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Oral administration of *Lactococcus chungangensis* inhibits 2,4-dinitrochlorobenzene-induced atopic-like dermatitis in NC/Nga mice

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ABSTRACT

Interest is increasing in the potentially beneficial role of probiotics in the prevention and treatment of atopic diseases. In this study, we investigated the protective effects of *Lactococcus chungangensis* CAU 28^T against atopic dermatitis using murine macrophage RAW 264.7 cells, human keratinocyte HaCaT cells, human mast cell line HMC-1 cells, and a 2,4-dinitrochlorobenzene-induced atopic dermatitis model (Nc/Nga mice). The results showed that *L. chungangensis* CAU 28^T exhibited potent antiinflammatory activity by inhibiting the production of the proinflammatory mediators nitric oxide and prostaglandin E_2 in lipopolysaccharide-stimulated RAW 264.7 cells. Treatment with *L. chungangensis* CAU 28^T reduced the release of β-hexosaminidase and histamine in HMC-1 cells stimulated with mast cell activator compound 48/80. In addition, the back skin and ears of NC/Nga mice exhibited reduced histological manifestations of atopic skin lesions such as erosion, hyperplasia of the epidermis and dermis, and inflammatory cell infiltration. Oral administration of *L. chungangensis* CAU 28^T suppressed the production of IL-4, IL-5, IL-12, IFN-γ, tumor necrosis factor-α, and thymus- and activation-regulated chemokine (TARC) in skin lesions, indicating that it strongly drives the local immune system with efficacy comparable to that of tacrolimus, a topical immunomodulatory drug used for the treatment of atopic dermatitis. The findings indicate that *L. chungangensis* CAU 28^T could be a novel probiotic candidate for controlling the symptoms of atopic dermatitis.

Key words: *Lactococcus*, anti-atopic dermatitis, antiinflammatory, antiallergy

INTRODUCTION

Atopic dermatitis (**AD**) is a multifactorial inflammatory skin disease, with complex interactions of innate and adaptive immune responses, based on pharmacological, immunological, environmental, and psychological predisposition, and that is triggered by a variety of factors (Grewe et al., 1998). Atopic dermatitis induces edema, erythema, itching, skin pigmentation, thickening, eczematous lesions, and excoriation of the skin.

The disease is caused by a combination of genetic and environmental factors. The prevalence of allergy tends to be lower in families with a greater number of siblings. The incidence of allergic disease increases when one moves from a low- to a high-prevalence area and is higher in urban areas than rural areas. These observations highlight the importance of environmental factors in the development of impaired epithelial barrier functions (Williams et al., 1999).

Acute AD skin lesions reveal an important role of the type 2 immune response, characterized by the infiltration of $CD4^+$ T cells and the secretion of IL-4 and IL-5; in contrast, in chronic AD skin lesions, T-helper 1 type immune responses, characterized by the production of IL-12, IFN-γ, and tumor necrosis factor-α (**TNF-α**) by T-helper 1 cells, are involved (Werfel et al., 1996; Grewe et al., 1998; Spergel et al., 1999; Sieling et al., 2003). Thymus- and activation-regulated chemokine (**TARC**), a chemokine involved in T-helper 2 cell migration, was recently found to be closely associated with AD (Kakinuma et al., 2001; Furusyo et al., 2007). Serum TARC levels were significantly elevated in patients with AD, particularly in those severely affected by the disease, compared with patients with other inflammatory skin diseases and with healthy individuals (Hijnen et al., 2004; Tamaki et al., 2006).

Histamine is a preformed mediator that is stored primarily in the cytoplasmic granules of basophils and mast cells. It is released in response to activation of IgE receptors on these cell types or by stimulation with degranulating agents, such as complement components, neuropeptides, and cytokines. Production of IgE is

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closely related to allergic dermatitis and AD, and serum IgE levels have been reported to be elevated in allergic sensitization and AD patients compared with healthy individuals (Ogawa et al., 1971; Burrows et al., 1989).

Recently, 2,4-dinitrochlorobenzene (**DNCB**) has been used on the basis of evidence of its efficacy in inducing AD-like skin lesions and IgE hyperproduction in mice models (Kitagaki et al., 1995). 2,4-Dinitrochlorobenzene induces hemorrhages, itching, edema, scarring, dryness, and AD-like skin lesions on the face, nose, ears, neck, and back of BALB/c and NC/Nga mice (Lee et al., 2010; Choi et al., 2012).

Currently, topical corticosteroids are the main therapeutic strategy for treatment of AD; however, they can only be used for a short time and in limited skin regions because of serious adverse effects such as aggravation and recurrence of AD. Among them, tacrolimus ointment, a novel topical immunomodulatory drug, is used for the treatment of AD in both adults and children. Tacrolimus exerts its therapeutic effect on AD by inhibiting production of proinflammatory cytokines (Nasr, 2000; Reitamo et al., 2000).

Among their potential health-promoting properties, the ability of probiotic bacteria to modulate the host immune system, either by modulating the intestinal microbiota or by direct signaling, is currently an area of intense research. The beneficial role of probiotics in clinical and developmental immunology, especially *Bifidobacterium* and *Lactobacillus* strains in AD, has gained significant interest in recent years with both animal studies and human clinical trials (Isolauri et al., 2000; Isolauri, 2001; Kalliomaki et al., 2001; Abrahamsson et al., 2007; Di Felice et al., 2008; Wickens et al., 2008; Bickert et al., 2009). The probiotic therapeutic approach holds great promise for the treatment and prevention of clinical conditions associated with inflammatory and allergic responses (Meneghin et al., 2012).

Lactococci, a member of lactic acid bacteria (**LAB**), are frequently used in the manufacture of cheese and fermented food products because of their generally recognized as safe (GRAS) status. Moreover, they are assumed incapable of surviving in the gastrointestinal tract. Therefore, previous studies have shown that some lactococcal strains can tolerant a low pH and a high concentration of bile in the gastrointestinal tract (Kimoto et al., 1999, 2000, 2003). *Lactococcus lactis* exhibits antipathogen activity through production of bacteriocins and acidification of media (Parente and Ricciardi, 1999). For example, *L. lactis* C59 was found to improve cytokine balance for preventing IgE-dependent allergic diseases (Yoshida et al., 2011).

Lactococcus chungangensis CAU 28^T, a strain of nondairy origin, was isolated from activated sludge as a sixth member of the genus *Lactococcus* (Cho et al., 2008). Previously, we have reported that this strain can be applied to the manufacture of dairy products and it has been found to possess functional activities such as alcohol dehydrogenase and aldehyde dehydrogenase, and other enzyme activities such as amylase, proteinase, and lipase (Konkit et al., 2014, 2015, 2016; Konkit and Kim, 2016). However, the effect of *L. chungangensis* CAU 28^T on AD remains to be evaluated. In the present study, we evaluated the antiinflammatory, antiallergic, and anti-AD activities of *L. chungangensis* CAU 28^T in relation to the pathogenesis of AD and compared them with those of with tacrolimus, a routinely used AD drug.

MATERIALS AND METHODS

Reagents and Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (**MTT**) reagent, Griess reagent, dimethyl sulfoxide, mast cell activator compound 48/80 (**C48/80**), *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, DNCB, and hematoxylin and eosin solution were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (**DMEM**) and Iscove's modified Dulbecco's medium (**IMEM**) were purchased from Lonza (Walkersville, MD). Fetal bovine serum and gentamycin were purchased from GIBCO BRL (Grand Island, NY). Histamine assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI). Tryptic soy broth was purchased from Difco (Detroit, MI).

