

New Functional Probiotic *Lactobacillus sakei* Probio 65 Alleviates Atopic Symptoms in the Mouse

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ABSTRACT The purpose of this study was to investigate the improvement of allergic dermatitis in chemical allergen-induced mice by *Lactobacillus sakei* probio 65. *L. sakei* probio-65 was isolated from kimchi, a traditional Korean fermented food. This strain was resistant to gastric acidity, bile, and several antibiotics and possessed antimicrobial activity against several pathogenic microorganisms. To investigate whether the probiotic activity of *L. sakei* probio 65 was effective for treating allergic dermatitis, the organism was supplied to mice triggered by allergen (1-chloro-2,4-dinitrobenzene). Mice that received *L. sakei* probio 65 showed a more rapid recovery compared to control mice, as assessed by visual evaluation of the severity of allergic dermatitis and levels of immunoglobulin (Ig) E and interleukin (IL)-4. *L. sakei* probio 65 exhibited good probiotic properties *in vitro* and in mice and was effective in reducing allergen-induced skin inflammation through the regulation of both elevated IgE and IL-4 in sensitized mice.

KEY WORDS: • allergic dermatitis • immunoglobulin E • interleukin-4 • *Lactobacillus sakei* probio 65 • probiotics

INTRODUCTION

ATOPIC DERMATITIS (AD), also referred to as atopic eczema, is a chronic inflammatory disease of the skin that affects millions of individuals worldwide.¹ Symptoms include pruritus and chronic or relapsing eczematous lesions typically localized to the face and extensor sides of the extremities in children and at the flexural sides in adults.² Acute AD is associated with a cutaneous inflammatory response resembling that in the airways of patients with allergic rhinitis and asthma.³ T-cells, mainly of the CD4 lineage, are present in large numbers in skin lesions of AD patients,⁴ and cutaneous lymphocyte-associated antigen (CLA)-bearing T-cells are present both in the circulation and locally in dermal lesions.³ When these CD4⁺CLA⁺ T-cells are activated, cytokines are released into the serum and skin, promoting allergic inflammation.⁵ The T-helper 2 (Th2) cytokine pattern predominates in acute AD, whereas a T-helper 1 pattern is present in chronic AD.^{4,6} The cytokines released by Th2 cells up-regulate antigen-presenting cells expressing

the immunoglobulin (Ig) E receptor, which contains FcεRI and has a high affinity for IgE. The up-regulation of FcεRI on dendritic cells has been observed in AD lesions.^{6,7}

IgE is the largest immunoglobulin monomer and is present in the serum of healthy individuals at extremely low levels.⁸ However, IgE levels rise in response to parasitic infections and are high in atopic individuals and patients who show immediate hypersensitivity to allergens. Enhanced IgE production is probably a consequence of Th2 type-skewed immune responses.^{9,10} Several studies have suggested that lactic acid bacteria may act as prospective dietary immunoregulators. For example, *Lactobacillus casei* Shirota, *Lactobacillus plantarum* L-137, and *Lactobacillus paracasei* KW3110 have been found to down-regulate antigen-induced serum IgE production in mice.^{10–12} Recently, it was shown that *Staphylococcus aureus* is present on the skin of AD patients.² This bacterium secretes proteins that promote lymphocyte activation independent of allergens and may be important in flares of AD.⁶ The household dust mite, *Dermatophagoides pteronyssinus*, has also been shown to precipitate symptoms of AD and other atopic diseases, such as asthma and allergic rhinitis.¹³

Probiotics are defined as “living microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition.”¹⁴ The development of pro-

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biotics during the past decade signals an important advance in the food industry towards the development of functional food.¹⁵ A potential regulatory role of probiotics in allergic disease was first suggested by the demonstration that they have a suppressive effect on lymphocyte proliferation and interleukin (IL)-4 generation *in vitro*.¹⁶ Subsequently, it was shown that probiotics can regulate the immunoinflammatory cytokines IL-10¹⁷ and transforming growth factor- β ,¹⁸ allergic inflammation in the gut,¹⁹ and mucosal dysfunction caused by inflammation.¹⁵ In infants given probiotic-supplemented elimination diets, there was a significant improvement in the course of atopic eczema.^{19,20}

Here, we isolated and characterized a novel probiotic strain that we designated as *Lactobacillus sakei* probio 65. To determine whether this strain can improve AD, we induced AD in mice chemically. We found that feeding with *L. sakei* probio 65 accelerated recovery from AD in the mice and that this was associated with reductions in serum IgE and splenocyte IL-4 levels.

