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Title: Anti-allergic effects of *Lactobacillus crispatus* KT-11 strain on ovalbumin-sensitized BALB/c mice

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Running head: ANTI-ALLERGIC EFFECTS OF *L. CRISPATUS* KT-11

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ABSTRACT

In this study, we investigated the effects of oral ingestion of *Lactobacillus crispatus* KT-11 strain (KT-11) on the immune response in an allergic rhinitis mouse model, ovalbumin (OVA)-sensitized BALB/c. Sneezing activity in mice that were administered a KT-11-supplemented diet was significantly lower than that in mice administered a KT-11-free diet (control diet) at age of 11 weeks. We found that serum OVA-specific immunoglobulin E levels and total number of interleukin (IL)-4$^+$CD4$^+$ spleen cells in mice that were administered a KT-11-supplemented diet was significantly lower than that in mice administered control diet. The ratio of spleen interferon-γ$^+$CD4$^+$/IL-4$^+$CD4$^+$ cells was higher in the mice administered the KT-11-supplemented diet compared to that in mice administered the control or *L. rhamnosus* GG-supplemented diet. In contrast, the number of CD11b$^+$CD80$^+$ and FcεRIα$^+$CD117$^+$ cells was significantly lower in mice administered the KT-11-supplemented diet. These results suggested that KT-11 reduced OVA-induced allergic symptoms in BALB/c mice via the adjustment of the T helper type 1/T helper type 2 balance, and a decrease in the number of antigen-presenting cells and high affinity IgE receptor-positive mast cells.

KEYWORDS: *anti-allergic effect, antigen-presenting cells, Lactobacillus crispatus KT-11, mast cells, Th1/Th2 balance.*

INTRODUCTION

The number of patients diagnosed with allergic diseases such as atopic dermatitis, allergic asthma and allergic rhinitis has increased significantly in many countries worldwide. These type I allergic diseases are generally characterized by an elevation in serum immunoglobulin (Ig) E levels (Dreborg 2002). The production of IgE is thought to be due to a skewed T helper type 1 (Th1)/T helper type 2 (Th2) cell balance (Shirakawa *et al.* 1997; Prescott *et al.* 1999; Hopkin 2002). Several investigations have demonstrated that numerous lactic acid bacteria and their cellular components reduce allergic symptoms by shifting the Th1/Th2 balance from a Th2-dominant state to a Th1-dominant state (Fujiwara *et al.* 2004; Iliev ID *et al.* 2008; Segawa *et al.* 2008). Kitazawa *et al.* (2008) and Koizumi *et al.* (2008) have also reported that lactic acid bacteria and their cellular components induce the Th1 immune response via activation of Toll-like receptors (TLRs).

Lactobacillus acidophilus is one of the major bacterial inhabitants of the gastrointestinal tract. Thus, *L. acidophilus* is extensively used in yogurt starters, toddler formula and dietary supplements (Klein *et al.* 1998; Roy *et al.* 2000; Sanders & Klaenhammer 2001). *L. crispatus* is also a member of the *L. acidophilus* group, which comprises *L. acidophilus*, *L. amylovorus*, *L. gallinarum*, *L. gasseri and L. johnsonii* (Cato *et al.* 1983). Recently, we demonstrated that the *L. crispatus* KT-11 strain (KT-11) isolated from healthy infant feces exhibited anti-allergic effects in NC/Nga mice that develop atopic dermatitis and IgE hyperproduction (Tobita *et al.* 2009). The effects included a shift in the Th1/Th2 balance from a Th2-dominant to a Th1-dominant state via activation of TLR2, nucleotide-binding oligomerization domains 1 and 2.

However, very little is known about the precise mechanisms underlying KT-11 activity in acquired allergic responses.

In this study, we found that KT-11 reduces acquired allergic symptoms via not only the adjustment of the Th1/Th2 balance, but also via a decrease in the total number of antigen-presenting cells and high affinity IgE receptor positive mast cells present in the allergic rhinitis mouse model, ovalbumin (OVA)-sensitized BALB/c mice.

MATERIALS AND METHODS

Materials. Phycoerythrin (PE)-labeled anti-mouse interleukin (IL)-4 monoclonal antibody (mAb, clone 11B11), PE-labeled anti-mouse interferon (IFN)-γ mAb (clone XMG1.2), PE-labeled anti-mouse IL-10 mAb (clone JES5-16E3), PE-labeled anti-mouse CD80 mAb (clone 16-10A1), biotin-labeled anti-mouse CD4 mAb (clone RM4-5), biotin-labeled anti-mouse CD45R/B220 (B220) mAb (clone RA3-6B2), biotin-labeled anti-mouse CD117 (clone 2B8), biotin-labeled anti-mouse CD11b mAb (clone M1/70) and phycoerythrin/cyanine 5 (PE/Cy5)-labeled streptavidin were purchased from BioLegend (San Diego, CA, USA). Anti-mouse FcεRIα (clone G-14) and PE-labeled anti-goat IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). OVA, brefeldin A (BFA), ionomycin, streptomycin and phorbol 12-myristate 13-acetate (PMA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). IntraPrep was obtained from Beckman Coulter (Marseille, France). Defined fetal bovine serum (FBS) was obtained from HyClone Laboratories (Road Logan, UT, USA). Penicillin was purchased from MP Biomedicals (Costa Mesa, CA, USA). RPMI-1640 was purchased

from Nissui Pharmaceutical (Tokyo, Japan). Horseradish peroxidase (HRP)-labeled anti-mouse IgE and IgG were obtained from Bethyl Laboratories (Montgomery, TX, USA). 3,3',5,5'-Tetramethyl benzidine (TMB) was purchased from KPL (Gaithersburg, MD, USA). Bovine serum albumin (BSA) and aluminum hydroxide gel were obtained from Sigma-Aldrich (St. Louis, MO, USA). All chemicals used in this study were of the highest analytical grade commercially available.