Bacterial Culture

Lactococcus chungangensis CAU 28^T was cultured in tryptic soy broth at 30°C for 24 h, and cell density was measured using a microplate reader (Tecan, Männedorf, Switzerland). Bacterial cells were harvested by centrifugation (5 min at 10,000 × *g*), washed twice with sterile PBS (pH 7.4), and resuspended in PBS to obtain a final concentration of 10^9 cfu/mL.

Cell Culture

Murine macrophage cell line RAW 264.7, human keratinocyte cell line HaCaT, and human mast cell line HMC-1 were obtained from the Korean Cell Line Bank (Seoul, Korea). The RAW 264.7 cells, HaCaT cells, and HMC-1 cells were grown in DMEM and IMEM containing 10% fetal bovine serum at 37°C in a water-jacketed 5% CO_2 incubator (model 3111, Thermo Fisher Scientific, Waltham, MA). The density and viability of cells

were measured by standard microscopic observations, using a hemocytometer after staining with trypan blue.

Cell Viability Assay

Cell viability was determined with 10^9 cfu/mL of *L. chungangensis* CAU 28^T. The RAW 264.7 cells and HaCaT cells at a density of 5×10^5 cells/well were seeded in 24-well plates. After 24 h, the cells were subjected to the MTT assay. Medium was replaced with 500 μL of fresh serum-free medium containing 0.5 mg/mL of MTT. After 30 min of incubation at 37°C in a 5% CO_2 incubator, the MTT reagent containing medium was removed, and the reduced formazan dye was solubilized by adding 500 μL of dimethyl sulfoxide to each well. After gentle mixing, absorbance was determined at 590 nm using a microplate reader.

Nitrite and PGE_2 Assay

The RAW 264.7 cells were plated at a density of 5×10^5 cells in 24-well cell culture plates with 1 mL of culture medium and incubated for 24 h. The cells were treated with 10^9 cfu/mL of *L. chungangensis* CAU 28^T in LPS (0.1 μg/mL) and further incubated for 24 h. The concentration of nitrite generated was measured using the Griess reagent system. Equal volumes of the culture supernatant and Griess reagent were mixed and incubated for 10 min at room temperature. Absorbance was measured at 540 nm using a spectrophotometer and compared with a nitrite standard curve to determine the concentration of nitrite in the microplate reader. The level of prostaglandin E_2 (**PGE_2**) in RAW 264.7 cell culture medium was determined using an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Absorbance was determined at 450 nm by using a microplate reader and compared with a standard curve to determine the level of PGE_2 in RAW 264.7 cell culture medium.

Measurement of β-Hexosaminidase Release and Histamine Assay

The inhibitory effects of *L. chungangensis* CAU 28^T on the release of β-hexosaminidase from HMC-1 cells were evaluated by using the following modified method (Chen et al., 2010). First, HMC-1 cells were distributed at a density of 2×10^6 cells in 48-well cell culture plates, treated with Tyrode's buffer (0.4 m*M* NaH_2PO_4, 1.8 m*M* $CaCl_2$, 2.7 m*M* KCl, 5.6 m*M* glucose, 11.9 m*M* $NaHCO_3$, 137 m*M* NaCl, pH 7.2), and incubated with *L. chungangensis* CAU 28^T (10^9 cfu/mL) for 30 min. Briefly, cells were treated with 6 μg/mL of C48/80 for 30 min. After 30 min, the degranulation reaction was terminated by placing the cells on ice. To determine the activity of β-hexosaminidase released from the cells, 20 μL of each sample was incubated with 30 μL of 1 m*M* *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide dissolved in 0.1 *M* citrate buffer, pH 4.5, in a 96-well plate at 37°C for 1 h. The reaction was terminated by adding 0.1 *M* Na_2CO_3/0.1 *M* $NaHCO_3$ buffer. Histamine levels in HMC-1 cells were measured using a histamine assay kit according to the manufacturer's instructions (Cayman Chemical Company). The absorbance was determined at 450 nm by using a microplate reader.

Animal Experiments

Female 6-wk-old NC/Nga mice were purchased from Jungang Lab Animal Inc. (Seoul, Korea) and Charles River Japan (Tokyo, Japan). Mice were kept under conventional circumstances, including a 12-h light:12-h dark cycle, and food and water were freely available. The controlled room was maintained at 22 ± 2°C with humidity of 55 ± 10% (Suto et al., 1999; Aioi et al., 2001). The NC/Nga mice were divided into 5 groups (n = 10 per group): (1) control, (2) DNCB, (3) topical application of *L. chungangensis* CAU 28^T, (4) oral administration of *L. chungangensis* CAU 28^T, and (5) topical application of tacrolimus. All groups except the control group were treated with DNCB to induce AD-like skin lesions; DNCB was applied to the dorsal skin and ears and was dissolved in an acetone:olive oil mixture (3:1, vol/vol). After complete removal of dorsal hair within an area of approximately 10 cm^2, 200 μL of 1% DNCB solution was applied 3 times per week for 2 wk. In the topical *L. chungangensis* group, *L. chungangensis* CAU 28^T (10^9 cfu/mouse) was topically applied to the dorsal skin and ears of NC/Nga mice 8 times for 4 wk. In the oral *L. chungangensis* group, *L. chungangensis* CAU 28^T (10^9 cfu/mouse) was administered orally to NC/Nga mice 8 times for 4 wk. In the final group, tacrolimus (100 mg/kg) was applied to the dorsal skin and ears of NC/Nga mice 8 times for 4 wk. All handling and care of animals, and experimental procedures were performed according to the guidelines approved by the Animal Care and Use Committee of Chung-Ang University of Korea.

Evaluation of Skin Severity

The severity of AD on the dorsal skin and ear lesions was evaluated after treatment with DNCB for 1 wk (2 times/week). Skin severity scores were defined as the sum of (1) erythema or hemorrhage, (2) dryness or scarring, (3) edema, and (4) erosion or excoriation and

was scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe). The total dermatitis score was defined as the sum of the individual scores (Kunz et al., 1997). The skin was photographed once a week.

Histopathological Studies

Tissue lesions were sliced and ear slices were fixed in 10% (vol/vol) neutral buffered formalin for 24 h. The tissue samples were embedded in paraffin and thin sections (5 μm thick) were obtained. The sections were stained with hematoxylin and eosin solution, and histopathological changes were examined by light microscopy.

Total Serum IgE

Total serum IgE levels were measured using an ELISA kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Blood was obtained from NC/Nga mice and the sera were centrifugally separated at 1,200 × g for 15 min. Absorbance was measured at 450 nm by using a microplate reader.

Reverse Transcription-PCR

The expression of mRNA transcripts for chemokines and cytokines in the ear tissue from NC/Nga mice was determined by using reverse transcription-PCR. The primers are listed in Supplementary Table S1 (http://dx.doi.org/10.3168/jds.2016-11301). The tissue was homogenized, and total RNA was isolated by using the TRI method (MRC, Cincinnati, OH), according to the manufacturer's instructions. The reverse transcription-PCR reaction mixture contained AMV-reverse transcriptase, deoxynucleotide triphosphates (Promega, Madison, WI), oligo dT primers, and diethyl pyrocarbonate-treated water; the final volume was 20 μL. The mixture was incubated at 42°C for 60 min. Thereafter, cDNA were amplified with specific primers for the genes. After 10 min of incubation at 95°C, PCR amplification was performed for 30 cycles with the following steps: denaturation at 95°C for 30 s, annealing at 55 to 65°C for 30 s, and extension at 70°C for 3 min. The PCR was performed using primers for IL-4, IL-5, IL-12, IFN-γ, TNF-α, TARC, and GAPDH. The sequences of the primers are indicated in Supplementary Table S1. The PCR products were analyzed using the GelDoc XR+ imaging system (BioRad, Hercules, CA).