MATERIALS AND METHODS

Isolation of lactic acid bacteria from kimchi

Lactic acid bacteria were isolated from homemade kimchi by homogenizing it and then filtering the homogenate through a sieve (100 mesh). The filtered homogenate was diluted by decimal dilution, and an appropriate volume of the dilution was spread onto MRS agar. The plate was incubated at 37°C for 1 day. Colonies of lactic acid bacteria were then isolated by sequential subcultivation.

16S rDNA sequencing and phylogenetic analysis of isolated lactic acid bacteria

Bacterial cells were collected and resuspended in 100 μ L of STES buffer (0.4 M NaCl, 0.2 M Tris-HCl [pH 7.6], 0.01 M EDTA, and 1% sodium dodecyl sulfate). Glass beads were added to the suspension, and it was vigorously mixed for 5 minutes with a microtube mixer (model MT-360,

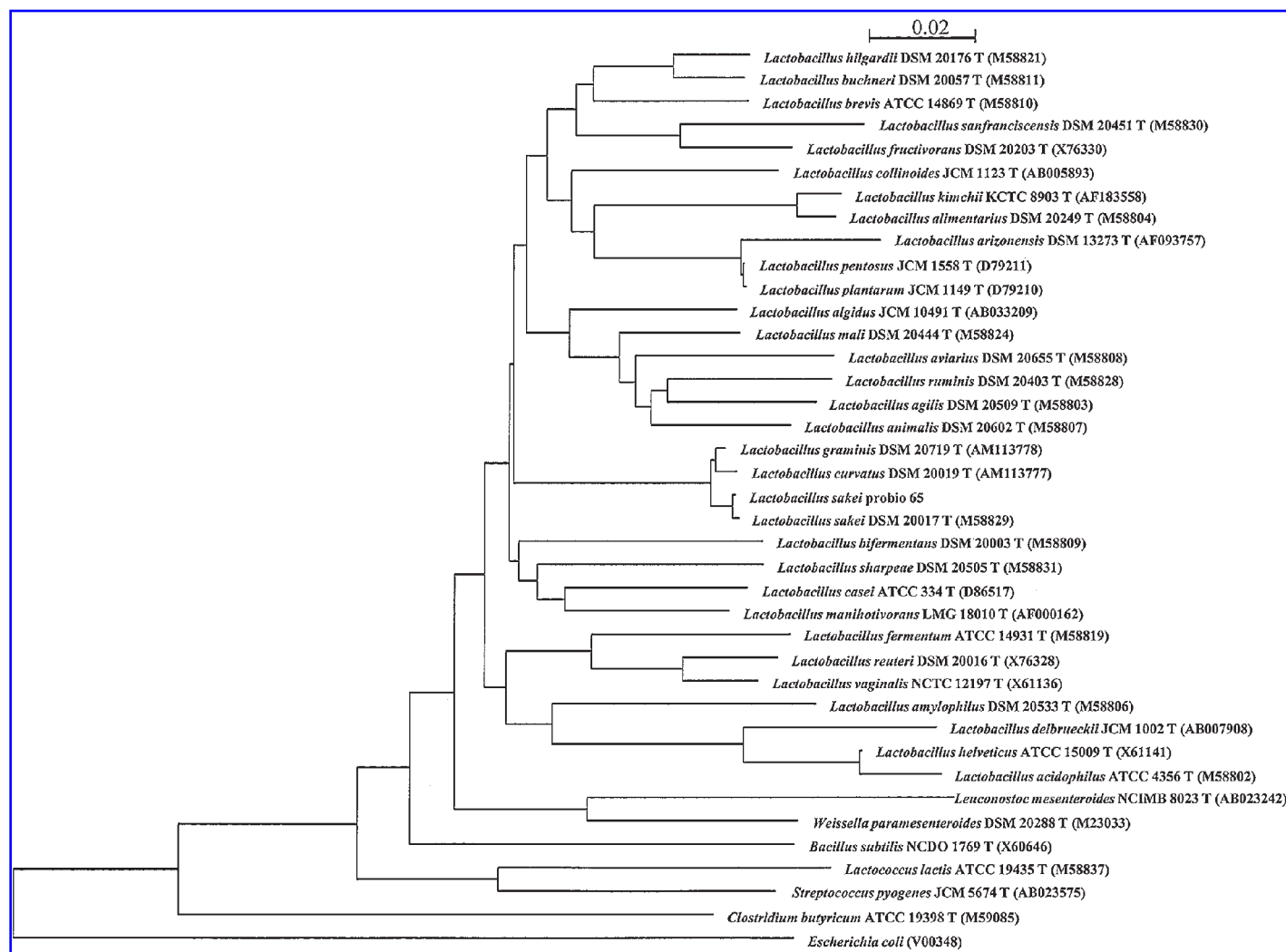


FIG. 1. Phylogenetic tree based on 16S rDNA showing the relationship of *L. sakei* probio 65 to other species of *Lactobacillus*. Bar indicates substitution per site.

TOMY, Tokyo, Japan) to lyse the cells. Cell lysates were subjected to phenol extraction, and then 5 μ L of RNase A was added to the supernatant. After incubation at 37°C for 1 hour, a second phenol extraction/ethanol precipitation was performed.

