

ORIGINAL ARTICLE

Atopic dermatitis-mitigating effects of new *Lactobacillus* strain, *Lactobacillus sakei* probio 65 isolated from KimchiJ.-Y. Kim¹, B.-K. Park¹, H.-J. Park¹, Y.-H. Park², B.-O. Kim³ and S. Pyo¹¹ School of Pharmacy, Sungkyunkwan University, Suwon, Korea² Department of Applied Microbiology, Yeungnam University, Gyeongsan, Korea³ Department of Applied Biology, Kyungpook National University, Sangju, Korea**Keywords**atopic dermatitis, chemokine, cytokine, IgE, *Lactobacillus sakei*.**Correspondence**Suhkneung Pyo, School of Pharmacy,
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Abstract**Aims:** Atopic dermatitis (AD) is an inflammatory skin disease. Probiotics have been reported to modulate immune responses and thus are now being suggested as potential treatments for allergies. In this study, we investigated the inhibitory effects of *Lactobacillus sakei* probio 65 isolated from Kimchi on artificially inducing AD in NC/Nga mice.**Methods and Results:** Oral administration of viable or heat-inactivated *Lact. sakei* probio 65 improved the condition of skin and reduced scratching frequency. Serum levels of IgE and cutaneous T-cell-attracting chemokine (CTACK) were significantly decreased by this therapy. Dead *Lact. sakei* probio 65 also decreased IL-4 and IL-6 serum concentrations. Moreover, both live and dead *Lact. sakei* probio 65 inhibited the expression of Thymus and activation-regulated chemokine and CTACK in AD-like skin lesions. The increased levels of Foxp3 expression in the lesional skin and ears were also suppressed by *Lact. sakei* probio 65. In addition, *Lact. sakei* probio 65 inhibited β -hexosaminidase release and the secretion of IL-4, TNF- α and IL-6 from RBL-2H3 cells.**Conclusions:** Oral treatment with both viable and heat-inactivated *Lact. sakei* probio 65 inhibits skin inflammation and AD-like skin lesions, as well as mast cell activation.**Significance and Impact of the Study:** *Lactobacillus sakei* probio 65 has an inhibitory effect on atopic dermatitis-like skin lesions and may represent an effective new anti-inflammatory agent.**Introduction**

Atopic dermatitis (AD) is a chronic inflammatory skin disorder often characterized by erythematous eruption, associated pruritus and/or chronic relapsing eczematous lesions (Leung and Bieber 2003). In early AD, the skin barrier is disrupted and/or damaged by pruritus-induced scratching. This type of mechanical injury increases the production of proinflammatory cytokines, which activates various immune cells, ultimately initiating the AD inflammatory cycle (Kang *et al.* 2007).

Allergic diseases like AD are characterized by Type 2 helper T-cell-dominated responses. Th2 cells mediate immunoglobulin E (IgE) production through the release

of cytokines and other chemical mediators (Karki *et al.* 2012). Specifically, Th2 cells produce interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-10 (IL-10) and interleukin-13 (IL-13), all of which relate to specific immune cell functioning (Romagnani 1991). For example, IL-4 plays an important role in IgE isotype switching within B cells and also promotes the differentiation of naïve T cells into allergic-type Th2 cells (Joo *et al.* 2009; Karki *et al.* 2012). Mast cells, which are present in all epithelial barriers, also play a significant role in AD through the secretion of large quantities of cytokines, including IL-4, IL-5, IL-6 and TNF- α (Han *et al.* 2009). In addition to various cytokines, chemokines, including the predominant Th2 chemokines, have a crucial role in regulating the infiltration of

inflammatory cells into skin lesions in AD (Nakazato *et al.* 2008). Thymus and activation-regulated chemokine (TARC)/CCL17 and cutaneous T-cell-attracting chemokine (CTACK)/CCL27 are two skin-associated chemokines exclusively produced by epidermal keratinocytes that are critical in T-cell migration to the skin and allergen-specific skin inflammation (Homey *et al.* 2006). New research evaluating the role of regulatory T cells (Treg) in AD shows that Treg cells are characterized by the dominant induction of forkhead (winged helix) transcription factor forkhead box p3 (Foxp3) and thus likely control immune tolerance by balancing the immune response in inflammation (Lee and Cho 2011). In the setting of AD, Treg cells regulate Th2 response to allergens and maintain functional tolerance (Larché 2007).

Probiotics are generally defined as live microbial food supplements that are thought to confer health benefits (Furrie 2005). Previous studies have demonstrated that probiotics may prevent antibiotic-associated diarrhoea, pouchitis and atopic disease in children and may have a therapeutic role in acute infectious diarrhoea in children (Gill and Guarner 2004). Interestingly, probiotics composed of dead organisms may also produce similar pharmacologic effects. In this way, probiotics consisting of live cells, dead cells or even the resulting microbial metabolites may help prevent inflammatory bowel disease, acute infectious diarrhoea and allergic disease (Adams 2010). Currently, the most commonly used probiotics are lactobacilli and bifidobacteria. Of *Lactobacillus* species, *Lactobacillus sakei* has been shown to exert potent inhibitory activity against *Staphylococcus aureus*, a pathogenic organism frequently present on lesional atopic skin which secretes proteins that promote lymphocyte activation (Park *et al.* 2008; Woo *et al.* 2010). Emerging evidence now suggests that *Lact. sakei* probio 65 isolated from Kimchi – a traditional Korean fermented vegetable dish – may suppress serum levels of IgE and reduce splenocytes IL-4 secretion and skin inflammation in mice (Park *et al.* 2008). In this study, we investigated the inhibitory effects of viable and heat-inactivated *Lact. sakei* probio 65 on cutaneous allergic response both *in vivo* and *in vitro*.