Serum Levels of Chemokine and Cytokine

Serum levels of IL-4, IL-5, IL-12, IFN-γ, TNF-α, and TARC were measured using an ELISA kit (R&D Systems) according to the manufacturer's instructions. Absorbance was determined at 450 nm by using a microplate reader and was compared with a standard curve to determine the serum levels of chemokines and cytokines.

Statistical Analysis

All measurements were conducted 3 times and were expressed as the mean ± standard error (SE). Statistical analyses of the differences between samples were performed by one-way ANOVA, followed by a post hoc multiple comparison with Duncan test and t-test using the predictive analytics software (PASW) statistics package for Windows (SPSS Inc./IBM Corp., Armonk, NY). Differences at $P < 0.05$ were considered statistically significant.

RESULTS

Cytotoxicity

The RAW 264.7 and HaCaT cells were treated for 24 h with 10^9 cfu/mL of *L. chungangensis*, and cytotoxicity was assessed using the MTT assay. The MTT method is a useful alternative to radioisotopic methods for quantitating macrophage cytotoxicity for actively growing in vitro targets (Ferrari et al., 1990). The MTT assay measures the mitochondrial activity of cells, which is considered indicative of cell viability. *Lactococcus chungangensis* CAU 28^T did not exert cytotoxic effects on RAW 264.7 and HaCaT cells, as indicated by the MTT assay after 24 h of incubation (Supplemental Figure S1; http://dx.doi.org/10.3168/jds.2016-11301).

Effects on LPS-Induced Production of NO and PGE_2

The inhibitory effects of *L. chungangensis* CAU 28^T on the production of nitrite were comparatively evaluated by measuring the levels of nitrite, a stable metabolite of NO, and PGE_2 in LPS-stimulated RAW 264.7 cells. Levels of NO and PGE_2 in the cell supernatants were measured by using the Griess assay and ELISA. *Lactococcus chungangensis* CAU 28^T effectively suppressed LPS-induced NO production. Consistent with this observation, *L. chungangensis* CAU 28^T markedly inhibited PGE_2 production. The NO level in LPS-stimulated RAW 264.7 cells exposed to 10^9 cfu/mL of *L. chungangensis* CAU 28^T was 30.7 ± 6.4% of the control (Figure 1A). Further, PGE_2 production in cells exposed to 10^9 cfu/mL of *L. chungangensis* CAU 28^T was 46.8 ± 6.1% of the control (Figure 1B). These results showed that *L. chungangensis* CAU 28^T exerted

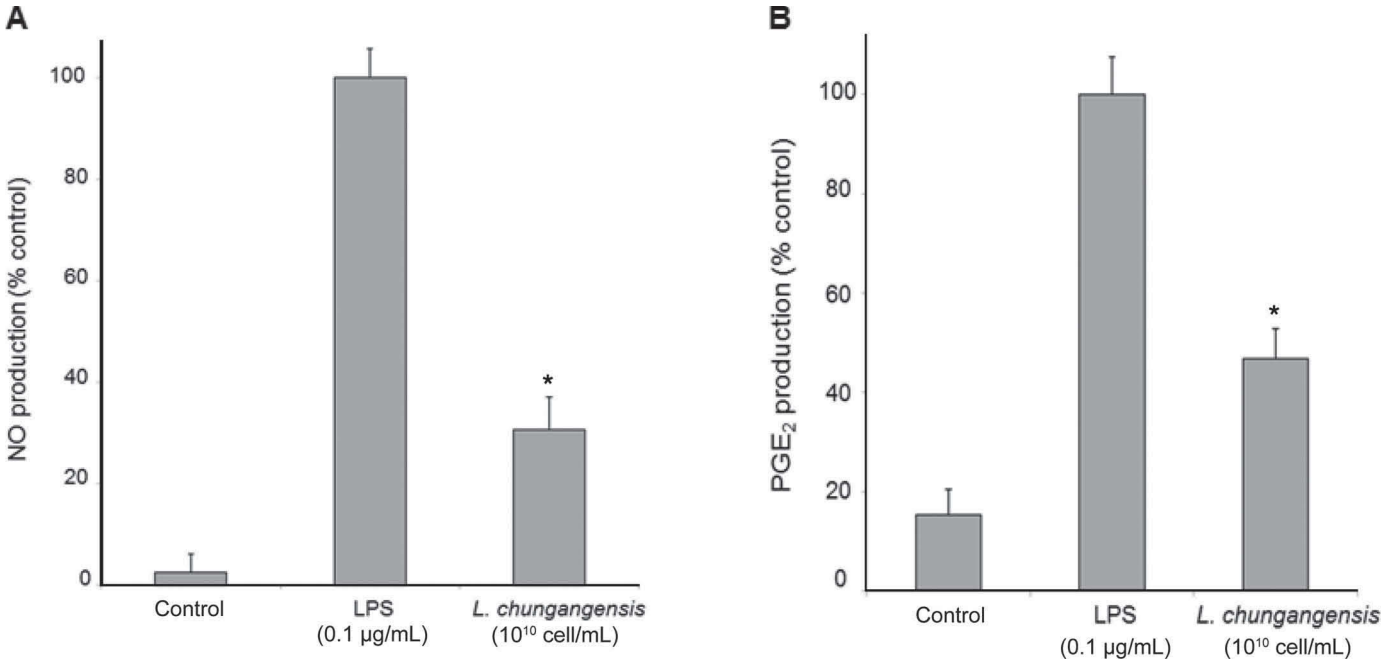

Figure 1. Suppression of LPS-induced production of (A) NO and (B) prostaglandin E_2 (PGE_2) by *Lactococcus chungangensis*, assessed in the supernatant of RAW 264.7 cells. Error bars represent standard errors. $^*P < 0.05$: *L. chungangensis* treatment compared with LPS (panels A and B).

an antiinflammatory effect by ameliorating the production of the inflammatory mediators NO and PGE_2.

Effects on β-Hexosaminidase and Histamine Release

The release of β-hexosaminidase was used as a marker of mast cell degranulation to evaluate the antiallergic activity of *L. chungangensis* CAU 28^T on C48/80-stimulated HMC-1 cells. The HMC-1 cells (1×10^6) and HMC-1 cells pretreated with *L. chungangensis* CAU 28^T (at the indicated concentrations) for 30 min were challenged with C48/80 (5 μg/mL). The release of β-hexosaminidase was measured as a percentage of the control, and results are shown in Figure 2A. The C48/80-stimulated HMC-1 cells treated with 10^9 cfu/mL of *L. chungangensis* CAU 28^T showed a release of β-hexosaminidase at a level 35.1 ± 1.0% of the control. Thus, *L. chungangensis* CAU 28^T inhibited the release of β-hexosaminidase (i.e., degranulation) from C48/80-induced HMC-1 cells.