16S rDNAs were amplified by polymerase chain reaction (PCR) using two universal primers: forward primer 5'-GA GTT TGA TCC TGG CTC AG-3' (*Escherichia coli* numbering system, positions 9–27) and reverse primer 5'-GGT TAC CTT GTT ACG ACT T-3' (*E. coli* numbering system, positions 1,492–1,510).²¹ The conditions for PCR were as follows: 30 cycles consisting of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 1.5 minutes. PCR products were purified using an AccuPrep PCR Purification Kit (Bioneer, Daejeon, Republic of Korea), and 16S rDNA sequences were determined using the Perkin-Elmer ABI Prism 310 Genetic Analyzer kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions.

The 16S rDNA sequence of the isolate was aligned with representatives of related genera using the CLUSTAL W software program.²² Evolutionary distance matrices were calculated based on the two-parameter method of Kimura,²³ and the phylogenetic tree was constructed using the neighbor-joining method²⁴ with programs of the PHYLIP package version 3.572.²⁵

Characterization of *L. sakei* probio 65

Scanning electron microscopy. An aliquot of a *L. sakei* probio 65 MRS culture was spread on an MRS agar plate and incubated for 20 hours at 37°C. The cultured cells and agar were cut to a size of 25 mm² and then incubated in a solution containing 2.5% glutaraldehyde for 30 minutes. Fixed cells were incubated in 2% osmic acid for 60 minutes, after washing in 0.01 M cacodylate buffer. Samples were then dehydrated with ethanol. A solution containing



FIG. 2. Scanning electron microscopic image of *L. sakei* probio 65. Magnification $\times 5,000$.