Materials and methods

Animals and reagents

Twenty-five male 6-week-old NC/Nga mice were purchased from SLC Inc. (Shizuoka, Japan) and kept in standard plastic cages in a controlled environment at a temperature of $23 \pm 1^\circ\text{C}$, humidity of $55 \pm 5\%$, 10 to 18 circulations per hour and a 12 h light/dark cycle. All mice were supplied with a basal diet and sterilized water

without any restrictions during the experiment. The animals were randomly divided into five groups: a normal control group (group 1), a vehicle-treated group (group 2), a dexamethasone-treated group (group 3), a live *Lact. sakei* probio 65-treated group (group 4) and a dead *Lact. sakei* probio 65-treated group (group 5). Each experimental group consisted of five mice. The animal experiment was conducted one time. All research described here adheres to the animal facility guidelines of Sungkyunkwan University. All reagents were purchased from Sigma-Aldrich Co. (St Louis, MO, USA) unless otherwise stated.

Cell culture

The RBL-2H3 rat basophilic leukaemia mast cell line was purchased from ATCC (Rockville, MD, USA) and grown in minimum essential medium with Eagle's salt (EMEM) supplemented with 15% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1 mmol l^{-1} sodium pyruvate in a humidified incubator with a 5% CO_2 /95% air atmosphere at a temperature of 37°C . Prior to subculture, cells were detached using 0.125% trypsin containing 0.01 mol l^{-1} EDTA.

Induction of atopic dermatitis-like skin lesion in mice

The dorsal fur of all NC/Nga mice was removed by electric shaver prior to atopic dermatitis-like skin lesion induction. Specifically, 1-chloro-2,4-dinitrobenzene (DNCB) was used as an AD inducer (Yun *et al.* 2008). Briefly, 1% DNCB was freshly dissolved in acetone:olive oil (4 : 1) and then applied topically to the shaved area on the dorsal skin of the mice twice weekly for 2 weeks (four times in total).

Preparation of *Lactobacillus sakei* probio 65

Lactobacillus sakei probio 65 was isolated from home-made Kimchi using previous method (Park *et al.* 2008). First, the Kimchi was homogenized, and the homogenate was filtered through a sieve (100 mesh). This filtered homogenate was then diluted by decimal dilution and spread onto MRS agar. The plates were then incubated at 37°C for 1 day. The resulting colonies of lactic acid bacteria including *Lact. sakei* probio 65 were isolated by sequential subcultivation. These bacterial cells were lysed by STES buffer [0.4 mol l^{-1} NaCl, 0.2 mol l^{-1} Tris-HCl (pH 6.6), 0.01 mol l^{-1} EDTA and 1% sodium dodecyl sulfate (SDS)], and phenol was added to cell lysates for DNA extraction. After phenol extraction, the supernatant was incubated with RNase A at 37°C for 1 h, and a second phenol extraction/ethanol precipitation was

performed. 16S rDNAs were amplified by polymerase chain reaction (PCR), and PCR products were purified using an AccuPrep PCR purification kit (Bioneer, Daejeon, Korea). Sequences of 16S rDNA were determined by the Perkin–Elmer ABI Prism 310 Genetic Analyzer kit (Applied Biosystems, Foster City, CA, USA), following manufacturer's instruction. The 16S rDNA sequence of each isolate was aligned with representatives of related genera by CLUSTAL W software program to identify the strain. Isolated *Lact. sakei* probio 65 was incubated in MRS broth for 24 h at 37°C with continuous agitation at 150 g. Dead *Lact. sakei* probio 65 was prepared by autoclaving bacteria cultured in MRS broth at 121°C for 15 min.

Lactobacillus sakei probio 65 administration

Lactobacillus sakei probio 65 was administered to the mice using a previously described procedure with modifications (Park *et al.* 2008). The test groups were orally administered 200 μ l of PBS containing either live or dead *Lact. sakei* probio 65 (5×10^9 colony-forming units ml^{-1}) every day for 2 weeks after the induction of atopic dermatitis-like skin lesion. The dexamethasone-treated mice were given 200 μ l of 0.06 mg ml^{-1} dexamethasone in 10% ethanol. As a control, the other two groups were administered distilled water.

Evaluation of scratching behaviour frequency

Scratching behaviour was observed after all treatments were completed. The mice were placed into cages, and scratching behaviour was counted for 10 min. Each measurement was repeated five times so that mice were observed for 50 min in total. Scratching of the ears and dorsal skin with the hind paw was quantified in this experiment.

Serum IgE, IL-4, IL-6 and CTACK measurements

Blood samples were collected from the inferior vena cava after completion of treatment, and the sera were separated. Serum concentrations of IgE, IL-4 and IL-6 were measured using an enzyme-linked immunoassay kit purchased from Biolegend, Inc. (San Diego, CA, USA). Serum concentrations of CTACK were determined via an enzyme-linked immunoassay kit purchased from Blue-Gene Biotech Co., Ltd. (Shanghai, China).