Lactic acid bacteria. KT-11 was purchased as a stock culture from Kitii Co., Ltd. (Kanagawa, Japan). *L. rhamnosus* GG (GG) was obtained from the American Type Culture Collection (Manassas, VA, USA). KT-11 and GG were inoculated in DeMan-Rogosa-Sharpe broth and cultivated for 24 h at 37°C, collected by centrifugation, washed three times with sterile water, heat-treated at 65°C for 30 min and lyophilized.

Feeding procedure. Five-week-old male BALB/c mice were obtained from Japan SLC (Shizuoka, Japan). As shown in **Figure 1**, the mice were first **Fig. 1.** assigned to different test regimens, administered a commercial mouse powder feed (MF, Oriental Yeast Co., Tokyo, Japan) for a week, and then given either MF (control diet), MF containing KT-11 (5×10^7 CFU/MF 1 g, KT-11-supplemented diet) or MF containing GG (5×10^7 CFU/MF 1 g, GG-supplemented diet) between 6 and 13 weeks of age ($n = 6$). The mice were then intraperitoneally injected with 200 μL saline containing 20 μg OVA and 2 mg aluminum hydroxide gel at 6 and 8 weeks of age. The mice were intranasally challenged by instillation with 10 μL distilled water containing

100 µg OVA three times a week between 9 and 13 weeks of age. The total number of sneezing events was then counted for 10 min, 1 min from the intranasal instillation. Food and water was supplied *at libitum* throughout the course of the experiment. The mice were housed at 23 ± 2°C under a standard 12-h light-dark cycle. Blood, spleen and Peyer's patch samples were collected immediately following a lethal dose of ether at 13 weeks of age. Serum was obtained by centrifugation at 450 x *g* for 60 min at 4°C and stored at -30°C until use. All animal experimentation undertaken during this study was conducted in accordance with the guidelines for the Regulation of Animal Experimentation at Shinshu University, and according to Law No. 105 and Notification No. 6 of the Japanese government.

Cell suspensions and functional analysis. Spleen and Peyer's patch samples were homogenized in RPMI-1640 medium containing 5% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. The cell suspensions were then washed three times in this medium and adjusted to 1 x 10^6 viable cells/mL. The cell surface markers for CD11b, CD117 and B220 were then observed using a specific biotin-conjugated anti-mouse mAb for 15 min at 4°C, followed by incubation with PE/Cy5-labeled streptavidin for 15 min at 4°C. CD11b$^+$CD80$^+$ cells were incubated with PE-labeled anti-mouse mAb specific for CD80 for 15 min at 4°C. For FcεRIα$^+$CD117$^+$ and IgE$^+$B220$^+$ cells, the cell surface antigens for FcεRIα or IgE were labeled using the specific anti-mouse antibodies for 15 min at 4°C, followed by incubation with PE-labeled anti-goat IgG for 15 min at 4°C. The cell numbers were then determined using a Guava personal cell functional analyzer (Guava PCA: Guava Technologies,

Hayward, CA, USA).

When observing intracellular cytokines in CD4$^+$ cells, spleen cells were incubated at 37°C in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 20 µg/mL BFA, 2 µg/mL ionomycin and 20 ng/mL PMA for 4 h. The cell surface marker antigens for CD4 were then labeled using a specific biotin-conjugated anti-mouse mAb for 15 min at 4°C, followed by incubation with PE/Cy5-labeled streptavidin for 15 min at 4°C. Intracellular cytokines were measured following permeabilization and labeling with a PE-labeled anti-mouse cytokine mAb specific for IL-4, IFN-γ or IL-10. In order to achieve this, cells were fixed with IntraPrep reagent 1 for 15 min, washed and then permeabilized with IntraPrep reagent 2. The cells were then incubated with the appropriate antibodies and intracellular cytokine levels determined using Guava PCA.

