthma. Allergic asthma is a chronic respiratory disease characterized by airway inflammation and remodelling. ***Objective:*** This study was aimed to evaluate the effect of oral administration of *Lactococcus lactis* NZ9000 on asthmatic airway inflammation and lung tissue remodelling in rats and its relation to the maintenance of an adequate intestinal barrier. ***Methods:*** Wistar rats were ovalbumin (OVA) sensitized and challenged and orally treated with *L. lactis*. Lung inflammatory infiltrates and cytokines were measured, and remodelling was evaluated. Serum OVA-specific immunoglobulin (Ig) E levels were assessed. We also evaluated changes on intestinal environment and on systemic immune response. ***Results:*** *L. lactis* diminished the infiltration of proinflammatory leucocytes, mainly eosinophils, in the bronchoalveolar compartment, decreased lung IL-4 and IL-5 expression, and reduced the level of serum allergen-specific IgE. Furthermore, *L. lactis* prevented eosinophil influx, collagen deposition, and goblet cell hyperplasia in lung tissue. In the intestine, *L. lactis*-treated asthmatic rats increased Peyer's patch and goblet cell quantity and mRNA expression of IgA, MUC-2, and claudin. Additionally, intestinal morphological alterations were normalized by *L. lactis* administration. Splenocyte proliferative response to OVA was abolished, and serum levels of transforming growth factor (TGF)-β were increased by *L. lactis* treatment. ***Conclusions:*** These findings suggest that *L. lactis* is a potential candidate for asthma prevention, and the effect is mediated by the improvement of intestinal barrier function and systemic TGF-β production.

Edited by: H.-U. Simon, Bern.

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Introduction

Allergic asthma is the most common and heterogeneous disease in which lower airways are compromised by chronic inflammation that progressively diminishes the functional capacity of the lungs. It is estimated that asthma affects approximately 300 million people worldwide [1]. The prevalence of asthma is continually increasing mainly in Western countries, where urbanization and exacerbated exposure to hygienic conditions increase the risk of allergic diseases [2, 3]. Constant exposure to allergens, among other environmental and genetic factors, stimulates immune cells to secrete cytokines that induce the activation of T cells, generating a Th2 response. Then, cytokines such as interleukin (IL)-4, IL-5, and IL-13 stimulate eosinophil migration to the lung parenchyma and allergen-specific immunoglobulin (Ig) E secretion, increase collagen deposition, and induce goblet cell hyperplasia [4].

An increasing number of works have focussed on studying the role of rural conditions and beneficial microbiota in the reduction of allergen-sensitization risk [5, 6]. Moreover, there is strong evidence that gut microbiota in early life appear to influence allergic disease onset later in life. Microbiota diversity and dysbiosis in neonates are associated with an increased risk of asthma development [7]. Additionally, the absence of early microbiota transmission from the mother in caesarean sections increases the risk of asthma in newborns [8]. This evidence suggests that beneficial bacteria with probiotic properties may regulate atypical immune responses by modulating the mucosal immune system. Based on these observations, a clinical trial in which *Lactobacillus rhamnosus* GG was administered to pregnant women with first-degree atopic ancestry prior to delivery and later to newborns demonstrated that the children's risk of developing eczema was reduced by 50% [9]. Moreover, analyses of accumulated data have demonstrated the preventive potential of probiotics in the reduction of allergic conditions in early life. In a meta-analysis conducted by Zuccotti and coworkers [10], children with high risk of developing eczema treated with probiotic mixtures had significantly reduced incidence of eczema although it is important to mention that other allergic pathologies like asthma or rhino-conjunctivitis were not prevented.

Lactococcus lactis (*L. lactis*) are lactic acid bacteria (LAB) that are generally regarded as safe. Strains of the *Lactococcus* genus of LAB are widely used as starter bacteria in manufacturing cheese and other fermented dairy products and are also found in fermented vegetables [11]. Various studies have demonstrated the probiotic and immunomodulatory properties of *L. lactis* in a strain-specific manner [12]. In in vitro assays, *L. lactis* HY 449 inhibits its *Streptococcus mutans* growth in cariogenic biofilms, and the strain NK34 decreases the production of nitric oxide and proinflammatory cytokines by lipopolysaccharide-activated macrophages [13, 14]. When orally administered, *L. lactis* NCC 2287 attenuates oesophageal eosinophilic inflammation in a rodent model of eosinophilic oesophagitis, and the G121 strain isolated from a cowshed on a farm diminishes allergic reactions in mice [15, 16]. Besides, oral administration of *L. lactis* C59 reduces serum IgE levels in allergen-sensitized mice models [17].

L. lactis presents a great biomedical potential mainly as a drug delivery system and a mucosal vaccine vector [18]. In particular, *L. lactis* NZ9000 is used in the production of heterologous proteins through the NICE expression system inserted in *L. lactis* MG1363 [19]. Recombinant *L. lactis* NZ9000 producing cytokines [20] or antigens [21] can modulate the host immune response. However, for this strain, very few studies have focussed on the study of per se immunomodulatory properties in a non-recombinant state. Evidence shows that nasal-administered *L. lactis* NZ9000 upregulates innate and specific immune responses in both local and systemic compartments [22]. The aim of this study was to analyze whether the oral administration of *L. lactis* NZ9000 ameliorates lung inflammation and airway remodelling in ovalbumin (OVA) sensitized and challenged rats and whether this effect is linked to changes in intestinal barrier function together with a systemic immunomodulatory response to propose a new strategy for the treatment of asthma.

Materials and Methods

Animal Conditions

Conventional male Wistar rats (8 weeks old, 150–200 g) were obtained from the Central Bioterium of the Autonomous University of Aguascalientes and kept in the local Animal Core Facility at 25°C with 12-h light/dark cycles. The rats were fed Laboratory Chow 5001 (Purina, Mexico City, Mexico) and provided tap water ad libitum. All animals were treated with enrofloxacin (0.06%) prepared in drinking water for 3 days. Finally, animals were kept under standard and hygienic conditions to acclimatize for 7 days before starting the asthma induction protocol.

L. lactis Strain and Culture Conditions

L. lactis strain NZ9000 (kindly donated by Oscar P. Kuipers from the University of Groningen) was grown in 10% glucose-M17 broth (BD Difco, Sparks, MD, USA) at 30°C. Cells were harvested at 9,000 *g* for 5 min. Finally, cell suspensions were adjusted to 5 ×

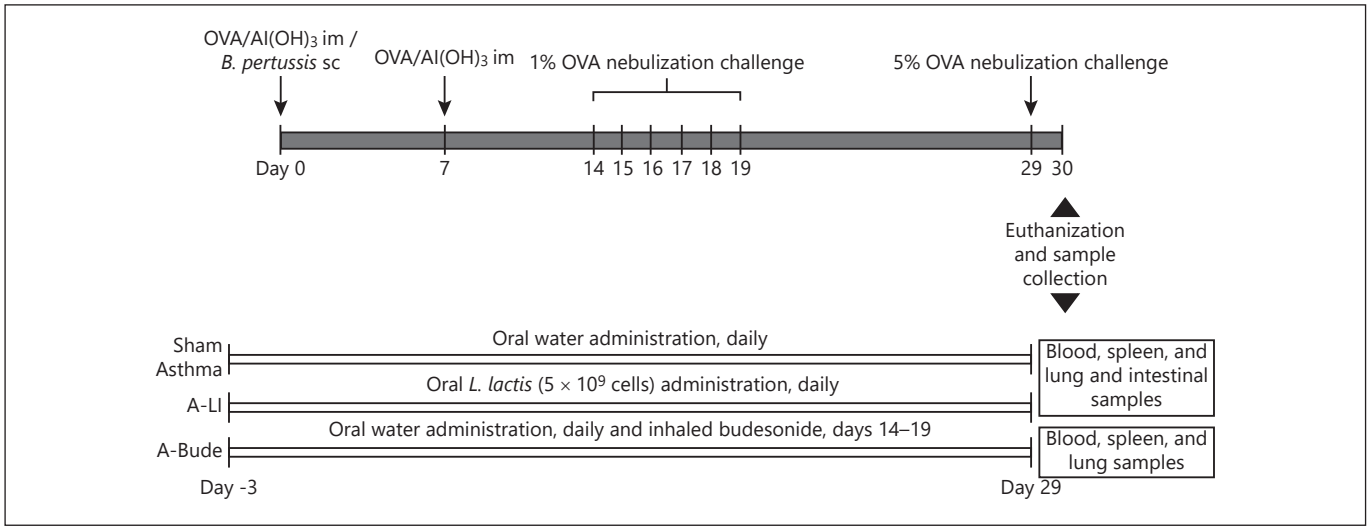

Fig. 1. Experimental design of OVA-induced asthma model and treatment. Asthma was induced (asthma, A-Ll, and A-Bude) by an IM injection of 1 mg of OVA in $Al[OH]_3$ gel at days 0 and 7. *B. pertussis* was injected SC only on day 0. Daily nebulization of OVA 1% was performed from days 14 to 19. At day 29, animals were nebulized with 5% OVA as a final challenge. Sham animals were injected only with adjuvants and nebulized with deionized water. *L. lactis* (A-Ll) or water (sham, asthma, and A-Bude) was orally administered from day −3 to 29. Besides, the A-Bude group was nebulized with 1 mg of budesonide 30 min after each 1% OVA challenge at days 14–19. On day 30, the animals were euthanized and samples were collected. OVA, ovalbumin; IM, intramuscular; SC, subcutaneously; *L. lactis*, *Lactococcus lactis*.

10^9 cells/mL in sterile water based on an optical density (OD) at 600 nm of 1.0, corresponding to 5×10^8 cells/mL.

