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# Inhibitory Effect of *Lactobacillus plantarum* K-1 on Passive Cutaneous Anaphylaxis Reaction and Scratching Behavior in Mice

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*Lactobacillus plantarum* K-1 (LP) inhibiting AP-1 (c-Jun) and NF-κB activations was isolated from kimchi, and its inhibitory activity against scratching behavior and passive cutaneous anaphylaxis reaction in mice was investigated. Heat-inactivated LP (heated at 60°C for 30 min) potently inhibited the expression of TNF-α and IL-4 as well as the activation of their transcription factors, NF-κB and c-jun, in phorbol 12'-myristate 13'-acetate-stimulated RBL-2H3 cells. LP ($1 \times 10^{10}$ CFU per mouse) showed a potent inhibition against passive cutaneous anaphylaxis reaction induced by the IgE-antigen complex in mice, inhibiting it by 87.5%. LP ($1 \times 10^{10}$ CFU/mouse) inhibited histamine-induced scratching behavior by 58.9% compared to the control group. LP significantly inhibited vascular permeability induced by histamine. The inhibitory activity of LP against vascular permeability was in proportion to its inhibition against scratching behavior. LP potently inhibited histamine-induced cytokine production: it ($1 \times 10^{10}$ CFU per mouse) inhibited IL-4, IL-1β, and TNF-α expression by 88.9%, 88.6%, and 98.9%, respectively. LP also inhibited IgE level increased by histamine by 85.3%. It inhibited histamine-induced the activations of their transcription factors, NF-κB and c-Jun. Based on these findings, LP may improve allergic diseases, such as anaphylaxis, atopic dermatitis, rhinitis, and pruritus by inhibiting the expression of IgE-switching cytokine IL-4 and proinflammatory cytokines IL-1β and TNF-α via NF-κB and AP-1 signaling pathways.

**Key words** *Lactobacillus plantarum* K-1, Allergy, Scratching behavior, Passive cutaneous anaphylaxis

**Selected by Editors**

## INTRODUCTION

Allergic diseases were first proposed to be less prevalent in children who were exposed frequently to infectious agents in 1989 (Strachan, 1989). This proposal may be strongly related to the increasing prevalence of immune disorder in industrialized countries, which has been attributed to reduced exposure to microbial stimuli (Guarner et al., 2006). It was supported by the finding that non-allergic children exhibit intensive colonization of aerobic bacteria or *Bacteroides* in their indigenous intestinal flora (Kalliomäki et al., 2001). Normal intestinal microflora consist of > 500 bacterial species and reach their highest concentrations in the terminal ileum, cecum and colon. Intestinal microflora produce toxic sources, such as gram-negative bacterial endotoxins, and harmful enzymes, such as β-glucuronidase and tryptophanase, which produce cytotoxic or carcinogenic agents. Cytotoxins and endotoxins are potent stimuli of innate immune responses, induce pro-inflammatory and IgE-inducing cytokines in colonic epithelial cells, and cause various diseases, such as colitis, carcinoma, anaphylaxis, and hypersensitivity. Therefore, researchers attempted to utilize symbiotic microorganisms in the treatment of immune disorders,

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such as anaphylaxis, pruritus, rhinitis, and atopic dermatis, and several *in vitro*, *in vivo* and clinical experiments have demonstrated the role of microorganisms as immune regulators (Majamaa and Isolauri, 1997; Isolauri, 2001; Ogden and Bielory, 2005).

