



***Pediococcus pentosaceus* Sn26 Inhibits IgE Production and the Occurrence of Ovalbumin-Induced Allergic Diarrhea in Mice**

Takeyuki MASUDA,¹ Makoto KIMURA,¹ Sanae OKADA,² and Hisako YASUI^{1,†}

¹*Sciences of Functional Foods, Graduate School of Agriculture, Shinshu University, 8304 Minamiminowa, Kamiina, Nagano 399-4598, Japan*

²*Culture Collection Center, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan*

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We investigated the anti-allergic effect of a new strain (*Pediococcus pentosaceus* Sn26, the Sn26 strain) among 59 strains isolated from Japanese fermented vegetable pickles, the Sunki pickle. The Sn26 strain increased Th1 type cytokine (IL-12 and IFN- γ) production of Peyer's patch (PP) cells in BALB/c mice, improved the Th1/Th2 balance, and inhibited IgE production of splenocytes of ovalbumin (OVA)-induced allergic diarrheic mice. Next we demonstrated, by neutralizing IL-12 and IFN- γ , that the Sn26 strain first induced IL-12, that IL-12 induced IFN- γ , and that decreases in IL-4 and IgE production followed. Furthermore, oral administration of the Sn26 strain decreased serum OVA-specific IgE levels and ameliorated the appearance of diarrhea in OVA-induced allergic diarrheic mice. Based on these results, it was assumed that oral administration of the Sn26 strain ameliorated type-1 allergies through improvement of the Th1/Th2 balance and decreases in IgE production.

Key words: *Pediococcus pentosaceus*; Th1/Th2 balance; allergic diarrhea; IgE; lactic acid bacteria

Recently, the number of people suffering from type-1 allergies, such as food allergies, pollen allergies, and atopic dermatitis, has been increasing all around the world. The development of type-1 allergy is directly related to excess production of IgE antibodies. Cross-linking of IgE intermediated by binding of multivalent antigens at the surfaces of mast cells triggers the release of many chemical mediators, including histamine, leukotrienes, serotonin, and prostaglandins from these cells, and these chemical mediators cause type-1 allergic diseases.¹⁾ Therefore, inhibition of IgE production is a useful approach to preventing allergic diseases. Cytokines derived from helper T (Th) cells are major modulators of IgE synthesis. Th cells can be classified into various subtypes, including type-1 helper T (Th1) cells, type-2 helper T (Th2) cells, type-3 helper T (Th3) cells, type-17 helper T cells, regulatory T (Treg) cells, *etc.*, that are involved in the immune system.²⁾ These Th cells produce various interleukins. Interleukin (IL)-4 produced by Th2 cells induces IgE-switching and plays an important role in IgE production.^{3,4)} On the other hand, IFN- γ produced by Th1 cells suppresses IgE production by inhibiting the proliferation of Th2 cells.⁵⁾

IL-12, produced mainly by macrophages, stimulates differentiation from naïve T cells to Th1 cells. The balance of activation between Th1 and Th2 cells is considered critical in IgE production.

Kweon *et al.* have reported that BALB/c mice primed systematically with ovalbumin (OVA) develop severe diarrhea after repeated oral challenge with OVA, and that this allergic diarrhea is based on a food allergy model that leans towards the dominance of Th2 cells and high levels of IgE in the serum.⁶⁾ Therefore, in order to improve treatment of this allergy, suppression of IgE production through improvement of the IFN- γ /IL-4 value, which represents the Th1/Th2 balance, is important.

On the other hand, beneficial effects, including regulation of the enteric environment,⁷⁾ prevention of *Helicobacter pylori* infection,⁸⁾ prevention of antibiotic associate, acute and traveler's diarrhea,⁹⁾ *etc.*, due to lactic acid bacteria (LAB) and their products have been reported. In addition, it has been reported that some strains of LAB have an immunomodulatory effect, an anti-tumor effect,¹⁰⁾ and several protective effects against the influenza virus and rotavirus infections.^{11,12)} It has also been reported that some strains of LAB have the ability to prevent and alleviate type-1 allergies, including food allergies, pollen allergies, and atopic dermatitis, by modulation of the immune system through improvement of the Th2 dominant state toward an appropriate Th1/Th2 balance.¹³⁻¹⁷⁾ However, these effects were mostly found for LAB isolated from fermented milk or the human intestine, as research on LAB isolated from fermented vegetables is very rare. The LAB in fermented vegetables are expected to enhance the immunomodulatory effect due to the change of the cell wall and cell membrane *etc.*, because they survive in poor nutrition and in extreme environments that contain antibiotic substances.

Hence, we researched the anti-allergic effects of LAB isolated from Japanese traditional fermented vegetable pickles, the Sunki pickle using OVA-induced allergic diarrheic model mice. First we screened four strains by comparing the cellular immunomodulatory function of 59 strains of LAB using mouse Peyer's patch (PP) cell culture. PP is lymphoid tissue in the lamina propria of the small intestine that plays an important role in the immune response to intestinal microorganisms and food antigens. Next we selected a strain, the Sn26 strain, that

[†] To whom correspondence should be addressed. Fax: +81-265-77-1428; E-mail: hisakoy@shinshu-u.ac.jp

Table 1. Species and Inductive Levels of Cytokines of 59 LAB Strains Isolated from Sunki Pickle