Histamine is the best-known pruritogen and is regarded as a primary target for antipruritic therapies. Similar to the inhibitory effect on β-hexosaminidase release, incubation with *L. chungangensis* CAU 28^T (10^9 cfu/mL) for 30 min before challenge with C48/80 significantly inhibited ($P > 0.05$) the release of histamine. As shown in Figure 2B, the release of histamine from C48/80-stimulated HMC-1 cells exposed to 10^9 cfu/mL of *L. chungangensis* CAU 28^T was 19.0 ± 6.9% of that of the control, indicating that the allergic responses were effectively diminished by *L. chungangensis*.

Suppression of DNCB-Induced AD-Like Skin Dermatitis in NC/Nga Mice

The NC/Nga mice (n = 50) were allocated to each of 5 groups (n = 10/group): control group, DNCB group, topical application of *L. chungangensis* CAU 28^T group, oral administration of *L. chungangensis* CAU 28^T group, and topical application of tacrolimus group. All groups except the control group were treated with DNCB to induce AD-like skin lesions. The DNCB was applied to the dorsal skin and ears. Repeated topical application of DNCB significantly increased AD-like skin symptoms such as hemorrhage, edema, scarring, dryness, and erosion in NC/Nga mice (Figure 3). Repeated topical application of DNCB significantly increased the dermatitis scores and the thickness of the skin or ear in the DNCB-treated group compared with those of the control group. Oral administration of *L. chungangensis* CAU 28^T significantly reduced the severity of DNCB-induced AD-like skin lesions and the thickness of the skin or ear (Figure 4A). The AD-like skin lesions of NC/Nga mice were healed with oral administration of *L. chungangensis* CAU 28^T, which were similar to that observed with topical application of tacrolimus, relative

to the control group (Figure 4B). In addition, topical application of *L. chungangensis* CAU 28^T resulted in slight reduction of AD symptoms compared with that of the DNCB group. These results demonstrate that *L. chungangensis* CAU 28^T was able to decrease AD-like skin symptoms in NC/Nga mice.

Suppression of Hyperproduction of IgE

Although AD has various definitions, it is generally defined by the presence of elevated levels of total and allergen-specific IgE in the serum, leading to positive skin-prick test reactions to common allergens. To examine the effect of *L. chungangensis* CAU 28^T on production of IgE in AD-induced NC/Nga mice, we performed ELISA to measure serum IgE levels as a diagnostic marker of AD. As shown in Figure 5, serum IgE levels in the DNCB group were significantly increased ($P < 0.05$) compared with that of the control group, implying that AD and immune system responses were successfully induced. The IgE level in NC/Nga mice subjected to oral administration of *L. chungangensis* CAU 28^T was 16.46 pg/mL, a level similar to that observed with topical application of tacrolimus, which was 14.72 pg/mL. These results indicated that *L. chungangensis* CAU 28^T was effective in suppressing IgE hyperproduction, and that the level of inhibition was equivalent to that of a commercial AD treatment.

Inhibition of mRNA Expression of Chemokines and Cytokines in Mouse Ear Tissue

The mRNA expression levels of IL-4, IL-5, IL-12, IFN-γ, TNF-α, and TARC were determined in the control and treatment groups. As shown in Figure 6, mRNA levels of IL-4, IL-5, IL-12, IFN-γ, TNF-α, and TARC were enhanced after DNCB treatment in ear tissue of NC/Nga mice. Oral administration of *L. chungangensis* CAU 28^T and topical application of tacrolimus dramatically suppressed mRNA expression of IL-4, IL-5, IL-12, IFN-γ, TNF-α, and TARC. The expression levels in NC/Nga mice subjected to oral administration of *L. chungangensis* CAU 28^T were similar to that of mice treated with topical application of tacrolimus.

Inhibition of Production of Chemokines and Cytokines

IL-4, IL-5, and IL-12. *Lactococcus chungangensis* CAU 28^T has the ability to inhibit production of chemokines and cytokines. The IL-4 level in NC/Nga mice subjected to oral administration of *L. chungangensis* CAU 28^T was 46.87 pg/mL, which was similar to that obtained with topical application of tacrolimus (44.78 pg/mL). This level was lower than that observed with topical application of *L. chungangensis* CAU 28^T and DNCB (Figure 7A). Further, the levels of IL-5 and IL-12 in NC/Nga mice orally administered with *L.*

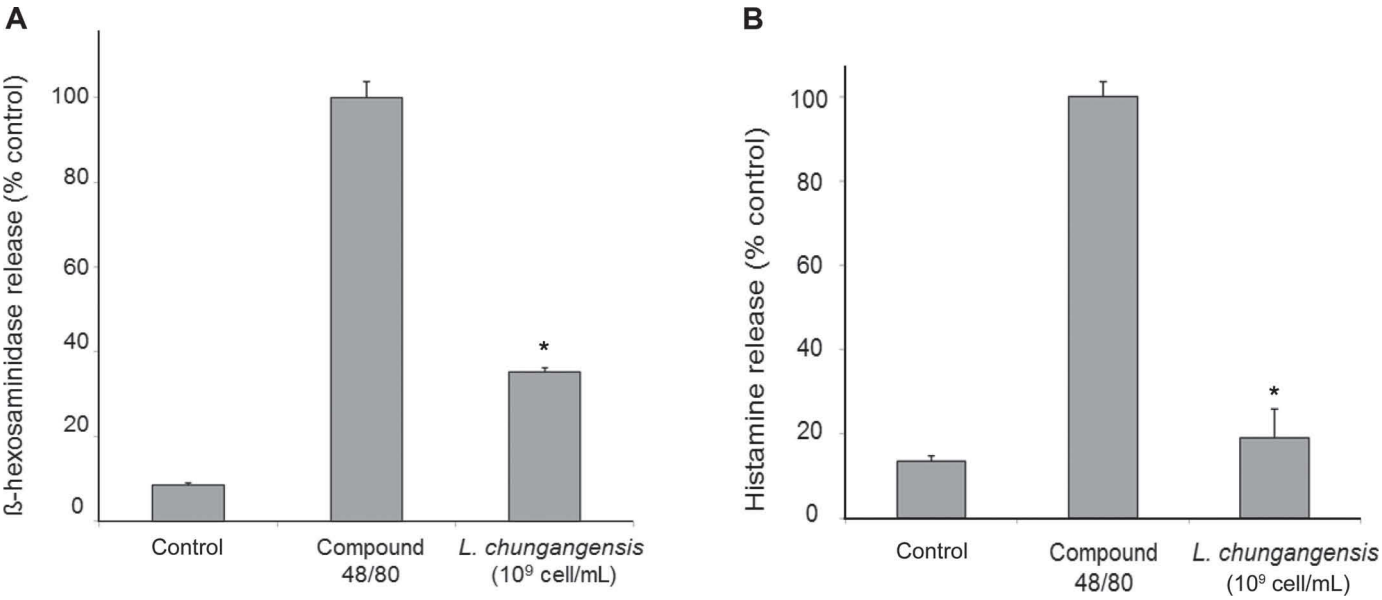

Figure 2. Inhibitory effect of *Lactococcus chungangensis* CAU 28^T on the release of (A) β-hexosaminidase and (B) histamine, assessed in the supernatant of mast cell activator compound 48/80-induced HMC-1 cells. Error bars represent standard errors. *$P < 0.05$: *L. chungangensis* treatment compared with compound 48/80 (panels A and B).

