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Inhibitory effects of *Lactobacillus crispatus* isolated from vaginal microbiota on atopic dermatitis

질 내 균총에서 유래한 락토바실러스 크리스파투스 균주의 아토피성 피부염 저해효과

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고 우 리

Abstract

Inhibitory effects of *Lactobacillus crispatus* isolated from vaginal microbiota on atopic dermatitis

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Atopic dermatitis (AD), is known to have chronic inflammatory symptoms in the skin. It induces disorder in the skin and initiates the allergic march including allergic rhinitis and asthma in the early life of a human. Probiotic *Lactobacillus* is reported to prevent the symptoms of the allergic diseases. There have been no reported vaginal *Lactobacillus* spp. associated with allergic diseases. To examine the possible anti-allergic effects of vaginal *Lactobacillus* spp., RBL-2H3 cells, EL4 cells, U266B1 cells and RAW 264.7 cells were used in this study. Several vaginal *Lactobacillus* strains including *L.crispatus, L.gasseri, L.jensenii,* and *L.sakei* were shown to inhibit the degranulation of basophil. Increased IgE level, which is key pathogenesis of the atopic disease, was found to be alleviated in U266B1 cell co-cultured with vaginal

L.crispatus, *L.jensenii* and *L.sakei* strains. Several strains of vaginal *L.crispatus, L.gasseri, and L.jensenii* prevented secretion of Th2 type cytokines including IL-4, IL-5 which mediate allergic response. Through determining the ratio of IL-10 to TNF-α and IL-10 to IL-6, the anti-inflammation capacity of vaginal *Lactobacillus* strains was measured. *L.crispatus* species were shown to have the highest anti-inflammatory capacity. To test *in vivo* therapeutic effects of vaginal *Lactobacillus* spp. on atopic dermatitis, NC/Nga mouse model was used. In the mice treated with vaginal *Lactobacillus crispatus* SNUV206, dermatitis score and scratching time were significantly decreased compared to the mice treated with PBS. Ear and dorsal skin thickness which are thickened in atopic dermatitis patients are also alleviated in SNUV206 treated mice. In conclusion, SNUV206 may have potential therapeutic effects on atopic dermatitis.

Keywords: Vaginal Bacteria, *Lactobacillus crispatus*, Allergy, Atopic Dermatitis

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I. Introduction

Atopic dermatitis (AD) represents an allergic inflammatory disease and is often the first disease present in a series of allergic diseases[1]. AD has increased in prevalence over the past several decades, particularly in developed countries[2]. During the early stage of allergy, antigens processed by antigen presenting cells (APC) activates T helper cells (Th cell), expressing Th2 type cytokines such as interleukin (IL)-4, IL-5 and IL-13[3][4]. Especially, IL-4 plays important role in switching immunoglobulin E (IgE) production in B cells[5][6]. Produced IgE reaches to the mast cells or basophil in tissue or blood and binds to the FcεRI to stimulate degranulation of the cells[7][8]. As a result of degranulation, pro-inflammatory compounds such as histamine, cytokines, and chemokines are released[9][7]. These molecules cause the symptoms of AD, the recruitment of inflammatory cells. Macrophage, which plays a critical role in innate immunity and also helps initiate adaptive immunity, can secrete various cytokines that promote and inhibit the inflammation. Representative pro-inflammatory cytokines are tumor necrosis factor α (TNF- α) and IL-6, and anti-inflammatory cytokine IL-10 is the key cytokine which can balance the Th1 and Th2 immune response[10].

Although the cause and mechanism of AD are not fully understood, antihistamine and corticosteroid treatment are used as therapies for AD-induced inflammation[11]. However, it has been previously shown that reduced diversity of the intestinal microbiota in infants is associated with increased risk of AD[12][13]. As a result, bacterial exposure has been suggested as a key factor in atopic diseases, potentially through intestinal colonization and immunomodulation[14]. Several trials have evaluated the role of probiotics in the prevention of atopic diseases and atopic sensitization, and there is compelling evidence for the effect on AD[15][16]. Furthermore, the mode of delivery (especially Cesarean section delivery) are reported to be associated with allergic disease incidence rate after birth as well as AD[17][18]. Therefore, it is assumed that microbial exposure through maternal vaginal microbiome during birth process may have a beneficial effect on the development of the immune system of infants in early life[19].

Thus, the objective of this research is to test the anti-allergic capability of *Lactobacillus* spp. originated from the vagina. To achieve this aim, in this study, we used several *in vitro* system which can evaluate the inhibitory effect of vaginal *Lactobacillus* spp. on degranulation, IgE secretion, Th2 cytokines production, and anti-inflammatory response.

We also used DNCB-induced NC/Nga murine model to investigate its therapeutic effect *in vivo*.

II. Materials and Methods

In vitro study

1. *Lactobacillus* spp. culture and preparation

The *Lactobacillus* strains isolated form human vagina and gut were used in this study (Table 1). The vaginal samples were obtained from three Korean women consisting of a monozygotic twin pair and their mother. The Strains originated from gut were isolated from fecal samples of Korean Population. All isolates were identified to the species level by sequencing of 16s rRNA. *Lactobacillus* spp. were cultured anaerobically in MRS broth (Thermo Fisher Scientific, USA) with 0.05% L-cysteine hydrochloride (Sigma-Aldrich, USA) for 18 h at 37℃. After being subcultured twice (1% v/v) from the stock, the bacterial cells were harvested in a centrifuge (4℃, 1500rpm, 3min) and washed twice with PBS. The number of bacteria was counted by Baclight Live/Dead Bacterial Viability Kit (Molecular Probes, USA) using an Accuri C6 flow cytometer (Accuri Cytometers, USA).

Table 1. *Lactobacillus* strains used in this study

***Lactobacillus* species**	**Isolated from**			
	vagina		**gut**	
	strain	**No. of isolates**	**strain**	**No. of isolates**
Lactobacillus crispatus	SNUV285 SNUV195 SNUV008 SNUV206 SNUV350-1 SNUV448 SNUV151-1 SNUV272-1 SNUV236 SNUV364-1 SNUV773	11		-
Lactobacillus fermentum	SNUV417	1	SNUG375	1
Lactobacillus gasseri	SNUV290 SNUV445 SNUV433-1	3	SNUG134	1
Lactobacillus jensenii	SNUV191 SNUV221 SNUV470-2	3		-
Lactobacillus sakei	SNUV494 SNUV502	2		-
Lactobacillus vaginalis	SNUV351	1		-
Total		21		2

Twenty-one vaginal *Lactobacillus* strains and two *Lactobacillus* strains isolated from the gut were used in this study.

2. Cell line culture

RBL-2H3 rat basophil cell line (KCLB No. 22256), EL4 mouse T lymphoma cell line (KCLB No. 40039) and RAW 264.7 mouse macrophage cell line (KCLB No. 40071) were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). U266B1 human B lymphoma cell line (ATCC® TIB 196™) was obtained from Korea Centers for Disease Control and Prevention (Ochang, Korea). RBL-2H3 cells, EL4 cells, and RAW 264.7 cells were cultured in DMEM medium (Gibco, USA) supplemented with 10% inactivated FBS, 100 IU/ml penicillin-streptomycin in 37℃ under 5% CO_2. U266B1 cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% inactivated FBS, 100 IU/ml penicillin-streptomycin in 37℃ under 5% CO_2.

