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Oral administration of *Lactobacillus plantarum* lysates attenuates the development of atopic dermatitis lesions in mouse models

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Lactobacillus plantarum **is a well-documented probiotic that has been used in clinical trials for the regulation of the immune system and treatment of gastrointestinal diseases. In this study, we evaluated the effects of *L. plantarum* cell lysates on the immune regulation through the *in vitro* and *in vivo* studies. *L. plantarum* lysates were prepared by sonication method, and we observed that the repetition of disruption step increased indicator components within the bacterial lysates. Indicator components might affect TNF-α production. *L. plantarum* lysates did not induce TNF-α production, while LPS-induced TNF-α production was dramatically inhibited in a sonication-dependent manner in THP-1 cells. Oral administration of *L. plantarum* lysates effectively attenuated the horny layer formation and decreased epidermal thickening in NC/Nga mice skin. The damage to barrier function after the 8 weeks oral administration was reduced by *L. plantarum* lysates as compared to that in the atopic dermatitis (AD) mice. Further study revealed that *L. plantarum* lysates polarized Th1 response via induction of IL-12 and IFN-γ production and inhibition of IL-4 and IgE production in NC/Nga mice. Together, our results suggest that *L. plantarum* lysates are remarkable material for host homeostasis and it could be used for the treatment of inflammatory diseases.**

Keywords: *Lactobacillus plantarum*, bacterial lysates, atopic dermatitis, cytokine, IgE, immune regulation

Introduction

Atopic dermatitis (AD) are chronic and relapsing inflammatory skin diseases caused by a skin barrier disorder. AD is increasing in prevalence and is frequently found in infants and children (Cooper, 1994). Although the pathophysiology of AD is not fully known, it involves a complicated interaction of environmental and genetic factors that induce abnormalities in the structure and function of the epidermal barrier and immune system (Sajić *et al.*, 2012). The involvement of mast cells and CD4+T cells in the skin lesions affect AD pathogenesis (Van Bever, 1992). The elevated serum immunoglobulin (Ig) E levels in patients with AD also suggest that T helper (Th) 2 cytokines may participate in disease pathogenesis (Chan, 2008). Interleukin (IL)-4 switches B cell signals to synthesis IgE, and IgE-mediated mast cell activation leads to the release of various kinds of chemical mediators, which results in infiltration of inflammatory cells into the skin lesion. On the other hand, IL-12 and Interferon (IFN)-γ repress B cell-mediated IgE synthesis (Snapper and Paul, 1987). These day, it is well established that moisturizers play an important role in preventing skin inflammation in AD, including reducing the amount of topical corticosteroid use (Saijic *et al.*, 2012; Varothai *et al.*, 2013). The presence of probiotic bacteria in the intestinal microbiota is also known to correlate with protection against AD. Probiotics, such as lactobacilli, are very promising evidence to recommend the addition of probiotics to foods for the prevention and treatment of allergic diseases (Ozdemir, 2010).

Probiotics are known for their health-promoting effects such as the non-specific enhancement of the immune system, protection against intestinal infection, reduction of serum cholesterol level, and antioxidant properties (Meydani and Ha, 2000). Several strains of probiotics also have effects on the production of cytokines, such as IL-12, IL-10, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, IL-8, and RANTES, and on cell proliferation in human intestinal epithelial cells (Wallace *et al.*, 2011). Among probiotics, *L. plantarum* is a well-documented probiotic that has been used in clinical trials for the regulation of the immune system and treatment of gastrointestinal diseases with other probiotics (Georgieva *et al.*, 2008). *L. plantarum* reduces serum cholesterol, LDL-cholesterol, and triglyceride levels (Xie *et al.*, 2011), and attenuates inflammatory bowel disease (IBD) (Schultz *et al.*, 2002). Studies using probiotics have been focused on the role of whole bacteria cells and bacteria cell wall components such as peptidoglycan (PGN) and lipoteichoic acid (LTA). These days, however, researchers are trying to apply the bacterial lysates to disease treatments. For example, bacterial lysates seem not to act through direct modification of colonization patterns in contrast to the application of viable bacteria. Clinical studies, as well as animal experiments, have indicated that bacterial lysates increases secretory IgA levels, which is the most important immunomodulating activity of bacterial lysates (Pfefferle *et al.*, 2013). Orally ad-