Antibody analysis. OVA-specific IgE and IgG levels were measured using ELISA. Briefly, the wells of microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 µL OVA (100 µg/mL) in 0.1 M carbonate buffer (pH 9.6) overnight at 4°C and washed with phosphate buffered saline containing 0.05% Tween 20 (PBS-T). The plates were then post-coated with 300 µL of 0.1 M carbonate buffer (pH 9.6) containing 0.4% BSA for 2 h at 25°C, and washed with PBS-T. The serum samples used for the examination of OVA-specific IgE and IgG were then diluted 50- or 1000-fold in PBS-T containing 0.4% BSA and added to each well in a volume of 100 µL. The plate was then incubated for 2 h at 25°C. After washing the plate as described above, 100 µL optimally diluted HRP-labeled anti-mouse IgE

or IgG in PBS-T containing 0.4% BSA was added to each well (IgE and IgG: 0.1 µg/mL), the plate incubated for 1 h at 25°C and washed with PBS-T. TMB (100 µL) was used to detect the peroxidase reaction. The plate was then incubated for 30 min at 25°C and the reaction stopped by the addition of 100 µL 4 N H_2SO_4. Total antigen levels were then read at 450 nm on a microplate reader (550; Bio-Rad Laboratories, Hercules, CA, USA). The OVA-specific antibody level was calculated using the following formula: Antibody level = ELISA value (A_{450}) × Dilution-fold of the test sample.

Statistical analysis. Data is presented as the mean ± standard deviation (SD). Statistical analyses were performed using the Dunnett's multiple comparison tests for one-way analysis of variance or Student's t-test. Differences were considered significant when P values were less than 0.05.

RESULTS

In this study, BALB/c mice were fed a control diet, KT-11-supplemented diet or a GG-supplemented diet between 6 and 13 weeks of age. Under the breeding period, there was no significant differences in body weight among the mice administered the control diet, KT-11-supplemented diet and GG-supplemented diet, and the average intake of feed was about 3.46 g/day/mouse (control), 3.43 g/day/mouse (KT-11) and 3.45 g/day/mouse (GG) (data not shown). However, the number of sneezing events was lower in the mice administered the KT-11- and GG-supplemented diets than in those administered the control diet (**Fig. 2**). The level of **Fig. 2.** OVA-specific IgE was significantly lower in the mice administered the

KT-11- and GG-supplemented diets than in those administered the control diet (**Fig. 3A**), although the level of OVA-specific IgG did not significantly differ between the mice groups (**Fig. 3B**). The total number of spleen IFN-γ^+CD4$^+$ cells was higher in the mice administered the KT-11- and GG-supplemented diets than in those administered the control diet (**Fig. 4A**), while the total number of spleen IL-4$^+$CD4$^+$ cells in the mice administered the KT-11-supplemented diet was lower compared with that in mice administered the control and GG-supplemented diets (**Fig. 4B**). The ratio of IFN-γ^+CD4$^+$/IL-4$^+$CD4$^+$ was also higher in the mice administered the KT-11-supplemented diet than in those administered the GG-supplemented diet (**Fig. 4C**). The total number of spleen CD11b$^+$CD80$^+$, FcϵRIα^+CD117$^+$, IL-10$^+$CD4$^+$ or IgE$^+$B220$^+$ cells and Peyer's patch IgE$^+$B220$^+$ cells were significantly lower in the mice administered the KT-11-supplemented diet than in those administered the control diet (**Fig. 5A-D, G**), although Peyer's patch CD11b$^+$CD80$^+$ and FcϵRIα^+CD117$^+$ cells did not significantly differ between the 2 mice groups (**Fig. 5E, F**).

Fig. 3.

Fig 4.

Fig 5.

DISCUSSION

It has been shown previously that allergic rhinitis may be induced by the continuous nasal administration of OVA after an intraperitoneal injection of OVA in BALB/c mice (Saito *et al.* 2001). Thus in this study, we induced an acquired allergic response via the continuous nasal administration of OVA in BALB/c mice. We observed that sneezing events was lower in the mice administered the KT-11-supplemented diet than in the mice administered the control diet from 11 to 13 weeks of age. In particular, sneezing events were

significantly lower in the mice administered the KT-11-supplemented diet than in the mice administered the control diet at 11 weeks of age. These results indicated that KT-11 suppressed the effector phase of allergic rhinitis. In particular, the levels of serum OVA-specific IgE in the mice administered the KT-11-supplemented diet was lower than that observed in the control-fed mice. Masuda *et al.* (2008) and Inoue *et al.* (2009) reported that levels of serum OVA-specific IgE in OVA-sensitized BALB/c mice administered *Tetragenoccocus* (*T*.) *halophilus* (1 x 10^9 CFU/day/mouse) and *Bifidobacterium* (*B*.) *breve* (5 x 10^8 CFU/day/mouse), respectively, were significantly lower than those in the mice not administered the bacteria. In our study, it is calculated that the mice administered the KT-11-supplemented diet ingest about 1.73 x 10^8 CFU KT-11/day/mouse. These results indicate that the reduction of serum IgE level may occur at a lower dose in *L. crispatus* than in *T. halophilus* and *B. breve*.

The Peyer's patches and the spleen play an important role in the mucosal and systemic immune systems, respectively. In addition, the IgE^+B220^+ cells have been shown to represent IgE-producing B cells (Manetz & Meade 1999). The total number of IgE^+B220^+ cells in the spleen and Peyer's patches in mice administered the KT-11-supplemented diet was lower than that observed in the control-fed mice. These results indicated that KT-11 supplementation resulted in a reduction in the total number of B cells that produce IgE in both the systemic and mucosal immune systems.