3. β-hexosaminidase secretion assay

RBL-2H3 cells were seeded in 6-well plates (1×10^6 cells/well). Three hours after seeding, the cells were sensitized with 0.5μg/ml DNP-specific IgE (Sigma-Aldrich, USA), and incubated overnight. After the cells were washed with siraganian buffer (119mM NaCl, 5mM KCl, 0.4mM $MgCl_2$, 25mM PIPES, 40mM NaOH, pH7.2) twice, they were exposed to the *Lactobacillus* spp. (1×10^9 cells/well) or ketotifen (20μg/ml) and incubated 37℃ for 20min. After incubation, the cells were stimulated with 1μg/mL of dinitrophenyl-human serum albumin (DNP-HSA) for 37℃ 10 min. The reaction was terminated by placing the plates on ice. The cell culture supernatant was collected to determine the level of IL-4 by ELISA (BD Biosciences, USA). 25μl of cell culture supernatant was incubated with an equal volume of 1mM substrate solution (1mM p-nitrophenyl-N-acetyl-β-D-glucosaminide) dissolved in 0.1 M citrate buffer, pH 4.5) at 37 °C for 90 min. Then, the enzyme reaction was terminated by adding 200μl of stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). Finally, absorbance was measured at 405 nm with a microplate reader.

4. Cytokine production assay

To measure the IL-4 and IL-5 inhibition effect of *Lactobacillus* spp. isolated from the vagina, EL4 cells were seeded in 24-well plates (5×10^4 cells/well) and incubated 37℃ overnight. EL4 cells were stimulated by 20ng/ml PMA (Sigma-Aldrich, USA) and 1μg/ml Ionomycin (Sigma-Aldrich, USA), and the *Lactobacillus* strains (5×10^5 cells/well) were added to the cells. After 24h, the supernatant was collected for measuring the level of IL-4 and IL-5 by ELISA (BD Biosciences, USA). To measure the effect on TNF-α, IL-6, and IL-10 of *Lactobacillus* spp. isolated from the vagina, RAW 264.7 cells were seeded in 24-well plates (1×10^5 cells/well) and incubated 37℃ overnight. RAW 264.7 cells were stimulated by 100ng/ml lipopolysaccharide (LPS, Sigma-Aldrich, USA), and the *Lactobacillus* strains (1×10^6 cells/well) were added to the cells. After 24h, the supernatant was collected for measuring the level of TNF-α, IL-6, and IL-10 by ELISA (BD Biosciences, USA).

5. IgE production assay

The IgE-inhibitory effects of *Lactobacillus* spp. isolated from the vagina in human B cells were measured by the following method. U266B1 cells were seeded in 24-well plates (1×10^5 cells/well) and incubated 37℃ overnight. U266B1 cells were stimulated by 50ng/ml IL-4 (Sigma-Aldrich, USA) and 100μg/ml LPS (Sigma-Aldrich, USA), and the *Lactobacillus* strains (1×10^6 cells/well) were added to the cells. After 48h, the IgE in supernatants was detected by using human IgE ELISA (Bethyl Laboratory Inc., USA).

In vivo study

6. Animals

Female NC/Nga mice (6 weeks old) were obtained from SLC Japan (Shizuoka, Japan). The mice were maintained on a 12 hours light/dark cycle with lights on at 9 a.m. 5 mice per group were used in all experiments. All the experimental procedures were approved by Seoul National University Institution Animal Care and Use Committees (IACUC).

7. Murine atopic dermatitis model

The dorsal hair of all mice was shaved by electric shaver prior to atopic dermatitis-like skin lesion induction. 1-chloro-2,4-dinitrobenzene (DNCB) was used as an AD inducer. In brief, 1% DNCB solution was freshly made in acetone : olive oil (3 : 1), and applied on the shaved area of dorsal skin of the mice twice a week from day 0 to end of the experiment. Five groups (DNCB/*Lactobacillus* spp.) of mice were administrated with *Lactobacillus* spp. at 1×10^9 CFU/200ul/day by oral gavage from day 22 to day 56, while the negative control group (DNCB/PBS) of mice was fed with 200μl of PBS. As a naïve control, mice without DNCB treatment were maintained by being fed with 200μl of PBS (Fig. 1). At the end of the experiment, ear thickness was measured

with a caliper, and spleen was sampled for evaluating the splenomegaly.

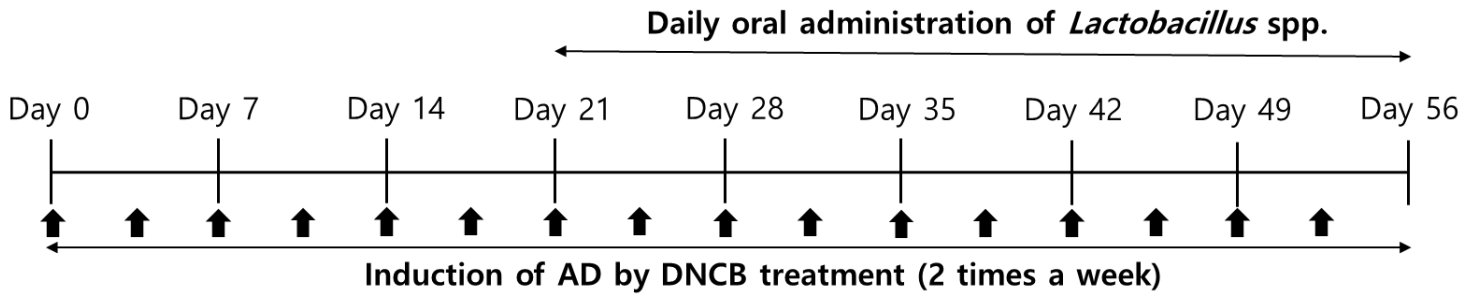

Figure 1. Experimental procedure of murine atopic dermatitis model

Experimental design. NC/Nga mice were divided into seven groups: (1) naïve, (2) DNCB treat (negative), (3) DNCB + SNUV195, (4) DNCB + SNUV8, (5) DNCB + SNUV206, (6) DNCB + SNUG375, (7) DNCB + SNUG134 (n=5 per group). To study the effect of vaginal *Lactobacillus* spp. in atopic dermatitis, mice were treated with DNCB for 8 weeks, and PBS or *Lactobacillus* was fed by oral gavage for 5 weeks from day 21. ⬆, topical DNCB treatment.

8. Evaluation of dermatitis score

The severity of dermatitis score was evaluated before sacrifice. Scores of 0 (none), 1 (mild), 2 (moderate), and 3 (severe) were measured for each of the four symptoms: 1) erythema/hemorrhage, 2) scarring/dryness, 3) edema, and 4) excoriation/erosion. The sum of the individual scores indicating clinical severity was taken as the dermatitis score.

9. Evaluation of scratching behavior

The AD-like behavioral change was evaluated by measuring the time that mice spent scratching their nose, ears, and dorsal skin with their paws for 20 min on the last day of the experiment (on day 56).

10. Histological analysis

To measure the skin thickening, the dorsal skin of each mouse was excised on day 56, fixed with neutral buffered formalin, and embedded in paraffin. The skin sections were cut from the blocks at 5μm thickness. After staining with hematoxylin and eosin (H&E), the epidermal thickness was observed under a microscope at 40× magnification.

11. Flow cytometry

To investigate the immune response of AD-induced NC/Nga mice treated with *Lactobacillus* spp., mesenteric lymph node (MLN) of the mice were excised and minced into single cell suspension passing through a 20-μm

cell strainer (Celltrix). Suspended cells were centrifuged at 1500rpm for 5 min. The pellet was resuspended in fluorescence-activated cell sorting (FACS) buffer (2 mM EDTA, 2% fetal bovine serum in PBS). The cells were then incubated with labeled monoclonal antibodies (eBioscience) and analyzed by flow cytometry using a FACSverse flow cytometer (Becton Dickinson). Data were analyzed with FlowJo software (Tree Star, Inc). We validated the flow cytometric identification of $CD3^+$ (T cells), $B220^+$ (B cells), $CD3^+CD4^+$ (T helper cells), and $CD3^+CD4^+CD25^+$ (activated T cells) among mesenteric lymph node cells as previously described.[20]

12. Statistical analysis

All data were analyzed with Prism 5 (GraphPad Software, CA). Statistical significance was measured using Mann-Whitney test when comparing two groups, two-way ANOVA with Bonferroni post-test comparing various groups.