Strain	Species	(ng/ml)		Strain	Species	(ng/ml)	
		IL-12	IFN- γ			IL-12	IFN- γ
cont		0.469	1.451	Sn18	<i>Lb. plantarum</i>	3.677	5.852
no. 1	<i>Leuc. gelidum</i>	3.898	8.445	Sn19	<i>Ped. acidilactici</i>	3.003	6.007
no. 2	<i>Lb. delbrueckii</i>	0.701	6.304	Sn21	<i>Lb. fermentum</i>	11.096	5.582
no. 3	<i>Lb. fermentum</i>	7.603	5.622	Sn24	<i>Lb. plantarum</i>	4.916	7.431
no. 4	<i>Lb. plantarum</i>	0.449	2.809	Sn26	<i>Ped. pentosaceus</i>	7.624	6.948
no. 6	<i>Lb. delbrueckii</i>	2.612	8.065	Sn28	<i>Lb. plantarum</i>	5.825	6.474
no. 7	<i>Lb. plantarum</i>	1.859	7.811	Sn32	<i>Lactobacillus sakei</i>	5.960	5.696
no. 8	<i>Lb. plantarum</i>	1.627	6.939	Sn34	<i>Ped. acidilactici</i>	2.272	6.481
no. 9	<i>Lb. plantarum</i>	1.191	6.122	Sn42	<i>Lb. plantarum</i>	4.125	7.176
no. 10	<i>Lb. fermentum</i>	22.833	5.793	Sn47	<i>Lb. plantarum</i>	5.549	6.515
no. 11	<i>Lb. delbrueckii</i>	2.198	7.346	Sn50	<i>Lb. plantarum</i>	7.106	6.675
no. 12	<i>Lb. fermentum</i>	0.744	2.997	Sn52	<i>Lb. brevis</i>	8.260	5.394
no. 13	<i>Lb. plantarum</i>	0.540	2.702	Sn53	<i>Lb. plantarum</i>	0.628	5.629
no. 14	<i>Lb. brevis</i>	7.694	5.534	Sn57	<i>Lb. plantarum</i>	4.538	5.659
no. 15	<i>Lb. brevis</i>	6.485	5.731	Sn58	<i>Lb. coryatus</i>	1.446	5.003
no. 16	<i>Lb. fermentum</i>	13.927	6.308	Sn62	<i>Lb. fermentum</i>	1.283	3.443
no. 17	<i>Lb. fermentum</i>	12.086	5.797	Sn64	<i>Lb. delbrueckii</i>	4.480	5.544
no. 18	<i>Lb. fermentum</i>	11.056	5.578	Sn66	<i>Lb. plantarum</i>	1.381	5.447
no. 19	<i>Lb. fermentum</i>	0.675	3.271	Sn70	<i>Leuc. garlicum</i>	8.457	6.045
no. 20	<i>Lb. fermentum</i>	10.530	5.959	Sn72	<i>Lb. fermentum</i>	13.046	5.645
no. 21	<i>Lb. fermentum</i>	12.629	5.011	Sn74	<i>Leuc. mesenteroides</i>	6.991	6.152
Sn1	<i>Lb. plantarum</i>	5.768	5.493	Sn75	<i>Lb. fermentum</i>	0.813	2.541
Sn4	<i>Lb. plantarum</i>	5.053	4.830	Sn76	<i>Lb. plantarum</i>	1.292	5.348
Sn5	<i>Lb. plantarum</i>	4.057	6.225	Sn78	<i>Leuc. gelidum</i>	4.374	5.526
Sn6	<i>Leuc. citreum</i>	11.272	4.809	Sn80	<i>Leuc. gelidum</i>	6.947	5.749
Sn11	unidentified	2.732	6.491	Sn81	<i>Leuc. gelidum</i>	5.871	5.467
Sn12	<i>Leuc. citreum</i>	9.319	5.374	Sn82	<i>Leuc. gelidum</i>	4.548	5.519
Sn13	<i>Lb. plantarum</i>	1.214	5.281	Sn83	<i>Leuc. gelidum</i>	6.235	5.369
Sn15	<i>Lb. plantarum</i>	2.340	5.960	Sn84	<i>Ped. pentosaceus</i>	4.961	5.401
Sn16	<i>Lb. plantarum</i>	1.062	4.903	Sn86	<i>Lb. plantarum</i>	1.850	5.469

Fifty-nine LAB strains isolated from Sunki pickle were identified by the 16S rRNA method. The strains were killed by heating at 121 °C for 15 min. PP cells were cultured with 1 µg/ml of these strains in a 37 °C, 5% CO₂ incubator. The culture supernatants were collected at 3 d to measure the cytokine (IL-12 and IFN- γ) levels by ELISA.

improved the Th1/Th2 balance and inhibited the IgE production of splenocytes of ovalbumin (OVA)-induced allergic diarrheic mice among the strains. Spleen produces serum IgE, which causes the occurrence of OVA-induced diarrhea. We found that oral administration of the Sn26 strain ameliorated allergic diarrhea through decreases in the IgE level in serum.

Materials and Methods

Mice. Female BALB/c mice were purchased from SLC (Hamamatu, Japan) and were used at 6 weeks or 8 weeks of age. All animal experiments followed the guidelines for the regulation of animal experiments of Shinshu University.

Microorganisms. Fifty-nine LAB strains were isolated from Sunki pickle and were identified by the 16S rRNA method (Table 1). These strains were incubated in de Man, Rogosa, and Sharpe (MRS) broth, cultivated for 48 h at 30 °C, harvested by centrifugation at 2,000 rpm for 15 min, washed 3 times with distilled water, and freeze-dried. Then the microorganisms were killed by heating at 121 °C for 15 min.