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ministered OM-85 BV, the best clinically evaluated bacterial lysates, is able to stimulate a selective TH1-driven response came from studies in neonate rats indicating that bacterial lysates can induce maturation of dendritic cells and skew the perinatal TH2 milieu toward TH1 maturation (Bowman and Holt, 2001). In another study, topical treatment with non-pathogenic bacterium *Vitreoscilla filiformis* lysate (Vf) reduced AD-like inflammation in NC/Nga mice. Vf also induced IL-10-producing DCs, which was dependent on Toll-like receptor 2 (TLR 2) activation. Vf-induced IL-10+ DCs primed naive CD4+ T helper cells to become regulatory IFN-γ (low) IL-10 (high) Tr1 (type 1 regulatory T) cells, indicating that innate sensing of bacterial lysates induces tolerogenic DCs and regulatory Tr1 cells suppressing T effector cells and cutaneous inflammation (Volz *et al*., 2014).

Here, we introduced an immune regulatory effect of *L. plantarum* lysates prepared by mechanical lysis. Mechanical lysis is thought to provide more immunogenic lysate components because no denaturation of proteins occurs during mechanical disruption of the cells. We evaluated inhibitory effect of TNF-α in THP-1 cells using *L. plantarum* lysates, and we also assessed the attenuation of AD with 2,4-Dinitrochlorobenzene (DNCB)-treated NC/Nga mice.

Materials and Methods

Preparation of *L. plantarum* cell lysates

L. plantarum K8 (KCTC 10887BP) was cultured in 10 L MRS broth (HiMedia Laboratories, India) at 37°C for 18 h, and then cells were harvested by centrifugation at 8,000 rpm for 10 min. After a wash in phosphate-buffered saline (PBS) three times, 50 g cells (wet weight) were disrupted by microfluidizer (10,000–13,000 psi, 3–10°C). To obtain indicator components from disrupted cells, additional disruption steps were performed (up to 9 times). Bacterial lysates were freeze-dried and packed under clean room conditions.

Mice

NC/Nga (6 weeks old) mice were purchased from Central Lab, Animal Inc. (Korea). They were kept in individual cages at 24±2°C and 50±10% moisture condition, and fed nutritionally balanced rodent food (Central Lab. Animal Inc.) and sterilized water. The mice were cared for and used in accordance with the guidelines of the animal ethics committee of Gyeonggi Bio Center. To develop a mouse model of atopic dermatitis (AD) using skin sensitization, each mouse was exposed onto the shaved back skin with 200 μl immune-disturbing material containing acetone: olive oil (3:1) and 2.5% 2, 4-dinitrochloro benzene (DNCB; Sigma). After 3 days exposure, mice were treated with 150 μl 1.0% DNCB at 3-day intervals. Mice were allocated to three random groups (n=6/group): normal control group, AD group, and AD-treated group. The treated group received orally administered *L. plantarum* lysates (250 mg/kg/day) once a day.

Material administration to mice

Mice were fed freeze-dried *L. plantarum* lysates (250/kg/day), which were dissolved in 200 μl of sterilized water for two weeks before the experiments were performed. Mice were administered with casein dissolved in 5:5 phosphate-buffered saline (PBS) and an alum solution (Sigma), 4, 7, 14, and 21 days after the sera were collected to detect and measure levels of immunoglobulin. Mice were sacrificed to obtain their peritoneal macrophages after 2 weeks of treatment.

Histopathological examination

The dorsal skins of the experimental mice were removed on the final day of the schedule and fixed in 10% neutral-buffered formalin and embedded in paraffin. Serial paraffin sections (5 μm) were stained with haematoxylin and eosin (H&E), and the skin layer was examined by NIS-Elements (Nikon).