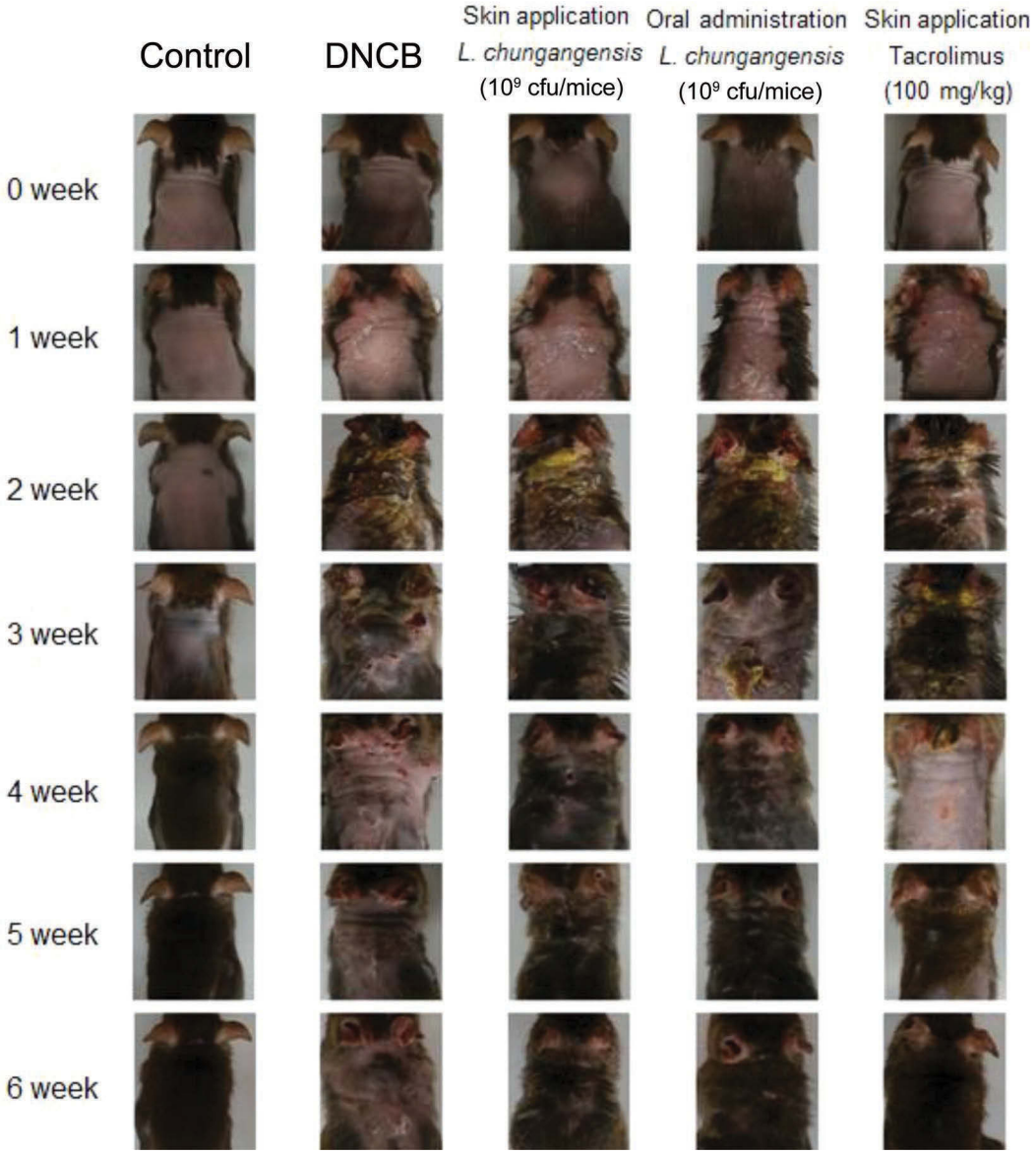

Figure 3. Effects of *Lactococcus chungangensis* on the symptoms of atopic dermatitis induced by 2,4-dinitrochlorobenzene (DNCB) in NC/Nga mice (a mouse model cell line for atopic dermatitis). Color version available online.

chungangensis CAU 28^{T} were 106.36 and 41.55 pg/mL, respectively. These levels were similar to those resulting from topical application of tacrolimus (98.40 and 40.13 pg/mL, respectively). In addition, the levels of IL-5 and IL-12 after oral administration of *L. chungangensis* CAU 28^{T} and topical application of tacrolimus were lower than those with topical application of *L. chungangensis* CAU 28^{T} and DNCB (Figure 7B and 7C).

IFN-γ, TNF-α, and TARC. The levels of IFN-γ, TNF-α, and TARC after oral administration of *L. chungangensis* CAU 28^{T} and topical administration of tacrolimus were lower than those after topical application of *L. chungangensis* CAU 28^{T} and DNCB. The IFN-γ level in NC/Nga mice subjected to oral administration of *L. chungangensis* CAU 28^{T} was 98.67 pg/mL, which is similar to that with topical application of tacrolimus (93.4 pg/mL) (Figure 7D). Furthermore, the levels of TNF-α and TARC in NC/Nga mice orally administered with *L. chungangensis* CAU 28^{T} were 354.81 and 36.04 pg/mL, respectively. These levels were similar to those with tacrolimus topical application, which were 351.26 and 33.58 pg/mL, respectively (Figure 7E and 7F).