Allergic diarrhea. Mice 8 weeks of age were primed by subcutaneous (sc) injection of 1 mg of OVA in Complete Freund Adjuvant (CFA). One week after systemic priming, mice were repeatedly challenged with 10 mg of OVA by oral administration 3 times per week for several weeks. Diarrhea was monitored visually 1 h after each oral OVA challenge.

Cell cultures. PP cells were prepared from PP by treatment with 1.10 unit/ml of dispase (Gibco, Grand Island, NY, USA) dissolved in Joklik modified MEM (Sigma-Aldrich, St. Louis, MO, USA), and they (2.5 × 10⁶ cells/ml) were then cultured in RPMI-1640 medium

(Sigma-Aldrich, St. Louis, MO, USA) that contained 5% fetal bovine serum (FBS; Biowest, Miami, Florida, USA), 100 U/ml of penicillin and 100 µg/ml of streptomycin without (cont) or with 1 µg/ml of heat-killed LAB on a 96well culture plate in a 37 °C, 5% CO₂ incubator.¹⁸⁾ Culture supernatants were harvested at 3 d to measure Th1-type cytokine (IL-12 and IFN- γ) production. The concentrations of IL-12 and IFN- γ in the supernatant were determined by ELISA. The ability to regulate cytokine production and to suppress IgE production of the four selected strains were evaluated by splenocytes isolated from OVA-induced allergic diarrheic mice which were orally challenged with OVA 6 times. The splenocytes (2.5 × 10⁶ cells/ml) of these mice, in which the serum IgE level increased to about 2,000 ng/ml, were cultured in RPMI-1640 medium that contained 10% FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin, on a 96well culture plate. These cells were cultured with 100 µg/ml of OVA in the absence (cont) and the presence of 1 µg/ml of the four strains, in a 37 °C, 5% CO₂ incubator. The supernatants were harvested at 3 d to measure cytokine (IL-12, IFN- γ , and IL-4) production, and at 14 d to measure IgE antibody (total and OVA-specific IgE). The concentrations of cytokines and IgE antibody in the supernatant were determined by ELISA.

Neutralization. To determine the effects of cytokines such as IL-12 and IFN- γ on IgE suppression, a neutralization test was performed. Zero, 0.1, 1, and 10 µg/ml of purified rat anti-mouse IL-12 monoclonal antibody (BD Biosciences, clone C17.8, San Diego, CA, USA) or purified rat anti-mouse IFN- γ monoclonal antibody (BD Biosciences, clone XMGI.2, San Diego, CA, USA) were added to splenocytes (2.5 × 10⁶ cells/ml) of OVA-allergic diarrheic mice, and these splenocytes were cultured in the presence of Sn26 strains (1 µg/ml) on a 96well culture plate in a 37 °C, 5% CO₂ incubator. As a control, the splenocytes were cultured in absence of the Sn26 strain and these antibodies. Supernatants were harvested at 3 d to measure cytokine (IL-12, IFN- γ , and IL-4) production, and at 14 d to measure total IgE

antibody. The concentrations of IL-12, IFN- γ , IL-4, and total IgE in the supernatant were determined by ELISA.

In vivo experiment. Oral administration of 1 mg of the Sn26 strain to Sn26 group or of phosphate buffer saline (PBS) to the control group was done 5 times per week from the beginning to the end of the experiment (10 and 11 mice per group respectively). Two weeks from the beginning of the experiment using mice of 6 weeks age, the mice were primed by sc injection of 1 mg of OVA in CFA. One week later, the mice were repeatedly administered 10 mg of OVA orally 3 times per week for several weeks. Diarrhea was monitored visually 1 h after each oral OVA challenge. We measured the total and OVA-specific IgE levels of serum by ELISA sometimes.

ELISA. Measurement of IL-12, IFN- γ , IL-4, total IgE, and OVA-specific IgE in the culture supernatant was done by sandwich ELISA. Purified rat anti-mouse IL-12 (p40/p70) monoclonal antibody (BD Biosciences, clone C15.6, San Diego, CA, USA), rabbit (polyclonal) anti-mouse/rat IFN- γ (Biosource, Camarillo, CA, USA), monoclonal antibody mouse IL-4 (Mabtec, clone 11B11, Nacka, Strand, Sweden), or anti-IgE (EIU, LO-ME-2 purified, Belgium) was coated on the wells of a 96well ELISA plate (Nunc, Roskilde, Denmark) using a carbonate buffer, and this was incubated overnight at 4°C.

After blocking of the unoccupied sites on a plate with bovine serum albumin (BSA), the test sample and standard recombinant mouse IL-12 (Techne Corp, Minneapolis, MN, USA), recombinant murine IFN- γ (Biosource, Camarillo, CA, USA), recombinant murine IL-4 (Strathmann Biotec, Hanover, Germany), purified mouse IgE κ monoclonal (BD Biosciences, San Diego, CA, USA), or hyper immune anti-serum to OVA was added. Hyper immune anti-serum to OVA was prepared after intraperitoneal injection of 20 μ g of OVA with alum adjuvant into the mice 3 times. We determined that 1 unit of anti-OVA-specific IgE was equivalent in A₄₅₀ value to 100 dilutions of hyperimmune antiserum to OVA.

Subsequently, for detection of cytokines and antibodies, biotinylated rat anti-mouse IL-12 (p40/p70) monoclonal antibody (BD Biosciences, clone C17.8, San Diego, CA, USA), mouse (monoclonal) anti-rat/mouse IFN- γ biotin Conjugate (BD Biosciences, clone DB-1, San Diego, CA, USA), biotin conjugated antibody mouse monoclonal IL-4 (Mabtec, clone BVD6-24G2, Nacka, Strand, Sweden), biotinylated anti-IgE (Techopharm Biotechnology, LO-ME-2 biotin, Frankfurt, Germany), or biotinylated OVA was used. Biotinylated OVA was done using an ECL™ Protein Biotinylation Module (GE Healthcare, Buckinghamshire, UK). Streptavidin-horseradish peroxidase conjugate was added to each well. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Sigma, St. Louis, MO, USA) was added to the wells, and then the reaction was stopped with 1 M H₂SO₄. The absorbance of the contents of the wells was read at 450 nm on a Microplate Reader (Bio-Rad Laboratories).

