



Note

Sake Lees Fermented with Lactic Acid Bacteria Prevents Allergic Rhinitis-Like Symptoms and IgE-Mediated Basophil Degranulation

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We tested the effect of oral administration of fermented sake lees with lactic acid bacteria (FESLAB) on a murine model of allergic rhinitis upon immunization and nasal sensitization with ovalbumin (OVA). We used *Lactobacillus paracasei* NPSRIk-4 (isolated from sake lees), and *L. brevis* NPSRIv-8 (from fermented milk) as starter strains to produce the FESLAB. Oral FESLAB administration resulted in the development of significantly fewer sneezing symptoms than those seen in sham control animals given sterile water. We also found that FESLAB suppressed the allergen-induced degranulation of RBL2H3 rat basophilic leukemia cells.

Key words: allergic rhinitis; degranulation; fermented sake lees; lactic acid bacteria; *Lactobacillus*

Sake lees (sake-kasu) are a byproduct generated in the production of Japanese sake (Japanese rice wine), and are used as a condiment for making such traditional Japanese foods as a soup (kasu-jiru) and pickled fishes/vegetables (kasuduke pickles). Several lines of evidence have suggested that the intake of sake lees could be beneficial for our health. Mochida *et al.* have reported that animals fed diets containing sake lees showed lower serum cholesterol levels.¹⁾ A sake lees-containing diet (50%) has also been shown to be hypolipidemic to rats with no side effect on their growth.²⁾ Manabe *et al.* have indicated that rats fed the sake lees-supplemented diets shows increased spontaneous locomotive activity.³⁾ More recently, Izu and co-workers have demonstrated that sake yeast (*Saccharomyces cerevisiae* Kyokai yeast), one of the main ingredients of sake lees, suppressed alcohol-induced liver injury in mice.⁴⁾ Indeed, sake lees and sake yeast contain such functional food components as angiotensin I-converting enzyme inhibitory peptides with antihypertensive activity⁵⁾ and S-adenosyl-L-methionine that may be beneficial in preventing various diseases.⁶⁾ However, our knowledge about the physiological effects of dietary sake lees is

still limited, and a large amount of sake lees is discarded as an industrial waste. The development of new functional foods utilizing sake lees would be an attractive recycling strategy for this waste. In the present study, we generated fermented sake lees with lactic acid bacteria (FESLAB) by fermenting sake lees with *Lactobacillus paracasei* and *L. brevis*. We show here that dietary supplementation with FESLAB prevented the development of nasal symptoms in a murine model of allergic rhinitis. We also provide evidence that FESLAB down-regulated the IgE-mediated type I allergic reaction.

L. paracasei NPSRIk-4, which was isolated from sake lees, and *L. brevis* NPSRIv-8, which was isolated from fermented milk, were used as starter strains to ferment the sake lees. We chose these strains mainly because of a good taste of resulting FESLAB upon fermentation with them. The other two salient features of these *Lactobacilli* are a gamma-aminobutyric acid (GABA)-producing ability (NPSRIv-8 cells) and a resistance to artificial gastric juice (NPSRIk-4 cells). These lactic acid bacteria were cultured in an MRS medium (Difco Laboratories, Detroit, MI, USA) at 30 °C for 48 h, and then inoculated into an SY medium (10% sake lees/2% yeast extract) for further cultivation at 30 °C for 48 h. These cultures were used as a starter for the FESLAB fermentation. Sake lees (containing 40% water and 10% ethanol, and prepared from Ginjo-sake brewing) were obtained from the Niigata Prefectural Sake Research Institute and stored at -20 °C until being used. Prior to their fermentation, the sake lees were steamed for 30 min to remove the alcohol and to sterilize them. After cooling to room temperature, the sake lees were dissolved in sterile water to make a 20% suspension and then pasteurized at 105 °C for 20 min. The *Lactobacilli* starter (3.2×10^7 cells/mL of *L. paracasei* NPSRIk-4 cells and 3.5×10^7 cells/mL of *L. brevis* NPSRIv-8 cells) was added to the resulting sake lees suspension, and the mixture was cultured at 30 °C for 24 h to obtain FESLAB. We observed an increase in the concentration

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; FESLAB, fermented sake lees with lactic acid bacteria; i.n., intranasal; i.p., intraperitoneal; mAb, monoclonal antibody; OVA, ovalbumin; PBS, phosphate-buffered saline

of NPSRIk-4 cells (5.1×10^7 cells/mL) and NPSRIv-8 cells (7.6×10^7 cells/mL) upon cultivation with the sake lees. Moreover, the pH value and glucose concentration of the culture broth decreased from 4.93 to 3.51 (pH), and 11.18 mg/mL to 6.01 mg/mL (glucose content). These results indicate successful lactic acid fermentation of the FESLAB culture.