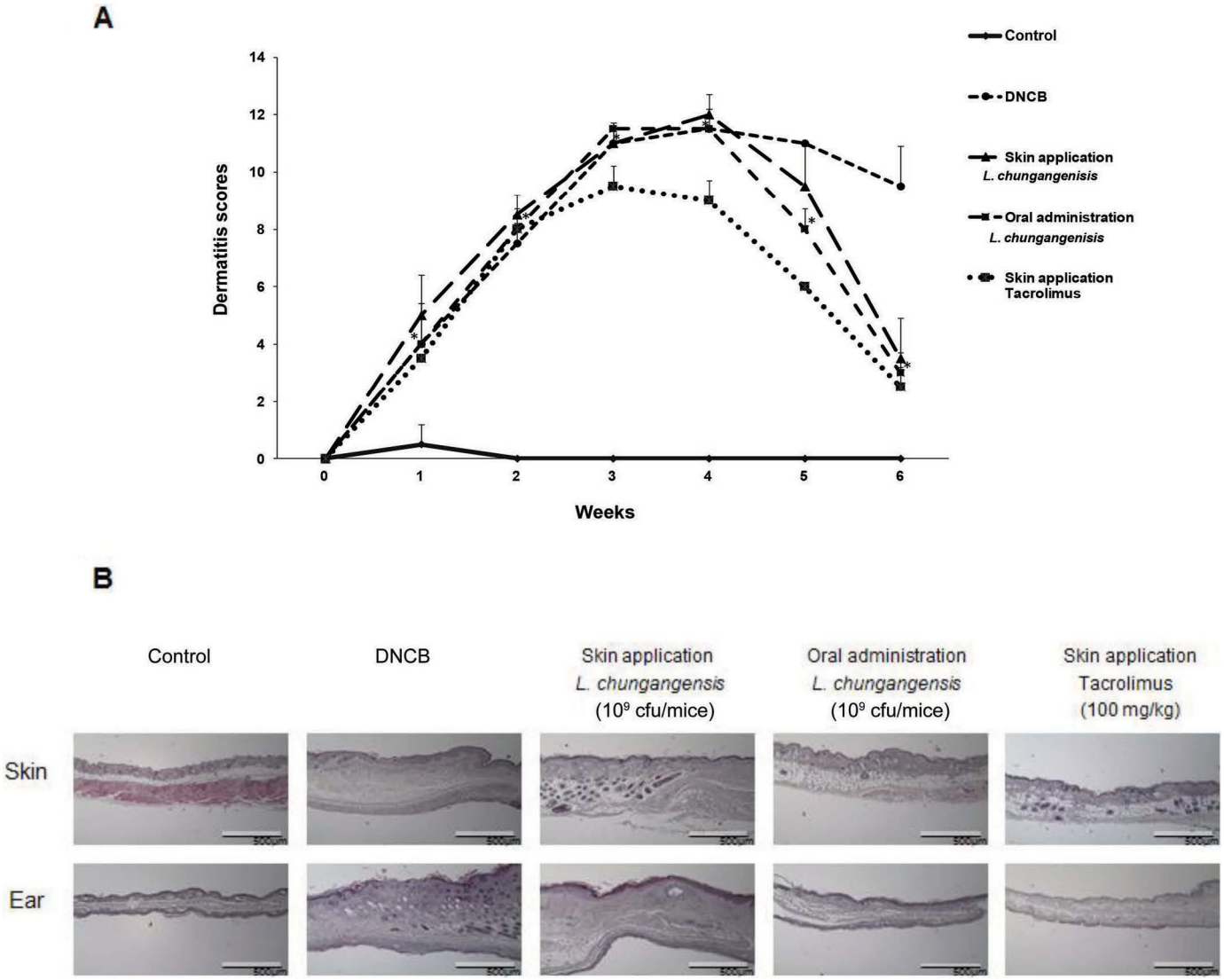

Figure 4. Inhibitory effects of *Lactococcus chungangensis* CAU 28^{T} on 2,4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis-like skin symptoms in NC/Nga mice (a mouse model cell line for atopic dermatitis). The dermatitis score was defined as the sum of the scores for 7 clinical criteria: erythema, hemorrhage, dryness, scarring, edema, erosion, and excoriation (A); histopathological changes were examined by light microscopy (B). Error bars represent standard errors. $^{*}P < 0.05$: oral administration of *L. chungangensis* compared with DNCB or skin applications of *L. chungangensis* or tacrolimus. Color version available online.

DISCUSSION

Probiotics are live microbial food supplements that are beneficial to human health by maintaining or improving their intestinal microbial balance (Fuller, 1989). Because of these health benefits, the application of probiotic bacteria has increased in dairy products such as yogurt and fermented milk (Saarela et al., 2000). Probiotic bacteria strains must survive in foods to reach the human gastrointestinal system and to further modify gut microbiota. Moreover, an interesting aspect of probiotic bacteria is that they can interact with the host mucosa and may beneficially modulate the immune system (Ziemer and Gibson, 1998; Naidu et al., 1999).

Currently, most probiotic strains belong to the genera *Lactobacillus* and *Bifidobacterium* (Smart et al., 1993; Prasad et al., 1998; Grill et al., 2000). However, *Lactococcus*, *Enterococcus*, *Saccharomyces* (Salminen and von Wright, 1998; Kim, 2014), and *Propionibacterium* (Grant and Salminen, 1998) are also considered as probiotic genera. The LAB exhibit antipathogenic activity through production of bacteriocins and acidification of media (Klaenhammer, 1988; De Vuyst and Leroy, 2007). *Lactococcus* are LAB that are usually used as starter cultures for the manufacture of cheese and other

fermented milk products. They have been assumed to be probiotics because they are capable of surviving in the gastrointestinal tract (Kimoto et al., 1999).

Lactococcus chungangensis CAU 28^T is the sixth member of the genus *Lactococcus* and has been found to contain potential functional genes relevant to the dairy industry (Konkit et al., 2014). In addition to genes that are useful for dairy products, *L. chungangensis* CAU 28^T also exhibits alcohol and aldehyde dehydrogenase activity, and reduces blood alcohol and aldehyde levels in mice (Konkit et al., 2015, 2016). However, the anti-AD, antiinflammatory, and antiallergy effect of *L. chungangensis* CAU 28^T has not been reported previously.

Atopic dermatitis is an inflammatory skin disease provoked by an imbalance between Th1 and Th2 immune response, and it is one of the most common skin lesions in pediatric patients, with a genetic predisposition (Uehara and Kimura, 1993; Stemmler et al., 2007). Atopic dermatitis induces edema, erythema, itching, skin pigmentation, thickening, eczematous lesions, and excoriation of the skin. These symptoms may be accompanied by sleep disturbance and depression and can affect the quality of life (Kiebert et al., 2002; Spergel and Paller, 2003). In addition, AD is frequently associated with food allergy, which complicates its management in approximately 40% of children (Eigenmann et al., 1998), and it is related to the hyperresponsiveness of lymphocytes to allergens. Furthermore, mast cell degranulation and production of histamine and IgE are closely linked with allergic reactions (Theoharides et al., 2012).

Generally, corticosteroid treatment (creams, ointments, or injections) has been used extensively because it is the most effective therapy for AD (Nakagawa, 2006). Despite their rapid and proven efficiency, corticosteroids can cause various side effects when applied for a long period, such as thinning of the skin, stretch marks, epidermal barrier dysfunction, and immunosuppression (Smith, 2000; Jensen et al., 2011; Berke et al., 2012). Tacrolimus is an example of a topical immunomodulatory ointment used for the treatment of AD in both adults and children (Kang et al., 2001). Tacrolimus exerts its therapeutic effect by inhibiting the production of proinflammatory cytokines (Nasr, 2000; Reitamo et al., 2000). In addition, tacrolimus has been reported to cause side effects such as headache, paresthesia, tremor, tinnitus, photophobia, blurred vision, gastrointestinal upset, nausea, vomiting, hyperkalemia, hypertension, and hyperuricemia (Atkison et al., 1995). This highlights the urgent need to develop new therapies that target the underlying causes of AD.

This study investigated the antiinflammatory, antiallergy, antibacterial, and anti-AD effects of *L.*