GG was first isolated in 1985, and now represents one of the most extensively studied strains of lactic acid bacteria (Gorbach 2000). GG has been shown to adjust the Th/Th2 balance and induce IL-10 production during

allergic responses (Pessi *et al.* 2000; Sawada *et al.* 2007; Ghadimi *et al.* 2008). It is also well established that IL-4, IL-5 and IL-13 that are produced by Th2 cells, stimulate IgE production (Platts-Mills 2001). In contrast, Th1 cells predominantly secrete IFN-γ, which inhibits IL-4 production in Th2 cells (Pène *et al.* 1988). It is generally considered that the Th1/Th2 balance during type I allergic disease patient functions in a Th2-dominant state, and the spleen is an effective organ in order to investigate the Th1/Th2 balance (DiMeo *et al.* 2008). Hence, we investigated the ratio of IFN-γ$^+$CD4$^+$/IL-4$^+$CD4$^+$ and the numbers of IL-10$^+$CD4$^+$ cells in spleens. The ratio of IFN-γ$^+$CD4$^+$/IL-4$^+$CD4$^+$ in the mice administered the KT-11-supplemented diet was higher than that in the mice administered the GG-supplemented diet. These results indicated that the KT-11-mediated anti-allergic effect is attributed in part to the adjustment of the Th1/Th2 balance, even though the total number of IL-10$^+$CD4$^+$ cells was decreased in the mice administered the KT-11-supplemented diet. Regulatory T cells that produce IL-10 are known to suppress inflammatory responses via inhibition of various cytokines and IgE production (Wu *et al.* 2007). These results suggested that the reduction in allergic symptoms following KT-11 and GG administration may be due to an adjustment in the Th1/Th2 balance, even though the reduction in allergic symptoms following KT-11 supplementation was not due to IL-10-producing regulatory T cells. Thus, we hypothesize that the anti-allergic mechanisms underlying KT-11 activity may be different to those underlying GG activity.

During allergic immune responses, the antigens invading the body are first taken up by macrophage and dendritic cells. The activated macrophages

and dendritic cells then stimulate Th2 cells by antigen-presentation to produce Th2 cytokines that in turn promote B cell proliferation and IgE class switching (Punnonen *et al.* 1994). It is well established that activation of Th cells requires interaction of co-stimulatory molecules, in addition to the T cell receptor and major histocompatibility complex molecules delivered by antigen-presenting cells. CD80 and CD86 generally represent the most important co-stimulatory molecules, and are involved in allergic immune responses (Hofer *et al.* 1998; Burastero *et al.* 1999). The up-regulation of CD80, but not CD86, on the surface of dendritic cells of OVA-immunized BALB/c mice also plays a pivotal role in allergic immune responses, namely Th2 responses (Cheng *et al.* 2003). The total number of CD11b$^+$CD80$^+$ cells present in the mice administered the KT-11-supplemented diet was found to be lower than that in control-fed mice. These results suggested that the anti-allergic effects of KT-11 administration were in part due to the suppression of antigen-presenting cell activation.

Type I allergic diseases are caused by degranulation in mast cells. Degranulation occurs following antigen cross-linking between IgE molecules bound to FcεRI, a high-affinity IgE receptor present on mast cells (Platts-Mills 2001). Mast cells are normally defined on the basis of cell surface expression of FcεRIα and CD117 (Brown *et al.* 2008). The total number of FcεRIα$^+$CD117$^+$ spleen cells present in the mice administered the KT-11-supplemented diet was lower than that in the control-fed mice. These results suggested that the anti-allergic effects of KT-11 were in part due to a decrease in the number of mast cells.

In conclusion, *L. crispatus* KT-11 strain appears to suppress acquired allergic responses via the adjustment of the Th1/Th2 balance and a reduction in antigen-presenting and mast cell numbers in OVA-sensitized BALB/c mice.

REFERENCES

Brown MA, Sayed BA, Christy A. 2008. Mast cells and the adaptive immune response. *Journal of Clinical Immunology* **28**, 671-676.

Burastero SE, Magnani Z, Confetti C, Abbruzzese L, Oddera S, Balbo P, Rossi GA, Crimi E. 1999. Increased expression of the CD80 accessory molecule by alveolar macrophages in asthmatic subjects and its functional involvement in allergen presentation to autologous TH2 lymphocytes. *Journal of Allergy and Clinical Immunology* **103**, 1136-1142.

Cato EP, Moore WEC, Johnson JL. 1983. Synonymy of strains of "*Lactobacillus acidophilus*" group A2 (Johnson et al. 1980) with the type strain of *Lactobacillus crispatus* (Brygoo and Aladame 1953) Moore and Holdeman 1970. *International Journal of Systematic Bacteriology* **33**, 426-428.

Cheng X, Wang C, Qian G, Zhu B. 2003. CD80, but not CD86 were up-regulated on the spleen-derived dendritic cells from OVA-sensitized and challenged BALB/c mice. *Immunology Letters* **89**, 31-38.

DiMeo D, Tian J, Zhang J, Narushima S, Berg DJ. 2008. Increased interleukin-10 production and Th2 skewing in the absence of 5-lipoxygenase. *Immunology* **123**, 250-262.

Dreborg S. 2002. The implications of nomenclature. *Annals of Allergy, Asthma and Immunology* **89**, 83-85.