III. Results

1. Effect of *Lactobacillus* spp. isolated from the vagina on β-hexosaminidase release in RBL-2H3 cells

β-hexosaminidase is a well-established degranulation marker resulting from the allergic response of RBL-2H3 cells[21]. The inhibitory effect of *Lactobacillus* spp. isolated from the vagina on β-hexosaminidase secretion was examined. Through primary screening of 49 *Lactobacillus* strains isolated from the vagina, 17 strains which had stronger inhibitory effects compared to ketotifen and 4 strains which had no inhibitory effect were selected (Fig. S1). After three independent sets of experiments, β-hexosaminidase secretion was significantly lower in RBL-2H3 cells treated with 17 *Lactobacillus* strains isolated from the vagina (Fig. 2). Ketotifen (20μg/ml) was also shown to significantly reduce β-hexosaminidase secretion induced by DNP-HSA. Some *Lactobacillus* species such as *L. crispatus, L. gasseri*, *L. jensenii and L. sakei* showed species-specific β-hexosaminidase suppression effects.

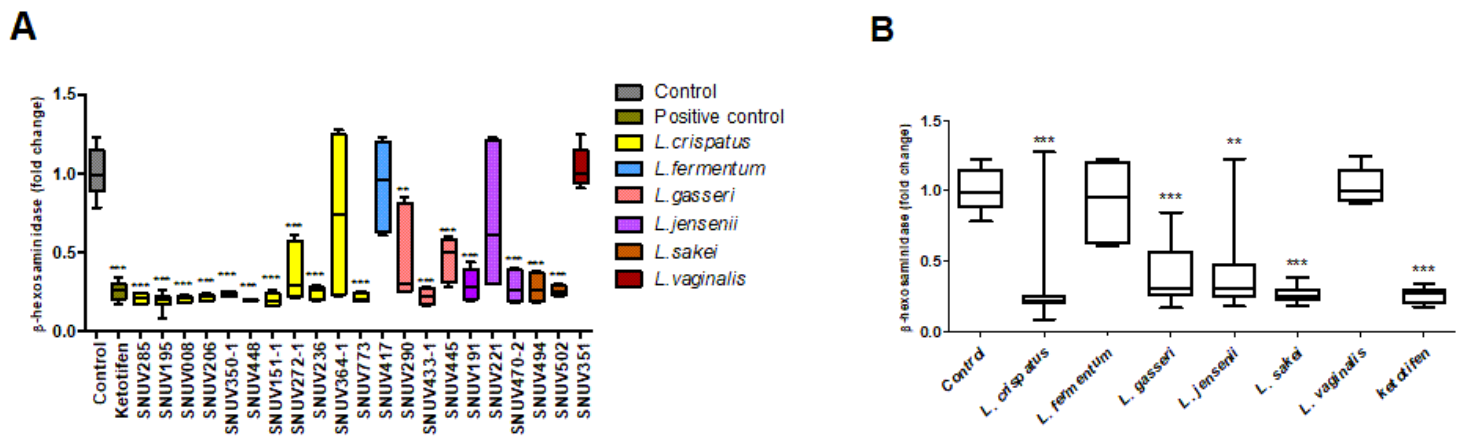

Figure 2. Effect of Lactobacillus spp. isolated from the vagina on β-hexosaminidase release in RBL-2H3 cells

Rat RBL-2H3 cells were cultured with *Lactobacillus* spp. for 20min. β-hexosaminidase release in the duplicate microplate wells was determined by colorimetric method. (A) Strain- and (B) species-specific effects of *Lactobacillus* spp. isolated from the vagina on β-hexosaminidase release in RBL-2H3 cells. The whiskers of each boxplot represent the 10 to 90 percentiles. Three independent sets of experiments were performed in each case. * $P < 0.05$, ** $P<0.01$, *** $P < 0.001$.

2. Effect of *Lactobacillus* spp. isolated from the vagina on the level of IgE secretion in U266B1 cells

To evaluate the effect of *Lactobacillus* spp. isolated from the vagina on IgE production in B lymphocytes, we selected 14 vaginal *Lactobacillus* strains which have consistent and repeatable inhibition capability in β-hexosaminidase release assay. U266B1 cells were stimulated with LPS and IL-4. Five strains of *L. crispatus* and two strains of *L. sakei* used in this study significantly inhibited the production of IgE and one strain of *L. jensenii* also showed inhibitory effect (Fig. 3).

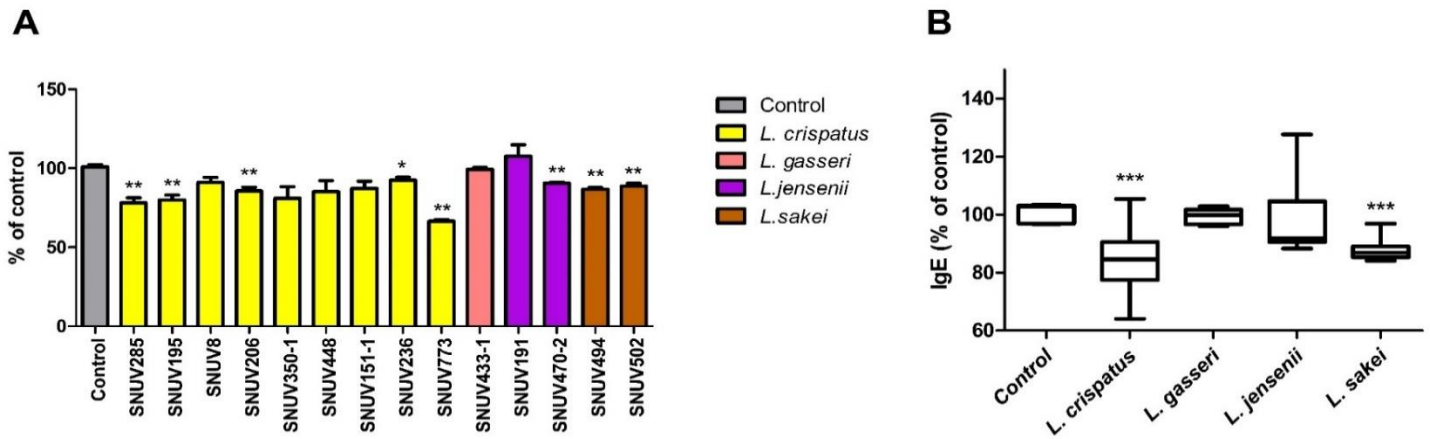

Figure 3. Effect of *Lactobacillus* spp. isolated from the vagina on the level of IgE secretion in U266B1 cells

Human U266B1 B cells were stimulated with LPS and IL-4 co-cultured with *Lactobacillus* spp. isolated from the vagina. After 48 hours, the level of secreted IgE was measured with an ELISA kit. (A) Strain- and (B) species-specific effects of *Lactobacillus* spp. isolated from the vagina on IgE release in U266B1 cells. Three independent sets of experiments were performed in each case. * P< 0.05, ** P<0.01, *** P<0.001.

3. Effect of *Lactobacillus* spp. isolated from the vagina on cytokine secretion in cell lines

To better understand the effect of *Lactobacillus* spp. isolated from the vagina on immune cell signaling, level of 5 cytokines were measured in the supernatant of cells co-cultured with *Lactobacillus* spp. isolated from the vagina. First, the IL-4 level was shown to decrease in comparison with the control in RBL-2H3 cell line in response to treatment with several strains of *L. crispatus, L. gasseri, L. jensenii* and *L. sakei*. Second, 6 strains of *L. crispatus* and 1 strains of *L. jensenii* showed significant suppressive activity on the production of IL-4, 7 strains of *L. crispatus* and 1 strains of *L. gasseri and L. jensenii* significantly inhibited the secretion of IL-5 in EL4 cell line. Lastly, the pro-inflammatory cytokines TNF-α, IL-6 and anti-inflammatory cytokine IL-10 were measured in Raw 264.7 cell line co-cultured with *Lactobacillus* spp. isolated from the vagina. Then the ratio of IL-10/TNF-α and IL-10/IL-6 were evaluated to determine anti-inflammatory effects of *Lactobacillus* spp. isolated from the vagina. All strains showed anti-inflammatory effect, but *L. crispatus* showed the highest significant increase in both indicators.