Allergen Sensitization and Challenge Protocol

Twenty male Wistar rats were randomly divided into 4 groups of 5 animals each as follows: (i) sensitization and challenge control (sham), (ii) OVA-induced asthma (asthma), (iii) OVA-induced asthma treated with *L. lactis* (A-Ll), and (iv) OVA-induced asthma treated with budesonide (A-Bude; AstraZeneca, London, UK). As previously described, we developed a rat model of asthma by OVA sensitization and inhalation [23]. In brief, the animals were sensitized intramuscularly with 1 mg of OVA (Sigma-Aldrich, St Louis, MO, USA) absorbed into 7.8 mg of aluminium (Imject™; Thermo Scientific, Waltham, MA, USA) in 1 mL of isosmotic saline solution at days 0 and 7; the sham group was administered an equal volume of isosmotic saline solution. As an adjuvant, all animals received $10–15 \times 10^9$ heat-killed bacilli/mL of *Bordetella pertussis* contained in 0.5 mL of the diphtheria-pertussis-tetanus vaccine (Biofarma, Bandung, Indonesia) by subcutaneous injection on day 0. Allergen challenges were performed by 1% OVA nebulization in an acrylic container (60 × 50 × 35 cm) with an ultrasonic nebulizer for 30 min on days 14–19 and a final OVA nebulization on day 29 with 5% OVA for 30 min. The sham group was nebulized with deionized water. The A-Ll group received 5×10^9 *L. lactis* cells in 1 mL of sterile water using an oesophageal catheter from day −3 until day 29. At the same time, the sham, asthma, and A-Bude groups received the same volume of tap water. Rats from the A-Bude group were nebulized with 1 mg of budesonide 30 min after each 1% OVA challenge (days 14–19). Samples were collected 24 h after a final 5% OVA challenge. For the evaluation of airway inflammation and remodelling, samples of the 4 experimental groups were processed, and budesonide was used as a standard asthma treatment for comparison with *L. lactis* results in relation to asthmatic features. In order to evaluate the intestinal parameters, samples of the sham, asthma, and A-Ll groups were analyzed. A-Bude group was not considered because the action mechanism of inhaled budesonide is restricted to airways [24]. The experimental protocol is summarized in Figure 1.

Specimen Collection

On day 30, the rats were anaesthetized by peritoneal injection of ketamine/xylazine at doses of 95 and 1 mg/mL/kg (PiSA, Hidalgo, Mexico) in order to obtain blood samples, bronchoalveolar lavage fluid (BALF), the spleen, and lung and intestinal tissues. In brief, blood samples were collected from the cava vein, centrifuged at 800 *g* for 8 min, and serum samples were aliquoted and frozen at −80°C. For BALF recovery, the thoracic cavity was exposed and opened, and a catheter was inserted into the left lung through a small incision in the trachea for perfusion with 1 mL of phosphate-buffered saline (PBS)/0.05% bovine serum albumin (BSA). BALF was recovered by gentle aspiration and centrifuged at 500 *g* for 5 min at 4°C. The resulting cell pellets were suspended in 100 μL of PBS (pH 7.4) for cellular determination. The spleen was removed aseptically from rats to spleen cell isolation. The right lung middle lobe was perfused with 1 mL of neutral formalin, dissected, and cut into 3 pieces, and the middle third was used for histological assays. The small intestine was dissected, and a 1-cm segment of the distal portion was immersed in neutral formalin. Both tissues were fixed for 24 h and later embedded in paraffin. Caecal contents were obtained and stored at −80°C. Besides, lung (right caudal lobe) and distal small intestine tissues were immersed in RNAlater (Ambion, Austin, TX, USA) and stored at −80°C until use.

Table 1. Oligonucleotides used in this study

Oligonucleotide	Sequence	Access number
IL-5	Fw: CAGTGGTGAAAGAGACCTTG Rv: GTATGTCTAGCCCCTGAAAG	NM_021834.1
IL-13	Fw: ATCGAGGAGCTGAGCAACAT Rv: ATCCGAGGCCTTTTGGTTAC	NM_053828.1
TGF-β	Fw: GACTCTCCACCTGCAAGACCAT Rv: CGGGTGACTTCTTTGGCGTA	NM_021578.2
Fc-IgA	Fw: CGGAACTATGAATGTGACCT Rv: GACTAAGGAGGGTTTTGGAC	AJ510151.1
MUC-2	Fw: GTATGTGCTCGCCTGTATGC Rv: TGACCTCCAGATGTGAGCAG	XM_017604244.1
Claudin-1	Fw: AACCTCTTACCCAACACCACG Rv: GCCAAGACCCTCATAGCCAT	NM_031699.2
Occludin	Fw: AGGACAGACCCAGACCACTA Rv: ACTCTTCGCTCTCCTCTCTG	NM_031329.2
FoxP3	Fw: CGGGAGAGTTTCTCAAGCAC Rv: CACAGGTGGAGCTTTTGTCA	NM_001108250.1
GATA-3	Fw: AGAAGGCAGGGAGTGTGTGA Rv: TTAGCGTTCCTCCTCCAGAG	NM_133293.1
RORγt	Fw: GCAGCAACGGGAACAAGTAG Rv: GGGCTATACTCAAGGTGGCA	XM_006232926.3
β-Act	Fw: GTCGTACCACTGGCATTGTG Rv: GCTGTGGTGGTGAAGCTGTA	NM_031144.3

TGF, transforming growth factor.

Cellularity of BALF

For total BALF cell quantification, 10 μL of suspended cell pellet was mixed with 10 μL of 0.4% trypan blue, and viable cells were counted in a Neubauer chamber under a light microscope. Differential cell counts were performed by smearing 50 μL of the suspended cell pellets for Wright staining (Hycel, Jalisco, Mexico). Differential cellular analysis was performed under a light microscope at ×400 magnification by identifying 100 cells in triplicate per sample. Absolute counts were calculated based on the total number of cells and the percentage of each cell type.

Quantification of OVA-Specific IgE

Levels of OVA-specific IgE were measured by ELISA. In brief, 50 μL of OVA (200 μg/mL) in carbonate/bicarbonate coating buffer (50/50 mM, pH 9.6) was added to all wells and incubated overnight at 4°C with gentle orbital shaking. The plates were washed 3 times with 150 μL of PBS-Tween 20 (PBST, pH 7.4). Blocking was performed with 150 μL of BSA 5% in carbonate/bicarbonate coating buffer for 30 min at room temperature. The plates were washed 3 times with PBST. Then, 50 μL of serum samples at a 1:5 dilution in PBST 1% BSA were added to each well in triplicate. The reaction was incubated for 1 h at 37°C with gentle orbital shaking and washed 3 times with PBST. Then, wells were incubated with 50 μL of biotin-labelled mouse monoclonal anti-rat IgE (1:100 diluted in PBST-BSA; Sigma-Aldrich). After washing 3 times with PBST, 50 μL of HRP-streptavidin (1:60,000 diluted in PBST-BSA; Sigma-Aldrich) was added, and the plate was incubated for 1 h at 37°C with gentle orbital shaking, followed by 3 washes with PBST. Subsequently, 50 μL of *o*-phenylenediamine (Sigma-Aldrich) was added, and the plate was incubated for 30 min at room temperature in a dark chamber. The reaction was stopped by the addition of 50 μL of 4 N sulphuric acid, and the colour produced was measured in an iMark Microplate reader (Bio-Rad, Hercules, CA, USA) at 490 nm. Data were presented in relation to the positive index [25], which was calculated as follows: positive index = OD mean of 10 control non-immune sera + 2 standard deviations. The net OD of each sample was divided by the positive index. Values below 1.0 were considered negative for anti-OVA IgE antibodies and those above 1.0 were considered positive.

Histological Analysis of Lung and Intestine Tissues

Fixed lung and intestine tissues were embedded in paraffin, and 5-μm sections were cut. The sections were stained with haematoxylin and eosin (H&E), Masson's trichrome, and periodic acid-Schiff for lung tissue and H&E and alcian blue/nuclear red staining for intestine tissue (all kits were purchased from Hycel). Prepara-

tions were observed with an AxioPlan microscope (Zeiss, Oberkochen, Germany) at ×1,000 or ×100 magnification. Measurements were performed using AxioVision Rel. 4.8 software (Zeiss). In slices stained with H&E, eosinophils were counted in the lung parenchyma and represented as cells per area of the pulmonary interstitium (40,000 μm^2). Collagen deposition was measured in slices stained with Masson's trichrome as the ratio of the area of collagen per subepithelial basement perimeter. For lung and intestine tissues, counted goblet cells in periodic acid-Schiff-stained slices were presented as cells per bronchial or intestinal perimeter, respectively. Intestinal histomorphometric parameters were evaluated in H&E-stained slices by measuring villus height and crypt depth, analyzing 5 villi and 5 crypts per slice and 3 slices/sample at ×100 magnification. Villus height was defined as the distance between the villus apex and villus-crypt junction, and the crypt depth was measured as the distance from the villus-crypt junction to the crypt base.

Measurement of Peyer's Patches

Peyer's patches were identified as whitish cumulus of protruding lymphoid tissue. Weight (*w*) and height (*h*) were measured with a digital Vernier. The area was determined as follows: $\pi \times (w \times h)/2$.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from intestine and lung tissues conserved in RNAlater using the SV Total RNA Isolation System (Promega, Madison, MI, USA) with the aid of the basic Ultra-Turrax Homogenizer system (Ika, Staufen, Germany) and then quantified with Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA). For cDNA synthesis, reverse transcription was carried out using the GoScript Reverse Transcription System (Promega) in a 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Quantitative real-time PCR was performed with the GoTaq qPCR master mix system (Promega) in a StepOne real-time PCR system (Applied Biosystems). Oligonucleotides used for the quantification of mRNA expression are listed in Table 1. Levels of gene expression were determined with the $2^{-\Delta\Delta Ct}$ method using β-actin as a housekeeping gene [26].

Determination of SCFAs in Caecal Samples

Caecal samples (100 mg) were thawed and suspended in 200 µL of sterile deionized water for 5 min by vortexing. After centrifugation at 16,000 *g* for 10 min, the supernatant was collected and measured in a gas chromatograph (6850 Network GC System; Agilent Technologies, Santa Clara, CA, USA) with an HP-5MS column (Agilent) and coupled to a 5975C VL MSD mass spectrometer with triple-axis detector (Agilent). The initial temperature in the oven was 100°C with an increment rate of 15°C/min. The injector and detector temperatures were both set at 270°C, and helium was used as the carrier gas at a 1.5 mL/min flow rate. Individual acetic and butyric acid concentrations were determined by comparing peaks with a standard curve made with the WSFA-2 standard (Sigma-Aldrich).