Fujiwara D, Inoue S, Wakabayashi H, Fujii T. 2004. The anti-allergic effects of lactic acid bacteria are strain dependent and mediated by effects on both Th1/Th2 cytokine expression and balance. *International Archives of Allergy and Immunology* **135**, 205-215.

Ghadimi D, Fölster-Holst R, de Vrese M, Winkler P, Heller KJ, Schrezenmeir J. 2008. Effects of probiotic bacteria and their genomic DNA on T_H1/T_H2-cytokine production by peripheral blood mononuclear cells (PBMCs) of healthy and allergic subjects. *Immunobiology* **213**, 677-692.

Gorbach SL. 2000. Probiotics and gastrointestinal health. *The American Journal of Gastroenterology* **95**, 2-4.

Hofer MF, Jirapongsananuruk O, Trumble AE, Leung DY. 1998. Upregulation of B7.2, but not B7.1, on B cells from patients with allergic asthma. *Journal of Allergy and Clinical Immunology* **101**, 96-102.

Hopkin JM. 2002. The rise of atopy and links to infection. *Allergy* **57**, 5-9.

Iliev ID, Tohno M, Kurosaki D, Shimosato T, He F, Hosoda M, Saito T, Kitazawa H. 2008. Immunostimulatory oligodeoxynucleotide containing TTTCGTTT motif from *Lactobacillus rhamnosus* GG DNA potentially suppresses OVA-specific IgE production in mice. *Scandinavian Journal of Immunology* **67**, 370-376.

Inoue Y, Iwabuchi N, Xiao JZ, Yaeshima T, Iwatsuki K. 2009. Suppressive effects of *Bifidobacterium breve* strain M-16V on T-helper type 2 immune responses in a murine model. *Biological and Pharmaceutical Bulletin* **32**, 760-763.

Kitazawa H, Tohno M, Shimosato T, Saito T. 2008. Development of molecular immunoassay system for probiotics via toll-like receptors based on food immunology. *Animal Science Journal* **79**, 11-21.

Klein G, Pack A, Bonaparte C, Reuter G. 1998. Taxonomy and physiology of probiotic lactic acid bacteria. *International Journal of Food Microbiology* **41**, 103-125.

Koizumi S, Wakita D, Sato T, Mitamura R, Izumo T, Shibata H, Kiso Y, Chamoto K, Togashi Y, Kitamura H, Nishimura T. 2008. Essential role of Toll-like receptors for dendritic cell and NK1.1[+] cell-dependent activation of type 1 immunity by *Lactobacillus pentosus* strain S-PT84. *Immunology Letters* **120**, 14-19.

Manetz TS, Meade BJ. 1999. Development of a flow cytometry assay for the identification and differentiation of chemicals with the potential to elicit irritation, IgE-mediated, or T cell-mediated hypersensitivity responses. *Toxicological Sciences* **48**, 206-217.

Masuda S, Yamaguchi H, Kurokawa T, Shirakami T, Tsuji RF, Nishimura I. 2008. Immunomodulatory effect of halophilic lactic acid bacterium *Tetragenococcus halophilus* Th221 from soy sauce moromi grown in high-salt medium. *International Journal of Food Microbiology* **121**, 245-252.

Pène J, Rousset F, Brière F, Chrétien I, Bonnefoy JY, Spits H, Yokota T, Arai N, Arai K, Banchereau J, De Vries EJ. 1988. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons γ and α and prostaglandin E2. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 6880-6884.

Pessi T, Sütas Y, Hurme M, Isolauri E. 2000. Interleukin-10 generation in atopic children following oral *Lactobacillus rhamnosus* GG. *Clinical and Experimental Allergy* **30**, 1804-1808.

Platts-Mills TA. 2001. The role of immunoglobulin E in allergy and asthma. *American Journal of Respiratory and Critical Care Medicine* **164,** 1-5.

Prescott SL, Macaubas C, Smallacombe T, Holt BJ, Sly PD, Holt PG. 1999.

Development of allergen-specific T-cell memory in atopic and normal children. *Lancet* **353,** 196-200.

Punnonen J, Aversa G, Cocks BG, de Vries JE. 1994. Role of interleukin-4 and interleukin-13 in synthesis of IgE and expression of CD23 by human B cells. *Allergy* **49**, 576-586

Roy D, Ward P, Vincent D, Mondou F. 2000. Molecular identification of potentially probiotic lactobacilli. *Current Microbiology* **40**, 40-46.

Saito H, Howie K, Wattie J, Denburg A, Ellis R, Inman MD, Denburg JA. 2001. Allergen-induced murine upper airway inflammation: local and systemic changes in murine experimental allergic rhinitis. *Immunology* **104**, 226-234.

Sanders ME, Klaenhammer TR. 2001. Invited review: the scientific basis of *Lactobacillus acidophilus* NCFM functionality as a probiotic. *Journal of Dairy Science* **84**, 319-331.

Sawada J, Morita H, Tanaka A, Salminen S, He F, Matsuda H. 2007. Ingestion of heat-treated *Lactobacillus rhamnosus* GG prevents development of atopic dermatitis in NC/Nga mice. *Clinical and Experimental Allergy* **37**, 296-303.