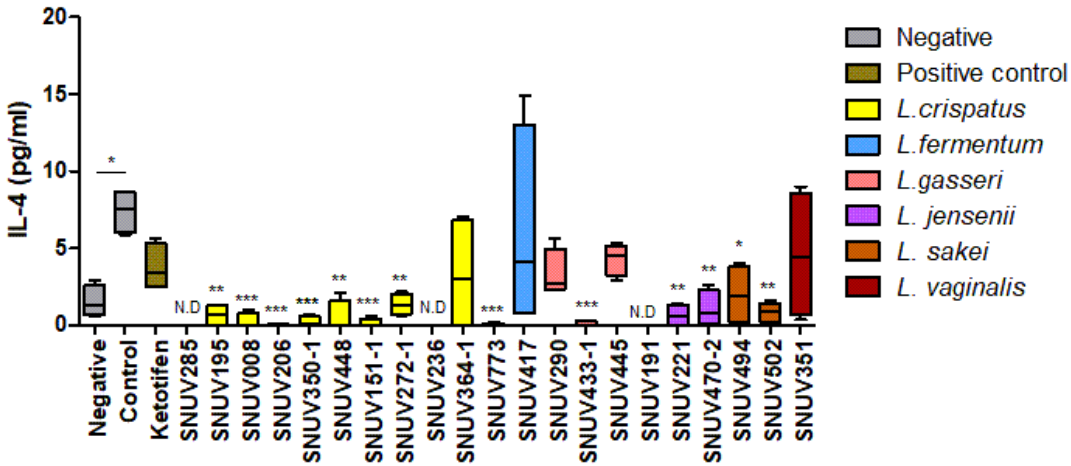

Figure 4. Effect of Lactobacillus spp. isolated from the vagina on the level of IL-4 in RBL-2H3 cells

Rat RBL-2H3 cells were cultured with *Lactobacillus* spp. isolated from the vagina for 24 h. The supernatant level of IL-4 was determined by ELISA kit. The whiskers of each boxplot represent the 10 to 90 percentiles. Three independent sets of experiments were performed in each case. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, N.D: not detected.

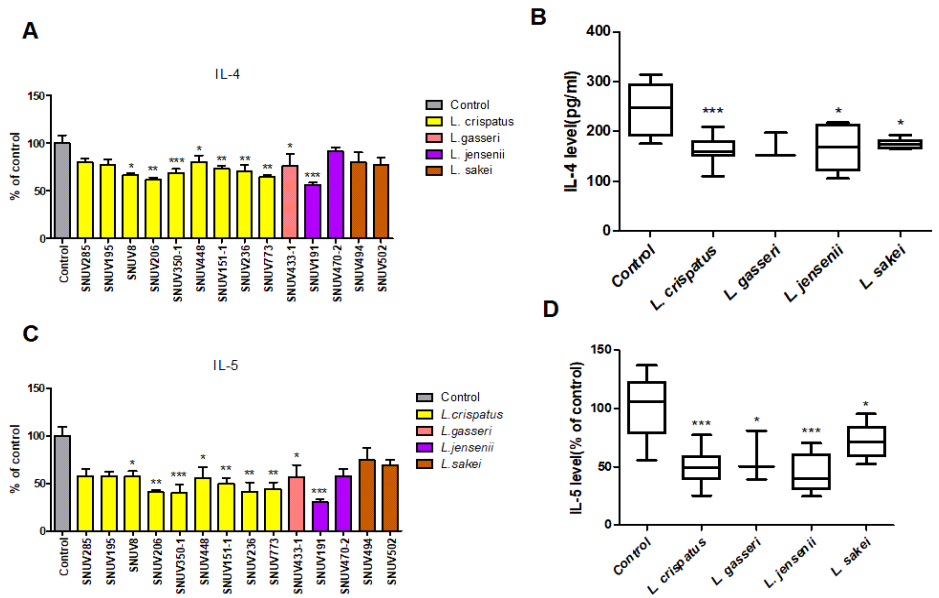

Figure 5. Effect of *Lactobacillus* spp. isolated from the vagina on the level of IL-4 and IL-5 in EL4 cells

EL4 cells were cultured with *Lactobacillus* spp. for 24 h. The supernatant level of IL-4 and IL-5 in the duplicate microplate wells were determined by ELISA kit. Several strains of *Lactobacillus* spp. isolated from the vagina effect on the supernatant (A) IL-4 and (C) IL-5 level in EL4 cells. Values are shown as mean±SD. Species-specific effect of *Lactobacillus* spp. isolated from the vagina on (B) IL-4 and (D) IL-5 secretion level in EL4 cells. The whiskers of each boxplot represent the 10 to 90 percentiles. Three independent sets of experiments were performed in each case. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

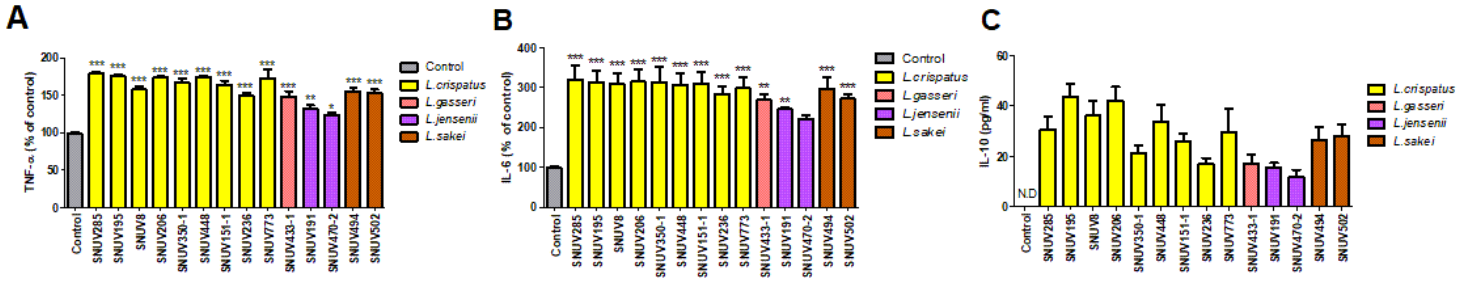

Figure 6. Effect of *Lactobacillus* spp. isolated from the vagina on the level of TNF- α, IL-6 and IL-10 in RAW 264.7 cells

RAW 264.7 cells were cultured with *Lactobacillus* spp. for 24 h. The supernatant level of TNF-α, IL-6, and IL-10 in the duplicate microplate wells were determined by ELISA kit. Several strains of *Lactobacillus* spp. isolated from the vagina effect on the supernatant (A) TNF- α (B) IL-6 and (C) IL-10 level in RAW 264.7 cells. Values are shown as mean±SD. Three independent sets of experiments were performed in each case.