Statistical analysis. The levels of various cytokines and total and OVA-specific IgE production on the splenocytes of the OVA-induced diarrheic mice were expressed as mean $\pm$ SD. Differences in the levels of these cytokines and IgE antibodies between the absence and the presence of the LAB strain on these splenocytes and between the absence and the presence of anti-IL-12 antibody and anti-IFN- γ antibody were analyzed by two-sided Student's *t* test. Differences in the symptom rates of diarrhea and total and OVA-specific IgE levels in serum between the absence and the presence of oral administration of the Sn26 strain were analyzed by the 2 $\times$ 2 contingency table and Two-sided student's *t*-test respectively. A probability value of less than 0.05 was considered statistically significant.

Results

Screening of LAB strains that increased IL-12 and IFN- γ production on the PP cells of BALB/c mice

We examined the cellular immunomodulatory function of 59 strains of LAB isolated from the Sunki pickle using the PP cell culture method (Table 1). We found some strains that induced large quantities of IL-12 or IFN- γ . The no. 10 strain induced IL-12 quite highly, but

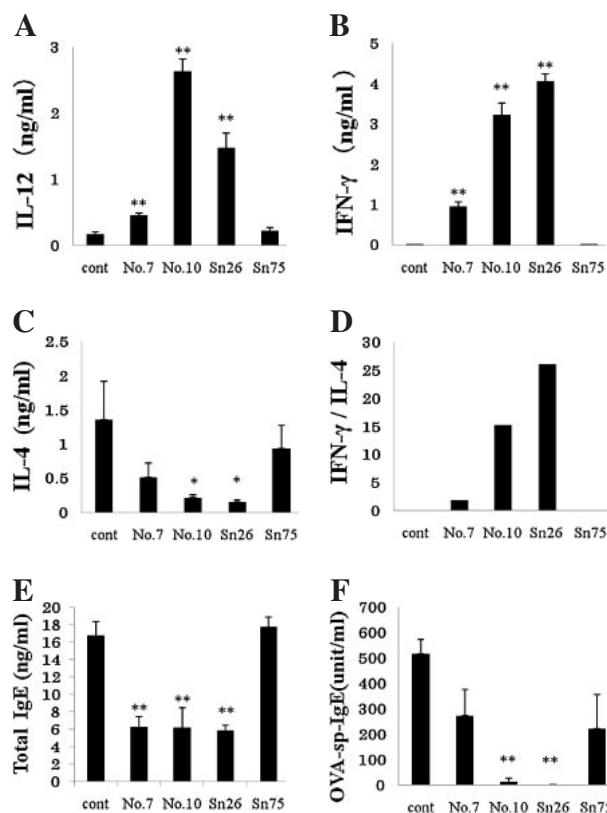


Fig. 1. Differences in IL-12, IFN- γ , IL-4, Total IgE, and OVA-Specific IgE Induction among the Strains Selected from 59 Strains. Splenocytes obtained from OVA-allergic diarrheic mice were cultured without (cont) or with 1 μ g/ml of four heat-killed LAB strains at 37°C, 5% in a CO₂ incubator. The supernatant was collected at 3 or 14 d to measure the cytokine (IL-12 (A), IFN- γ (B), IL-4 (C), IgE (total (E), or OVA-specific (F)) levels by ELISA. The Th1/Th2 balance was represented as the rate of IFN- γ /IL-4 levels (D) **p* < 0.05, ***p* < 0.01 vs. the value of cont.

the no. 2, no. 4, no. 12, no. 13, no. 19, Sn53, and Sn75 strains scarcely induced IL-12. All the strains of no. 10, no. 12, no. 19, and Sn75 were *Lb. fermentum*. As for IFN- γ induction, the no. 1, no. 6, no. 7, no. 11, Sn24, Sn26, and Sn42 strains were also very high, and the no. 4, no. 12, no. 13, no. 19, Sn62, and Sn75 strains were low. The no. 7, Sn24, and Sn42 strains, which induced large quantities of IFN- γ , and no. 4, which scarcely induced IFN- γ , were *Lb. plantarum*. Based on these results, it was found that the ability to induce IL-12 and IFN- γ secretion from PP cells depended on the strain of LAB, but did not depend on the species of LAB. We selected no. 7, which induced IL-12 weakly and IFN- γ strongly, no. 10, which induced IL-12 very strongly and IFN- γ strongly, the Sn26 strain, which induced IL-12 and IFN- γ strongly, and Sn75, which scarcely induced IL-12 or IFN- γ (Table 1).