Spleen Cell Isolation

Each spleen was perfused individually with cold saline solution, and the obtained cell suspension was centrifuged at 212 *g* for 10 min at 10°C and depleted of erythrocytes by incubation in hypotonic lysis buffer (0.17 M Tris, 0.15 M NH_4Cl, pH 7.2) for 5 min on ice; the reaction was stopped by adding cold saline solution and centrifugation at 212 *g* for 10 min at 10°C. Then, cells were washed twice in saline solution by centrifugation, and the pellet was suspended in the RPMI-1640 medium without phenol red (Sigma-Aldrich) supplemented with 10% foetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Sigma-Aldrich). Splenocyte suspensions were used to determine whether purity was ≥90% and viability by the trypan blue exclusion assay ≥98%. Cells were cultivated at a density of 2×10^5 splenocytes in 100 µL of the supplemented RPMI medium.

Splenocyte Proliferation Assay

Cultured cells were incubated with OVA (0.1 mg/mL), concanavalin A (ConA, 1 µg/mL; Sigma-Aldrich), or culture medium as basal condition for 96 h at 37°C under an atmosphere of 5% CO_2. Then, 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) solution (5 mg/mL) was added to each well, and the cells were incubated for an additional 4 h. The purple formazan crystals were solubilized with 0.1 N hydrochloric acid in isopropyl alcohol, and plates were mixed thoroughly and read at 595 nm on an iMark Microplate reader. Data were presented as the stimulation index (SI) that was defined as the ratio of OD of the test sample to that of the control sample.

Quantification of TGF-β

Levels of transforming growth factor (TGF)-β were measured in serum samples and in allergen-stimulated splenocyte supernatants according to the manufacturer's protocol with the commercially available rat ELISA kit (Invitrogen, Carlsbad, CA, USA). To stimulate splenocytes, cells were incubated with OVA (2 mg/mL) or culture medium as basal condition for 96 h at 37°C under an atmosphere of 5% CO_2.

Statistical Analysis

Statistical comparison of the means among groups was determined with the one-way analysis of variance (ANOVA) test with the Bonferroni post hoc test using GraphPad Prism v.5.01 software (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered significant at $p < 0.05$.

Results

L. lactis Reduces Airway Inflammation and Prevents Lung Remodelling

To analyze the effect on airway inflammation associated with allergic asthma, *L. lactis* was administered orally 3 days before and during OVA sensitization and OVA challenge in a rat model. As budesonide has high anti-inflammatory potency and is one of the primary alternatives for asthma treatment [24], we compared the effect of oral *L. lactis* with inhaled budesonide treatment on the lung inflammation. Total leucocyte numbers in BALF were significantly increased by 2.76-fold in asthmatic rats (Fig. 2a) compared to sham rats; the BALF cellularity in

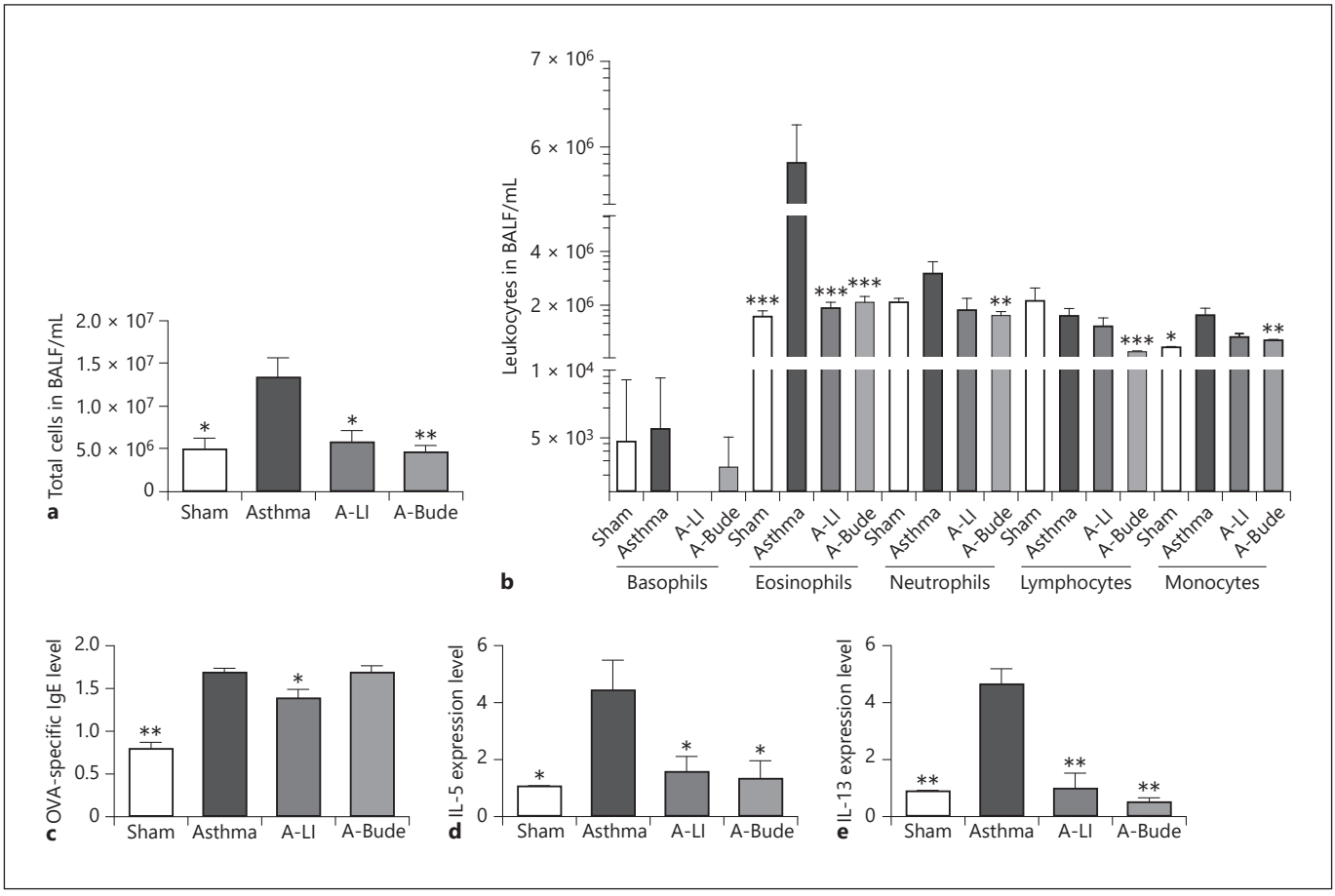

Fig. 2. Effect of orally administered *L. lactis* on total (**a**) and differential (**b**) leucocyte infiltration in the bronchoalveolar compartment, OVA-specific IgE levels in the serum of animals (**c**), and IL-5 (**d**) and IL-13 (**e**) mRNA expression in lung tissue. The results are presented as mean ± standard error of the mean. $N = 5$ animals per group; samples from each rat were analyzed in duplicate. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus the asthma group. A-Ll, OVA-induced asthmatic animals treated with *L. lactis*; A-Bude, OVA-induced asthmatic animals treated with budesonide. *L. lactis*, *Lactococcus lactis*; OVA, ovalbumin; BALF, bronchoalveolar lavage fluid.

asthmatic rats treated with *L. lactis* (A-Ll) was significantly reduced by 57.44% compared to that of asthmatic rats. As expected, budesonide treatment also reduced inflammatory cell infiltration in asthmatic rats by 66.05% in the bronchoalveolar compartment. *L. lactis* and budesonide treatments had similar effects since there was no significant difference between the 2 groups of treated rats. The asthma group was characterized by eosinophilic infiltration since the number of these cells was significantly increased by 3.79-fold than the sham group (Fig. 2b). The eosinophil count obtained in the A-Ll group was significantly reduced by 68.10% compared to that of the asthma group, and no significant change was observed between the A-Ll and A-Bude groups. Another cellular population that increased in the BALF of asthmatic animals was monocytes. *L. lactis* and budesonide treatment reduced the number of monocytes by 49.13 and 60.09%, respectively, although the effect was significant only in the A-Bude group. No significant changes in bronchoalveolar basophil, neutrophil, and lymphocyte numbers were observed in asthmatic animals compared to the sham animals.

In allergic asthma, high levels of allergen-specific IgE are present in the blood [4]. To verify that animals were allergen sensitized, we analyzed the levels of OVA-specific IgE. Sham animals were negative for OVA-specific IgE antibodies, which indicated that adjuvants alone did not induce an allergen-specific IgE response. On day 30, serum IgE levels in the asthma group were increased 2.12-fold, but *L. lactis* administration significantly reduced allergen-specific IgE levels by 17.74% (Fig. 2c). There was no effect on IgE levels with budesonide treatment. Another indica-