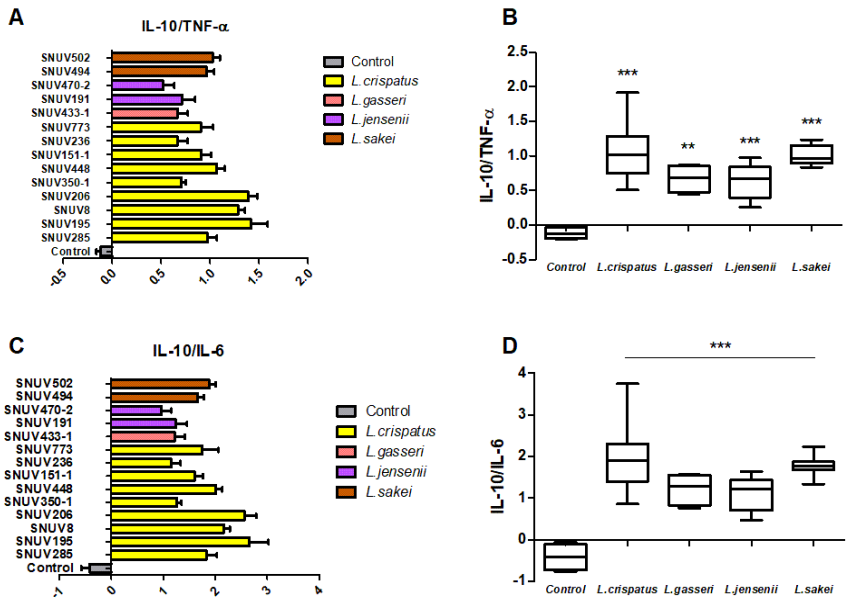

Figure 7. Effect of *Lactobacillus* spp. isolated from the vagina on the level of IL-10/ TNF-α and IL-10/IL-6 in RAW 264.7 cells

RAW 264.7 cells were cultured with *Lactobacillus* spp. for 24 h. The ratio of IL-10/ TNF-α and IL-10/IL-6 was evaluated. Several strains of *Lactobacillus* spp. isolated from the vagina effect on (A) IL-10/ TNF-α and (C) IL-10/IL-6 ratio in RAW 264.7 cells. Values are shown as mean±SD. Species-specific effect of *Lactobacillus* spp. isolated from the vagina on (B) IL-10/ TNF-α and (D) IL-10/IL-6 ratio in RAW 264.7 cells. The whiskers of each boxplot represent the 10 to 90 percentiles. Three independent sets of experiments were performed in each case. ** $P < 0.01$, *** $P < 0.001$.

4. Vaginal *L. crispatus* strain SNUV206 alleviated DNCB-induced AD-like symptoms in NC/Nga mice

Through *in vitro* assays above， we selected 3 vaginal *L.crispatus* strains which showed the most suppressive effect on allergic resp onse. 2 *Lactobacillus* strains originated from the human gut are also used in this animal study. SNUG375 strain is *L.fermentum* which showed inhibitory effect on AD in the previous study. SN UG134 strain is *L.gasseri* which used as a bacteria control. AD-like skin lesions were induced via the repeated topical application of DNCB in NC/Nga mice. A 5-week course of oral treatment of *Lactobacillus* spp. suppressed the development of AD-like skin lesions in comparison with the control group (Fig. 8). The dermatitis score of AD-like skin lesions between the negative control group and SNUV195, SNUV8, SNUG134 groups were not significantly different throughout the 6 weeks of treatment (Fig. S3). However, the SNUV206 and SNUG375 group showed a significant decrease in dermatitis score compared to the negative control group (Fig. 9A). Among groups tested in this study, we selected 4 meaningful groups in-depth study. As shown in Fig. 9C, the ear thickness of mice treated with SNUV206 was shown to be significantly reduced in comparison to those treated with PBS. The

scratching behavior of the naïve group was determined to be 4.4±3.2 seconds in 20 minutes. The score significantly increased in the negative group. By contrast, the scratching time of the SNUV206 (21.1±12.4) group was significantly lower than those of the negative (103.8±41.9) group (Fig. 9B). We compared the change of morphological features of the spleen among the groups. The mice receiving PBS and SNUG134 were found to have significantly enlarged spleens compared to those of the control group. However, spleen weight was reduced in mice treated with SNUV206 (Fig. 9D). No significant difference in body weight is observed among the experimental groups, implying that administration of *Lactobacillus* spp. is physiologically safe to the NC/Nga mice (Fig. S2)

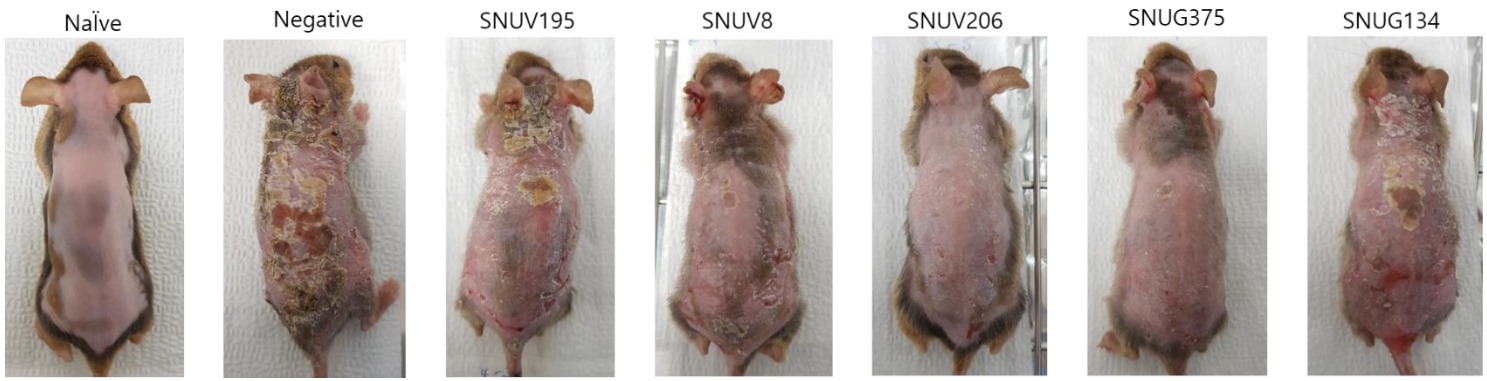

Figure 8. DNCB-induced AD-like skin lesions in NC/Nga mice

Effect of *Lactobacillus* spp. on DNCB-induced AD-like skin lesions in NC/Nga mice.

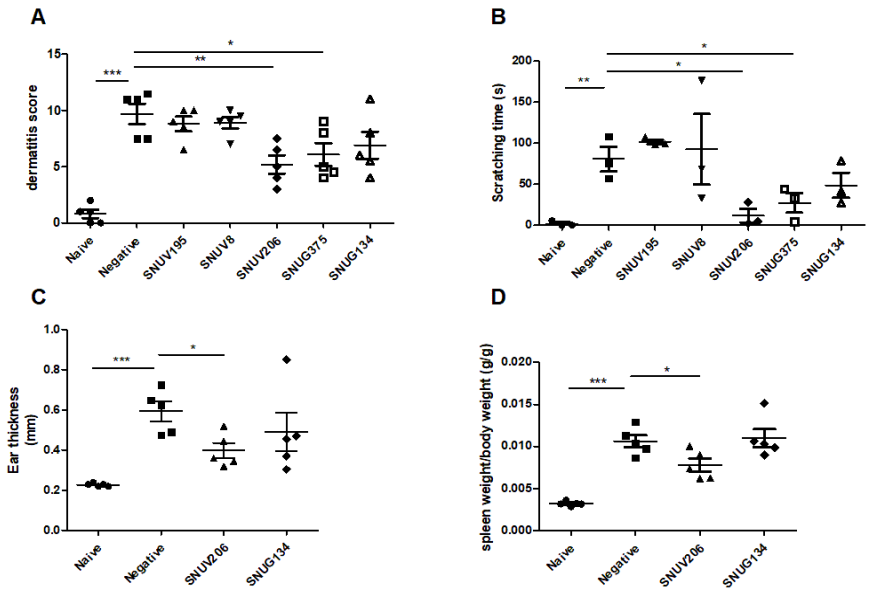

Figure 9. Effect of *Lactobacillus* spp. on DNCB-induced AD-like symptoms in NC/Nga mice

Effect of *Lactobacillus* spp. on DNCB-induced AD-like symptoms in NC/Nga mice. (A) The dermatitis score of AD-like skin lesions was measured concerning erythema/hemorrhage, scaling/dryness, edema, and excoriation/erosion at the end of the study. (B) Scratching behavior of mice was observed for 20 min after sensitization. (C) The average of the left and right ear thickness was measured. (D) After sacrifice, the spleen was isolated and weighed to examine splenomegaly alteration. Spleen weight-to-body weight ratio was evaluated. Results are expressed as the means±SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