Segawa S, Nakakita Y, Takata Y, Wakita Y, Kaneko T, Kaneda H, Watari J, Yasui H. 2008. Effect of oral administration of heat-killed *Lactobacillus brevis* SBC8803 on total and ovalbumin-specific immunoglobulin E production through the improvement of Th1/Th2 balance. *International Journal of Food Microbiology* **121**, 1-10.

Shirakawa T, Enomoto T, Shimazu S, Hopkin JM. 1997. The inverse association between tuberculin responses and atopic disorder. *Science* **275**,

77-79.

Tobita K, Yanaka H, Otani H. 2009. Heat-treated *Lactobacillus crispatus* KT strains reduce allergic symptoms in mice. *Journal of Agricultural and Food Chemistry,* **57**, 5586-5590.

Wu K, Bi Y, Sun K, Wang C. 2007. IL-10-producing type 1 regulatory T cells and allergy. *Cellular and Molecular Immunology* **4**, 269-275.

FIGURE LEGENDS

Figure 1. Experimental schedules. After pre-breeding for a week, BALB/c mice were administered a control, KT-11-supplemented (5×10^7 CFU/MF 1 g) or GG-supplemented diet (5×10^7 CFU/MF 1 g) between 6 and 13 weeks of age. The mice were then intraperitoneally injected with 200 µL saline containing 20 µg OVA and 2 mg aluminum hydroxide gel at 6 and 8 weeks of age and intranasally challenged by instillation with 10 µL saline containing 100 µg OVA three times a week between 9 and 13 weeks of age. Sneezing events were counted for 10 min after 1 min of the intranasal instillation. Blood, spleen and Peyer's patch samples were collected at 13 weeks of age.

Figure 2. The number of sneezing events in mice. Sneezing activity was counted for 10 min after 1 min of the intranasal instillation with OVA. ●, control diet; ▲, KT-11-supplemented diet; ■, GG-supplemented diet. Data is presented as mean ± SD. *P < 0.05 (compared to control using the Dunnett's multiple comparison test).

Figure 3. Serum levels of OVA-specific antibodies. The mice administered the control, KT-11-supplemented and GG-supplemented diets are presented as the filled, open and cross-hatched bars, respectively. OVA-specific IgE (A) and IgG (B) levels were determined using ELISA. Data is presented as the mean ± SD. *P < 0.05 (compared to control using the Dunnett's multiple comparison test).

Figure 4. Th1/Th2 cell balance in the spleen. The mice administered the

control, KT-11-supplemented and GG-supplemented diets are presented as the filled, open and cross-hatched bars, respectively. The total number of spleen IFN-γ^+CD4$^+$ (A) and IL-4$^+$CD4$^+$ (B) cells was determined using Guava PCA. The Th1/Th2 cell balance is presented as the mean IFN-γ^+CD4$^+$ cell number against the mean IL-4$^+$CD4$^+$ cell number (C). Data is presented as the mean ± SD. *$P < 0.05$, ***$P < 0.001$ (compared to control using Dunnett's multiple comparison test).

Figure 5. The total number of immunocompetent cells in the spleen and Peyer's patches. The mice administered the control and KT-11-supplemented diets are presented in the filled and blank bars, respectively. (A) Spleen CD11b$^+$CD80$^+$ cells; (B) spleen FcεRIα^+CD117$^+$ cells; (C) spleen IL-10$^+$CD4$^+$ cells; (D) spleen IgE$^+$B220$^+$ cells; (E) Peyer's patch CD11b$^+$CD80$^+$ cells; (F) Peyer's patch FcεRIα^+CD117$^+$ cells; (G) Peyer's patch IgE$^+$B220$^+$ cells. Data is presented as the mean ± SD. *$P < 0.05$, ***$P < 0.001$ (compared to control using the Student's t-test).