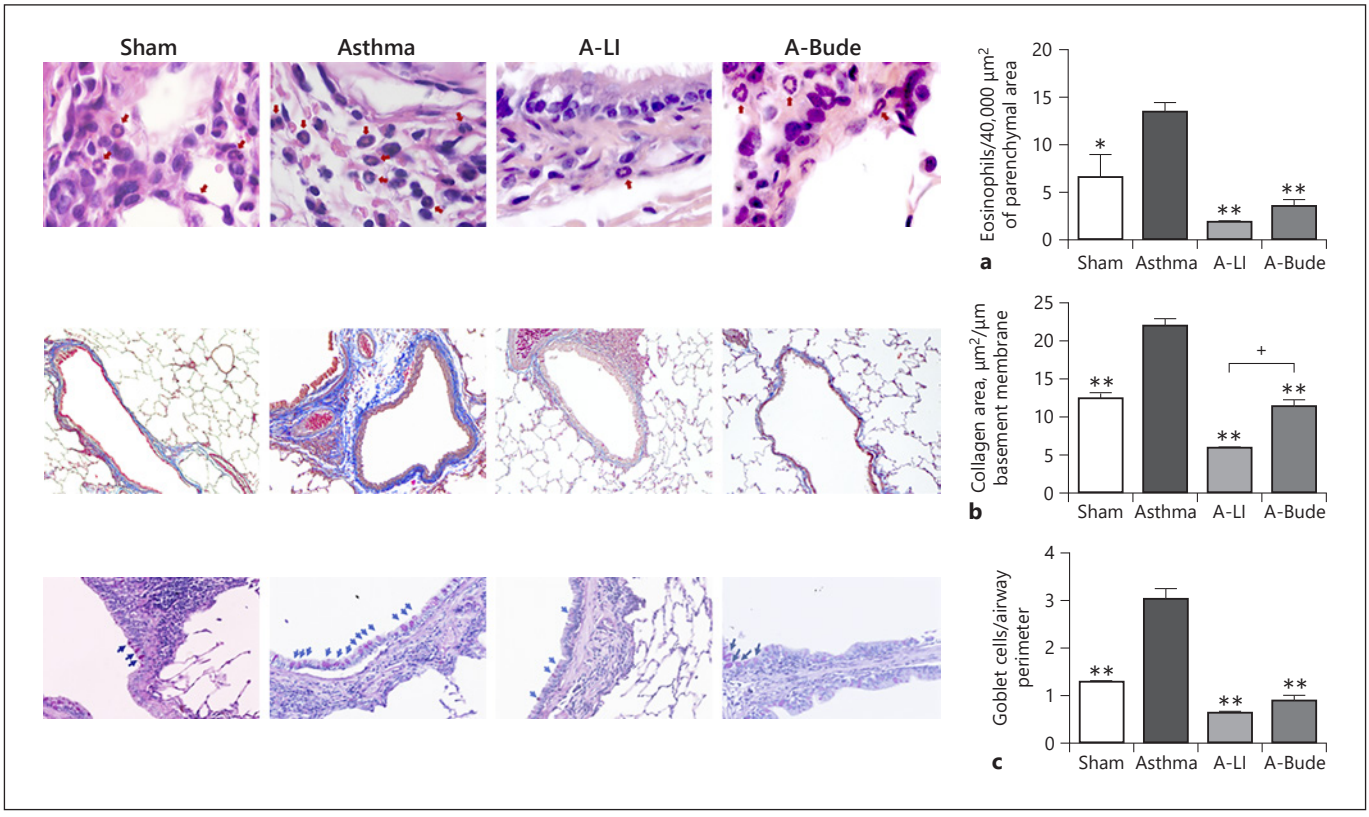

Fig. 3. Evaluation of airway inflammation and lung architecture changes after *L. lactis* administration. Recruitment of eosinophils (red arrows) (**a**), subepithelial collagen deposition (blue colouration) (**b**), and bronchial goblet cells (blue arrows) (**c**). Representative histological images were obtained at a magnification of ×1,000 for eosinophils and ×100 for collagen and goblet cells. The results are presented as mean ± standard error of the mean. $N = 4$ animals per group; samples from each rat were analyzed in triplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the asthma group. $^{+}p < 0.05$ between the indicated groups. A-Ll, OVA-induced asthmatic animals treated with *L. lactis*; A-Bude, OVA-induced asthmatic animals treated with budesonide. *L. lactis*, *Lactococcus lactis*; OVA, ovalbumin.

tor of the asthmatic airway inflammation in the lung is the production of Th2 cytokines. Since IL-5 is an important eosinophil activator and chemoattractant [27] and IL-13 induces IgE production and airway mucus secretion [4], we assessed the mRNA levels of both Th2 cytokines (Fig. 2d, e). The mRNA expression of IL-5 and IL-13 was significantly increased in the asthma group (4.39- and 5.30-fold, respectively). In the A-Ll group, the expression of both cytokines was significantly reduced, by 65.55% for IL-5 and by 78.57% for IL-13. Likewise, budesonide treatment also reduced the mRNA levels of IL-5 and IL-13 (70.67 and 88.97%, respectively). There was no significant difference in the mRNA expression of IL-5 and IL-13 between the A-Ll and A-Bude groups, demonstrating that *L. lactis* NZ9000 is as effective as budesonide in suppressing the expression of these cytokines.

Airway remodelling is driven by constant allergen insult and the consequent inflammatory process. Lung changes include eosinophil migration through parenchyma, extracellular matrix remodelling, and goblet cell hyperplasia, events that are characteristics in the onset of chronic asthma [4]. The histological analysis showed marked reconstruction of the lung architecture in the asthmatic group compared to the sham group, with significant increases in eosinophil influx, subepithelial collagen deposition, and goblet cell hyperplasia, of 2.01-, 1.74-, and 2.36-fold, respectively (Fig. 3). However, lung inflammation in the *L. lactis*-treated group was significantly suppressed compared to that of the asthma group since the peribronchial eosinophil number, collagen deposition, and goblet cell hyperplasia were reduced by 86.24, 72.80, and 79.17%, respectively. This activity was also observed in the A-Bude group. Surprisingly, although the effect of both treatments was equivalent in relation to the reduction in eosinophil and goblet cell counts, the decrease in collagen accumulation in the ex-

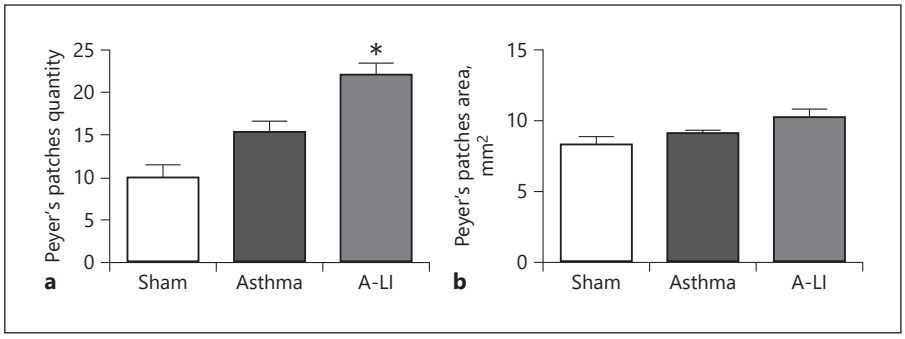

Fig. 4. Effect of L. *lactis* administration on Peyer's patches in asthmatic animals. Quantity (**a**) and size (**b**) of Peyer's patches. The results are presented as mean ± standard error of the mean. $N = 4$ for sham and asthma groups, and $N = 5$ for the A-Ll group. *$p < 0.05$ versus the asthma group. A-Ll, OVA-induced asthmatic animals treated with *L. lactis. L. lactis*, *Lactococcus lactis*; OVA, ovalbumin.

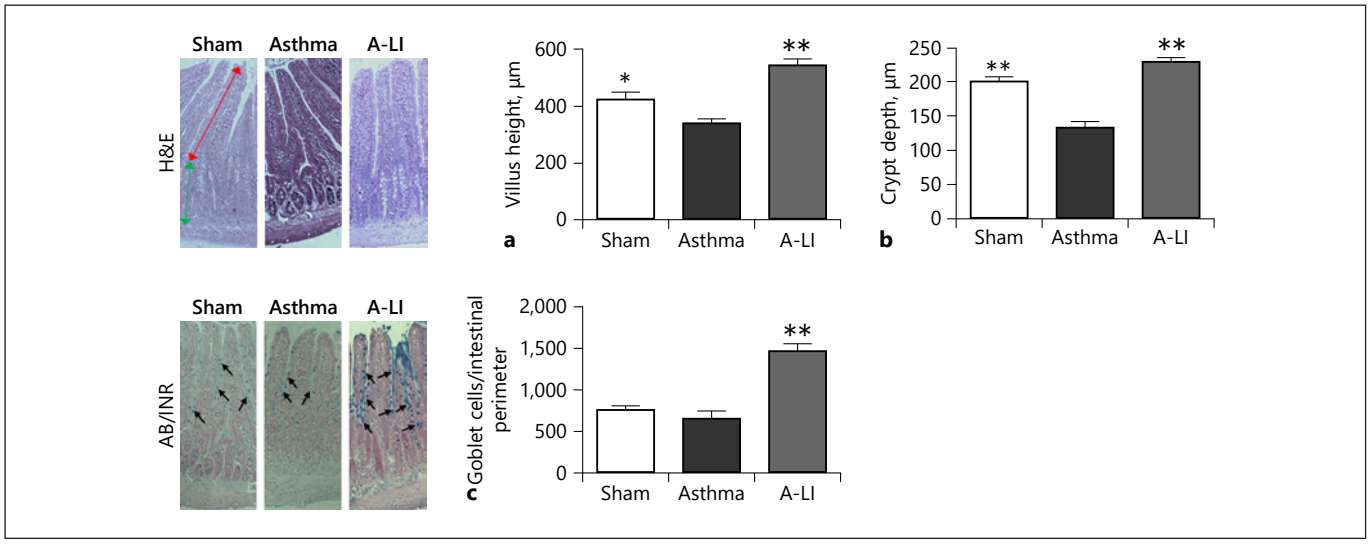

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Fig. 5. Analysis of intestinal morphology in experimental groups. Intestine villus height (red arrow) (**a**), crypt depth (green arrow) (**b**), and intestinal goblet cells (black arrows) (**c**). Representative histological images were obtained at a magnification of ×100. The results are presented as mean ± standard error of the mean. $N = 5$ per group. Samples from each rat were analyzed in triplicate. *$p < 0.05$ and **$p < 0.01$ versus the asthma group. A-Ll, OVA-induced asthmatic animals treated with *L. lactis. L. lactis*, *Lactococcus lactis*; OVA, ovalbumin; H&E, haematoxylin and eosin; AB/NR, alcian blue/nuclear red.

tracellular matrix was significantly increased in the A-Ll group (47.82% compared to A-Bude). Oral administration of *L. lactis* effectively inhibited the OVA-induced pulmonary inflammation and progression of lung architecture remodelling in allergic asthmatic animals in a similar manner to that of the standard treatment.

Intestinal Morphology Is Improved in Asthmatic Rats by L. lactis Administration

To investigate whether positive effects on asthma in orally administered *L. lactis* animals are accompanied by an improvement of intestinal morphology, we then measured the quantity and size of Peyer's patches and histomorphometric characteristics of the intestine. In the dysbiosis process, Peyer's patches are atrophied [28]. In asthmatic rats, the quantity of Peyer's patches was slightly, but not significantly, increased; however, in the A-Ll group, the number of Peyer's patches was significantly higher (1.44-fold) than that of the asthma group (Fig. 4a). Nevertheless, the Peyer's patch size remained unchanged among the experimental groups (Fig. 4b).