Lactic acid bacteria (LAB) are gram-positive, nonspore forming, non-respiring cocci or rods that ferment carbohydrates and produce lactic acid as the main product. (Simon and Gorbach, 1984; Collins and Gibson, 1999; Cho et al, 2006). The common LAB genera in fermented foods, such as cheese, yogurt, and kimchi, and intestinal microflora, are *Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp., *Pediococcus* sp., *Enterococcus* sp., *Streptococcus* sp., and *Bifidobacterium* sp. LAB is safe microorganisms that improve any disturbances of indigenous microflora (Perdigon et al., 1991; Campieri and Gionchetti, 1999), ameliorate the development of beneficial microflora (Collins and Gibson, 1999), have anticolitic effect (Campieri and Gionchetti, 1999; Lee et al., 2010), and induce non-specific activation of the host immune system (Perdigon et al., 1991). Recently, the enhanced presence of LAB in the intestinal microbiota has been correlated with atopy prevalence (Majamaa and Isolauri, 1997; Kalliomäki et al., 2003). There is insufficient but very promising evidence supporting the addition of LAB to foods for the prevention and treatment of allergic diseases, especially atopic dermatitis. LAB also potentiates tolerance induction via the sublingual route as adjuvants for sublingual allergy vaccines by increasing IL-12 and IL-10 expression in mice (van Overtvelt et al., 2010). Of LAB, *Lactobacillus plantarum* isolated from kimchi, a traditional Korean food, reduced allergen-induced, airway hyper-responsiveness and enhanced the expression of regulatory factors, such as Foxp3 and IL-10, in intestinal laminar propria cells (Hong et al., 2010). *L. plantarum* isolated from kimchi was also found to inhibit the anti-scratching behavioral effect induced by histamine by inhibiting IL-4 expression (Jang et al., 2011). However, its anti-allergic effect remains to be thoroughly studied.

Therefore, we isolated *L. plantarum* K-1 (LP), which most potently inhibited AP-1 and NF-κB activation in phorbol 12'-myristate 13'-acetate (PMA)-stimulated RBL-2H3 cells, from kimchi, and investigated its inhibitory activity against scratching behavior and passive cutaneous anaphylaxis (PCA) reaction in mice.

## MATERIALS AND METHODS

### Materials

Dulbecco's modified Eagles medium (DMEM), fetal bovine serum, dinitrophenol-human serum albumin (DNP-HSA), histamine, PMA, and Evans blue dye were purchased from Sigma Co. Azelastine was donated by Dr. Nam-Jae Kim, an adjunct professor at Kyung Hee University. Enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D systems. MRS was purchased from BD Co. Fifty LABs were isolated from kimchi according to the previous method of Lim and Im (2009).

### Animals

Male ICR and BALB/*c* mice (20 - 22 g, 5 weeks old) were obtained from the Charles River Orient Experimental Animal Breeding Center. All animals were housed in wire cages at 20-22°C, relative humidity of 50 ± 10%, air ventilation frequency of 15-20 times/h and 12-h illumination (07:00-19:00; intensity, 150-300 Lux), fed standard laboratory chow (Charles River Orient Experimental Animal Breeding Center), and allowed water *ad libitum*. All experiments were performed in accordance with the NIH and Kyung Hee University guides for Laboratory Animals Care and Use and approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University.

### Measurement of CA reaction

An IgE-dependent cutaneous reaction was measured according to the previous method of Choo et al. (2003). The male ICR mice were intradermally injected, with 10 μg of anti-DNP IgE, into each of two dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. Forty-eight hour later each mouse received an injection of 200 μL of 3% Evans blue in PBS, containing 200 μg of DNP-HAS, via the tail vein. LP ($1 \times 10^9$ CFU and $1 \times 10^{10}$ CFU/mouse) and azelastine (10 mg/kg) was administered once a day for 3 days. DNP-HSA injection was injected 1 h after the final administration of LP and azelastine. Thirty min after the DNP-HSA injection, the mice were sacrificed, their dorsal skins removed, and the pigmented area measured. After extraction with 1 mL of 1.0 M KOH and 4 mL of a mixture of acetone and 0.2 M phosphoric acid (13:5), the amount of dye was determined colorimetrically at 620 nm.

### Assay of scratching behavioral frequency in mice

Male BALB/*c* mice were placed in acrylic cages (22 × 22 × 24 cm) for about 10 min to become acclimatized. The behavioral experiments were performed according to the method of Shin et al. (2007). The rostral part of the skin on the back of the mice was clipped, and 300

μg/50 μL of histamine (dissolved in saline) was intradermally injected into each mouse. Control mice received a saline injection in the place of the histamine. Immediately after the intradermal injection, the mice (one animal/cage) were put back into the same cage and the scratching behaviors recorded using an 8-mm video camera (SV-K80, Samsung) under unmanned conditions. Scratching of the injected site with the hind paws was counted and compared with that of other sites, such as the ears. Each mouse was used for only one experiment. The mice generally showed several scratches per second, and a series of these behaviors was counted as one incident of scratching over a 60 min period. LP ($1 \times 10^9$ CFU and $1 \times 10^{10}$ CFU/mouse) and azelastine (10 mg/kg) were administered once a day for 3 days. The final administration of the test agents were performed 1 h before the scratching agent.