5. *Lactobacillus* spp. administration decreased skin thickness in NC/Nga mice

Figure 10A shows the changes in the histopathological features of the lesions on the dorsal skin of NC/Nga mice. The negative mice had highly thickened epidermis, a mean of 719.5μm compared with 432.0μm in naïve mice. In comparison with the negative group, SNUV206 and SNUG134 received groups showed less thickened epidermis (Fig. 10B). Administration of SNUV206 and SNUG134 resulted in epidermal thicknesses of 638.9 and 597.8μm, respectively. Furthermore, SNUV206 treated group tends to be decreased compared to the negative control group in the serum IgE level (Fig. S4). Through FACS analysis, *Lactobacillus* spp. administration did not alter the T cell population, B cell population, proportion of helper T cell, and cytotoxic T cell in the AD-induced mice (Fig. S5). Nevertheless, the number of $CD25^+$ cells in the SNUV206 treated mice was downregulated compared to the negative group mice while the number of $CD25^+$ cells in the negative group was significantly upregulated compared to the naïve group, implying that inflammatory tone may attenuate by treatment of SNUV206 to the AD-induced mice (Fig. S6).

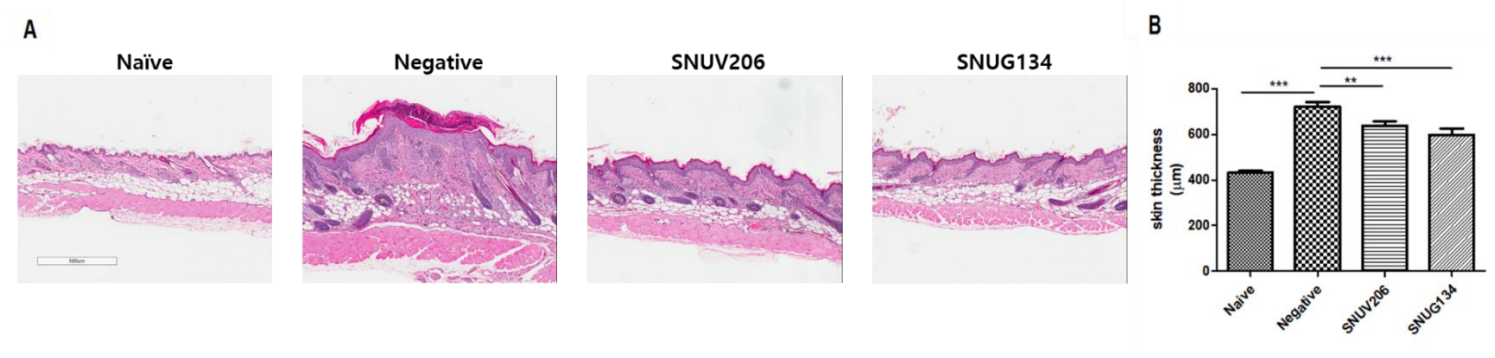

Figure 10. Histological changes in skin in AD-affected NC/Nga mice

(A) Histological features of dorsal skin lesions stained with hematoxylin and eosin (40x). (B) Epidermal thickness according to treatment group. Values are shown as mean±SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

IV. Discussion

In previous studies, the protective effects of probiotics isolated from the gut or food on allergic diseases have been demonstrated[22][23]. Recently, it has also been suggested that vaginal *Lactobacillus* spp. can have a probiotic effect [24]. Most studies related to vaginal *Lactobacillus* spp. are focused on vaginal health[25][26]. However, in this study, we focused on the capability of *Lactobacillus* spp. to exert a probiotic effect on allergic disease. 21 vaginal *Lactobacillus* strains previously isolated from healthy Korean women were used to test anti-allergic effect in this study. Using RBL-2H3 mast cells, EL4 T cells, U266B1 B cells and RAW264.7 macrophage cells, we demonstrated that treatment with vaginal *Lactobacillus* strains exerts beneficial effects on allergic responses *in vitro*. Through DNCB-induced NC/Nga mice, we demonstrated that oral treatment of a vaginal *Lactobacillus* strain has an inhibitory effect on AD-like skin lesions *in vivo*.

Mast cells respond with degranulation following crosslinking of IgE bound FcεRI by various allergens, with the release of newly synthesized mediators including histamine and cytokines that evoke a potent immune allergic response. They can be activated by IgE-dependent mechanisms, releasing inflammatory mediators including histamine and cytokines[7].

RBL-2H3 cells are the most commonly used mast cell line which can produce β-hexosaminidase in the process of degranulation. Degranulation of RBL-2H3 cells, as a consequence of IgE-FcεRI stimulation, reflects the behavior of both mast cells and basophils in respect of their response to immunological stimuli[27]. In this study, 17 out of 21 vaginal *Lactobacillus* strains inhibited the secretion of β-hexosaminidase in IgE-DNP-stimulated RBL-2H3 cells, which indicated the inhibitory activity of vaginal *Lactobacillus* spp. to mast cell degranulation.

As IgE is a key effector responsible for the progression of allergic reactions and contributes to activation of other cell types involved in allergic inflammation[28], IL-4- and LPS-stimulated U266B1 cells were treated with vaginal *Lactobacillus* spp. and the secreted IgE level was measured. All strains of *L. crispatus* and *L. sakei* strains tested in this study were shown to diminish IgE secretion. In accordance with this data, similar observations of *Phellinus linteus* extract inhibiting IgE production in U266B1 cells have been reported[29]. Still, this is the first research to use bacteria to inhibit IgE production in the U266B1 cell line.

Cytokines are produced by various types of immune cells and Th1 and Th2 type cytokines are known to be associated with allergic pathogenesis.

Some strains of *L. crispatus*, *L. gasseri,* and *L. sakei* regulated Th2-type cytokine production in EL4 T cell line, including IL-4 or IL-5. Pro-inflammatory cytokines (TNF-α, IL-6) were shown to be elevated in RAW 264.7 macrophage cell line after treatment with vaginal *Lactobacillus* spp. The anti-inflammatory cytokine IL-10 is secreted by the secretion of pro-inflammatory cytokines, and IL-10 inhibits the release of pro-inflammatory cytokines. All vaginal *Lactobacillus* strains promoted the release of IL-10 compared to the media control in RAW 264.7 cells. The IL-10/TNF-α and IL-10/IL-6 ratio are considered to be a good indicator of the anti-inflammatory effect of a probiotic strain[30]. The group of *L. crispatus* showed the highest level compared to the other vaginal *Lactobacillus* species in this study.

It has been reported that several *Lactobacillus* strains isolated from the gut have inhibitory effects on allergic diseases in animals and humans[31][22]. In this study, we used a DNCB-induced NC/Nga mouse model to screen for effective vaginal *Lactobacillus* strains. In order to confirm an inhibitory effect on atopic dermatitis, we used SNUG375 *Lactobacillus* strains derived from the gut as a positive control and SNUG134 *Lactobacillus* strains derived from the gut as a negative control. The result of the dermatitis scores shows that SNUV206 strain

has a treatment effect on the atopic dermatitis phenotype that is stronger than the positive control, SNUG375. In patients with AD, itch-associated scratching results in skin barrier defects and increases inflammation, both of which ultimately increase associated itching[32]. The results indicate that SNUV206, as well as SNUG375, significantly inhibit spontaneous scratching behavior resulting from DNCB treatment, suggesting that SNUV206 may exert an anti-allergic effect by reducing scratching behavior and preventing the aggravation of skin lesions. In the present study, the ear thickness was used as an indicator of edema, frequently found in the chronic AD. The ear dermal thickness in SNUV206-administered mice was lowered compared to that in mice in the PBS control group. It indicates that SNUV206 treatment improves the skin condition by decreasing edema and skin deformation.