Immunoblotting

The ears and dorsal skin of the mice were homogenized and centrifuged, and the cells were washed twice with PBS, suspended in 70 μ l of buffer A [10 mmol l^{-1}

HEPES (pH 7.9), 1.5 mmol l^{-1} MgCl_2 , 10 mmol l^{-1} KCl, 0.5 mmol l^{-1} DTT, 0.5 mmol l^{-1} PMSF and Protease Inhibitor Cocktail (Sigma-Aldrich Co)] and incubated on ice. After 15 min of incubation, 0.5% Nonidet P (NP)-40 was added to lyse the cells, during which time the solution was vortexed for 10 s. Next, lysates were obtained after the solution was centrifuged for 10 min at 1500 g at a temperature of 4°C. All protein concentrations were determined via the Bio-Rad protein assay (Bio-Rad Lab, Hercules, CA, USA), with BSA used as the standard. All fractionated proteins were electrophoretically transferred to a $0.2 \mu\text{m}$ microporous polyvinylidene difluoride (PVDF) membrane (Millipore Co., Billerica, MA, USA) and probed with the appropriate antibodies. In all immunoblotting experiments, blots were reprobed with anti- β -actin antibody as a protein loading control.

β -hexosaminidase release assay

RBL-2H3 cells ($1 \times 10^6 \text{ ml}^{-1}$) were attached to 6-well plates and sensitized with 500 ng ml^{-1} of DNP-specific IgE overnight. After the cells were washed twice with Siraganian buffer (119 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 0.4 mmol l^{-1} MgCl_2 , 25 mmol l^{-1} PIPES, 40 mmol l^{-1} NaOH, pH 7.2), they were exposed to live or dead *Lact. sakei* probio 65 (5×10^9 colony-forming units well^{-1}), dexamethasone (100 nmol l^{-1}) or ketotifen ($100 \mu\text{mol l}^{-1}$) and incubated at 37°C for 20 min. After incubation, the cells were challenged with 100 ng ml^{-1} of dinitrophenyl-human serum albumin (DNP-HSA) at a temperature of 37°C for 10 min. The degranulation of RBL-2H3 cells was then terminated by placing the cells on ice. Next, 25 μ l of the resulting supernatant was mixed with an equal volume of substrate (p-nitrophenyl-N-acetyl- β -D-glucosaminide 1 mmol l^{-1} in citrate 0.1 mol l^{-1} , pH 4.5) and incubated at 37°C for 1 h. This reaction was then terminated by adding 200 μ l of stopping solution (0.1 mol l^{-1} $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 10.0). Finally, the absorbance was measured using a microplate reader at 405 nm (Menlo Park, CA, USA).

Determination of cytokine production

The RBL-2H3 cells were first sensitized with 500 ng ml^{-1} of DNP-specific IgE overnight. Next, after two rounds of washing, the cells were incubated with live or dead *Lact. sakei* probio 65 (5×10^9 colony-forming units well^{-1}), dexamethasone (100 nmol l^{-1}), or ketotifen ($100 \mu\text{mol l}^{-1}$) at 37°C for 20 min. The RBL-2H3 cells were then stimulated with 100 ng ml^{-1} of dinitrophenyl-human serum albumin (DNP-HSA) at 37°C for 10 min, and the resulting supernatant was used for ELISA (R&D systems, Minneapolis, MN, USA).

Statistical analysis

All results are reported in the form of mean $\pm$ SEM. One-way analysis of variance was used to determine any significant differences among the groups, after which the modified *t*-test with Bonferroni correction was used to compare individual groups. All significant values are indicated with an asterisk (* $P < 0.05$).

Results

The effect of *Lactobacillus sakei* probio 65 on atopic dermatitis-like skin lesions and scratching behaviour frequency

AD-like skin lesions were induced in NC/Nga mice via the repeated topical application of DNCB (Fig. 1a). A 2-week course of oral *Lact. sakei* probio 65 (both live and dead) significantly suppressed the development of AD-like skin

lesions as compared with the control group. Similar results were also observed in the animals treated with dexamethasone, a well-known anti-inflammatory and immunosuppressant. Based on these findings, we contend that both live and heat-killed *Lact. sakei* probio 65 may have a similar pharmacologic activity as dexamethasone. We also examined the effects of live and dead *Lact. sakei* probio 65 on murine scratching behaviour. After treatment of the dorsal skin with DNCB, a 15-fold increase in scratching behaviour frequency was observed. However, scratching behaviour was noted to decrease significantly after treatment with both live and dead *Lact. sakei* probio 65 (Fig. 1b).

Effect of *Lactobacillus sakei* probio 65 on serum IgE, IL-4, IL-6 and CTACK

Certain characteristic symptoms of atopic dermatitis are believed to be due to the strong polarization of the Th2