### Measurement of vascular permeability

The increase in vascular permeability caused by scratching agents was assessed as reported by Choo et al. (2003). After the intradermal injection of 300 μg/50 μL of histamine into the rostral part of the back of each mouse, 0.2 mL of 1% saline solution of Evans blue dye was injected intravenously. LP ($1 \times 10^9$ CFU/mouse or $1 \times 10^{10}$ CFU/mouse) or azelastine (10 mg/kg) was administered once a day for 3 days. Mice were sacrificed by cervical dislocation 60 min after the final administration of test agents and the scratching agent-injection site was excised. The skin specimen was dissolved in 1 mL of 1 M KOH solution by overnight incubation, and 4 mL of 0.2 M phosphoric acid solution-acetone (5:13) mixture was added. After shaking, the precipitates were filtered off and the amount of dye was measured colorimetrically at 620 nm.

### ELISA and immunoblot

For the assay of cytokines in skin tissues by ELISA, histamine-induced skin tissue specimens were homogenized in ice-cold lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.05% Nonidet P-40, 1 mM EGTA, 1:100 protease inhibitor cocktail, and 1:100 phosphatase inhibitor cocktail). Lysed specimens were centrifuged at 2,700 × g for 10 min at 4°C. The supernatant containing the cytosol was further centrifuged at 20,800 × g for 15 min at 4°C to obtain the cytosolic fraction. The nuclei in the pellet were washed 3 times by gentle resuspension in wash buffer (10 mM PIPES, pH 6.8, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 25 mM NaCl, 1:100 protease inhibitor cocktail and 1:100 phosphatase inhibitor cocktail) and centrifuged at 2,700 × g for 5 min at 4°C (Jang et al., 2011). The supernatants (50 μL) were transferred to 96-well ELISA plates, and the concentrations of IL-4 and TNF-α were then determined using commercial ELISA kits (Pierce Biotechnology, Inc.).

For the assay of cytokines in RBL-2H3 cells by ELISA, the cells ($2 \times 10^5$ cells) previously cultured in DMEM were treated with 20 nM PMA. The cells (1.8 mL) were exposed to 0.2 mL of the test agents ($0.2 \times 10^9$ or $1 \times 10^9$ CFU of heated LP dissolved in 0.5% dimethyl sulfoxide) for 4 h, followed by treatment with 0.2 mL DNP-HSA (1 μg/mL) for 40 min at 37°C. The supernatant (50 μL) was transferred to 96-well ELISA plates, and the IL-4 and TNF-α concentrations were then determined using commercial ELISA kits.

For the immunoblot analysis of transcription factors, phospho-p65, phospho-c-Jun (p-AP-1), p65 NF-κB, and c-jun (AP-1) in the tissues and RBL-2H3 cells were performed as previously reported (Ryu et al., 2011). The protein fractions of each lysate were subjected to electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a nitrocellulose membrane. Phospho-p65, phospho-c-Jun, p65 NF-κB, and c-jun were assayed with the corresponding antibodies. Immunodetection was carried out using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific Inc.).

### Statistical analysis

All data were expressed as the mean ± S.D., with statistical significance analyzed using one-way ANOVA followed by a Student-Newman-Keuls test.

## RESULTS

### Inhibitory effect of LP on the activation of NF-κB and AP-1 in PMA-stimulated RBL-2H3 cells

To isolate antiallergic LAB, we isolated 50 LAB strains from kimchi and investigated their inhibitory effect against the expression of IL-4 and TNF-α and the activation of their transcription factors NF-κB and AP-1 in PMA-stimulated RBL 2H3 cells (Fig. 1). Treatment with PMA increased proinflammatory cytokine TNF-α and IgE-switching cytokine IL-4 expression. Furthermore, it activated transcription factors NF-κB and AP-1, which regulate the expression of those cytokines. Of isolated LAB strains, LP most potently inhibited the expression of those cytokines. At a concentration of $1 \times 10^{10}$ CFU/mL, LP inhibited IL-4 and TNF-α expressions by 92% and 28%, respectively. LP also potently inhibited NF-κB and AP-1 activations. However, treatment with *Escherichia coli* plus PMA induced the activations of NF-κB and AP-1 more potently than PMA alone (data not shown).