The spleen is composed of various immune cells and plays an important role in regulating immune responses. As splenomegaly indicates abnormality of immune system function in AD[33], we measured the ratio of mice spleen and body weight. The DNCB-treated mice demonstrated significantly heavier spleens compared to the non-induced group. The enlarged spleen effect was found to be alleviated in SNUV206-treated mice, but not in SNUG134 treated mice.

In conclusion, this research clearly shows that vaginal *Lactobacillus* spp., especially vaginal *L. crispatus,* have an inhibitory effect on allergy-related response through *in vitro* assay. Moreover, vaginal *L. crispatus* SNUV206 treatment significantly improves symptoms of dermatitis in NC/Nga mice. These results indicate that SNUV206 has potential capability to achieve normal skin in AD-induced NC/Nga mice. Several previous studies have reported that the AD-like skin lesions in NC/Nga mice are comparable to those of human AD[34]. Therefore, the therapeutic effect of vaginal *L. crispatus* on AD-induced mouse could potentially translate to human subjects. SNUV206 is now under mechanical study to evaluate its mechanism of inhibitory effect on AD.

An effective compound of vaginal *L. crispatus* SNUV206 was not clarified yet due to its multifunctional probiotic properties. It is clear that vaginal *L. crispatus* interacts with several immune responses related to an allergic reaction, and its effective bioactivity is reproducible and consistent both *in vitro* and *in vivo*. So far non-specific treatments such as antihistamine and corticosteroid have been commonly used as therapies for AD. In this study, we suggest vaginal *Lactobacillus* strain SNUV206 as a specific and safe treatment for AD, to further identify its effector molecule and specify its mode of action remain to be addressed.

V. References

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VI. Supporting Information

Supplementary figure 1. Primary screening of *Lactobacillus* spp. isolated from the vagina inducing β-hexosaminidase release in RBL-2H3 cells

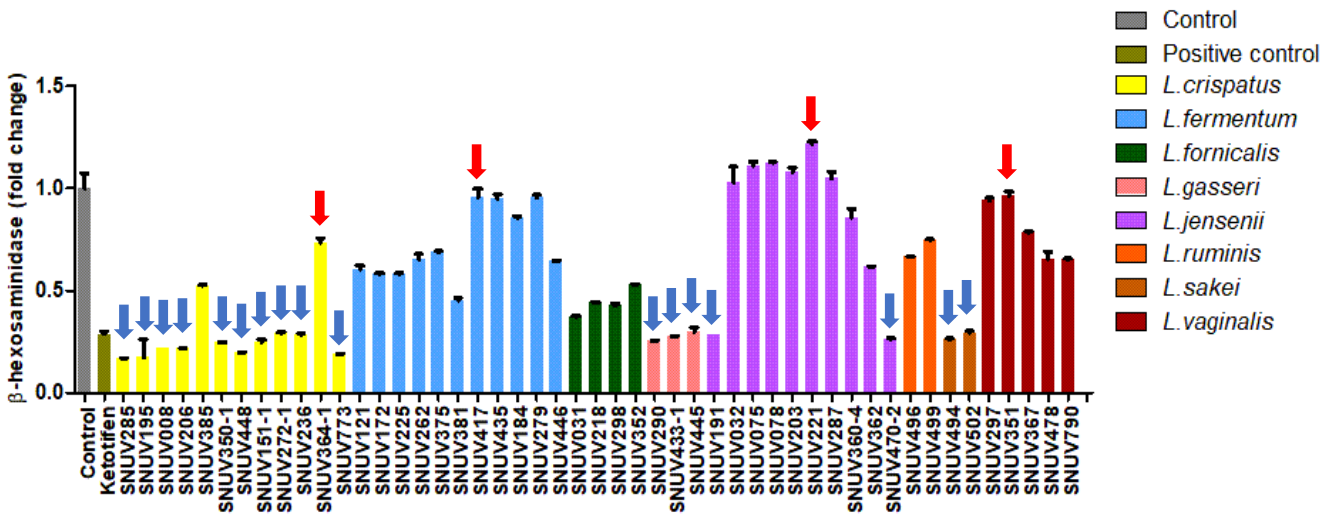
Supplementary figure 2. Body weight among the mice of experimental groups

Supplementary figure 3. The dermatitis score changes of AD-like skin lesions every week after administration of *Lactobacillus* spp.

Supplementary figure 4. Effect of *Lactobacillus* spp. on serum IgE levels of NC/Nga mice

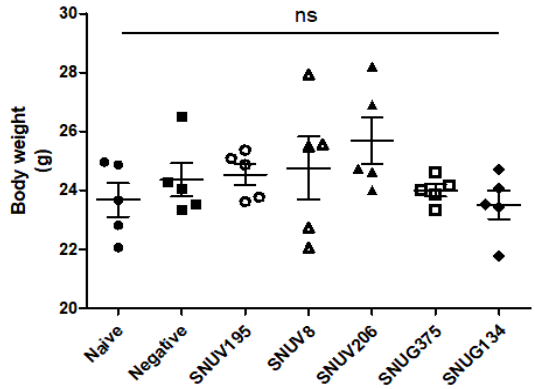
Supplementary figure 5. Effect of *Lactobacillus* spp. on lymphoid cell subtypes of NC/Nga mice

Supplementary figure 6. Effect of *Lactobacillus* spp. on the CD25 level within the CD3 CD4 T cell population of NC/Nga mice



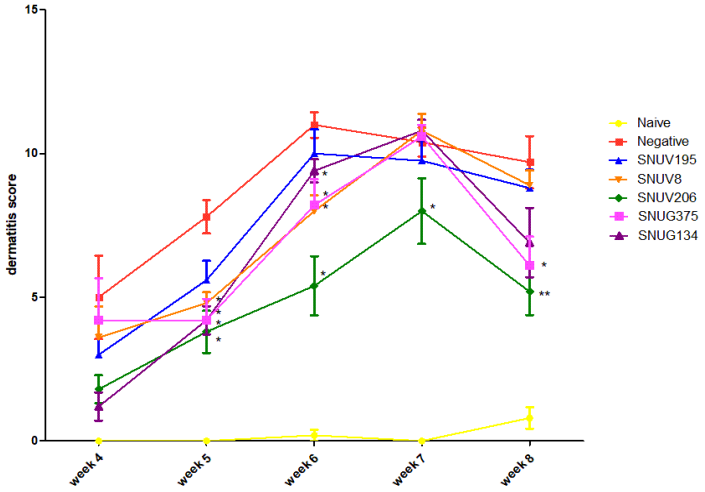
Supplementary figure 1. Primary screening of *Lactobacillus* spp. isolated from the vagina inducing β-hexosaminidase release in RBL-2H3 cells

49 *Lactobacillus* strains isolated from the vagina were tested β-hexosaminidase secretion in RBL-2H3 cells. 17 strains had stronger inhibitory effects compared to ketotifen which is positive drug control. 4 strains were selected as non-effective strain control (blue arrowheads, effective strains; red arrowheads, non-effective strains). Values are shown as mean±SD.



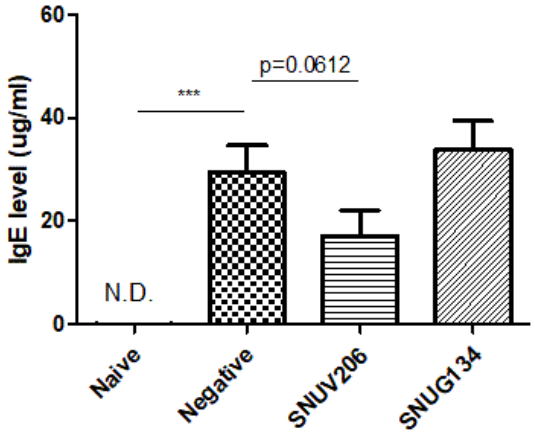
Supplementary figure 2. Body weight among the mice of experimental groups

No significant difference in body weight is observed among the experimental groups (N=5 per group), implying that administration of *Lactobacillus* spp. is physiologically safe to the NC/Nga mice.