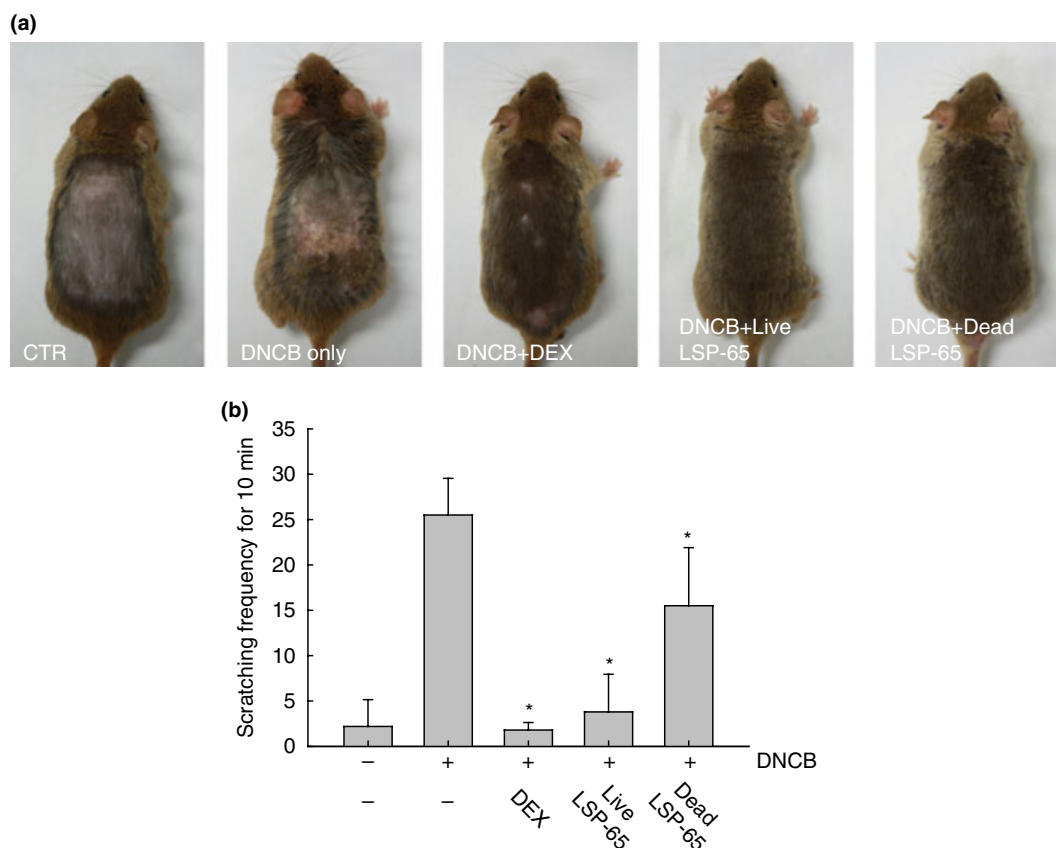


Figure 1 Atopic dermatitis (AD)-like skin lesions and scratching behaviours in DNCB-induced mice. (a) AD-like skin lesions were induced via the topical application of 1-chloro-2,4-dinitrobenzene (DNCB) four times, with oral treatment with *Lactobacillus sakei* probio 65 improving overall skin condition (CTR and DNCB only, fed with distilled water; DNCB+DEX, fed with 0.06 mg ml⁻¹ of dexamethasone, DNCB+Live LSP-65, fed with 5x10⁹ CFU ml⁻¹ of live *Lact. sakei* probio 65; DNCB+dead LSP-65, fed with 5x10⁹ CFU ml⁻¹ of heat-killed *Lact. sakei* probio 65). Skin features of mice were noted at the end of this experiment. Representative images from five mice. (b) Scratching behaviour was quantified after the completion of all treatments. In brief, the number of scratching behaviours was counted over 10 min. This measurement was then repeated five times (50 min in total). *Significantly different from DNCB-treated group ($P < 0.05$). CTR, control; DEX, dexamethasone; LSP-65, *Lact. sakei* probio 65.

immune response, thus resulting in IgE overproduction. As such, serum IgE concentrations were quantified in DNCB-induced mice. As shown in Fig. 2(a), serum IgE levels were significantly decreased in groups treated with dexamethasone, live *Lact. sakei* probio 65 and dead *Lact. sakei* probio 65 when compared with the DNCB-induced group. To evaluate the effect of *Lact. sakei* probio 65 on Th2 cytokine and chemokine production in DNCB-induced mice, serum levels of IL-4, IL-6 and CTACK/CCL27 were also quantified by ELISA. Interestingly, serum concentrations of IL-4 and IL-6 were significantly reduced by treatment with heat-killed *Lact. sakei* probio 65, but not by live *Lact. sakei* probio 65 (Fig. 2b, c). Serum CTACK concentrations were markedly inhibited in animals treated with dexamethasone, live *Lact. sakei* probio 65 and dead *Lact. sakei* probio 65 compared with the DNCB-induced control group (Fig. 2d).

Effect of *Lactobacillus sakei* probio 65 on the lesional skin and ears of AD-induced mice

To determine whether *Lact. sakei* probio 65 is able to suppress TARC and CTACK expression in DNCB-induced AD mice, DNCB-treated dorsal skin was homogenized, and the levels of chemokines in the homogenized skin were determined by Western blot analysis. As shown in Fig. 3(a), the expression of both TARC and CTACK increased remarkably in the DNCB-treated group. However, treatment with live and heat-killed *Lact. sakei* probio 65 resulted in a significant decrease in the expression of DNCB-induced TARC and CTACK.

To further determine the effects of live and dead *Lact. sakei* probio 65 on Foxp3 expression, murine dorsal skin and ears were homogenized, and Foxp3 protein expression was determined by Western blot