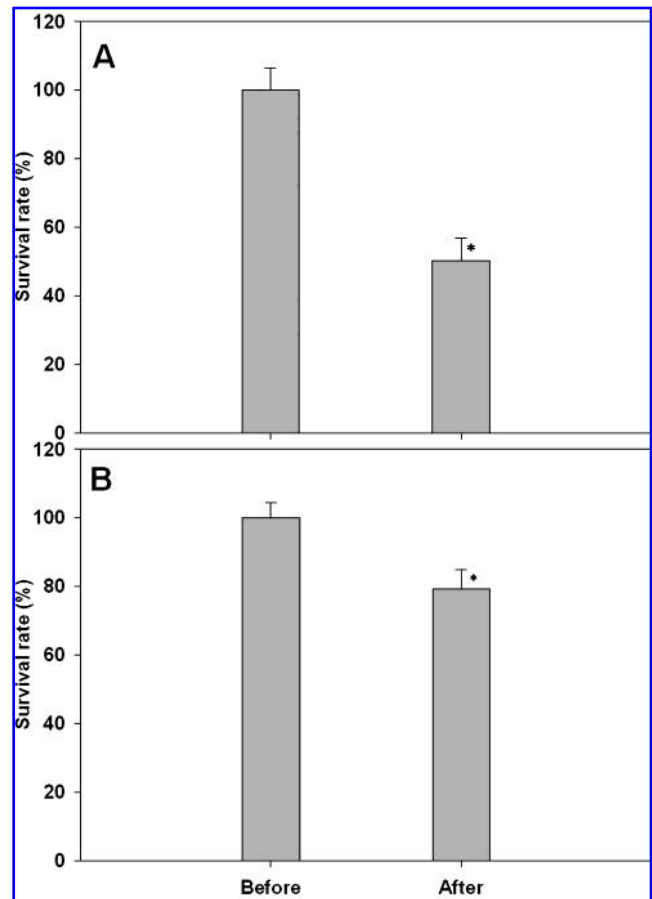


FIG. 3. Resistance of *L. sakei* probio 65 to (A) artificial gastric and (B) bile juice. "Before" and "After" indicate the value before treatment with artificial gastric acid and bile juice and after treating with the two reagents, respectively. Survival rate was determined by counting colony-forming units. * $P < .05$ compared with "Before" control.

amylacetate and ethanol (1:1 vol/vol) was applied to the samples for 20 minutes, and then the samples were soaked in 100% amylacetate twice for 20 minutes each. Afterwards, filter paper and mesh were used to remove amylacetate completely. After amylacetate was removed, the samples were dried by critical point drying (Hitachi, Tokyo) and coated (Eiko, Tokyo) with Au. Specimens were observed using a scanning electron microscope (Hitachi).

Resistance against artificial gastric juice and bile. Artificial gastric juice was manufactured by the method of Kobayashi *et al.*²⁶ MRS broth was adjusted to pH 2.5, and pepsin was then added to a final concentration of 1%. *L. sakei* probio 65 was incubated in this medium for 24 hours at 37°C with shaking (150 rpm) and then centrifuged at 6,000 g for 10 minutes. Bacterial cells were washed twice using 0.01 M phosphate-buffered saline (PBS). Artificial gastric juice (equal to the initial culture volume) was added, and bacteria were incubated for an additional 3 hours at 37°C. Survival rate was measured using the decimal dilution method on MRS agar at 37°C for 36 hours.

TABLE 1. ANTIBIOTIC SUSCEPTIBILITY OF *L. sakei* PROBIO 65

Antibiotic	Concentration ($\mu\text{g/mL}$)	Resistant (mm)	Diameter (mm)	Results
Ampicillin	10	<28	19	R
Carbenicillin	100	<19	24	S
Clindamycin	2	<14	20	S
Doxycycline	30	<16	22	S
Erythromycin	15	<21	19	R
Gentamicin	10	<15	9	R
Kanamycin	30	<18	0	R
Lincomycin	2	<24	11	R
Minocycline	30	<14	26	S
Neomycin	30	<12	9	R
Penicillin G	10	<19	20	R or S
Streptomycin	10	<14	0	R
Sulfisoxazole ^a	250	<24	0	R
Tetracycline	30	<19	20	R

R, resistant; S, sensitive.

^aIndicated concentration of sulfisoxazole (mg/mL).