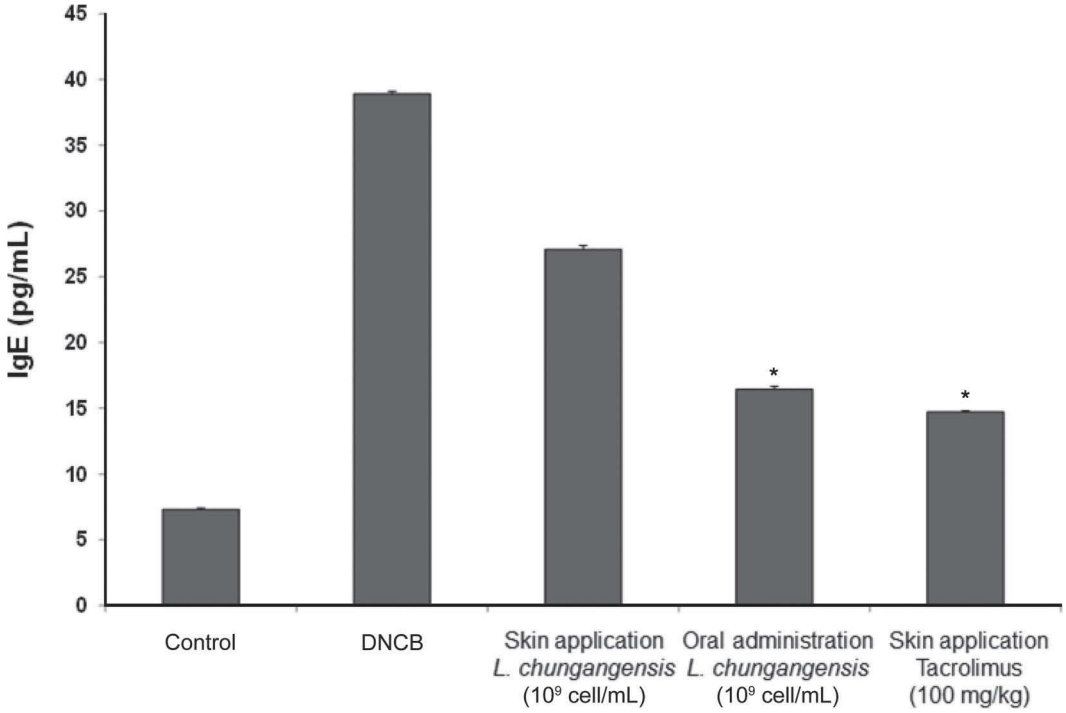

Figure 5. Inhibitory effects of *Lactococcus chungangensis* CAU 28^T on 2,4-dinitrochlorobenzene (DNCB)-induced serum levels of IgE in NC/Nga mice (a mouse model cell line for atopic dermatitis). Error bars represent standard errors. $^*P < 0.05$: oral administration of *L. chungangensis* or skin application of tacrolimus compared with skin application of *L. chungangensis* or DNCB.

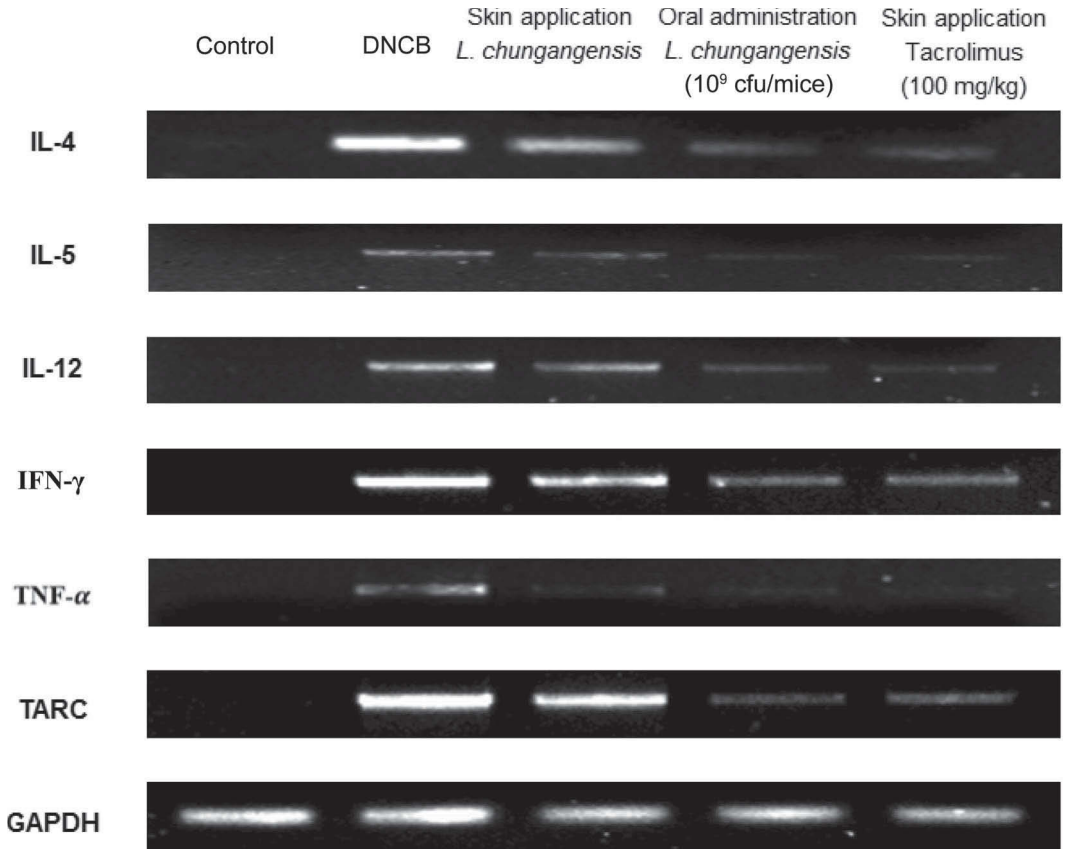

Figure 6. The inhibitory effects of *Lactococcus chungangensis* CAU 28^{T} on the mRNA expression of chemokines and cytokines in the ear tissue of NC/Nga mice (a mouse model cell line for atopic dermatitis). TNF-α = tumor necrosis factor-α; TARC = thymus- and activation-regulated chemokine.

chungangensis CAU 28^{T} in murine macrophage cell line RAW 264.7, human keratinocyte cell line HaCaT, human mast cell line HMC-1, and a murine model of DNCB-induced AD. Furthermore, NC/Nga mice were used because of their ability to exhibit characteristics similar to human AD in term of increased serum levels of IgE, chemokines, and cytokines, chronic dryness, and severe pruritus (Jin et al., 2009). After 24 h of incubation, cytotoxicity of *L. chungangensis* CAU 28^{T} was not observed. *Lactococcus chungangensis* CAU 28^{T} has shown potential antiinflammatory activity by inhibiting the production of NO and PGE_2, which are proinflammatory mediators, in LPS-stimulated macrophage RAW 264.7 cells (Grahames et al., 1999). In addition, *L. chungangensis* CAU 28^{T} inhibits the release of β-hexosaminidase and histamine during mast cell degranulation (Cheong et al., 1998).

In particular, the effect of oral administration of *L. chungangensis* CAU 28^{T} was similar to that of topical application of tacrolimus. Oral administration of *L. chungangensis* CAU 28^{T} suppressed the production of IL-4, IL-5, IL-12, IFN-γ, TNF-α, and TARC in skin lesions, which are local immune responses that strongly drive AD. This result is consistent with previous findings (Takahashi et al., 2006; Thomas et al., 2011), which found that the level of IL-4 was significantly lower in mice treated with probiotics than in untreated mice, and that the secretion of proinflammatory cytokines IFN-γ, TNF-α, and IL-12 was reduced.

Most studies have suggested that LAB strains exert an antiallergic effect by regulating the Th1–Th2 balance by inducing IL-12 production. On the other hand, some strains have the potential to suppress antigen-specific IgE production by inducing regulatory T cells (Ohno et al., 2005; Torii et al., 2007; Nonaka et al., 2008). Consequently, LAB strains have great potential in targeting allergic diseases.