Assessment of epidermal permeability barrier function

To determine whether epidermal permeability barrier function was altered in AD mice, we measured transepidermal water loss (TEWL) using a Vapometer® (Delfin Technologies Ltd.). To examine the thickness of mouse skin, the back skin of AD mice were examined with D-squame® Black Tape. The image of the collected dead skin samples was magnified 70 times using Charm View (Moritex) and analyzed with Image-Pro® Plus software (Media Cybernetics Inc.).

Quantitation of cytokine expression level

TNF-α, IL-12, IFN-γ, and IL-4 were measured using ELISA kits (R&D Systems) and immunoglobulins were detected using ELISA kits (BD). To measure casein-specific IgE, we used the method detailed by Sato *et al.* (2004). Standard serum for comparison was obtained from mice immunized three times with 20-μg casein and the alum solution. Standard serum was considered to contain 100% of each immunoglobulin.

Measurement of spontaneous scratching behavior in NC/Nga mice

Spontaneous scratching behavior by skin-lesioned NC/Nga mice was measured for 24 h (from 3:00 pm to 3:00 pm the next day) as described previously (Elliott *et al.*, 2000). For measurements, using a magnet (1 mm diameter; 3 mm length) was implanted subcutaneously into both hind paws of each ether anesthetized 2 h before the measurement of scratching. The mouse was placed in an observation chamber surrounded by a round coil. The electric current induced in the coil by the movement of magnets attached to the hind paws was amplified and recorded. The number of scratching bouts was automatically measured using a MicroAct (Neuroscience).

Detection of indicator components by LC-MS/MS

After disruption of bacteria, indicator components were detected by LC-MS/MS (Agilent Technologies 6410 Triple Quad). Freeze-dried *L. plantarum* lysates were dissolved in distilled water, and subjected to reverse-phase C18 column (2.1 mm × 150 mm). Solvents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) were added to the top of the column. The flow rate was 0.2 ml/min and gra-

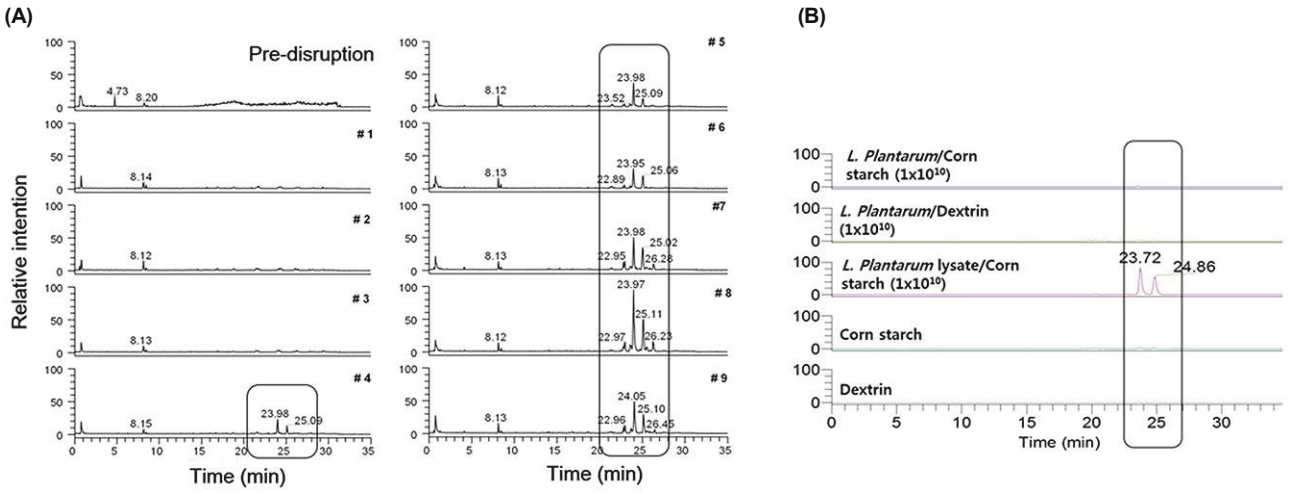

Fig. 1. Repeated sonication increased indicator components in *L. plantarum* lysates. (A) *L. plantarum* was disrupted with microfluidizer. Disruption step was repeated by 9 times and indicator components were detected by LC-MS/MS. (B) indicator components was detected by LC-MS/MS with intact whole *L. plantarum*, *L. plantarum* lysates, corn starch, and dextrin.