Effects of the four strains on cytokine and IgE production by splenocytes in OVA-induced diarrheic mice

Next we examined the ability of the four strains to improve the Th1/Th2 balance and to suppress secretion of IgE in splenocytes obtained from OVA-induced diarrheic mice. Figure 1 shows the differences in cytokine and IgE induction on splenocytes of OVA-induced allergic diarrheic mice among the strains. Due to PP cells in the BALB/c mice, the no. 10 and Sn26

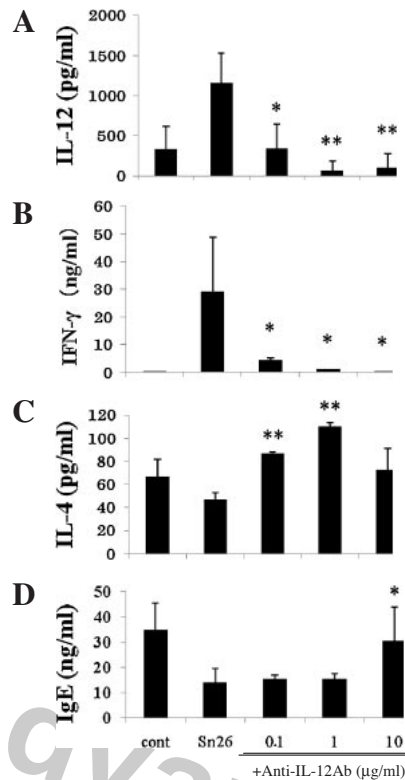


Fig. 2. Effects of Anti-IL-12 Antibody on the Sn26-Mediated Responses of IL-12, IFN- γ , IL-4, and IgE Production of Splenocytes in OVA-Allergic Diarrhea Mice.

Splenocytes obtained from OVA-allergic diarrhea mice were cultured in the absence (cont) and the presence (Sn26) of the Sn26 strains (1 μ g/ml). Anti-IL-12 antibody (0.1, 1, and 10 μ g/ml) was added in the presence of the Sn26 strains at the beginning of the culture period. Three or 14 d later, cytokine (IL-12 (A), IFN- γ (B), IL-4 (C)) or total IgE (D) levels in the culture supernatant were measured by ELISA. * p < 0.05, ** p < 0.01 vs. the value for Sn26.

strains induced IL-12 and IFN- γ very highly, but Sn75 scarcely induced them (Fig. 1A, B). The capacity of IFN- γ induction of no. 7 on splenocytes in the allergic diarrheic mice was lower than that on PP cells in the BALB/c mice (Fig. 1B, Table 1). This may depend on the difference in immune response between normal mice and allergic ones. Furthermore, no. 10 and Sn26 strongly suppressed IL-4 production and improved the Th1/Th2 balance (IFN- γ /IL-4) through a shift to Th1 (Fig. 1C, D). Three strains, not including the no. 75 strains, strongly inhibited total IgE production as compared with not adding LAB (cont) (p < 0.01) (Fig. 1E). In particular, no. 10 and Sn26 strongly inhibited OVA-specific IgE production (p < 0.01) (Fig. 1F). However, the ability to improve the Th1/Th2 balance and the inhibition of OVA-specific IgE production in the Sn26 strains were stronger than in the no. 10 strain. Finally, the Sn26 strain was screened as the LAB strain expected to have the strongest anti-allergy function among the 59 strains, and was used in further experiments.

Mechanism of the Sn26-mediated response of IgE production by splenocytes in OVA-induced diarrheic mice

By neutralizing IL-12 or IFN- γ induced by the Sn26 strain, the mechanism of IgE suppression of the Sn26 strain was analyzed. With the addition of the anti-IL-12

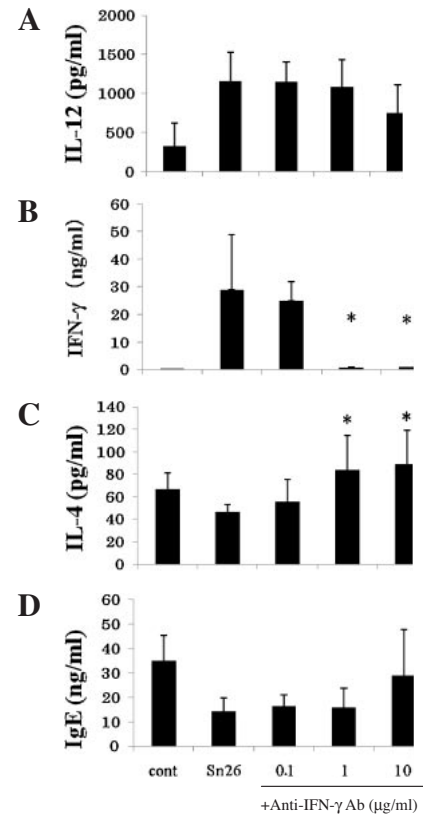


Fig. 3. Effects of Anti-IFN- γ Antibody on the Sn26-Mediated Responses of IL-12, IFN- γ , IL-4, and IgE Production of Splenocytes in OVA-Allergic Diarrhea Mice.

Splenocytes obtained from OVA-allergic diarrhea mice were cultured in the absence (cont) and the presence (Sn26) of the Sn26 strains (1 μ g/ml). Anti-IFN- γ antibody (0.1, 1, and 10 μ g/ml) was added in the presence of the Sn26 strains at the beginning of the culture period. Three or 14 d later, cytokine (IL-12 (A), IFN- γ (B), IL-4 (C)) or total IgE (D) levels in the culture supernatant were measured by ELISA. * p < 0.05, ** p < 0.01 vs. the value of Sn26.

antibody, the IL-12 level decreased significantly, and the production of IFN- γ was depressed significantly, too (Fig. 2). From this result it was clear that IL-12 participated in the production of IFN- γ . Furthermore, the addition of the anti-IL-12 antibody significantly increased the production of IL-4 and IgE. With the addition of the anti-IFN- γ antibody, the IFN- γ level decreased and the IL-4 level increased significantly (Fig. 3), but the IL-12 level was not affected. The IgE level, which was suppressed by the Sn26 strains, showed a tendency to increase with the addition of the anti-IFN- γ antibody. From these results, it was found that IFN- γ was induced by IL-12 and that IFN- γ suppressed IL-4 and IgE production.