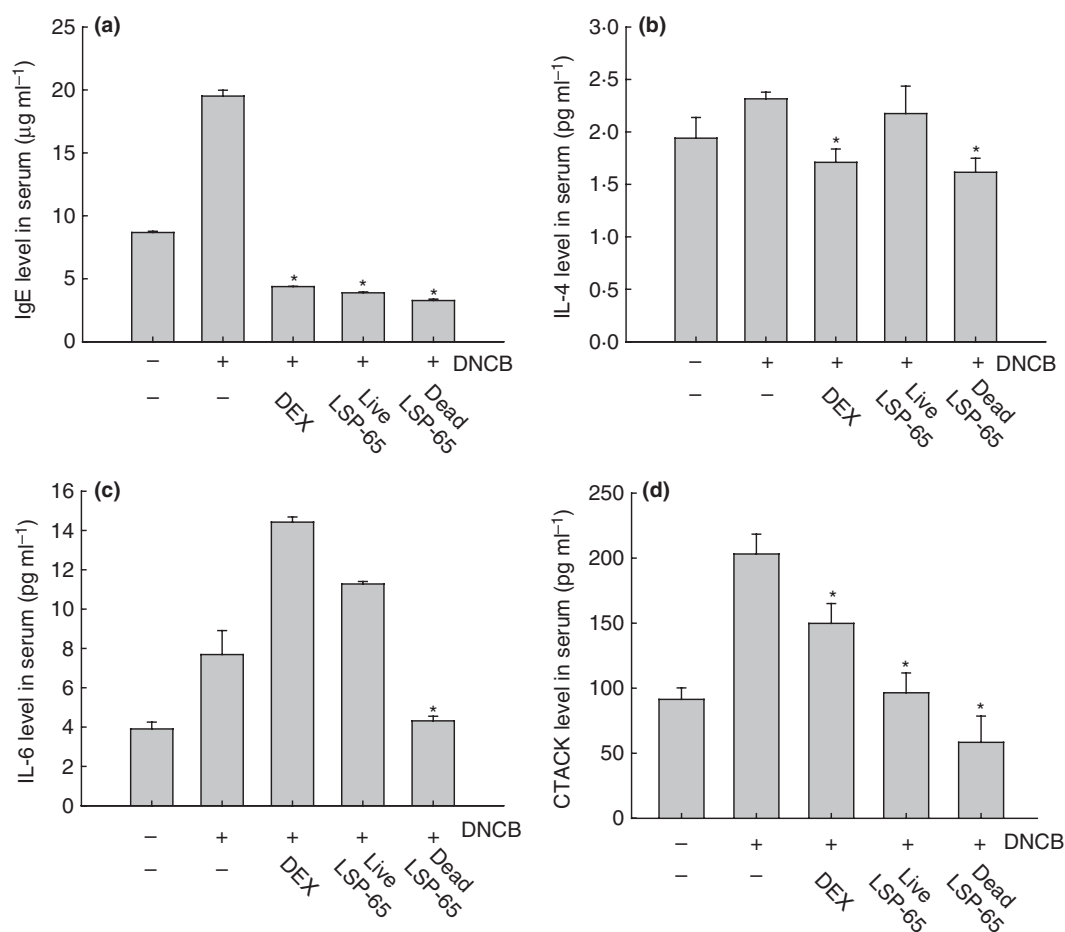


Figure 2 The effect of *Lactobacillus sakei* probio 65 on serum levels of IgE, IL-4, IL-6 and cutaneous T-cell-attracting chemokine (CTACK) in DNCB-induced mice. After the completion of all treatments, blood samples were collected from the inferior vena cava, and the sera was separated. The serum concentrations of IgE (a), IL-4 (b), IL-6 (c) and CTACK (d) were quantified by ELISA. All data are presented in the form of mean $\pm$ SEM. *Significantly different from DNCB-treated group ($P < 0.05$). DEX, dexamethasone; LSP-65, *Lact. sakei* probio 65.