dient using solvent B was applied every 5 min (5, 15, 30, 60, 100, 100, 5, and 5% of solvent B). MS detection was performed by general condition (+ESI, sim mode; 350°C gas temperature; 4,000 capillary voltage, 40 psig, 190 fragmentor voltage).

Statistical analysis

All experiments were performed at least three times. The data shown are representative results of the means ± SD of triplicate experiments. Differences were considered statistically significant when the P value was <0.05.

Results

Indicator components were increased by repeated disruption in *L. plantarum* cell lysates

To prepare the *L. plantarum* lysates, a general disruption method was applied. About 50 g *L. plantarum* (wet weight) was re-suspended in distilled water, and then subjected to microfluidizer. The disruption using microfluidizer was repeated 9 times. Interestingly, the repetition of the disruption step increased the indicator components within the disrupted *L. plantarum* (Fig. 1). The amount of indicator components was peaked after the 8th disruption and slightly decreased with the 9th disruption step. The peaks were only detected from disrupted *L. plantarum*, but not from intact *L. plantarum*, corn starch, or dextrin (Fig. 1B). Unfortunately, these indicator components were not identified in this study. The size of *L. plantarum* lysates was reduced by repeated disruption step (Fig. 2A). To examine the activity of indicator components from *L. plantarum*, THP-1 cells were stimulated with *L. plantarum* lysates, and then TNF-α level was examined by ELISA. The inhibitory effect of *L. plantarum* lysates against LPS was increased by repeated disruption (7 to 9 times) as compared to fewer disruption steps (fewer than 6 times), indicating that more indicator components may be extracted from the cells by increasing the disruption number (Fig. 2B). On the other hand, there was no significant induction of TNF-α was shown by *L. plantarum* lysates (Fig. 2C). These data suggest that *L. plantarum*

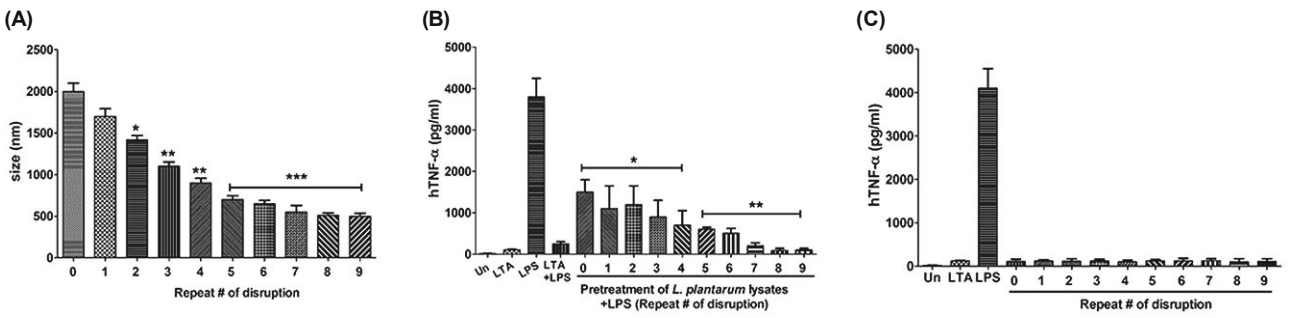

Fig. 2. TNF-α production by *L. plantarum* lysates in THP-1 cells. (A) Size of *L. plantarum* lysates was examined by electron microscope. (B) THP-1 cells were pre-treated with *L. plantarum* lysates (#1–9) for 24 h, and then LPS was retreated for 6 h. (C) THP-1 cells were treated with *L. plantarum* lysates (#1–9) for 6 h. TNF-α production was estimated by ELISA with culture supernatants. LPS and were used as a control. LTA+LPS indicates that pretreatment of LTA for 24 h and retreatment of LPS for 6 h. Un indicates untreated control. * P<0.05 compared to LPS only.