Effects of oral administration of Sn26 on the occurrence of OVA-induced allergic diarrhea

Next we examined the effects of oral administration of Sn26 on allergic diarrhea. The occurrence of allergic diarrhea in mice fed Sn26 (the Sn26 group) was delayed significantly as compared with that of the mice fed PBS (the control group) (Fig. 4A). The occurrence of diarrhea in the control group appeared 5 weeks after the start of the experiment, and the rate reached as high as 80% at the end of experiment. However, the diarrhea of the Sn26 group appeared for the first time at 7 weeks,

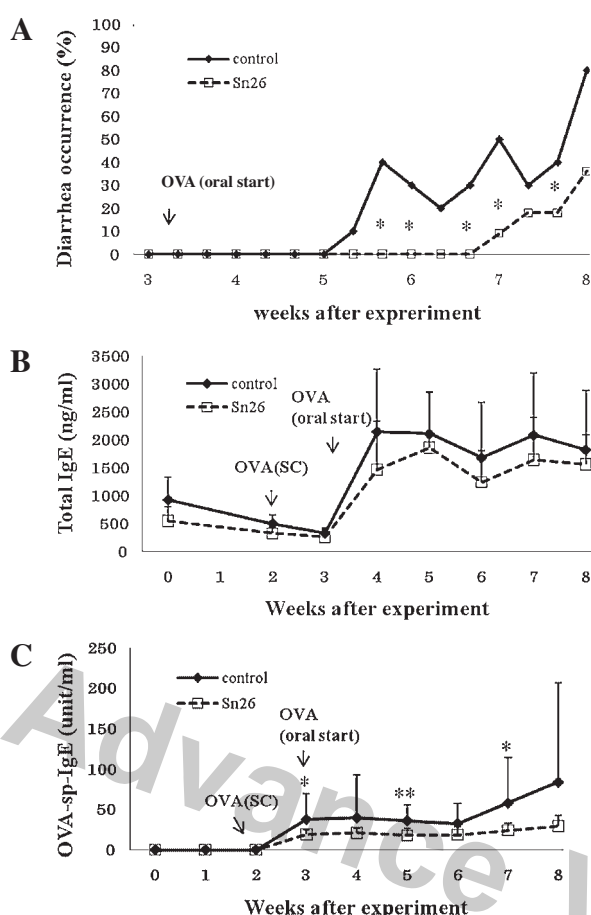


Fig. 4. The Effects of Oral Administration of the Sn26 Strain on OVA-Induced Diarrhea.

The mice were divided into two groups and were orally administrated 1 mg of the Sn26 strain (Sn26, $n = 10$) or PBS (control, $n = 11$) 5 times per week from the beginning to the end of the experiment. The mice were primed by subcutaneous injection of 1 mg of OVA in complete Freund's adjuvant. One week later, the mice were repeatedly challenged with 10 mg of OVA by oral administration 3 times per week for several weeks. Diarrhea was monitored visually 1 h after oral OVA-challenge (A). Total IgE (B) and OVA-specific IgE levels (C) in the serum were measured at intervals by ELISA. * $p < 0.05$, ** $p < 0.01$ vs. the value of control.

and the rate was 36% at the end of the experiment. In addition, the level of total IgE antibodies in serum in the control group tended to be higher than in the Sn26 group all the time after oral administration of OVA (Fig. 4B). The OVA-specific IgE level in the Sn26 group became significantly lower than that of the control group (Fig. 4C). From these results, it was guessed that OVA-induced allergic diarrhea ameliorated through inhibition of IgE production by oral administration of the Sn26 strain.

Discussion

Various LAB strains were isolated from the intestinal tracts of animals and fermented dairy products, such as yogurt and cheese, and beneficial effects, especially anti-allergic,¹⁴ anti-tumor,¹⁰ anti-influenza,¹¹ and anti-rotavirus effects,¹² due to the immunomodulatory effect have been reported. Various LAB strains are isolated from traditional fermented vegetables, including miso, soy sauce, and vegetable pickles in Japan. However, the beneficial effects, such as the immunomodulatory effect,

have not been studied sufficiently. It has been reported that the immunomodulatory effect of LAB depends on the strength of the bacterial structure or the quantity of peptidoglycan in the cell wall.^{15,19,20} These are almost entirely controlled by bacterial genes. But it is also assumed that the cell structure and the cell wall of these LAB isolated from vegetable fermented foods become solid and thick in order to adapt to severe growing conditions, high osmotic pressure, low nutrition, and the presence of antibacterial substances, and that these microorganisms have strong immunostimulating functions. In this study, we tried to find a new LAB strain that has a strong immunomodulatory effect and a strong ameliorative effect against type-1 allergies from the Sunki pickles.