In contrast, villus height and crypt depth (Fig. 5a, b) were significantly reduced in the asthma group, with values 20.81 and 34.57% lower than those in the sham animals, respectively. This phenomenon was prevented by the administration of *L. lactis* since the villus height in the A-Ll group was increased by 1.61-fold and the crypt depth by 1.75-fold. In relation to the number of intestinal goblet cells, there was no difference between the sham and asthmatic rats; however, the A-Ll group exhibited a marked

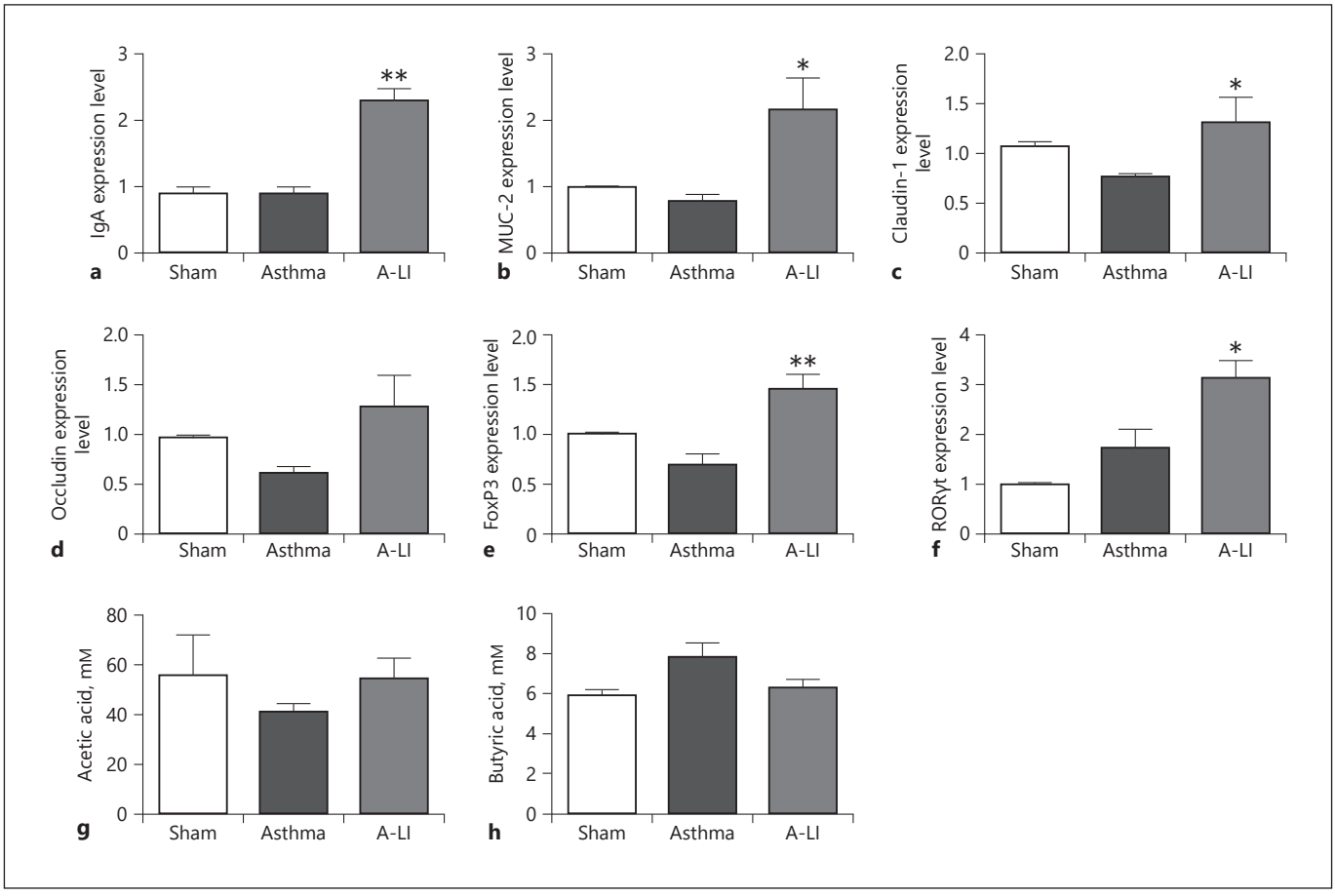

Fig. 6. Relative mRNA expression of intestinal barrier function and immunoregulatory transcription factor genes and caecal SCFA quantification in experimental groups. Total IgA (**a**), MUC-2 (**b**), claudin-1 (**c**), occludin (**d**), FoxP3 (**e**), and RORγt (**f**) in intestinal tissue. Levels of acetic (**g**) and butyric (**h**) acid in the caecum. The results are presented as mean ± standard error of the mean. $N = 4$ per group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$ versus the asthma group. A-Ll, OVA-induced asthmatic animals treated with *L. lactis*; SCFA, short-chain fatty acid; *L. lactis*, *Lactococcus lactis*; OVA, ovalbumin.

increase in goblet cell number, being 2.30-fold greater than that of the asthma group (Fig. 5c). Taken together, these results show that *L. lactis* improves intestinal morphology which is associated with a proper barrier function.

L. lactis Treatment Enhances the Expression of Genes Related to Intestinal Barrier Integrity and Immune Regulation

Probiotics have the potential to improve the mucosal intestinal barrier [29]. Therefore, we evaluated whether orally administered *L. lactis* promotes the mRNA expression of IgA, MUC-2, and tight junction-associated proteins claudin-1 and occludin to prevent a remote allergen-induced inflammatory process (Fig. 6a–d). Total IgA did not change between the asthmatic and sham rats, and MUC-2 and claudin-1 mRNA expression in the asthma group was slightly minor than that of the control animals. The *L. lactis*-treated group showed a 2.58-, 2.84-, and 1.74-fold increase in expression of total IgA, MUC-2, and claudin-1 in contrast to the asthma group (Fig. 6a–c). Although the occludin mRNA expression was 2.10-fold higher in the *L. lactis*-treated group than that of the asthma group, this increase was not significant (Fig. 6d). These findings suggest that *L. lactis* administration improves the intestinal mucosal barrier.

Since probiotic usage suppresses allergy symptoms by inducing Treg cells [30] and Th17 cells are involved in gut homeostasis [31], we analyzed the mRNA expression levels of the transcription factors FoxP3 (Treg) and RORγt (Th17) (Fig. 6e, f). In intestinal tissue, the asthma group did not show significant changes in the expression

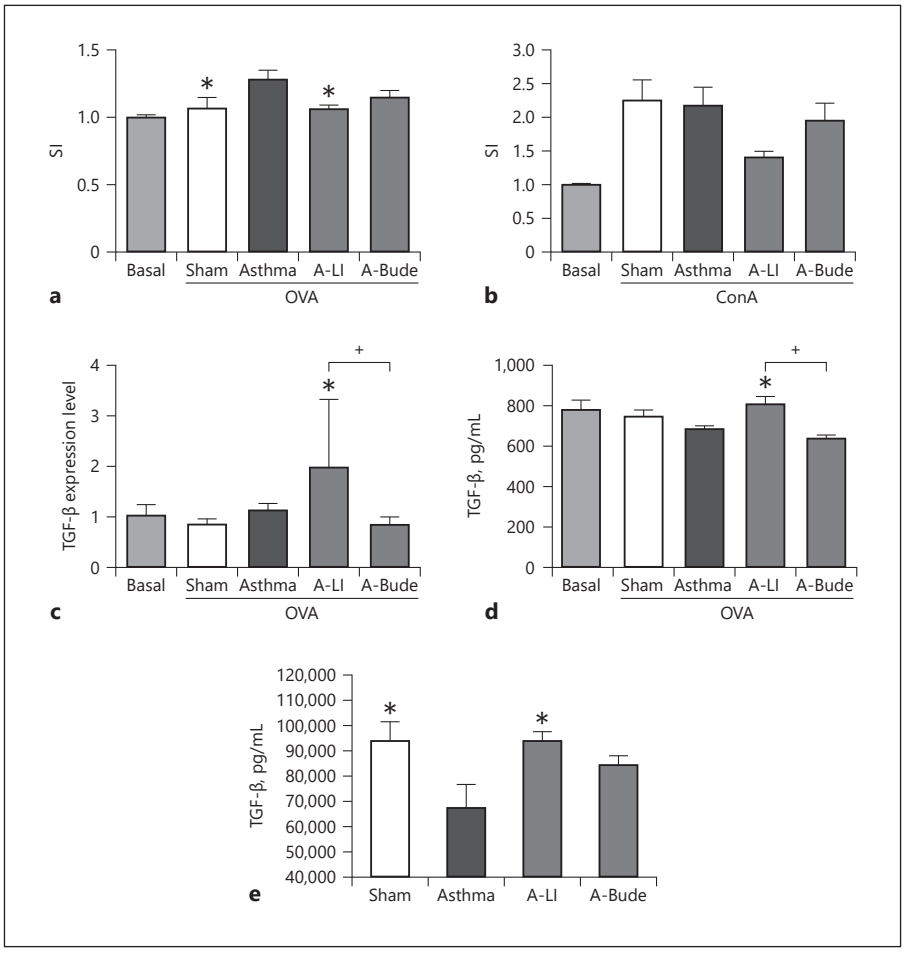

Fig. 7. Effect of *L. lactis* administration on spleen cell proliferation and TGF-β expression and on serum level of TGF-β. Splenocytes from experimental animals were stimulated with OVA 0.1 mg/mL (**a**) or ConA 1 μg/mL (**b**), and SI was calculated. Basal represents cellular proliferation without any stimulus. Splenocytes from experimental animals were stimulated with OVA 2 mg/mL, and TGF-β mRNA expression in cells (**c**) and secreted TGF-β in the supernatant (**d**) were evaluated. Basal represents mRNA expressed or cytokine quantity without any stimulus. TGF-β serum level (**e**) of experimental animals. The results are presented as mean ± standard error of the mean. $N = 5$ animals per group. $^*p < 0.05$ versus the asthma group. $+p < 0.01$ between the indicated groups. A-Ll, OVA-induced asthmatic animals treated with *L. lactis*; A-Bude, OVA-induced asthmatic animals treated with budesonide. *L. lactis*, *Lactococcus lactis*; TGF, transforming growth factor; OVA, ovalbumin; ConA, concanavalin A; SI, stimulation index.

levels of these transcription factors; only a slight reduction in FoxP3 expression and an increased RORγt expression tendency were observed in the asthma group compared to that of the sham group. In the A-Ll group, the mRNA expression levels of the transcription factors FoxP3 and RORγt were 2.09- and 2.11-fold higher than those of the asthma group. Thus, this immune environment induced by *L. lactis* in the intestine of treated rats might reflect in the lung through the regulation of asthmatic inflammation. Because LAB are responsible for the production of short-chain fatty acids (SCFAs), which have anti-inflammatory and immunoregulatory effects [30], acetic and butyric acid levels were assessed by gas chromatography. The results showed that levels of acetic acid (Fig. 6g) and butyric acid (Fig. 6h) were similar among experimental groups, indicating that the production of these two SCFAs in the caecum is not promoted during asthma development nor as a consequence of *L. lactis* administration.