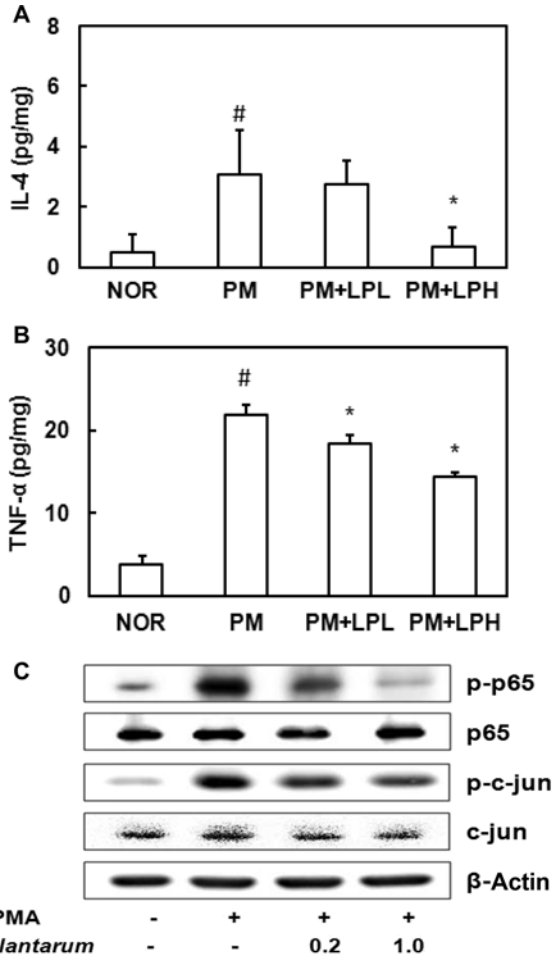


**Fig. 1.** Inhibitory effect of LP on the expressions of IL-4 (**A**) and TNF-α (**B**) and the activations of their transcription factors, AP-1 and NF-κB, (**C**) in PMA-stimulated RBL-2H3 cells. RBL-2H3 cells ($2 \times 10^5$) were stimulated with 20 nM PMA (PM) and then heat-inactivated LP (LPL, $0.2 \times 10^9$ CFU/well; LPH, $1 \times 10^9$ CFU/well) was treated. Normal group (NOR) was treated with vehicle alone instead of LP. TNF-α and IL-4 were assayed by ELISA and NF-κB and AP-1 were determined by immunoblot analysis. Mean ± S.D. (n = 5). $^{\#}p < 0.05$ *vs* normal control group. $^{*}p < 0.05$ *vs* PMA-treated group.

## Inhibitory effect of LP on IgE-antigen complex-induced passive cutaneous anaphylaxis in mice

LP was found to inhibit AP-1 and NF-kB activations in basophil cells. Therefore, the PCA reaction-inhibitory effect of LP in mice was measured (Fig. 2). The PCA reaction was induced by an injection of IgE and antigen, with LP administered orally once a day for three days prior to the challenge with antigen. The IgE-antigen complex potently induced the PCA reaction. LP showed potent inhibition against the PCA reaction. It was inhibited by 85.7% at a dose of $1 \times 10^{10}$ CFU/mouse.