Female BALB/c mice (Charles River Japan, Yokohama, Japan) were maintained under specific pathogen-free conditions on a CRF-1 diet (Oriental Yeast, Tokyo, Japan) to test the *in vivo* effect of dietary FESLAB. All animals were housed in an animal facility kept at $22 \pm 3^\circ\text{C}$ with a 12-h light/12-h dark cycle. The oral administration procedure for the FESLAB suspension (100 μL /d/head) involved daily intragastric inoculation of the BALB/c mice ($n = 6$, 4 weeks old) for 51 d. We administered distilled sterile water or an unfermented sake lees suspension for sham control experiments. All animal studies were carried out under the protocols reviewed and approved by the Committee of Animal Experimentation at Hiroshima University.

A murine model of allergic rhinitis was created by previously described procedures with some modifications.^{7,8} Briefly, at 6 and 8 weeks of age (*i.e.*, 2 and 4 weeks after starting the oral administration of FESLAB), BALB/c mice were intraperitoneally immunized with either phosphate-buffered saline (PBS) or 20 μg of ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO, USA) emulsified in 2.25 mg of an alum adjuvant (LSL, Tokyo, Japan) in a 100 μL of total volume. Two weeks after the second OVA immunization, the mice were intranasally challenged with an OVA solution (25 mg/mL solution in PBS, 20 μL /head) by a daily instillation without anesthesia for 9 d. The mice were each placed in an observation cage (one animal/cage) just after the daily OVA challenge to evaluate nasal symptoms, and the number of sneezes was counted for 5 min under blinded conditions. Twelve hours after the ninth nasal challenge, blood samples were collected, and plasma IgE levels were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) as described.⁹ Plasma OVA-specific IgE, IgG1, and IgG2a titers were also analyzed by ELISA as described.¹⁰

Rat basophilic leukemia cell line RBL2H3 (RIKEN Cell Bank, Tsukuba, Japan) was maintained in an MEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS; Biowest, Rue de la Caille, Muaille, France) at 37°C and 5% CO_2 /95% air. A degranulation assay was conducted on RBL2H3 cells (3×10^5 cells/mL) that had been precultured in 24-well cell culture plates (Iwaki Glass, Funabashi, Japan) for 8 h, and then sensitized with the mouse anti-dinitrophenol (DNP) IgE monoclonal antibody (mAb; 10 μg /mL in 5% FBS/MEM; Seikagaku Biobusiness, Tokyo, Japan) by further culturing for 16 h. The cells were then washed twice with a siraganian buffer [119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl_2 , 1 mM CaCl_2 , 25 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), 0.1% bovine serum albumin (BSA) and 40 mM NaOH, at pH 7.2], and incubated with serially-diluted FESLAB samples (1:0, 1:2, and 1:4 dilutions) at 37°C for 15 min. Each sample dilution was buffered by adding a 10 $\times$ concentrate of the siraganian buffer. Allergen-driven mediator release was induced by stimulating with the

DNP-BSA antigen (Cosmo Bio, Tokyo, Japan) for 30 min. Calcium ionophore A23187 (Sigma-Aldrich) was used as a positive control stimulant for basophil degranulation. Total intracellular mediator release was carried out by cell lysis with the 0.2% Triton X-100/siraganian buffer. To determine the enzymatic activity of the released β -hexosaminidase, 50 μL of the supernatant was mixed with 50 μL of 1 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Sigma-Aldrich; in a 0.1 M citric acid buffer, at pH 4.5) in 96-well plates, and the mixture was incubated at 37°C for 30 min. The reaction was stopped by adding 100 μL of 0.1 M $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ (pH 10.0), and the absorbance at 405 nm (A405 nm) was analyzed by using a Wallac 1420 ARVOsx Multilabel Counter (Perkin Elmer Life Sciences, Boston, MA, USA). Mediator release (%) was calculated by using the following formula: β -hexosaminidase release (%) = supernatant β -hexosaminidase activity (A405 nm)/total intracellular β -hexosaminidase activity (A405 nm) $\times 100$. All the statistical analyses in this study were performed by using Student's *t*-test, with $p < 0.05$ being accepted as the level of significance.

We first tested the effect of an oral FESLAB administration on the nasal symptoms in the OVA-sensitized mice by monitoring their sneezing behavior. A sham control group, which was administered sterile water and then given an intraperitoneal (*i.p.*) injection and intranasal (*i.n.*) challenge with OVA (OVA/OVA), showed a marked sneezing count (shown in the blue plot in Fig. 1). Another control OVA/OVA group fed unfermented sake lees also developed the rhinitis symptoms that were statistically indistinguishable from those of the water-OVA/OVA group (shown in black). In contrast, the development of those nasal symptoms

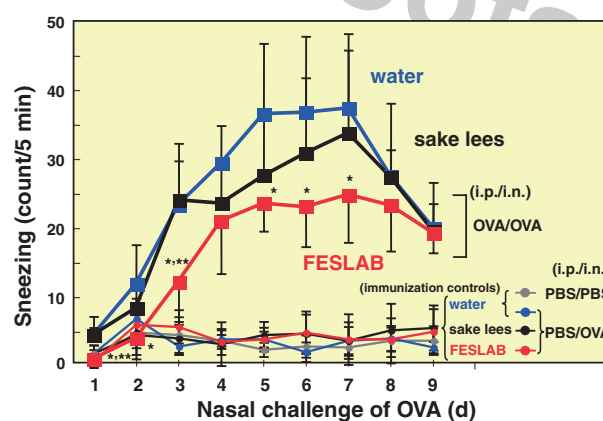


Fig. 1. Oral Administration of FESLAB Prevents the Development of Sneezing in BALB/c Mice upon Immunization and Nasal Challenge with OVA.