L. lactis Administration Reduces Allergen-Specific Proliferation of Spleen Cells

To investigate whether *L. lactis* treatment is modulating the systemic immune response in asthmatic animals, splenocyte proliferative response was evaluated. Spleen cells were prepared from asthmatic rats with or without treatments and cultured with the allergen or mitogen ConA. Stimulation of splenocytes from the asthma group with OVA resulted in a significantly enhanced proliferative response compared to that of sham animals, which proves the development of a systemic allergen-specific immune response (Fig. 7a). Remarkably, oral *L. lactis* administration abolished the OVA-induced proliferation of splenocytes. When proliferation by mitogen was assessed (Fig. 7b), both the sham and asthma animals showed an increased response in relation to basal condition, with an SI 1.7-fold greater than that stimulated by OVA in asthma rats. Although spleen cells from rats treated with *L. lactis* showed a slight reduction in the SI to the mitogen ConA

compared to asthma animals, it remains not significant. This datum suggests that *L. lactis* can induce mechanisms of allergen-specific immune tolerance, without suppressing immune competence in the animals. Proliferative response of splenocytes from asthmatic rats treated with budesonide was also evaluated to confirm that the action mechanism of inhaled budesonide is restricted to airways, as previously reported [24]. The SI of the A-Bude group in response to OVA or ConA was unchanged compared to that of the asthma group (Fig. 7a, b), showing that in our experimental conditions, inhaled budesonide is only modulating the allergic response at airways.

L. lactis Administration Increases TGF-β Production by Spleen Cells and TGF-β Level in Serum

TGF-β has been proposed as the principal initiator of the immune regulatory mechanisms after probiotic treatments [30]. To determine whether *L. lactis* administration influences TGF-β production by splenocytes *in vitro*, spleen cells isolated from asthmatic animals with or without treatments were stimulated by OVA, and the regulatory cytokine was quantified as mRNA and protein levels. There was no statistical difference in TGF-β mRNA or protein expression between the sham and asthma groups. Oral treatment with *L. lactis* resulted in a significant increase in mRNA expression of TGF-β by splenocytes in response to the allergen, which was accompanied by a significant rise in TGF-β secretion (Fig. 7c, d). TGF-β expression was not modified in the A-Bude group in comparison with asthmatic animals without treatment. Finally, we evaluated whether *L. lactis* administration impacts on TGF-β level in serum of asthmatic animals. As shown in Figure 7e, the quantity of TGF-β was significantly diminished by 28.72% in the serum of asthmatic animals. *L. lactis* treatment recovered serum TGF-β level to that of sham animals. The A-Bude group showed a slight increase in TGF-β level, but it was not significant. Taken together, the results suggest that *L. lactis* administration exerts the systemic immunomodulatory effect by inducing the TGF-β production.

Discussion and Conclusion

New therapies that act on the immunological mechanisms of allergic disease are required. Probiotics are interesting candidates in the prevention and treatment of allergic disorders, as recent evidence points to dysbiosis of the gut microbiota as a risk for atopy [7]. It is reported that the immunomodulatory effects of probiotics are strain dependent [32], and the mechanisms of action of probiotics are multi-faceted, as each probiotic may have specific functions on the host [33]. In this study, we investigated the effect of orally administered *L. lactis* NZ9000 on the prevention or relief of inflammatory processes and airway remodelling in OVA-sensitized rats and its relationship with the intestinal environment.

Allergic asthma is characterized by a Th2 immune response, with high serum concentrations of allergen-specific IgE and Th2 cytokines [34]. In our experimental model, high levels of IgE specific to OVA were detected in the serum of allergic rats. Serum levels of OVA-specific IgE were significantly decreased in asthmatic rats by *L. lactis* NZ9000 administration, indicating that the treatment downregulates humoral response to the allergen. In a previous report, the intranasal administration of *L. lactis* G121, from 10 days before and during the experimental asthma model development, does not modify allergen-specific IgE although it decreases allergen-specific IgG1 level indicating that the probiotic is modulating the humoral response associated with asthma [15]. However, our result differs from those previously reported on *L. lactis* NZ9000 or the related strain MG1369, in which probiotic pre-treatment before allergen sensitization does not modify levels of allergen-specific IgE in animals [35, 36]. Altogether, these results support a highly timing and route administration- and strain-dependent effect of *L. lactis* on the allergic humoral response. We also show that *L. lactis* NZ9000 treatment decreases IL-13 expression in the lung. As IgE production is highly dependent on IL-13 in asthmatic patients [4], reduction in IL-13 levels by *L. lactis* NZ9000 may be related to the decreased allergen-specific IgE.

The infiltrating leucocytes during asthmatic airway inflammation induce lung damage with the consequent remodelling of the tissue. Asthmatic pulmonary pathophysiology includes eosinophil and neutrophil influx into the parenchyma, subepithelial accumulation of extracellular matrix proteins, such as collagen type I, and goblet cell hyperplasia [37]. We demonstrate that orally administered *L. lactis* NZ9000 attenuates the asthmatic airway inflammation in OVA-sensitized rats, as it reduces the number of leucocytes, mainly eosinophils, in the bronchoalveolar compartment and lung parenchyma, and the expression of IL-5 and IL-13 in the lung. IL-5 is essential for the development of pulmonary eosinophilia, as well as the subsequent onset of lung damage [38]; IL-13 is the major effector cytokine of goblet cell hyperplasia, airway hyper-responsiveness, and inflammation in asthma [39]. Thus, the downregulation on airway in-

flammation mediated by *L. lactis* NZ9000 is consistent with the attenuated remodelling of the lung architecture since collagen accumulation and goblet cell hyperplasia were diminished in our asthmatic rats treated with the probiotic. In a previous study, orally pre-treated mice with *L. lactis* NZ9000 before sensitization were used as control to study the effect of pre-treatment with a recombinant *L. lactis* NZ9000 expressing the major dust mite allergen Der p2 on later sensitization. The authors did not report any improvement in airway remodelling with the non-recombinant strain [40]. Timing and duration of *L. lactis* NZ9000 supplementation differ between this and our study, key parameters in probiotic treatment to achieve beneficial effects in the prevention of allergic diseases [32]. In this context, our results are concordant with those of preceding studies that demonstrate that *L. lactis* strains can regulate inflammatory cell infiltration and airway remodelling in experimental models. Thus, in a murine model of eosinophilic oesophagitis, animals supplemented with the *L. lactis* strain NCC 2287 showed a reduction in oesophageal and bronchoalveolar eosinophilia [16]; in a mice model of induced allergic airway inflammation by means of intranasal transfer of *L. lactis* G121-treated OVA-pulsed BMDCs, a significant reduction in the number of eosinophils in BALF and lung tissue, together with a decrease in mucus production, was evidenced [41].

Inhaled budesonide is one of the main pharmacological strategies for asthma treatment because it is targeted to diminish airway inflammation and remodelling [24]. Oral *L. lactis* NZ9000 administration reduced pulmonary inflammation and attenuated airway remodelling as efficiently as inhaled budesonide. In our experimental conditions, *L. lactis* NZ9000 treatment was more effective than budesonide in modulating the allergic systemic immune response, as it decreased allergen-specific IgE levels. Since budesonide has a high ratio of local to systemic anti-inflammatory activity, after aerosolization, it remains in therapeutically effective doses locally [42]. In this context, a study in asthmatic children shows that certain doses of inhaled budesonide have no effects on total and specific IgE serum levels after 6 months of treatment although they improve the lung function [43]. In the same studio, a slight increase in specific IgE was detected after 3 months of budesonide administration. These results suggest that the effect of budesonide in serum levels of IgE may be different depending on the dose and period of treatment.

The maintenance of a healthy gut barrier is one of the mechanisms through which the probiotics regulate the immune system [44]. Intestinal barrier function is mediated by a delicate and dynamic equilibrium among different elements, such as microbiota, IgA production, Peyer's patch development, and tight junctions between intestinal epithelial cells [28]. An adequate mucous layer and small intestine morphology are crucial for the maintenance of mucosal homeostasis, as well as a healthy gut [45]. Increased gut permeability has been found in children with food allergies [46] as well as in asthmatic patients [47], indicating that an altered intestinal barrier is involved in the development or propagation over time of asthma or other allergic conditions. In our experimental asthma model, villus height and crypt depth were significantly reduced, and a slight decrease in MUC-2 and tight junction protein expression was observed. Our findings indicate that orally administered *L. lactis* NZ9000 enhances the expression of secretory IgA, MUC-2, and claudin-1 and promotes healthy intestinal morphology. Few studies have examined the effect of anti-allergic probiotics on intestinal barrier function. Among them, a mixture of *Lactobacillus* strains that reduces allergen sensitization in Bet v1 pollen-sensitized mice upregulates the expression of occludin and the TJ molecule ZO-1, increases total intestinal and serum IgA levels, and improves the function of the gut epithelial barrier [48]; *Clostridium butyricum* and *Lactobacillus reuteri* treatments which downregulate allergic immune response in a rat model of food sensitization decrease intestinal permeability and increase tight junction expression [49]. Additionally, it has been demonstrated that probiotic lactobacilli (*L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246) reduce small intestinal permeability in children with atopic dermatitis [50]. Taken together, our study and other studies demonstrate that although probiotics are orally administered and believed to temporarily colonize the intestine and colon where they improve intestinal barrier function, its immunomodulatory activity is exerted in a distant organ, such as the lung. Besides, in the intestinal tissue of our asthmatic rats, a slight reduction in FoxP3 expression and an increased RORγt expression tendency were observed. It is known that in the immunological environment associated with intestinal homeostasis, Treg and Th17 cells predominate [51]. Treatment with *L. lactis* NZ9000 upregulates FoxP3 and RORγt expression in the gut tissue of asthmatic rats. These results demonstrate that intact and enhanced intestinal barrier function might play an important role in the regulation of lung inflammatory processes by *L. lactis* NZ9000. As oral administration of probiotics with anti-allergic properties, such as *L. rhamnosus* and *Bifidobacterium lactis*, promotes the stimulation of regulatory T

cells [30] and *L. lactis* NZ9000 increases FoxP3 expression, we suggest that regulatory T cells might be participating in anti-asthmatic effects of *L. lactis* NZ9000. FoxP3+ regulatory T cells induced by probiotics at the intestine can migrate to the sites of pathological inflammation [52], as have been recently described in asthmatic mice treated with *L. rhamnosus* [53].