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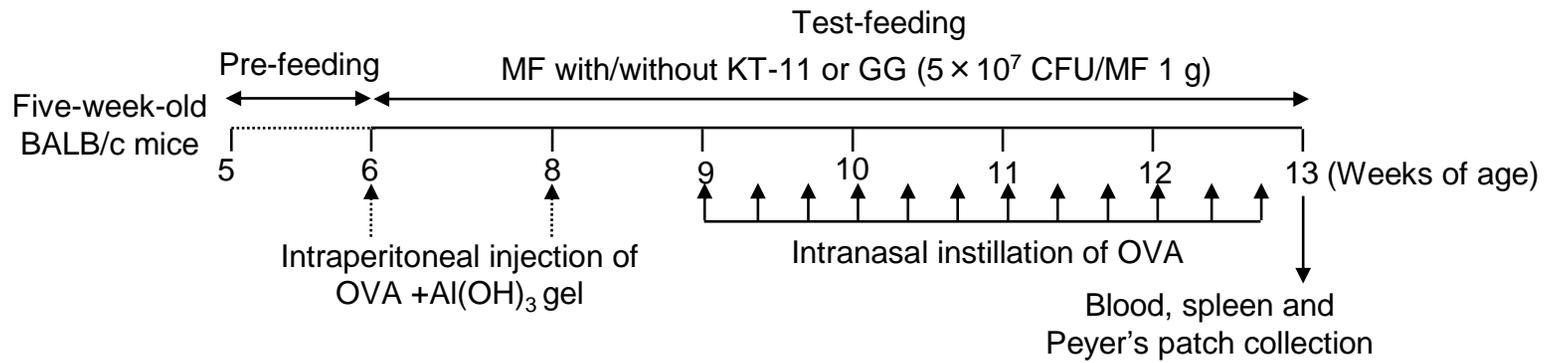
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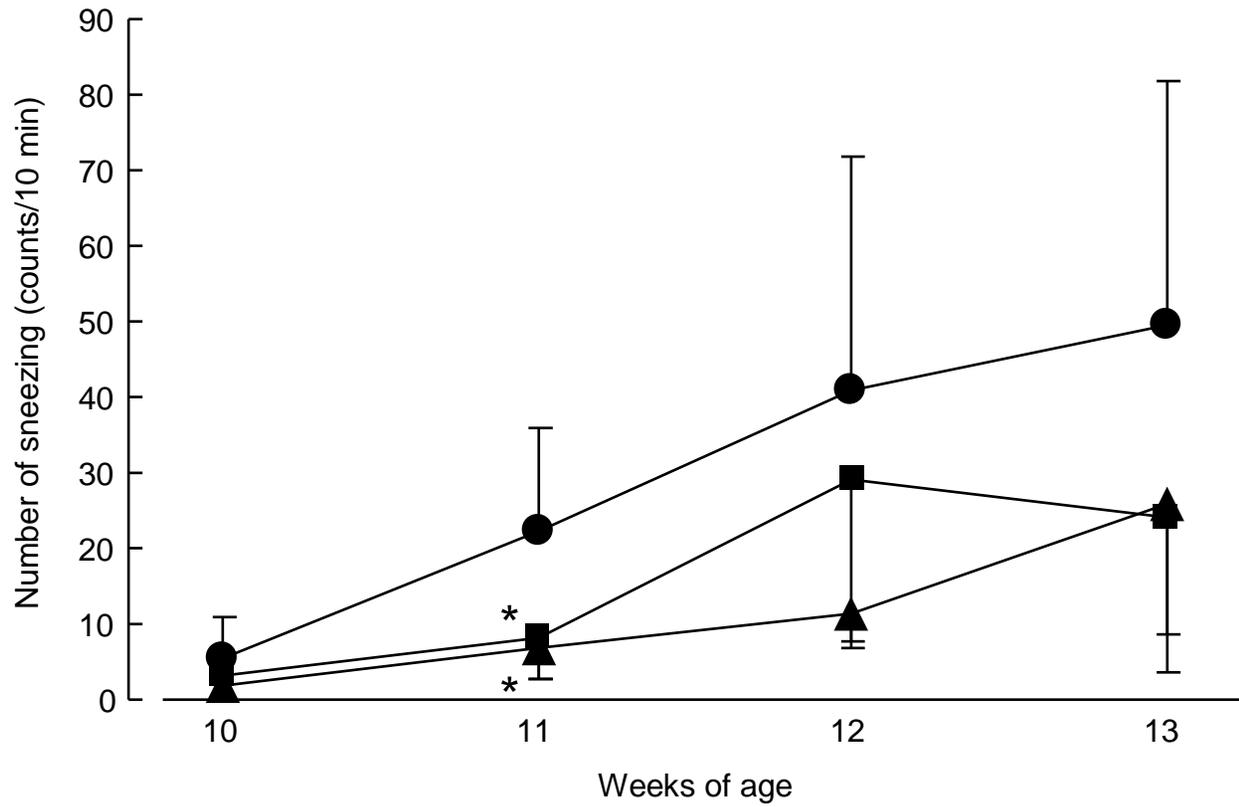
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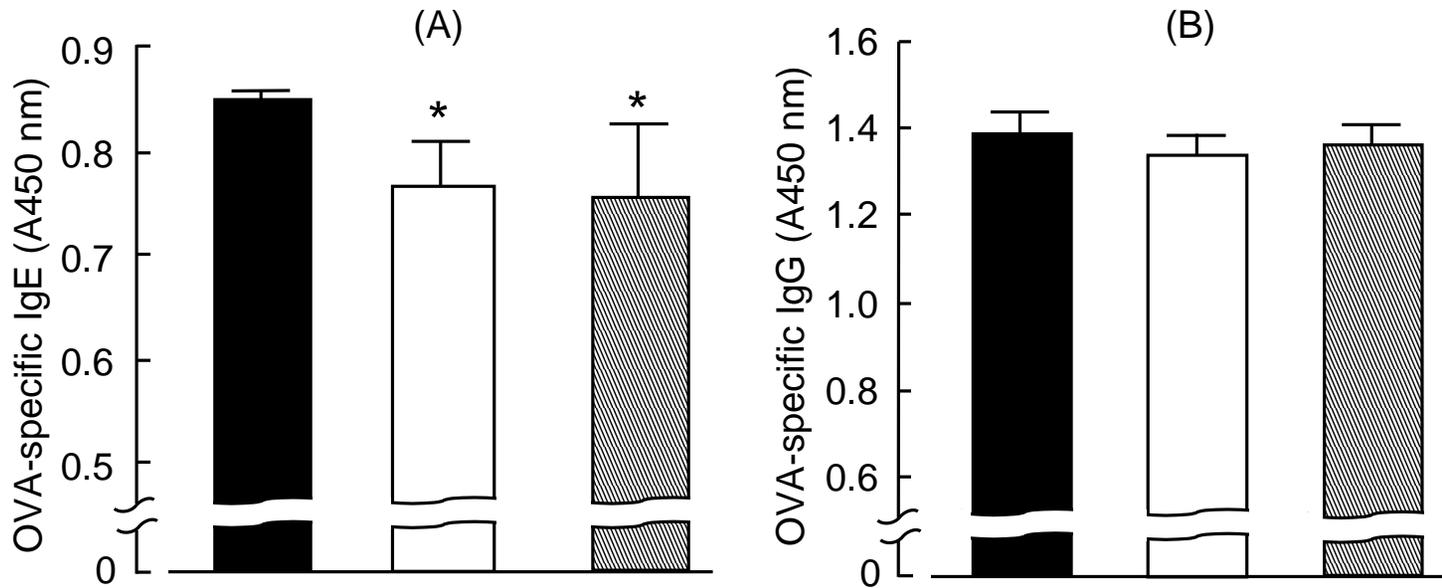
Figure 3. (Tobita *et al.*)