PP in the small intestine is the lymphoid organ that ingested foods first encounter. The food antigens are phagocytosed by the M cells of PP, and the antigens are presented in antigen-presenting cells (APCs), such as macrophages. APCs induce the differentiation of Th cells and this leads to various immunological responses. Th cells are one of the most important types of cells in the immune system, and they are divided into several types. They usually interact with each other and maintain a balance among themselves. Type-1 allergies develop when Th2 cells become superfluous and the production of IgE is promoted. On the other hand, activated APCs induce IL-12, which activates Th1 cells. The Th1 cells inhibit production of IgE through IFN- γ secretion, in contrast with Th2 cells, which promote IgE secretion through IL-4 and IL-5. It has been reported that some strains of LAB increase IL-12 and IFN- γ production and decrease IgE production and ameliorate type-1 allergy disease.¹⁶

Hence we researched the difference in IL-12 and IFN- γ induction in PP cells among the 59 strains isolated from the Sunki pickle, and found some strains that induce a large quantity of IL-12 and IFN- γ production (Table 1). Some strains did not induce IL-12 production but induced IFN- γ strongly. Though the mechanisms are not clear, these strains might activate Th1 cells through pathways in which APC do not participate. Shida *et al.* reported that the *Lb. casei* group and the *Lb. fermentum* group have a unique solid structure and induce IL-12 production strongly.¹⁹ As for our results, the no. 10, no. 16, and Sn72 strains that strongly induced IL-12 production were *Lb. fermentum* (Table 1). On the other hand, the no. 12, no. 19, and Sn75 strains that were *Lb. fermentum* had only minor effects. Furthermore, the capacity for IFN- γ induction also was different among various strains of the same species. Our results indicate that immunomodulatory activity depended on the strain but did not depend on the species of LAB. This is similar to the results found by Fujiwara *et al.*²¹ It has been reported that the cell wall of LAB becomes thick under high salt conditions and that induction of IL-12 production is related to the level of the salt condition.^{22,23} Sashihara *et al.* also found a positive relationship between the amount of peptidoglycan of LAB and the inductive activity of IL-12.¹⁵ Based on these reports, it was assumed that the difference in immunomodulatory activity among the strains contributes to the quality and quantity of the cell wall, which changed under environmental conditions, too.

It has been reported that the suppressive mechanism of type-1 allergies by some strains of LAB decreases IgE production by improvement of the Th1/Th2 balance through a shift from the Th2 type to the Th1 type. The Th1/Th2 balance was evaluated using an IFN- γ /IL-4 or a IL-12/IL-4 value.^{21,22,24} However, the correlative relationship between IL-12 and IFN- γ production was not clear in our study. Hence we examined the IgE suppressive effect of selected strains, that have a characteristic immunological function using splenocytes of allergic diarrheic mice (Fig. 1). Allergic diarrheic mice induced by OVA have been classified as having type-1 allergies, which are the result of excessive serum IgE production through the Th2 predominant response.²⁵ Hence we prepared splenocytes of the allergic diarrheic mice in which the serum IgE level increased to about 2,000 ng/ml, and then cultured them with the four selected strains. Then we compared the effect of the Th1/Th2 balance and the suppressive effect of IgE production among the strains. In this study, we used splenocytes because they produce serum IgE, which causes OVA-induced diarrhea. We focused on no. 10 and Sn26, which have strong suppressive effects on IgE production (Fig. 1E, F), and confirmed improvement in the Th1/Th2 balance. As for this result, the improvement in the Th1/Th2 balance of the Sn26 strain was stronger than that of the no. 10 strain. Hence we selected the Sn26 strain and decided to study the mechanism of IgE suppression and the anti-allergic effects of the strain.

We examined the suppressive mechanism of IgE production of the Sn26 strains by neutralizing IL-12 and IFN- γ . IL-12 and IFN- γ production was suppressed by neutralizing IL-12. The Sn26 strains had very strong capacity for IFN- γ induction, but the IFN- γ production decreased depending on the concentration of anti-IL-12 antibody (Fig. 2B). This result suggests that IL-12 induced by Sn26 can lead to IFN- γ induction. This is similar to the result of Yoshida *et al.*²⁶ Neutralizing by 1 μ g/ml of anti-IL-12 antibody decreased IL-12 and IFN- γ production significantly and increased IL-4 significantly but did not increase IgE. Neutralizing by 10 μ g/ml of anti-IL-12 antibody improved the IFN- γ /IL-4 balance and increased IgE production. A high concentration of anti-IL-12 antibody may be needed to cancel suppression of IgE production by the Sn26 strain. On the other hand, neutralization of IFN- γ lead to decreases in IFN- γ and increases in IL-4 and IgE production (Fig. 3B, C, D), but did not affect IL-12 production (Fig. 3A). This made it clear that the inductive ability of IFN- γ by Sn26 was due to IL-12. Type-1 cytokines (IL-12 and IFN- γ) contributed to inhibit IgE production. This mechanism is similar to that of the *Lb. casei* Shirota strain as reported by Shida *et al.*²⁷

Finally, oral administration of the Sn26 strain decreased IgE, especially the OVA-specific IgE level, in serum, and suppressed allergic diarrhea (Fig. 4). It has been reported that this allergic diarrhea in mice was treated successfully by removing IL-4 or IgE antibody which were produced excessively.⁶ Our experiment also showed a positive relationship between the serum IgE level, especially the serum OVA-specific IgE level, and the occurrence of allergic diarrhea.

The mechanism of allergic diarrhea has been reported to be as follows: Cross-linking of IgE intermediated by the binding of multivalent antigens at the surface of mast cells and/or basophiles triggers the release of many chemical mediators (degranulation). Brandt *et al.* have reported that the important chemical mediator is mucosal mast cell protease (MMCP)-1 or serotonin.²⁸ Other researchers have found that MMCP-1 correlated strongly with the occurrence of allergic diarrhea.^{29,30} We guessed that the Sn26 strains decreased those chemical mediators by inhibiting the degranulation of cells through decreases in the IgE antibody. It has also been reported that amelioration of allergic diseases is related not only to decreases in the IgE level through improvement of the Th1/Th2 balance, but also to increases in IL-10 from Treg and TGF- β from Th3.^{31,32} Further studies are now in progress.