Artificial bile juice was prepared as described in the Difco (Detroit, MI) manual. Pancreatin (Wako Fine Chemicals, Osaka, Japan) was added to MRS broth at a final concentration of 1%. The broth was adjusted to pH 6.8 using sterile 10% oxagall solution (Difco). Resistance against bile was tested by the modified method of Oh *et al.*²⁷ *L. sakei* probio 65 was pre-exposed to artificial gastric juice before being incubated in artificial bile juice to mimic the *in vivo* conditions of the gastrointestinal system. In brief, bacteria were incubated at 37°C for 24 hours, collected by centrifugation, and exposed to artificial gastric juice for 1 hour. After exposure, cells were collected by centrifugation, washed twice with 0.01 M PBS, and then incubated in artificial bile juice (equal to the initial culture volume) at 37°C for 3 hours. Survival rate was measured on MRS agar.

Antibiotic susceptibility. The antibiotic susceptibility test for the isolates was done using the disc diffusion method. An aliquot of *L. sakei* probio 65 culture was spread on an MRS agar plate, and discs containing various antibiotics were placed on the agar. After incubation for 34 hours at 37°C, the diameter of the clear zone around the disc was measured.

Antimicrobial activity. The antimicrobial activity of *L. sakei* probio 65 against 10 pathogenic microorganisms was tested using the paper disc method.²⁸ Ten pathogenic bacteria were cultivated in appropriated medium and at optimal temperature. One hundred microliters of a bacterial culture solution was spread on a Muller-Hinton agar plate with a sterile swab, and a sterile 8-mm-diameter paper disc (Toyo Roshi Kaisha, Ltd., Tokyo) was placed on the agar. Two hundred microliters of a 10-fold concentrate of supernatant from *L. sakei* probio 65 was dropped onto the disc, and the plates were incubated for 12 hours at 37°C. After the plate was incubated, the diameter of the clear zone was determined.

Preparation of animal and reagent for *in vivo* mouse test.

Four-week-old CD-1(ICR) class female mice were purchased from Orient Co. Ltd. (Seongnam, Republic of Korea), and fed a standard diet for mice (Superfeed). The mice were housed in an animal room maintained at $24 \pm 2^\circ\text{C}$, with a 12-hour light/dark cycle. Mice were acclimatized to the animal facility for 1 week before experimentation. 1-Chloro-2,4-dinitrobenzene (DNCB) (Sigma, St. Louis, MO) was used as the contact allergen.

Chemical allergen challenge and treating with *L. sakei* probio 65

The mice were divided into three groups. The negative control group ($n = 6$) did not receive *L. sakei* probio 65 and was not treated with the contact allergen DNCB. The posi-

TABLE 2. ANTIMICROBIAL ACTIVITY OF *L. sakei* PROBIO 65 AGAINST VARIOUS PATHOGENIC MICROORGANISMS

Pathogenic microorganism	Clear zone (mm)
<i>E. coli</i> KCTC 2441	27
<i>Salmonella typhimurium</i> SG	31
<i>Salmonella gallinarum</i> ST	28
<i>S. aureus</i> KCTC 1621	28
<i>Enterobacter cloacae</i> KCTC 2361	28
<i>Staphylococcus epidermidis</i> KCTC 1917	24
<i>Klebsiella pneumoniae</i> KCTC 2208	41
<i>Shigella flexneri</i> KCTC 2008	26
<i>Citrobacter freundii</i> KCTC 2006	28
<i>Bacillus megaterium</i> KCTC 1096	27

The clear zone was determined as the diameter including that of the paper disc.

tive control group ($n = 12$) did not receive *L. sakei* probio 65 but was treated with DNCB. The test group ($n = 12$) received *L. sakei* probio 65 and was then treated with DNCB, after which the mice received feed mixed with *L. sakei* probio 65, as follows. In brief, the test group was given 200 μL of culture broth containing *L. sakei* probio 65 (1×10^8 colony-forming units/mL) orally every day for 2 weeks to allow the bacteria to colonize the murine intestine. The other two groups received 200 μL of PBS instead. DNCB-induced skin sensitization was performed over 8 days according to the method of Renstrom *et al.*²⁹ In brief, $12 \pm 2 \text{ cm}^2$ of hair on the mice was removed by using a razor and hair remover and treated with $95 \pm 5 \mu\text{L}$ of 1% DNCB daily for 3 days. After 48 hours, the skin was treated with $120 \pm 10 \mu\text{L}$ of 2% DNCB daily for 3 days. After skin sensitization was evoked, the test group mice were again fed *L. sakei* probio 65 by giving them feed containing *L. sakei* probio 65 (15 mL of a *L. sakei* probio 65 culture [1×10^8 colony-forming units/mL] was mixed with 40 g of feed). The mice were allowed to eat freely, and their weight and coat conditions were measured at 3-day intervals. The experiment was performed for 2 weeks.