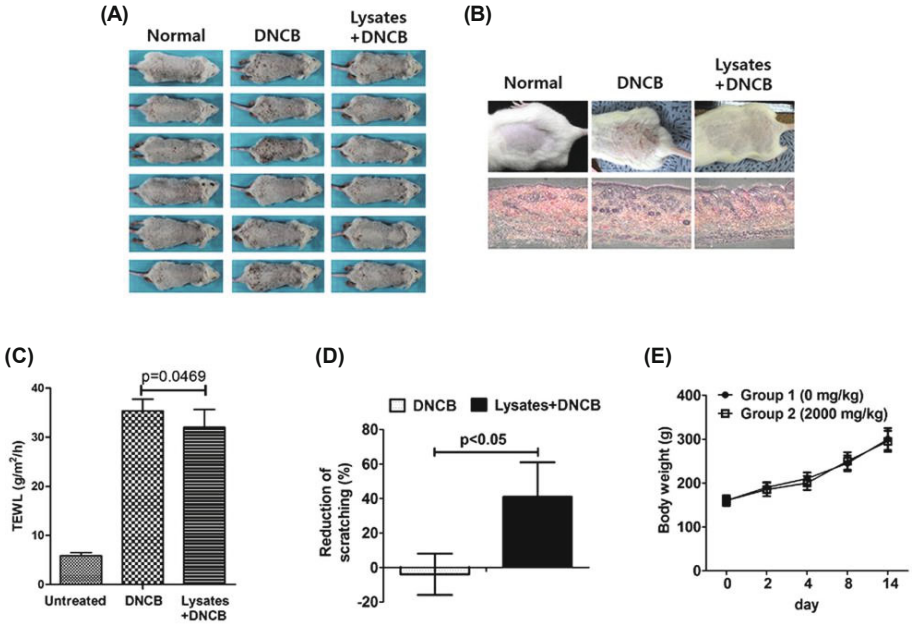

Fig. 3. Attenuation of AD lesions in NC/Nga mice. AD in NC/Nga mice (n=6) was induced by DNCB sensitization, and *L. plantarum* lysates were orally administered. AD lesions are visualized. (B) Epidermal layer of NC/Nga mice (n=6) skin was stained, and a representative figure is shown. (C) The differences in TEWL value between DNCB only and lysates-treated groups are shown. (D) The reduction of scratching movements by NC/Nga mice was measured for up to 24 h after DNCB treatment. (E) Mouse body weight was examined after administration of 0 mg/kg or 2,000 mg/kg *L. plantarum* lysates for indicated days.

lysates have an anti-inflammatory effect against pathogenic infection.

L. plantarum lysates attenuated AD lesions in mouse models

Clinical assessment of AD was performed by measuring the epidermal thickness of mice skin. The horny layer increased significantly in the DNCB-treated NC/Nga mice group compared with the negative control, while oral administration of *L. plantarum* lysates effectively attenuated the horny layer formation (Fig. 3A). Epidermal thickening in DNCB-treated NC/Nga mice was decreased by *L. plantarum* lysates (Fig. 3B). The thickness of mouse skin was increased in DNCB-treated group by 280% after oral administration for 8 weeks as compared to control. On the other hand, oral administration of *L. plantarum* lysates decreased skin thickness by 30% (P<0.05) in comparison to skin thickness in the DNCB-treated group. Next, epidermal permeability barrier function was assessed by measuring TEWL. TEWL is the rate at which water vapour is lost from the body across the skin. Exposure of the skin to chemicals and physical conditions generally results in an increase of TEWL. Thus, the measurement of TEWL by evaporimeter is a powerful non-invasive method for determining the efficiency of the barrier function of skin (Barel and Clarys, 1995). TEWL increased significantly in the DNCB-treated group by 420% after oral administration for 8 weeks compared with that in the negative controls. The damage to barrier function after the 8 weeks oral administ-