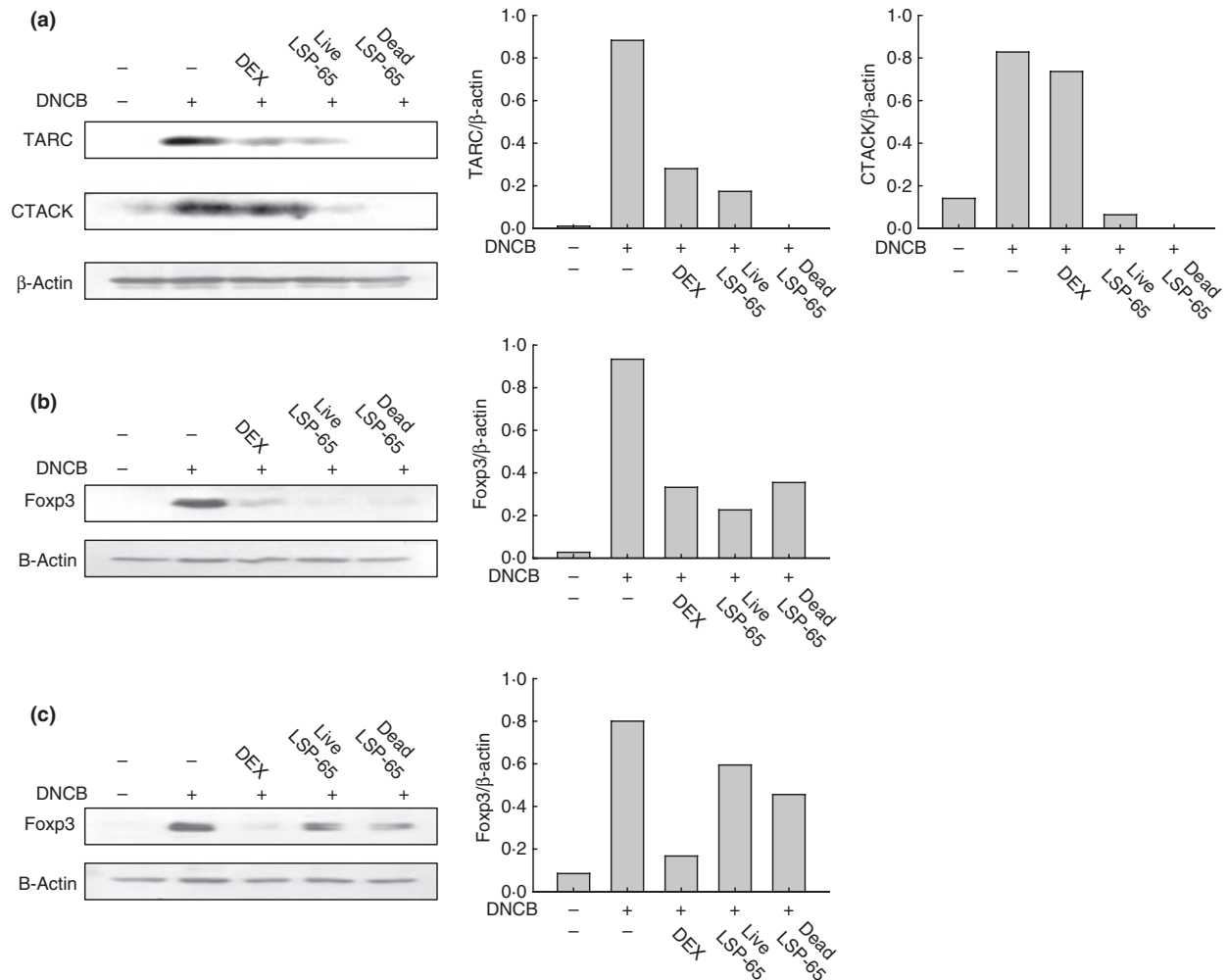


Figure 3 The effect of *Lactobacillus sakei* probio 65 on Thymus and activation-regulated chemokine (TARC) and cutaneous T-cell-attracting chemokine (CTACK) expression in dorsal skin and Foxp3 protein expression in the dorsal skin and ears of DNCB-induced mice. After the completion of all treatments, the dorsal skin and ears of mice were homogenized. The chemokines in the homogenized skin (a), as well as the protein expression of Foxp3 in the homogenized skin (b) and ears (c), were measured via Western blot analysis, with the protein expression of β -actin used as an internal control. The results illustrated are from a single experiment and are representative of three separate experiments. The expressions of TARC, CTACK and Foxp3 are expressed in arbitrary units, and the data are normalized to β -actin protein. DEX, dexamethasone; LSP-65, *Lact. sakei* probio 65.

analysis. As shown in Fig. 3(b,c), the protein expression of Foxp3 was remarkably increased in both dorsal skin and the ears of DNCB-induced mice when compared with the control animals. However, Foxp3 expression was significantly suppressed in both locations in mice treated with dexamethasone as well as live and dead *Lact. sakei* probio 65.

In vitro effects of *Lactobacillus sakei* probio 65 on IgE-sensitized RBL-2H3 cells

β -hexosaminidase secretion – a well-established degranulation marker – is a hallmark of allergic reactions resulting

from exposure of mast cells to allergens (Joo *et al.* 2009). The data presented here demonstrate that *Lact. sakei* probio 65 has an inhibitory effect on AD-like skin lesions in an animal model. Based on these findings, further *in vitro* experiments were performed to examine the influence of *Lact. sakei* probio 65 on mast cells. First, the inhibitory effect of *Lact. sakei* probio 65 on β -hexosaminidase secretion was examined. DNP-HSA significantly induced degranulation in IgE-sensitized RBL-2H3 cells compared with untreated cells. Conversely, β -hexosaminidase secretion was significantly lower in cells treated with either live or heat-killed *Lact. sakei* probio 65 (Fig. 4a). Ketotifen ($100 \mu\text{mol l}^{-1}$) and dexamethasone (100 nmol l^{-1}) were