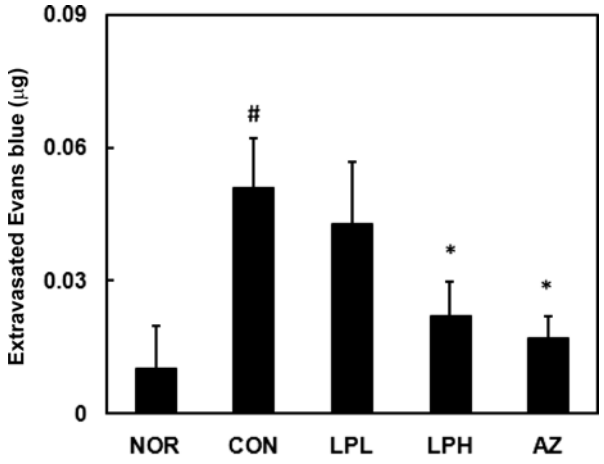


**Fig. 2.** Inhibitory effect of LP on IgE-induced passive cutaneous anaphylaxis reaction in mice. The passive cutaneous anaphylaxis reaction in two dorsal skin sites of mice was induced by an intradermal injection of anti-DNP-HSA. K-1 and azelastine were orally administered once a day for three days. The amounts of extravasated Evan blue from the dorsal skin (1 × 1 cm) were measured 1 h after the final administration of test agents. NOR, normal group treated with vehicle alone; CON, control treated with IgE-antigen complex alone; LPL, $1 \times 10^9$ CFU of LP with IgE-antigen complex; LPH, $1 \times 10^{10}$ CFU of LP with IgE-antigen complex; AZ, 10 mg/kg azelastine with IgE-antigen complex. Mean ± S.D. (n = 5). $^{\#}p < 0.05$ *vs* normal control group. $^{*}p < 0.05$ *vs* IgE-antigen complex-treated group.

## Antiscratching behavioral effect of *L. plantarum* in histamine-treated mice

The inhibitory effect of LP against histamine-induced scratching behavior in mice was investigated (Fig. 3A). LP potently inhibited scratching behavior induced by histamine. LP inhibited the scratching behavior by 58.9% at a dose of $1 \times 10^{10}$ CFU/mouse compared to the effects in the control group. The agent also inhibited the scratching behavior induced by histamine. When histamine was used as an inducer for scratching, the histamine increased vascular permeability as well as induced scratching behavior. LP significantly inhibited vascular permeability induced by histamine (Fig. 3B). The inhibitory activity of LP against vascular permeability was in proportion to its inhibition against scratching behavior.

To confirm the anti-scratching behavioral effect of LP, we investigated its inhibitory effect against IL-4 and TNF-α protein expressions and NF-κB and AP-1 activations and IgE production in mouse skin stimulated with histamine (Fig. 4). Histamine increased the expressions of IgE-switching cytokine, IL-4, and pro-inflammatory cytokines, IL-1β and TNF-α, by 1.9-fold,

1.7-fold, and 2.1-fold, respectively (Fig. 4). Histamine also increased the IgE level 1.9-fold. LP potently inhibited histamine-induced cytokine production: at $1 \times 10^{10}$ CFU/mouse, IL-4, IL-1β and TNF-α expressions were inhibited by 88.9%, 88.6%, and 98.9%, respectively. LP also inhibited IgE level increased by histamine by 85.3%. It also inhibited histamine-induced activations of the transcription factors, NF-κB and c-jun, which regulate TNF-α and IL-4 expressions, respectively (Fig. 5).