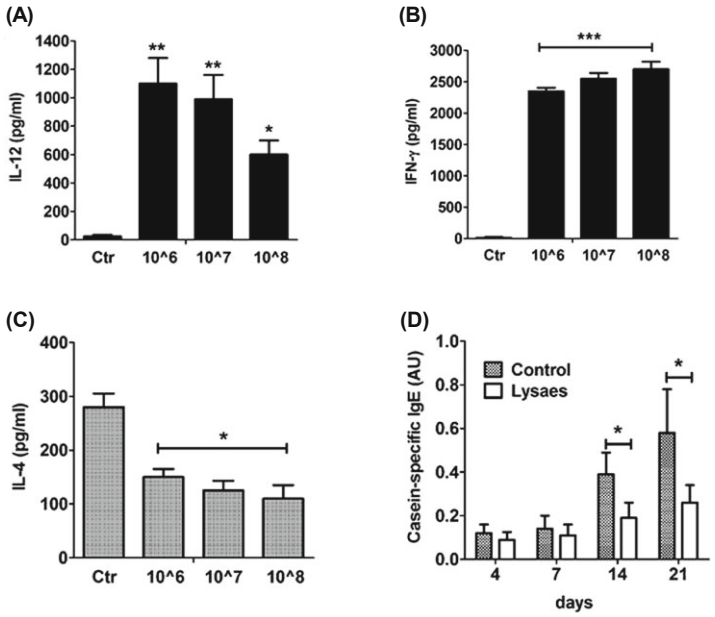

Fig. 4. Th1 polarization by *L. plantarum* lysates. Mouse macrophages were isolated from peritoneal cavity, and treated with *L. plantarum* lysates for 24 h. IL-12 (A), IFN-γ (B), and IL-4 (C) level was examined by ELISA from culture supernatants. (D) Mice were orally administered with *L. plantarum* lysates for 2 weeks before casein immunization. Blood was collected 4, 7, 14, and 21 days after casein immunization and casein-specific IgE levels were determined by ELISA. The data are represented as the mean ± SD from five mice per group. Statistical P values were determined using a two-tailed t-test: *P<0.05; **P<0.01; ***P<0.001 compared to the control.

ration was reduced by *L. plantarum* lysates (~14%, P=0.0469) compared with that in the AD mice that received DNCB treatment only (Fig. 3C). Topical application of DNCB increased scratching behavior by NC/Nga mice for up to 12 h after treatment compared with un-treatment. Compare to DNCB-treated only group, *L. plantarum* lysates-administered group reduced spontaneous scratching behavior by about 43% (Fig. 3D). The body weight of NC/Nga mice which administered *L. plantarum* lysates was not significantly altered as compared to vehicle (sterilized water) administration (Fig. 3E) and we did not observe abnormal behavior in *L. plantarum* lysates-administered mice, indicating that *L. plantarum* lysates are harmless to mice. Taken together, these results suggest that oral administration of *L. plantarum* lysates improves the clinical signs of AD and reduces damage to epidermal barrier function in mice.

L. plantarum lysates induced Th1 polarization

To analyze the role of *L. plantarum* lysates on cytokine production, mouse macrophages were isolated from peritoneal cavity, and cells were treated with *L. plantarum* lysates for 24 h. IL-12 production was significantly increased by 10^6 CFU/ml *L. plantarum* lysates and it was decreased by 50% in 10^8 CFU/ml *L. plantarum* lysates-treated cells (Fig. 4A). IFN-γ production was also significantly increased by *L. plantarum* lysates (Fig. 4B). IL-4 production from peritoneal macrophages, however, was decreased (Fig. 4C). These data suggest that *L. plantarum* lysates drive T helper (Th) 1 polarization via induction of inflammatory cytokines, such as IL-12 and IFN-γ, and reduction of anti-inflammatory cytokine, IL-4. To detect antigen specific IgE, we fed NC/Nga mice with *L. plantarum* lysates for 14 days before casein immunization. Seven days after immunization, blood was collected and the levels of anti-casein specific IgE were measured. Blood from the *L. plantarum* lysates-fed group showed significantly decreased anti-casein IgE levels as compared to the control group (Fig. 4D), indicating that AD lesions were attenuated by *L. plantarum* lysates in NC/Nga mice.