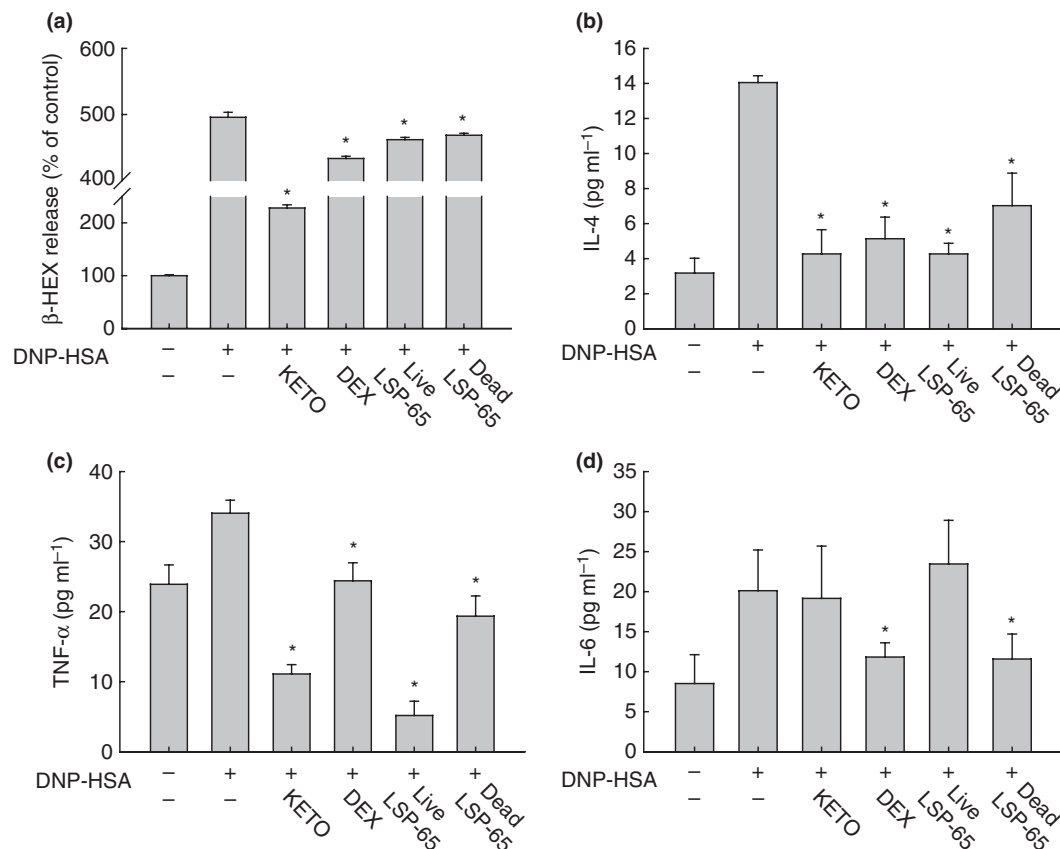


Figure 4 The effect of *Lactobacillus sakei* probio 65 on antigen-induced degranulation and cytokine release in RBL-2H3 cells. In brief, RBL-2H3 cells were sensitized overnight with 500 ng ml⁻¹ of DNP-specific-IgE. These IgE-primed cells were then pretreated with either live or dead *Lact. sakei* probio 65 (5 × 10⁹ CFU well⁻¹), dexamethasone (100 nmol l⁻¹) or ketotifen (100 μ mol l⁻¹) for 20 min, then stimulated with 100 ng ml⁻¹ of DNP-HSA for 10 min. The release of β -hexosaminidase (a) from IgE-sensitized RBL-2H3 cells was quantified using a microplate reader at 405 nm. The concentrations of IL-4 (b), TNF- α (c) and IL-6 (d) in the supernatant were measured by ELISA. The results of three independent experiments are expressed in the form of mean $\pm$ SEM. *Significantly different from DNCB-treated group ($P < 0.05$). DEX, dexamethasone; KETO, ketotifen; LSP-65, *Lact. sakei* probio 65.

also noted to significantly reduce β -hexosaminidase secretion induced by DNP-HSA.

As a large number of cytokines – including IL-4, IL-6 and TNF- α – are believed to be crucial for allergic inflammation, we quantified the effect of *Lact. sakei* probio 65 on these particular cytokines. As shown in Fig. 4(b,c), ketotifen, dexamethasone and *Lact. sakei* probio 65 significantly inhibited IL-4 and TNF- α secretion induced by DNP-HSA. Interestingly, IL-6 release was suppressed by dexamethasone and heat-killed *Lact. sakei* probio 65, although not by live *Lact. sakei* probio 65 (Fig. 4d), suggesting that *Lact. sakei* probio 65 has differential effects on cytokine production.

Discussion

In the present study, we describe the effects of live and dead *Lact. sakei* probio 65 on AD-like lesions both *in vivo*

and *in vitro*. The use of probiotics and prebiotics in the treatment of allergic disease has been reported to be effective in young children with atopic dermatitis and is now widely recommended (Gill and Guarner 2004; Woo *et al.* 2010). Although a large number of organisms in probiotics have been studied in the past, certain strains of lactobacilli and bifidobacteria – including *Lactobacillus GG*, *Bifidobacterium lactis*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri* and *Lactobacillus fermentum* – are now believed to exert some degree of favourable effect on the extent and severity of atopic dermatitis (Matricardi *et al.* 2003; Prescott and Björkstén 2007). Using NC/Nga mice and RBL-2H3 mast cells, we demonstrated that the administration of live and dead *Lact. sakei* probio 65 does convey beneficial effects on AD-like skin lesions.

Atopic dermatitis is a chronic inflammatory skin disease associated with extreme pruritus. Significant increases in serum IgE were observed in NC/Nga mice with AD-like

skin lesions induced via repeated DNCB challenge. As IgE activates other key effector cell types involved in allergic inflammation and contributes to other aspects of the allergic response, the elevated serum IgE levels observed in AD-induced mice suggested that these animals were more susceptible to manifesting other inflammatory reactions (Zheng *et al.* 2011). The data also indicated that both the severity of skin lesions and elevations in serum IgE concentrations were significantly reduced by treatment with both live and dead *Lact. sakei* probio 65. Moreover, we observed that the topical application of DNCB also increases Th2 cytokine levels, further suggesting that Th2-dominant responses result from the overproduction of both Th2 cytokines (IL-4, IL-5 and IL-14) and IgE in the NC/Nga mouse model (Vestergaard *et al.* 1999; Jin *et al.* 2009). Some previous data have suggested that serum IL-6 levels are also elevated in AD-induced mice (Yun *et al.* 2010; Zheng *et al.* 2011), with our DNCB treatment data also supporting this association. However, our results demonstrate that the DNCB-induced increase in serum Th2 cytokines (IL-4 and IL-6) was significantly inhibited by heat-killed *Lact. sakei* probio 65, whereas IL-4 and IL-6 concentrations were not altered by treatment with live *Lact. sakei* probio 65. Taken together, this data suggest that improvement in AD-like skin lesions after oral treatment with both live and heat-killed *Lact. sakei* probio 65 may be a result of the inhibitory effects of these organisms on IgE and/or Th2-specific cytokines.