Probiotic supplementation has demonstrated to increase colonic SCFA levels, mainly acetate, propionate, and butyrate, after fermentation of undigested food substances [54]. In healthy volunteers, *Lactobacillus plantarum* consumption favours an increase in SCFAs, such as acetate and propionate [55]. Moreover, a mix of probiotic supplements (*Bifidobacterium bifidum* W23, *Bifidobacterium animalis* subsp. *lactis* W52, and *L. lactis* W58) in the first 12 weeks after birth induces high levels of SCFAs in children, which results in a protective effect against the development of eczema [56]. Our results show that *L. lactis* NZ9000, by itself, does not increase caecal SCFA concentrations in asthmatic rats, indicating that they are not involved in the immunomodulatory effects of *L. lactis* on asthma. Studies on gut microbiota had reported that acetate induces intestinal IgA production [57]. We demonstrate that *L. lactis* NZ9000 promotes IgA production without increasing acetate levels, so other mechanisms might mediate its upregulating effect on IgA levels. As Peyer's patches produce secretory IgA to maintain an appropriate intestinal barrier function [28, 58], the augmentation in Peyer's patch quantity demonstrated in the rats herein may be mediating the IgA increase.

Nowadays, there is increasing evidence supporting the use of orally administered probiotics as a treatment in various systemic diseases [59, 60]. One of the major mechanisms of probiotics to prevent or ameliorate clinical symptoms in patients with allergic diseases is through increasing TGF-β responses [60]. TGF-β is a pleiotropic cytokine with remarkable immunoregulatory and anti-inflammatory properties. Since long ago it was described that TGF-β controls the *in vitro* proliferation of T cells [61]. In *Tgfb1*$^{-/-}$ mice, the inflammatory process is exacerbated and mainly presented in the heart and lung, with infiltration of lymphocytes and macrophages [62]. Also, the eosinophil survival is broadly affected by *in vitro* exposition to TGF-β [63]. Our results show that treatment with *L. lactis* NZ9000 leads to a decrease in splenocyte proliferation in response to OVA, which may be explained by the *in vitro* upregulation of TGF-β. The reduction of airway inflammation in asthmatic mice treated with either *L. rhamnosus* GG or *B. lactis* is associated with a suppressed allergen-induced splenocyte proliferative response and an increased number of TGF-β-secreting T cells in mesenteric lymph nodes [30]. Serum levels of TGF-β were also increased in *L. lactis* NZ9000-treated animals. Probiotic supplementation in atopic dermatitis patients improves the production of TGF-β in serum, which was accompanied by symptom mitigation [64]. Hence, it is possible that the systemic TGF-β production might be responsible for the prevention of asthmatic signs in our study. Importantly, *L. lactis* NZ9000 treatment does not affect the ConA stimulation of lymphocytes, which indicates the maintenance of the capacity of immune response in animals.

In conclusion, we found that *L. lactis* NZ9000 possesses a prophylactic effect on the development of allergic asthma. *L. lactis* NZ9000 effectively decreases serum allergen-specific IgE, suppresses the number of total leucocytes and eosinophils in BALF, and diminishes the number of eosinophils infiltrated in the lung interstitium. Goblet cell hyperplasia and collagen deposition are also attenuated by *L. lactis* NZ9000 treatment. The beneficial effect of the probiotic in airways is associated with the downregulation of IL-5 and IL-13 in lung tissue. The pulmonary effect of *L. lactis* NZ9000 is related to an enhancement of intestinal barrier function with the induction of a regulatory and homeostatic mucosal environment, to the abolishment of allergen-induced spleen cell proliferation, and to a systemic recuperation of TGF-β production. This study provides the first experimental basis for the potential use of *L. lactis* NZ9000 in the prevention of airway inflammation and remodelling associated with allergic asthma.

Acknowledgements

We would like to acknowledge M.V.Z. Karen E. Sánchez Hernández for breeding and providing animals used in this study and Mr. Manuel Tinajero for his excellent assistance in the histological procedures.

Statement of Ethics

All animals were manipulated and treated in strict accordance with the Institutional Normative Welfare Standards of the Autonomous University of Aguascalientes, and experimental protocols were approved by the Ethical Committee for the Use of Animal in Teaching and Research. All participants and animal handlers were committed to manipulating animals sensibly in order to reduce stress or suffering.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was supported by grants PIBB 17-11 and PIBB-15-9 N awarded by the Autonomous University of Aguascalientes and CONACYT grant 240921.

Author Contributions

D.C.G. and E.S. designed the experimental strategy, analyzed data, and wrote the paper; M.J. and C.E.R.S. performed the surgical lung process and histopathological analysis; P.G.A., A.H.M., and L.S.S.P. performed experiments to evaluate lung inflammation, gene expression, and intestinal environment; M.J.L.A., O.S.C., and R.M.L. analyzed data and provided advice on experiments.

References

1 Masoli M, Fabian D, Holt S, Beasley R; Global Initiative for Asthma (GINA) Program. The global burden of asthma: executive summary of the GINA Dissemination Committee report. Allergy. 2004 May;59(5):469–78.
2 Kay AB. Allergy and allergic diseases. First of two parts. N Engl J Med. 2001 Jan 4;344(1):30–7.
3 Timm S, Frydenberg M, Janson C, Campbell B, Forsberg B, Gislason T, et al. The urban-rural gradient in asthma: a population-based study in Northern Europe. Int J Environ Res Public Health. 2015 Dec;13(1):E93.
4 Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. Nature. 2008 Jul;454(7203):445–54.
5 Kauffmann F, Oryszczyn MP, Maccario J. The protective role of country living on skin prick tests, immunoglobulin E and asthma in adults from the Epidemiological Study on the Genetics and Environment of Asthma, Bronchial Hyper-responsiveness and Atopy. Clin Exp Allergy. 2002 Mar;32(3):379–86.
6 Nilsson L, Castor O, Löfman O, Magnusson A, Kjellman NI. Allergic disease in teenagers in relation to urban or rural residence at various stages of childhood. Allergy. 1999 Jul;54(7):716–21.
7 Arrieta MC, Stiemsma LT, Dimitriu PA, Thorson L, Russell S, Yurist-Doutsch S, et al. Early infancy microbial and metabolic alterations affect risk of childhood asthma. Sci Transl Med. 2015 Sep;7(307):307ra152.
8 Sevelsted A, Stokholm J, Bisgaard H. Risk of asthma from cesarean delivery depends on membrane rupture. J Pediatr. 2016 Apr;171:38–4.
9 Kalliomäki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet. 2001 Apr;357(9262):1076–9.
10 Zuccotti G, Meneghin F, Aceti A, Barone G, Callegari ML, Di Mauro A, et al. Probiotics for prevention of atopic diseases in infants: systematic review and meta-analysis. Allergy. 2015 Nov;70(11):1356–71.
11 Kelly WJ, Davey GP, Ward LJ. Characterization of lactococci isolated from minimally processed fresh fruit and vegetables. Int J Food Microbiol. 1998 Dec;45(2):85–92.
12 Suzuki C, Kimoto-Nira H, Kobayashi M, Nomura M, Sasaki K, Mizumachi K. Immunomodulatory and cytotoxic effects of various Lactococcus strains on the murine macrophage cell line J774.1. Int J Food Microbiol. 2008 Mar;123(1–2):159–65.
13 Han KJ, Lee NK, Park H, Paik HD. Anticancer and anti-inflammatory activity of probiotic Lactococcus lactis NK34. J Microbiol Biotechnol. 2015 Oct;25(10):1697–701.
14 Kim YJ, Lee SH. Inhibitory effect of Lactococcus lactis HY 449 on cariogenic biofilm. J Microbiol Biotechnol. 2016 Nov;26(11):1829–35.
15 Debarry J, Garn H, Hanuszkiewicz A, Dickgreber N, Blümer N, von Mutius E, et al. Acinetobacter lwoffii and Lactococcus lactis strains isolated from farm cowsheds possess strong allergy-protective properties. J Allergy Clin Immunol. 2007 Jun;119(6):1514–21.
16 Holvoet S, Doucet-Ladevèze R, Perrot M, Barretto C, Nutten S, Blanchard C. Beneficial effect of Lactococcus lactis NCC 2287 in a murine model of eosinophilic esophagitis. Allergy. 2016 Dec;71(12):1753–61.
17 Yoshida A, Aoki R, Kimoto-Nira H, Kobayashi M, Kawasumi T, Mizumachi K, et al. Oral administration of live Lactococcus lactis C59 suppresses IgE antibody production in ovalbumin-sensitized mice via the regulation of interleukin-4 production. FEMS Immunol Med Microbiol. 2011 Apr;61(3):315–22.
18 Wyszyńska A, Kobierecka P, Bardowski J, Jagusztyn-Krynicka EK. Erratum to: lactic acid bacteria: 20 years exploring their potential as live vectors for mucosal vaccination. Appl Microbiol Biotechnol. 2015 Apr;99(10):4531–77.
19 Linares DM, Kok J, Poolman B. Genome sequences of Lactococcus lactis MG1363 (revised) and NZ9000 and comparative physiological studies. J Bacteriol. 2010 Nov;192(21):5806–12.
20 Bermúdez-Humarán LG, Langella P, Cortes-Perez NG, Gruss A, Tamez-Guerra RS, Oliveira SC, et al. Intranasal immunization with recombinant Lactococcus lactis secreting murine interleukin-12 enhances antigen-specific Th1 cytokine production. Infect Immun. 2003 Apr;71(4):1887–96.
21 Enouf V, Langella P, Commissaire J, Cohen J, Corthier G. Bovine rotavirus nonstructural protein 4 produced by Lactococcus lactis is antigenic and immunogenic. Appl Environ Microbiol. 2001 Apr;67(4):1423–8.
22 Medina M, Villena J, Salva S, Vintiñi E, Langella P, Alvarez S. Nasal administration of Lactococcus lactis improves local and systemic immune responses against Streptococcus pneumoniae. Microbiol Immunol. 2008 Aug;52(8):399–409.
23 Roldán NR, Jiménez M, Cervantes-García D, Marín E, Salinas E. Glycomacropeptide administration attenuates airway inflammation and remodeling associated to allergic asthma in rat. Inflamm Res. 2016 Apr;65(4):273–83.
24 Pelaia G, Vatrella A, Busceti MT, Fabiano F, Terracciano R, Matera MG, et al. Molecular and cellular mechanisms underlying the therapeutic effects of budesonide in asthma. Pulm Pharmacol Ther. 2016 Oct;40:15–21.
25 Cardona NI, Lora F, Gomez JE. Standarization of an enzyme-linked immunosorbet assay (ELISA) for detection of specific anti-Toxoplasma gondii IgG in mice. Parasitol Latinoam. 2005;60:97–101.
26 Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101–8.
27 Amin K, Janson C, Bystrom J. Role of eosinophil granulocytes in allergic airway inflammation endotypes. Scand J Immunol. 2016 Aug;84(2):75–85.
28 Kim SH, Jeung W, Choi ID, Jeong JW, Lee DE, Huh CS, et al. Lactic acid bacteria improves Peyer's patch cell-mediated immunoglobulin A and tight-junction expression in a destructed gut microbial environment. J Microbiol Biotechnol. 2016 Jun;26(6):1035–45.
29 Sanchez B, Delgado S, Blanco-Miguez A, Lourenço A, Gueimonde M, Margolles A. Probiotics, gut microbiota, and their influence on host health and disease. Mol Nutr Food Res. 2017 Jan;61(1):1600240.
30 Feleszko W, Jaworska J, Rha RD, Steinhausen S, Avagyan A, Jaudszus A, et al. Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma. Clin Exp Allergy. 2007 Apr;37(4):498–505.