Discussion

In this study, we evaluated the effects of oral administration of *L. plantarum* cell lysates on the TNF-α inhibition in human THP-1 cells and attenuation of AD lesions in mouse model. AD is a common chronic inflammatory skin disease that is increasing in prevalence, and is frequently found in infants and children (Cooper, 1994). The pathogenesis of AD is not completely understood. Pathogenesis of AD involves a complicated interaction of environmental and genetic factors that induce the abnormalities in the structure and function of the epidermal barrier and immune system (Saijic *et al.*, 2012). The elevated serum IgE levels in patients with AD also suggest that Th2 cytokines may participate in disease pathogenesis (Chan, 2008). Our previous study to detect blood Ig levels in the OVA-challenged mice revealed that *L. plantarum* represses the Th1 immune response. The levels of total OVA-specific IgG and total IgE were not changed significantly in *L. plantarum*-fed mice but the OVA specific IgE level was slightly increased. This result may represent the increase in the Th2 immune response and repression of Th1 polarization. Unlike previous study, in the current study using *L. plantarum* lysates, we observed decreased IL-4 in peritoneal macrophages and casein-specific IgE level from blood, indicating that *L. plantarum* lysates repress Th2 response. Th1-response cytokines, such as IL-12 and IFN-γ, were increased by *L. plantarum* lysates. Unfortunately, in this study, we did not compared the biological effects between whole cell bacteria and disrupted bacteria. Thus, more investigation is needed to clarify the differences between whole cell *L. plantarum* and *L. plantarum* lysates.

Researches using probiotics have been focused on their physiological effects in host immune regulation and undisrupted bacterial whole cells are used in the studies. Recently, cell wall components such as peptidoglycan and lipoteichoic acid are emerged to identify fine mechanism. For examples, it is known that Pneumococcal LTA has a completely different chemical structure compared with that of *Staphylococcus aureus* (Morath *et al.*, 2001; Seo *et al.*, 2008). Draing *et al.* (2006) have shown that LTA of two different strains of *Streptococcus pneumoniae*: R6 (serotype 2) and Fp23 (serotype 4) resulted in different immunostimulatory potencies due to their different LTA structure. Peptidoglycan also can be purely isolated from bacteria cell wall (Rosenthal and Dziarski, 1994). Peptidoglycan is recognized by the nucleotide-binding oligomerization domain (NOD) proteins NOD1 and NOD2, which enable detection of intracellular bacteria and promote their clearance (Philpott *et al.*, 2014). Individual component of bacteria cell wall has some benefits by distributing accurate and effective process to identify fine mechanism and to manufacture immune stimulating reagents including vaccine adjuvant. In certain point, however, isolation of single component from bacteria is annoying since it is cost and time consuming works. In this study, we introduced a simply way to avoid this problem by providing bacterial lysates. There are some benefits for *L. plantarum* cell lysates: i) easy preparation ii) potential combination effects iii) long term storage. On the other hand, bacterial lysates have some difficulties in the identification of fine mechanism and there will be a dose limit. Thus, immune stimulating effect of bacterial lysates may be less than that of single component.

L. plantarum lysates, which disrupted by repeated microfluidizer process decreased LPS-induced TNF-α production in THP-1 cells. Oral administration of *L. plantarum* lysates effectively attenuated the AD lesions in NC/Nga mice skin. *L. plantarum* lysates also polarized Th1 response via induction of IL-12 and IFN-γ production and inhibition of IL-4 and casein-specific IgE production in NC/Nga mice. Taken together, our results suggest that *L. plantarum* lysates are remarkable material for host homeostasis and it could be used for the treatment of inflammatory diseases.

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