Determination of IgE in murine serum

Blood was collected from the tail vein at the end of the experiment. Two hundred microliters of blood was allowed to stand at room temperature for 2 hours, and then serum was collected by centrifugation at 1,000 g for 20 minutes. Total IgE in serum was measured using the IgE enzyme-linked immunosorbent assay kit for the mouse (Shibayagi, Shibukawa, Japan) according to the manufacturer's instructions. In brief, 50 μL of buffer solution and 5 μL of serum were added to antibody-coated wells. The mixture was in-

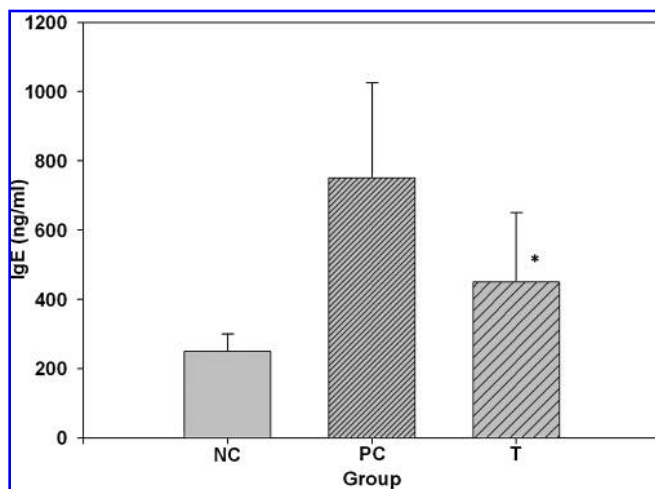


FIG. 4. Change in IgE level in serum from DNCB-induced mice. NC, negative control; PC, positive control; T, test group supplemented with *L. sakei* probio 65. * $P < .05$ compared with NC and PC by Duncan's multiple range test.

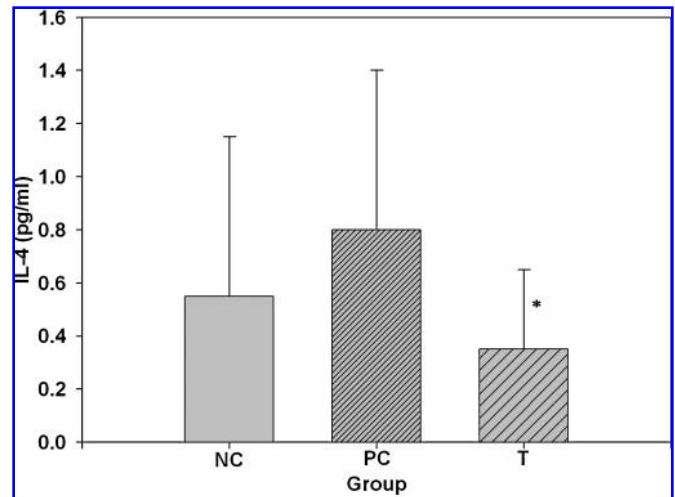


FIG. 5. Changes in IL-4 levels in splenocytes from DNCB-induced mice. NC, negative control; PC, positive control; T, test group supplemented with *L. sakei* probio 65. * $P < .05$ compared with NC and PC by Duncan's multiple range test.

cubated for 2 hours at room temperature and then discarded carefully, and 50 μL of horseradish peroxidase (HRP)-conjugated antibody solution was added to each well. After incubation for 2 hours at room temperature, the HRP solution was discarded, and 50 μL of HRP-avidin solution was added to each well. Following incubation, this solution was also discarded, and 50 μL of chromogenic substrate solution (tetramethylbenzidine) was added to each well. After 20 minutes, 50 μL of reaction stopper (1 M H_2SO_4) was added, and absorbance was measured at 450 nm.