References

- 1) Puxeddu I, Piliponsky AM, Bachelet I, and Levi-Schaffer F, *Int J. Biochem. Cell Biol.*, **35**, 1601–1607 (2003).
- 2) Romagnani S, *Inflamm. Bowel Dis.*, **5**, 285–294 (1999).
- 3) Rousset F, Robert J, Andary M, Bonnin JP, Souillet G, Chretien I, Briere F, Pene J, and de Vries JE, *J. Allergy Clin. Immunol.*, **87**, 58–69 (1991).
- 4) Yanagihara Y, Ikizawa K, Kajiwaru K, Koshio T, and Akiyama K, *J. Allergy Clin. Immunol.*, **96**, 1145–1151 (1995).
- 5) Murosaki S, Muroyama K, Yamamoto Y, Kusaka H, Liu T, and Yoshikai Y, *Biosci. Biotechnol. Biochem.*, **63**, 373–378 (1999).
- 6) Kweon MN, Yamamoto M, Kajiki M, Takahashi I, and Kiyono H, *J. Clin. Invest.*, **106**, 199–206 (2000).
- 7) Colombel JF, Cortot A, Neut C, and Romond C, *Lancet*, **330**, 43 (1987).
- 8) Kabir AM, Aiba Y, Takagi A, Kamiya S, Miwa T, and Koga Y, *Gut*, **41**, 49–55 (1997).
- 9) Gismondo MR, Drago L, and Lombardi A, *Int. J. Antimicrobial Agents*, **12**, 287–292 (1999).
- 10) Takeda K and Okumura K, *J. Nutr.*, **137**, 791S–793S (2007).
- 11) Hori T, Kiyoshima J, Shida K, and Yasui H, *Clin. Diagn. Lab. Immunol.*, **9**, 105–108 (2002).
- 12) Isolauri E, Kaila M, Mykkanen H, Ling WH, and Salminen S, *Dig. Dis. Sci.*, **39**, 2595–2600 (1994).
- 13) Yasui H, Shida K, Matuzaki T, and Yokokura T, *Antonie Van Leeuwenhoek*, **76**, 383–389 (1999).
- 14) Ishida Y, Bandou I, Kanzato H, and Yamamoto N, *Biosci. Biotechnol. Biochem.*, **67**, 951–957 (2003).
- 15) Sashihara T, Sueki N, and Ikegami S, *J. Dairy Sci.*, **89**, 2846–2855 (2006).
- 16) Segawa S, Hayashi A, Nakakita Y, Kaneda H, Watari J, and Yasui H, *Biol. Pharm. Bull.*, **31**, 884–889 (2008).
- 17) Watanabe T, Hamada K, Tategaki A, Kishida H, Tanaka H, Kitano M, and Miyamoto T, *J. Nutr. Sci. Vitaminol.*, **55**, 271–278 (2009).
- 18) Yasui H, Nagaoka N, and Hayakawa K, *Clin. Diagn. Lab. Immunol.*, **1**, 244–246 (1994).
- 19) Shida K, Kiyoshima-Shibata J, Nagaoka M, Watanabe K, and Nanno M, *J. Dairy Sci.*, **89**, 3306–3317 (2006).
- 20) Lawrnce C and Naucuel C, *Infect. Immun.*, **66**, 4947–4949 (1998).
- 21) Fujiwara D, Inoue S, Wakabayashi H, and Fujii T, *Int. Arch. Allergy Immunol.*, **135**, 205–215 (2004).
- 22) Masuda S, Yamaguchi H, Kurosawa T, Shirakami T, Tsuji RF, and Nishimura I, *Int. J. Food Microbiol.*, **121**, 245–252 (2008).
- 23) Piuri M, Sanchez-Rivas C, and Ruzal SM, *J. Appl. Microbiol.*, **98**, 84–95 (2005).
- 24) Segawa S, Nakakita Y, Takata Y, Wakita Y, Kaneko T, Kaneda H, Watari J, and Yasui H, *Int. J. Food Microbiol.*, **121**, 1–10 (2008).
- 25) Hino A, Kweon MN, Fujihashi K, McGhee JR, and Kiyono H, *Am. J. Pathol.*, **164**, 1327–1335 (2004).

- 26) Yoshida T, Hirano A, Wada H, Takahashi K, and Hattori M, *Int. Arch. Allergy Immunol.*, **133**, 239–247 (2004).
- 27) Shida K, Makino K, Morishita A, Takamizawa K, Hachimura S, Ametani A, Sato T, Kumagai Y, Habu S, and Kaminogawa S, *Int. Arch. Allergy Immunol.*, **115**, 278–287 (1998).
- 28) Brandt EB, Strait RT, Hershko D, Wang Q, Muntel EE, Scribner TA, Zimmermann N, Finkelman FD, and Rothenberg ME, *J. Clin. Invest.*, **112**, 1666–1677 (2003).
- 29) Vaali K, Puumalainen TJ, Lehto M, Wolff H, Rita H, Alenius H, and Palosuo T, *Scand. J. Gastroenterol.*, **41**, 1405–1413 (2006).
- 30) Pemberton AD, Wright SH, Knight PA, and Miller HR, *J Immunol.*, **176**, 899–904 (2006).
- 31) Hayashi A, Kimura M, Nakamura Y, and Yasui H, *J. Dairy Res.*, **76**, 158–164 (2009).
- 32) Nonaka Y, Izumo T, Izumi F, Maekawa T, Shibata H, Nakano A, Kishi A, Akatani K, and Kiso Y, *Int. Arch. Allergy Immunol.*, **145**, 249–257 (2008).

Advance View Proofs