CONCLUSIONS

The present findings indicate that *L. chungangensis* CAU 28^{T} is a novel therapeutic candidate for an anti-atopic agent to reduce AD symptoms. Moreover, the results strongly showed that *L. chungangensis* CAU 28^{T}

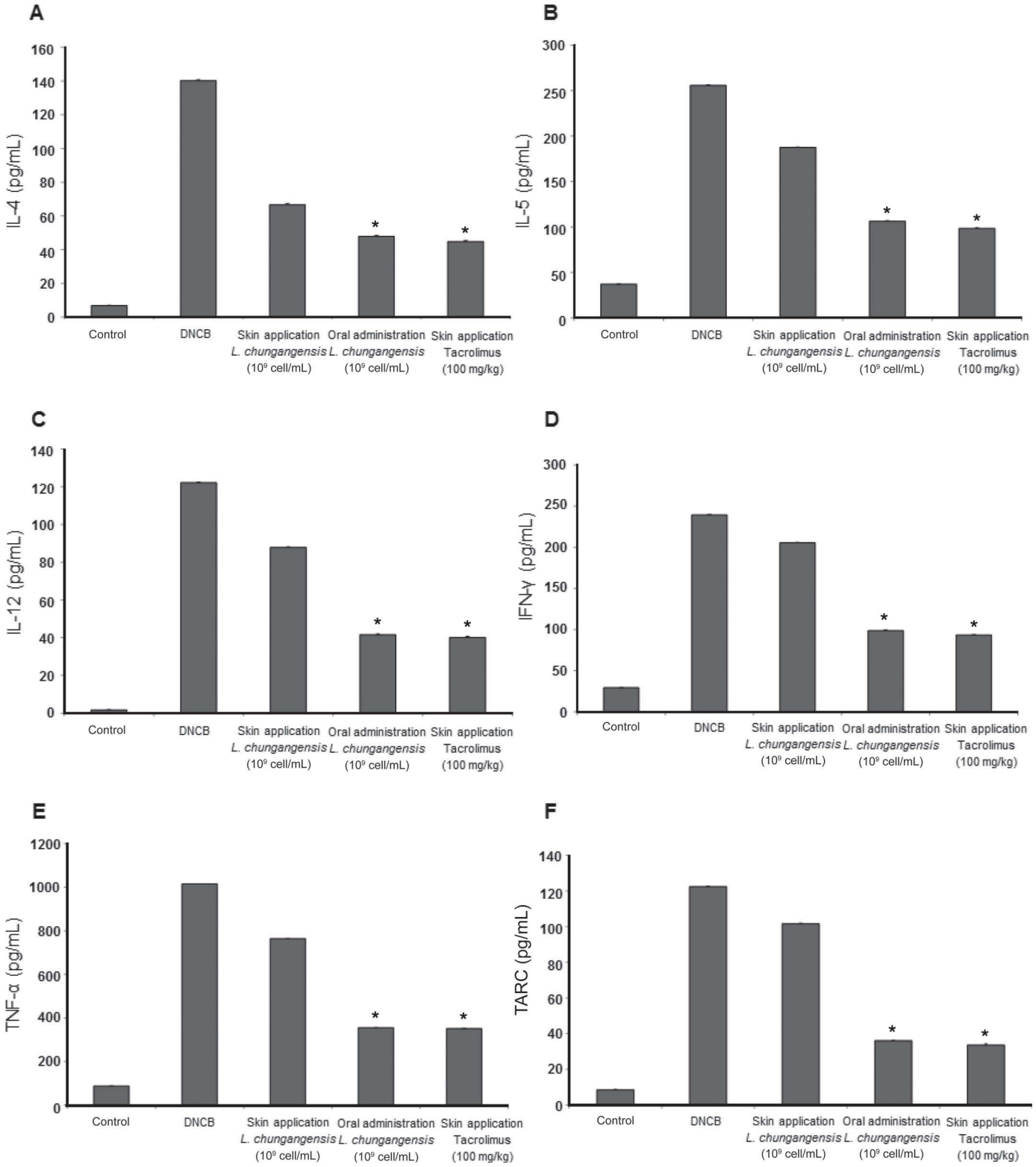

Figure 7. Inhibitory effects of *Lactococcus chungangensis* CAU 28^{T} on 2,4-dinitrochlorobenzene (DNCB)-induced production of chemokines and cytokines measured in serum obtained from NC/Nga mice (a mouse model cell line for atopic dermatitis): (A) IL-4, (B) IL-5, (C) IL-12, (D) IFN-γ, (E) tumor necrosis factor-α (TNF-α), and (F) thymus- and activation-regulated chemokine (TARC). Error bars represent standard errors. $^{*}P < 0.05$: oral administration of *L. chungangensis* or skin application of tacrolimus compared with skin application of *L. chungangensis* or DNCB.

could inhibit the release of allergy-associated proteins, highlighting its potential as a therapeutic for the prevention of allergic reactions and diseases. Further studies are needed to establish the specificity of this activity and other probiotic benefits in functional dairy foods.

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Supplementary Table S1. Primer sequences used in the RT-PCR

Gene	Primer Sequences (5′–3′)
IL-4	TCAACCCCCAGCTAGTTGTCA
	CATCGAAAAGCCCGAAAGAG
IL-5	AGCACAGTGGTGAAAGAGACCTT
	TCCAATGCATAGCTGGTGATTT
IL-12	GACACGCCTGAAGAAGATGAC
	CGCCATTCCACATGTCACTGC
IFN-γ	AACGCTACACACTGCATCT
	GAGCTCATTGAATGCTTGG
TNF-α	CAGGCGGTGCCTATGTCTC
	CGATCACCCCGAAGTTCAGTAG
TARC	CAGGAAGTTGGTGAGCTGGTATA
	TTGTGTTCGCCTGTAGTGCATA
GAPDH	AAGCTGTGGCGTGATGGCCG
	TGGGCCCTCAGATGCCTGCT

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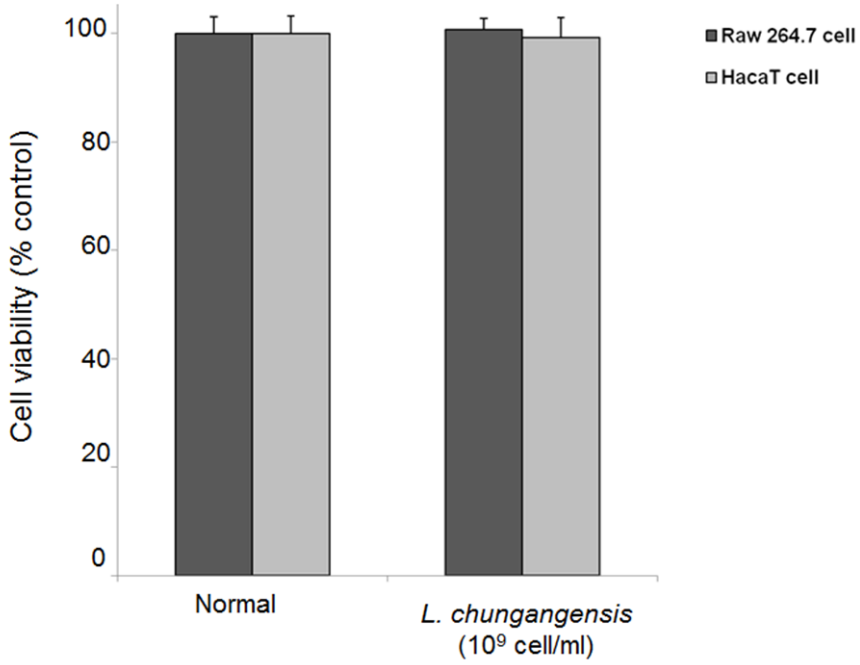
Supplemental Figure S1. The cytotoxicity of RAW 264.7 cells and HaCaT cells treated with *L. chungangensis*.