31 Ohnmacht C. Tolerance to the intestinal microbiota mediated by ROR(γt)(+) cells. Trends Immunol. 2016 Jul;37(7):477–86.
32 Wang HT, Anvari S, Anagnostou K. The role of probiotics in preventing allergic disease. Children. 2019 Feb;6(2):E24 .
33 Walker WA. Mechanisms of action of probiotics. Clin Infect Dis. 2008 Feb 1;46(Suppl 2): S87–51.
34 Bogdanov IV, Finkina EI, Melnikova DN, Tagaev AA, Ovchinnikova TV. Analysis of the serum cytokine profile in allergic patients opens a way to personalized treatment of allergy. Bull Exp Biol Med. 2019 Apr;166(6): 770–3.
35 Adel-Patient K, Ah-Leung S, Creminon C, Nouaille S, Chatel JM, Langella P, et al. Oral administration of recombinant Lactococcus lactis expressing bovine beta-lactoglobulin partially prevents mice from sensitization. Clin Exp Allergy. 2005 Apr;35(4):539–46.
36 Repa A, Grangette C, Daniel C, Hochreiter R, Hoffmann-Sommergruber K, Thalhamer J, et al. Mucosal co-application of lactic acid bacteria and allergen induces counter-regulatory immune responses in a murine model of birch pollen allergy. Vaccine. 2003 Dec;22(1):87–95.
37 Zhou-Suckow Z, Duerr J, Hagner M, Agrawal R, Mall MA. Airway mucus, inflammation and remodeling: emerging links in the pathogenesis of chronic lung diseases. Cell Tissue Res. 2017 Mar;367(3):537–50.
38 Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. J Exp Med. 1996 Jan;183(1):195–201.
39 Hershey GK. IL-13 receptors and signaling pathways: an evolving web. J Allergy Clin Immunol. 2003 Apr;111(4):677–91.
40 Ai C, Zhang Q, Ren C, Wang G, Liu X, Tian F, et al. Genetically engineered Lactococcus lactis protect against house dust mite allergy in a BALB/c mouse model. PLoS One. 2014 Oct;9(10):e109461.
41 Stein K, Brand S, Jenckel A, Sigmund A, Chen ZJ, Kirschning CJ, et al. Endosomal recognition of Lactococcus lactis G121 and its RNA by dendritic cells is key to its allergy-protective effects. J Allergy Clin Immunol. 2017 Feb; 139(2):667–78.e5.
42 Berger WE. Budesonide inhalation suspension for the treatment of asthma in infants and children. Drugs. 2005;65(14):1973–89.
43 Stelmach I, Bobrowska-Korzeniowska M, Majak P, Stelmach W, Kuna P. The effect of montelukast and different doses of budesonide on IgE serum levels and clinical parameters in children with newly diagnosed asthma. Pulm Pharmacol Ther. 2005;18(5):374–80.
44 Özdemir O. Various effects of different probiotic strains in allergic disorders: an update from laboratory and clinical data. Clin Exp Immunol. 2010 Jun;160(3):295–304.
45 Greig CJ, Alper A, Goodman AL, Cowles RA. Mucosal homeostasis is altered in the ileum of gnotobiotic mice. J Surg Res. 2018 Nov;231: 331–7.
46 Järvinen KM, Konstantinou GN, Pilapil M, Arrieta MC, Noone S, Sampson HA, et al. Intestinal permeability in children with food allergy on specific elimination diets. Pediatr Allergy Immunol. 2013;24(6):589–95.
47 Walker J, Dieleman L, Mah D, Park K, Meddings J, Vethanayagam D. High prevalence of abnormal gastrointestinal permeability in moderate-severe asthma. Clin Invest Med. 2014 Apr;37(2):E53–7.
48 Kozakova H, Schwarzer M, Tuckova L, Srutkova D, Czarnowska E, Rosiak I, et al. Colonization of germ-free mice with a mixture of three lactobacillus strains enhances the integrity of gut mucosa and ameliorates allergic sensitization. Cell Mol Immunol. 2016 Mar; 13(2):251–62.
49 Tulyeu J, Kumagai H, Jimbo E, Watanabe S, Yokoyama K, Cui L, et al. Probiotics prevents sensitization to oral antigen and subsequent increases in intestinal tight junction permeability in juvenile-young adult rats. Microorganisms. 2019 Oct;7(10):E463.
50 Rosenfeldt V, Benfeldt E, Valerius NH, Paerregaard A, Michaelsen KF. Effect of probiotics on gastrointestinal symptoms and small intestinal permeability in children with atopic dermatitis. J Pediatr. 2004 Nov;145(5):612–6.
51 Omenetti S, Pizarro TT. The Treg/Th17 axis: a dynamic balance regulated by the gut microbiome. Front Immunol. 2015 Dec;6:639.
52 Kwon HK, Lee CG, So JS, Chae CS, Hwang JS, Sahoo A, et al. Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. Proc Natl Acad Sci U S A. 2010 Feb; 107(5):2159–64.
53 Zhang J, Ma JY, Li QH, Su H, Sun X. Lactobacillus rhamnosus GG induced protective effect on allergic airway inflammation is associated with gut microbiota. Cell Immunol. 2018 Oct;332:77–84.
54 Macfarlane GT, Macfarlane S. Bacteria, colonic fermentation, and gastrointestinal health. J AOAC Int. 2012 Jan–Feb;95(1):50–60.
55 Wang L, Zhang J, Guo Z, Kwok L, Ma C, Zhang W, et al. Effect of oral consumption of probiotic Lactobacillus planatarum P-8 on fecal microbiota, SIgA, SCFAs, and TBAs of adults of different ages. Nutrition. 2014 Jul–Aug;30(7–8):776–83.e1.
56 Kim HK, Rutten NB, Besseling-van der Vaart I, Niers LE, Choi YH, Rijkers GT, et al. Probiotic supplementation influences faecal short chain fatty acids in infants at high risk for eczema. Benef Microbes. 2015;6(6):783–90.
57 Wu W, Sun M, Chen F, Cao AT, Liu H, Zhao Y, et al. Microbiota metabolite short-chain fatty acid acetate promotes intestinal IgA response to microbiota which is mediated by GPR43. Mucosal Immunol. 2017 Jul;10(4):946–56.
58 Lycke NY, Bemark M. The role of Peyer's patches in synchronizing gut IgA responses. Front Immunol. 2012 Nov;3:329.
59 Clemente JC, Manasson J, Scher JU. The role of the gut microbiome in systemic inflammatory disease. BMJ. 2018 Jan;360:j5145.
60 Eslami M, Bahar A, Keikha M, Karbalaei M, Kobyliak NM, Yousefi B. Probiotics function and modulation of the immune system in allergic diseases. Allergol Immunopathol. 2020 Aug;S0301–0546(20):30113–0.
61 Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarez-Mon M, Derynck R, et al. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. J Exp Med. 1986 May;163(5):1037–50.
62 Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, et al. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci U S A. 1993 Jan;90(2):770–4.
63 Alam R, Forsythe P, Stafford S, Fukuda Y. Transforming growth factor beta abrogates the effects of hematopoietins on eosinophils and induces their apoptosis. J Exp Med. 1994 Mar 1;179(3):1041–5.
64 Inoue Y, Kambara T, Murata N, Komori-Yamaguchi J, Matsukura S, Takahashi Y, et al. Effects of oral administration of Lactobacillus acidophilus L-92 on the symptoms and serum cytokines of atopic dermatitis in Japanese adults: a double-blind, randomized, clinical trial. Int Arch Allergy Immunol. 2014;165(4): 247–54.