Determination of IL-4 from murine splenocytes

Splenocytes were dissociated and cultivated according to the modified method of Kedar *et al.*³⁰ In brief, mice were sacrificed by cervical vertebral dislocation, and the spleen was extracted under aseptic conditions. The extracted spleen was transferred to 15 mL of 0.01 M PBS, and the fat and connective tissues were removed. The spleen was then transferred to an 87-mm-diameter Petri dish containing 3 mL of 0.01 M PBS, and the spleen was ruptured by using the base of a 10-mL syringe plunger. A single-cell suspension was then prepared by passing the splenocytes through a 21-gauge needle three times and then through a 26-gauge needle twice. The single-cell suspension was transferred to a 15-mL tube and centrifuged at 500 g for 10 minutes at 4°C. The supernatants were removed, and the splenocytes were incubated in 0.83% Tris-Cl (pH 7.2) for 10 minutes at 37°C to destroy the red blood cells. After being washed twice with 0.01 M PBS, the splenocytes were resuspended in RPMI 1640 medium. To measure the IL-4 levels in the splenocytes, the mouse IL-4 kit (R&D Systems, Minneapolis, MN) was used according to the manufacturer's instructions. Absorbance was measured at 540 nm.

Visual evaluation of allergic dermatitis

The severity of allergic dermatitis induced in the mice was assessed by the clinical visual evaluation method, which has generally been used in clinical trials.³¹ Extent was estimated as intensity, which was the sum of the scores for erythema, pruritus and dry skin, edema and excoriation, erosion, and lichenification. Each item was graded as nil (0), weak (1), moderate (2), or severe (3).

Statistical analysis

Experimental results are expressed as mean $\pm$ standard deviation. The SAS software (SAS Institute Inc., Cary, NC) was used for analyzing the statistical data. A value of $P < .05$ was considered statistically significant.

Nucleotide sequence accession number

The 16S rDNA from *L. sakei* probio 65 has been deposited in the GenBank database under accession number DQ631413.

RESULTS AND DISCUSSION

Isolation of *L. sakei* probio 65

Three hundred strains of lactic acid bacteria were isolated from crushed kimchi using cultivation on MRS agar. One strain was selected that showed the best survival rate in artificial gastric acid and bile. The 16S rDNA sequence of this isolate showed 100% similarity to that of *L. sakei* ssp. *sakei* DSM20017^T. Therefore, the strain is closely related to this taxon. This isolate was identified and designated as *L. sakei* probio 65 (Fig. 1). The bacteria were Gram-positive and appeared rod-shaped under scanning electron microscopy (Fig. 2).

L. sakei is one of the most important bacterial species involved in meat preservation and fermentation.³² It is also a predominant bacterium in the fermentation of kimchi, a traditional fermented cabbage product in the Republic of Korea.^{33,34} The presence of *L. sakei* in kimchi, a salty preservative food for the winter season in Korea, is due mostly to its ability to adapt to suboptimal temperatures and high salt concentrations, which promotes long-term survival.³⁵

TABLE 3. CHANGE IN DEGREE OF ALLERGIC DERMATITIS IN MICE AS DETERMINED BY VISUAL EVALUATION

Group	Allergen	Day				
		0	3	6	9	12
NC	—	0	0	0	0	0
PC	DNCB	10.7	11.33	11.17	4.33	3.5
T	DNCB	10	12	10.83	3.67	0.83

NC, negative control; PC, positive control; T, test mouse group supplemented with *L. sakei* probio 65 in feed.



FIG. 6. Improvements in skin of DNCB-induced mice by oral administration of *L. sakei* probio 65.