In patients with AD, itch-associated scratching results in skin barrier defects and increases inflammation, both of which ultimately increase associated itching (Mihara *et al.* 2004; Han *et al.* 2012). The results presented here indicate that both live and dead *Lact. sakei* probio 65 significantly inhibit spontaneous scratching behaviour resulting from DNCB treatment, suggesting that *Lact. sakei* probio 65 may exert an anti-allergic effect by reducing scratching behaviour and preventing the aggravation of skin lesions. There is some data to suggest that AD-associated pruritus results from increased levels of histamine, proteases and cytokines produced by various cells (Ständer and Steinhoff 2002). After antigen stimulation, mast cells release β -hexosaminidase as well as many other chemical mediators and cytokines that promote acute and chronic allergic reactions, including histamine, IL-4, IL-5, IL-6 and TNF- α (Bradding *et al.* 1994; Gilfillan and Tkaczyk 2006). In the present study, our results demonstrate that live and dead *Lact. sakei* probio 65 significantly inhibited β -hexosaminidase secretion in DNP-HSA-stimulated mast cells. These results also suggest that *Lact. sakei* probio 65 may represent a novel therapeutic agent for atopic dermatitis. Among the cytokines produced by activated mast cells, IL-4 is responsible for increasing plasma cells IgE production and promoting

naïve T cells to switch to Th2 cells. TNF- α is mainly produced by activated macrophages and T cells, although it is also synthesized and secreted by mast cells in response to IgE challenge (Han *et al.* 2009). IL-6 secreted by mast cells and basophils is also believed to contribute to cutaneous inflammatory and allergic reactions (Krüger-Krasagakes *et al.* 1996), while IL-6 and TNF- α have been reported to recruit neutrophils to the site of mast cell activation (El-Feky 2011). The data presented here show that treatment with live or heat-killed *Lact. sakei* probio 65 significantly resulted in selective inhibition of antigen-induced secretion of IL-4, IL-6 and TNF- α production, indicating that *Lact. sakei* probio 65 may exert some anti-atopic activity by inhibiting Th2 cytokine production in mast cells. Furthermore, these *in vitro* data correlate well with the cytokine levels observed in animal models. However, the mechanism(s) by which live and heat-killed *Lact. sakei* probio 65 exerts differential effects on cytokine production in mast cells or animal model are not fully understood. It is plausible that live and heat-killed *Lact. sakei* probio 65 may differentially affect the intracellular signal transduction pathway of cytokine production induced by DNCB because ingested application DNCB has been known to especially increase the production of Th1- and Th2-related cytokines (Van Och *et al.* 2002).

Lesional CD4⁺ T-cell infiltration is now considered one of the major characteristics of atopic dermatitis. T-cell trafficking is regulated by specific chemokines, with TARC (a ligand for CCR4) and CTACK (which binds to the CCR10 receptor) now believed to play important roles in AD (Hijnen *et al.* 2004). Specifically, TARC and CTACK are thought to act consecutively, with TARC responsible for recruiting T cells in the early stages and CTACK for inducing T-cell migration into the upper layers of the skin (Imai *et al.* 1999). Other recent data suggest that TARC and CTACK represent two important severity markers for AD by reflecting disease activity (Kakinuma *et al.* 2001, 2003). Previous studies have demonstrated that elevated serum levels of CTACK/CCL27 correlate significantly with AD severity (Nakazato *et al.* 2008). Our data support this association, as we observed that serum CTACK concentrations increased significantly with DNCB treatment. Elevated serum CTACK levels were also significantly reduced by oral treatment with both live and dead *Lact. sakei* probio 65. Moreover, oral treatment with live and heat-killed *Lact. sakei* probio 65 significantly decreased the expression of TARC and CTACK in skin lesions induced by DNCB. Taken together, these data suggest that *Lact. sakei* probio 65 may represent a novel therapeutic agent for AD by reducing Th2-specific inflammatory chemokines.

Recently, Treg cells have been suggested as representing an important regulator of T-helper type 2 responses

(Larché 2007). In one murine AD model, the absence of Foxp3 – a transcription factor involved in Treg cell development and function – resulted in increases in serum IgE levels, eosinophils and elevated Th2 cytokine production (Lin *et al.* 2005). Furthermore, the absence of Foxp3 + Treg cells was also suggested as resulting in the increased recruitment of inflammatory cells, as well as an increase in the expression of Th2 cytokines and serum IgE, all of which ultimately aggravated skin inflammation (Fyhrquist *et al.* 2012). In the results described here, we observed that Foxp3 protein expression was significantly elevated in the ears and skin of DNCB-induced mice. Oral treatment with both live and dead *Lact. sakei* probio 65 significantly reduced the expression of Foxp3 at both sites, thus suggesting that *Lact. sakei* probio 65 may exert an inhibitory effect on the pathogenesis of atopic dermatitis by regulating Treg cell populations.

Data from previous studies also suggest that immune stimulation with certain microbial products may prevent or treat allergic disease (Gill and Guarner 2004; Prescott and Björkstén 2007). Specifically, some studies suggest that nonviable microbes – such as heat-killed or ultraviolet-inactivated organisms – or even microbial metabolic products may represent superior agents, as the administration of live organisms has been associated with unwanted side effects (Boyle *et al.* 2006; Besselink *et al.* 2008; Kataria *et al.* 2009). Conversely, it has also been reported that only live *Lact. rhamnosus* strain GG (LGG) was effective in the management of atopic disease, while heat-inactivated LGG was not (Kirjavainen *et al.* 2003). In the present study, our data demonstrate that both live and dead *Lact. sakei* probio 65 may have some inhibitory effect on atopic dermatitis. Based on these findings, the viability of *Lact. sakei* probio 65 was not significant in this study, with dead *Lact. sakei* probio 65 as effective as live *Lact. sakei* probio 65 in modulating allergic inflammation.

In conclusion, oral treatment with both viable and heat-inactivated *Lact. sakei* probio 65 inhibits skin inflammation and AD-like skin lesions *in vivo*, as well as mast cell activation *in vitro*. These results suggest that *Lact. sakei* probio 65 may represent a potential novel therapeutic agent in the treatment of atopic dermatitis.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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