## DISCUSSION

Mast cells and basophils are well-known critical par-

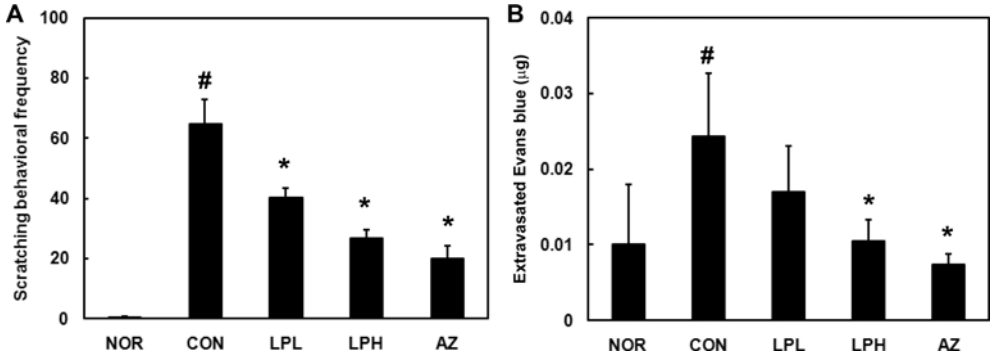


**Fig. 3.** Effect of LP on histamine-induced scratching behavior in mice. (**A**) Effect on scratching behavior. The scratching behaviors in the normal control group treated with saline alone and in the histamine-treated groups with and without test agents were counted for 1 h. LP ($1 \times 10^9$ CFU/mouse and $1 \times 10^{10}$ CFU/mouse) and azelastine (10 mg/kg) was administered once a day for three days. The final administration of test agents were performed 1 h before histamine administration. NOR, normal group; CON, control treated with histamine alone; LPL, $1 \times 10^9$ CFU of LP with histamine; LPH, $1 \times 10^{10}$ CFU of LP with histamine; AZ 10 mg/kg azelastine with histamine. (**B**) Effect on vascular permeability. The vascular permeability was increased by histamine in mice. Mice were treated with or without the oral administration of test agents 1 h before the intradermal injection of 300 μg/50 μL of histamine into the skin on the backs of mice. In the vascular permeability assay, the amount of Evan blue extravasated from the dorsal skin (1 × 1 cm) of mice was measured. Mean ± S.D. (n = 5). $^{\#}p < 0.05$ *vs* normal control group. $^{*}p < 0.05$ *vs* histamine-treated group.

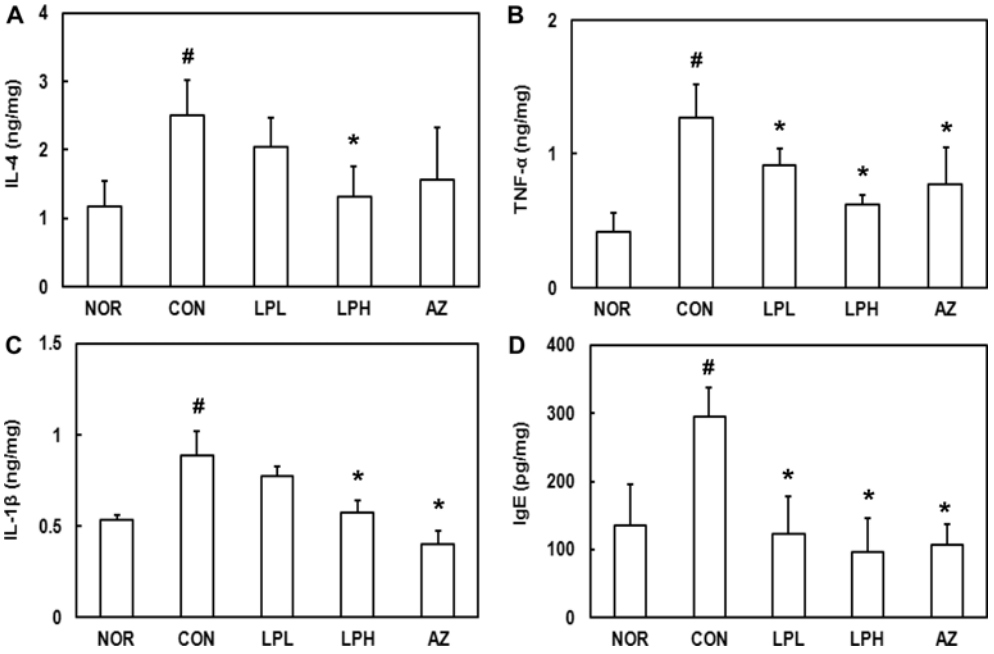


**Fig. 4.** Inhibitory effect of LP on the expressions of IL-4 (**A**) and TNF-α (**B**), IL-1β (**C**), and IgE (**D**) in histamine-stimulated mouse skin tissues. TNF-α and IL-4, IL-1β and IgE were assayed by ELISA. LP and azelastine were orally administered to mice once a day for three days: NOR, normal group; CON, control treated with histamine alone; LPL, $1 \times 10^9$ CFU of LP with histamine; LPH, $1 \times 10^{10}$ CFU of LP with histamine; AZ, 10 mg/kg azelastine with histamine. Mean ± S.D. (n = 5). $^{\#}p < 0.05$ *vs* normal control group. $^{*}p < 0.05$ *vs* histamine-treated group.