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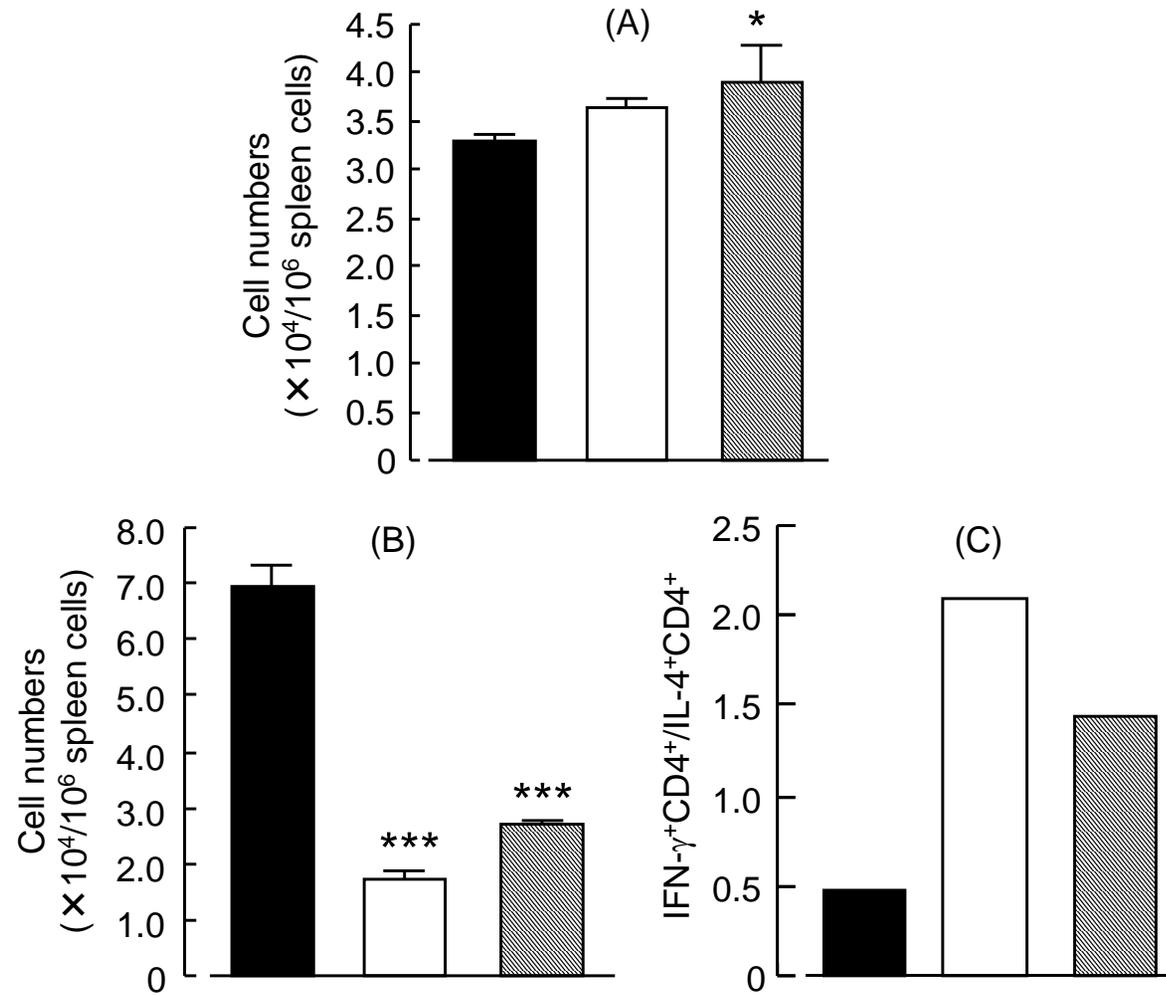
Figure 4. (Tobita *et al.*)

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Figure 5. (Tobita *et al.*)