Resistance to artificial gastric juice and bile

The survival rate of *L. sakei* probio 65 after exposure to artificial gastric juice and bile was 50% and 79.21%, respectively (Fig. 3). Probiotics that are resistant to artificial gastric juice and bile are important *in vivo*.^{14,36} These characteristics of probiotics are exemplified by the probiotic bacteria that colonize the intestine,³⁶ and these probiotics could be administered orally.¹⁴ Therefore, we conclude that *L. sakei* probio 65 has the potential to act in a probiotic manner.

Antibiotic susceptibility and antimicrobial activity of *L. sakei* probio 65

The susceptibility of *L. sakei* probio 65 to 10 antibiotics is summarized in Table 1. Erythromycin and kanamycin, which are used as a treatment for skin inflammation, could not inhibit the growth of *L. sakei* probio 65.

The antimicrobial activity of *L. sakei* probio 65 is summarized in Table 2. *L. sakei* probio 65 showed broad-spectrum bacteriocidal activity against various pathogenic microorganisms and also strongly inhibited the growth of *S. aureus*, which is known to be a factor in severe AD.

Determination of IgE and IL-4 levels in murine serum and splenocytes

After the experiment was ended, IgE and IL-4 were determined from murine serum and splenocytes, respectively (Figs. 4 and 5). The IgE level of the test group, which was supplemented with *L. sakei* probio 65, was 451.71 ± 185.59 ng/mL, which was lower than that (741.67 ± 287.58 ng/mL) of the positive control group. This pattern was also observed

for IL-4 levels. The IL-4 level of the test group was 0.37 ± 0.39 pg/mL, which was lower than that (0.80 ± 0.66 pg/mL) of the positive control group.

Improvement of allergic dermatitis in chemical allergen-induced mice by L. sakei probio 65

The body weights of the test and positive control mice did not differ over the course of the experiment (data not shown). However, while both groups initially showed severe AD after DNCB treatment, the skin of the test group improved significantly faster (Table 3). A significant difference was observed in the scores after 12 days; although the positive control still had a score of 3.5, the test group score had decreased to 0.83 (Fig. 6).

In conclusion, we used a mouse model of skin inflammation to show that *L. sakei* probio 65 ingestion reduces serum IgE and splenocyte IL-4 levels that are induced by AD. This reduction was matched by significantly more rapid improvement in skin inflammation. These observations are consistent with previous studies, which have shown that probiotic strains can down-regulate IgE and IL-4 production. With regard to the effect of probiotic strains on serum IgE levels, oral administration of *Lactobacillus gasseri* TMC 0356 was shown to significantly reduce the serum IgE levels in patients with high serum IgE levels and perennial allergic rhinitis.³⁷ Moreover, oral administration of *Lactobacillus* GG decreased the serum IgE levels and severity scoring of AD in IgE-sensitized infants.³⁸ In addition, the *Lactobacillus* strain *L. casei* inhibits allergen-triggered production of IgE in murine splenocytes by promoting IL-12 secretion by macrophages.²⁰ With regard to the effect of probiotic strains on IL-4 production, it has been shown that probiotic strains down-regulate the IL-4 production of Th2-skewed splenocytes *in vitro*.³⁹ In addition, preincubation of mononuclear cells with different strains of lactic acid bacteria (*L. plantarum*, *Lactobacillus lactis*, *L. casei*, and *Lactobacillus rhamnosus*), but not the Gram-negative bacterium *E. coli*, inhibited their production of the Th2 cytokines IL-4 and IL-5.⁴⁰ The latter inhibitory effect of lactic acid bacteria was shown to be mediated by IL-12 and interferon- γ since neutralization of these cytokines restored IL-4 production. Animal models have also shown that lactobacilli induce interferon- γ production and thereby reduce allergen-stimulated production of IL-4 and IL-5.^{16,41}

In summary, our observations suggest that *L. sakei* probio 65 may be a good probiotic strain and that oral supplementation of humans with this strain may improve allergen-induced skin inflammation by down-regulating IgE and IL-4 production. Further studies will be needed to confirm this. In particular, it will be of interest to explore the effect of *L. sakei* probio 65 on the expression of transcriptional factors related to the immune response, as this may help elucidate the immunological mechanisms by which probiotic strains improve skin inflammation and AD.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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