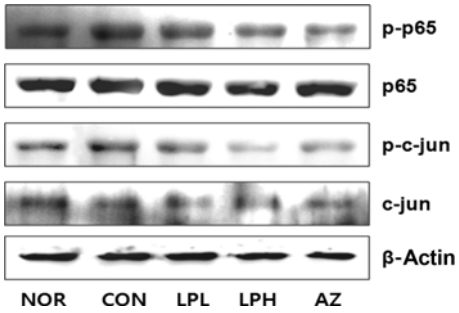


**Fig. 5.** Inhibitory effect of LP on the activations of transcription factors NF-κB and c-jun in histamine-induced mouse skin tissues. NF-κB, c-jun, and β-actin were determined by immunoblot analysis. LP and azelastine were orally administered to mice once a day for three days: NOR, normal group; CON, control treated with histamine alone; LPL, $1 \times 10^9$ CFU of LP with histamine; LPH, $1 \times 10^{10}$ CFU of LP with IgE-antigen complex; AZ, 10 mg/kg azelastine with histamine.

ticipants in various biological processes of allergic diseases (Maintz and Novak, 2007; Reich and Szepietowski, 2007). These cells express surface membrane receptors, with high affinity and specificity for IgE. The interaction of antigen-bound IgE in surface membrane receptors induces the release of histamine, prostaglandins, leukotrienes and cytokines (Kuraishi et al., 1995), and finally cause pruritus, inflammation, rhinitis, atopic dermatitis, asthma and food allergies. These allergic diseases are now rapidly increasing chronic health problem in most countries (Mitre and Nutman, 2006).

Antiallergic agents, such as anti-histamines, steroids, immunosuppressants, and quercetin, have been used against allergic diseases (Simons, 1992; Schafer-Korting et al., 1996; Sakuma et al., 2001), but improving these diseases is very difficult. Therefore, LAB has been advanced for allergic diseases, and their effectiveness has been given increasing attention.

LAB is recognized as beneficial microorganisms and have been demonstrated to exert preventive and therapeutic effects on Th2-bias diseases such as atopic dermatitis or food allergies (Kalliomäki et al., 2003; van Overtvelt et al., 2010). Fermented foods, such as yogurt, cheese, and kimchi, are considered to be good sources of microorganisms; a variety of LAB strains have been detected in those foods (Cho et al., 2006) and are known to regulate host defense mechanisms (Lee et al., 2005; Cho et al., 2007). Particularly, *L. plantarum* isolated from kimchi may have beneficial effects in allergic diseases, such as asthma, by enhancing the expressions of regulatory factors, such as Foxp3 and IL-10, in intestinal laminar propria cells (Cho et al., 2006; Hong et al., 2010). *L. plantarum* inhibited scratching behaviors caused by histamine or compound 48/80 via inhibiting IL-4 expression in mice (Jang et al., 2011). *L. rhamnosus* GG was noted to exert suppressive effects on asthma, and this may be correlated with increased numbers of Treg cells (Feleszko et al., 2007). *L. paracasei* was used to treat human patients suffering from allergic rhinitis (Peng and Hsu, 2005). These results demonstrated that *Lactobacillus* species might modulate dendritic cells or T cell-mediated responses *in vitro* by boosting the production of immunosuppressive cytokines, IL-10 and TGF-β (Khoury et al., 1992; von der Weid et al., 2001).

In the present study, LP potently inhibited IgE-induced PCA reaction in mice. LP also inhibited the expressions of proinflammatory cytokines TNF-α and IL-1β and the IgE-switching cytokine IL-4 as well as the activations of their transcription factors NF-κB and AP-1 in HMC-1 cells. Furthermore, LP inhibited histamine-induced scratching behavior as well as vascular permeability in mice. LP inhibited the expressions of IL-4, TNF-α, IL-1β, and IgE in mouse skin stimulated by histamine

Based on these findings, LP may attenuate scratching behavior by regulating the expression of IL-4, which induces IgE production and inflammatory diseases, such as colitis, by suppressing the expressions of proinflammatory cytokines, IL-1β and TNF-α via NF-κB and AP-1 activation. Furthermore, LP may improve allergic diseases, such as anaphylaxis, atopic dermatitis, rhinitis, and pruritus.

## ACKNOWLEDGEMENTS

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