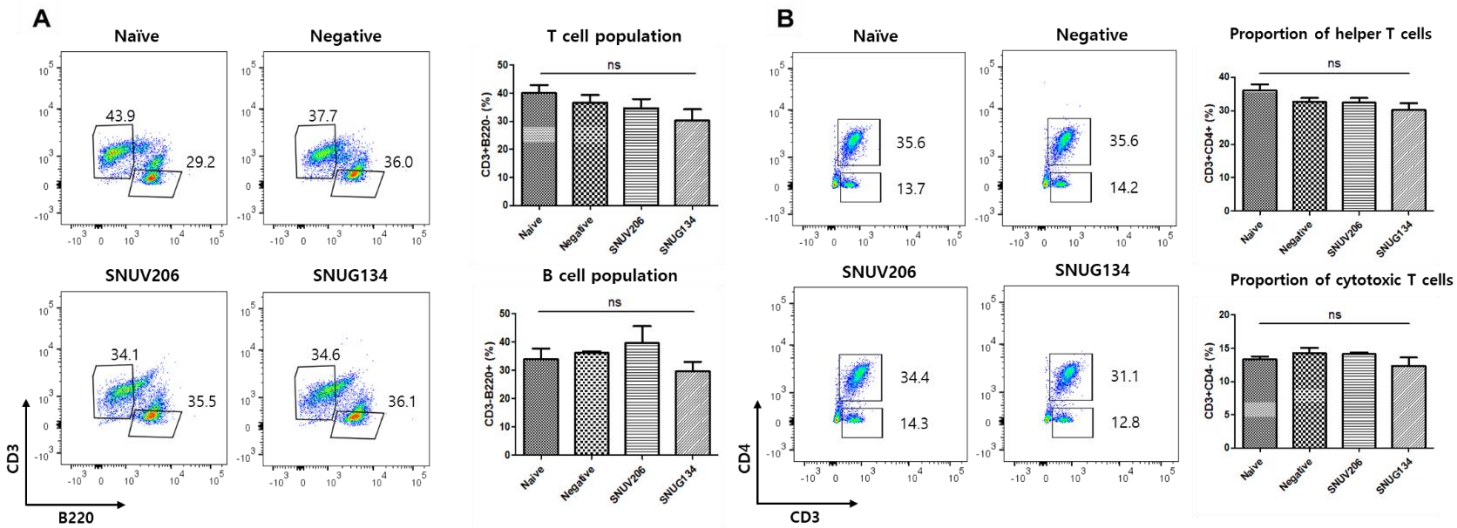
Supplementary figure 3. The dermatitis score changes of AD-like skin lesions every week after administration of *Lactobacillus* spp.

The dermatitis score of AD-like skin lesions was measured concerning erythema/hemorrhage, scarring/dryness, edema, and excoriation/erosion every week.



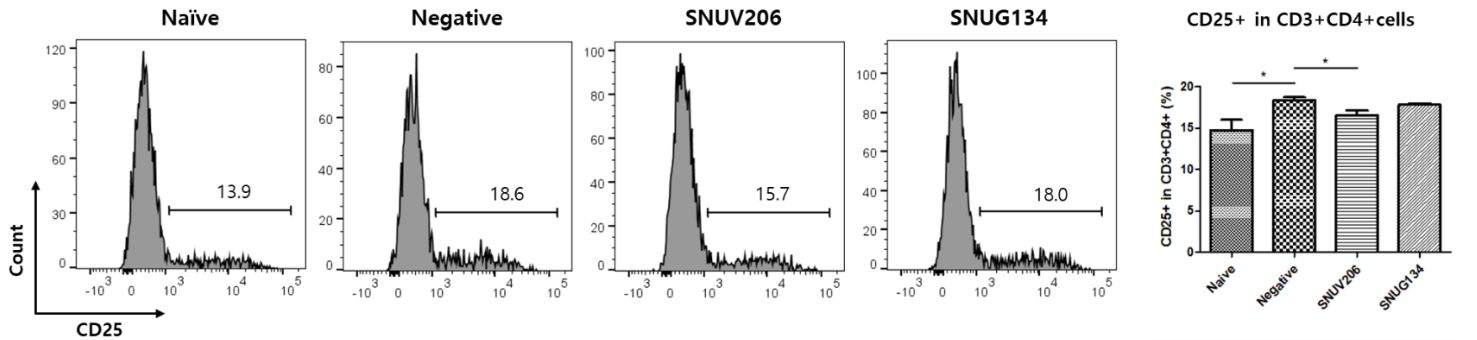
Supplementary figure 4. Effect of *Lactobacillus* spp. on serum IgE levels of NC/Nga mice

Blood was obtained from NC/Nga mice at the end of the study. IgE serum levels were determined using ELISA. SNUV206 treated group tends to be decreased compared to the negative control group in the serum IgE level. Values are shown as mean±SD. N.D not detected, *** $P < 0.001$.



Supplementary figure 5. Effect of *Lactobacillus* spp. on lymphoid cell subtypes of NC/Nga mice

Lactobacillus spp. administration did not alter the population of (A) T and B cell, (B) helper T cell, and cytotoxic T cell in the AD-induced mice. Values are shown as mean±SD. ns not significant.



Supplementary figure 6. Effect of *Lactobacillus* spp. on the number of CD25$^+$ cells among the CD3$^+$CD4$^+$ T cell population in NC/Nga mice

Percentage of CD25$^+$ cells among the CD3$^+$CD4$^+$ T cell population was investigated. The number of CD25$^+$ cells in the SNUV206 treated mice was downregulated compared to the negative group mice while the number of CD25$^+$ cells in the negative group was significantly upregulated compared to the naïve group, implying that inflammatory tone may attenuate by treatment of SNUV206 to the AD-induced mice. Values are shown as mean±SD. * $P < 0.05$.

국문초록

질 내 균총에서 유래한 락토바실러스 크리스파투스 균주의 아토피성 피부염 저해효과

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고우리

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아토피성 피부염은 피부에서 일어나는 만성 염증질환으로, 피부조직에서 염증을 일으키고 비염, 천식 등 다른 알레르기 반응이 함께 유발되는 알레르기 행진의 첫 시작으로써 유아기 때 많이 발생한다. 락토바실러스는 알레르기 질환을 예방 및 치료한다고 보고되고 있으나, 현재까지는 질 유래 락토바실러스를 이용한 알레르기 질환의 예방 및 치료에 대한 연구는 아직 보고된 바가 없다. 이 연구에서는 RBL-2H3 세포, EL4 세포, U266B1 세포 그리고 RAW 264.7 세포를 사용하여 질 유래 락토바실러스 균주의 항알레르기 효과를 실험하였다. 몇 개의 균주들이 베타-헥소스 아미니다아제 (β-hexosaminidase), 인터루킨 4 (IL-4), 인터루킨 5 (IL-5) 그리고 면역글로불린 E (IgE)의 분비를 억제하였고, 항 염증성 사이토카인인 인터루킨 10 (IL-10)의 분비를 촉진하였다. 질 유래 락토바실러스 균주의 아토피성 피부염 치료효과를 실험하기 위해 NC/Nga 마우스 모델이 사용되었다. 질 유래 락토바실러스 크리스파투스 SNUV206 균주를 처리한 마우스 그룹에서 아토피성 피부염의 여러 증상이 완화됨을 확인하였다. 결론적으로 본 연구에서는 알레르기와 관련된 면역 마커들을 측정하여 질 유래 락토바실러스의 알레르기 반응 억제 능력을 확인하였고, 동물실험을 통해 아토피성 피부염 증상 완화를 확인하여 질 유래 락토바실러스 SNUV206 균주의 아토피성 피부염 치료 가능성을 제시하였다.

주요 단어: 질 유래 균, 락토바실러스 크리스파투스, 알레르기, 아토피성 피부염