4-week-old BALB/c mice were intragastrically inoculated with a FESLAB suspension daily for 51 d. Two weeks after starting the FESLAB administration, those mice were *i.p.* immunized and *i.n.* challenged with OVA (shown as OVA/OVA, in red) as described in the text. The number of sneezes in 5 min was counted just after the daily nasal challenge with OVA. Each value is expressed as the mean $\pm$ standard deviation (SD) with six mice per group. * $p < 0.05$ (versus OVA/OVA mice given sterile water, in blue); ** $p < 0.05$ (versus OVA/OVA mice orally administered unfermented sake lees, in black). Data obtained from the immunization control groups (a PBS/PBS group given water and three PBS/OVA groups) show baselines for this assay, clearly demonstrating that our rhinitis model was appropriately conducted.

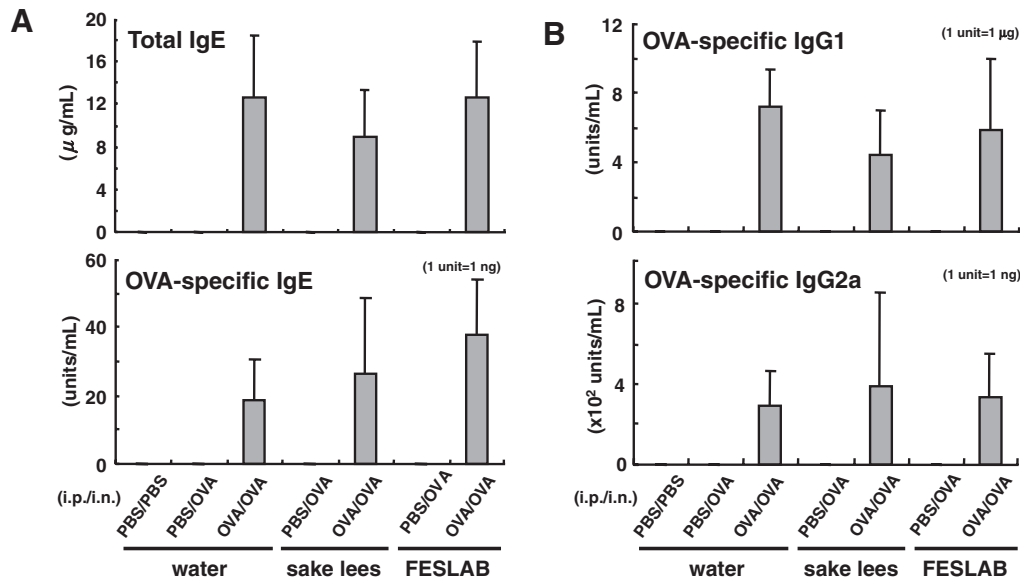


Fig. 2. FESLAB Administration Has No Effect on Plasma Total IgE Levels Nor on the OVA-Specific Antibody Response.

A, After the ninth nasal challenge with OVA, plasma samples were collected from each group of mice, and the total IgE concentration and OVA-specific IgE response were analyzed by sandwich ELISA and ELISA as described.^{9,10} The OVA-specific IgE titer (unit) is defined as the ELISA value for 1 ng of the mouse IgE isotype control antibody. B, OVA-specific IgG1 and IgG2a titers in these mice. The respective OVA-specific IgG1 and IgG2a responses (unit) correspond to the ELISA values for 1 μg of IgG1 and 1 ng of the IgG2a isotype controls.

was prevented in the FESLAB-administered OVA/OVA mice (shown in red); the significant anti-rhinitis effects were observed on the days 1, 2, 3, 5, 6, and 7 when compared with the water-OVA/OVA mice (shown by single asterisks in Fig. 1). We also found that the FESLAB-OVA/OVA group showed a significantly lower sneezing count on the days 1 and 3 in than the sake lees-OVA/OVA group (shown by double asterisks).

To explore the mode of the anti-rhinitis action of dietary FESLAB, we next examined the total plasma IgE concentration and allergen-specific antibody response in these mice. As shown in Fig. 2A (top view), oral FESLAB administration had no effect on the peripheral IgE levels in the OVA/OVA group. The OVA-specific IgE response of the FESLAB-fed OVA/OVA mice was also comparable to that of the sham control groups given either water or unfermented sake lees (Fig. 2A, bottom view). We also found no significant difference in OVA-specific IgG1 and IgG2a responses between the FESLAB-fed group and the two control groups (Fig. 2B). These results indicate that oral supplementation of FESLAB did not affect either the allergen-specific antibody response or the peripheral IgE concentration.

The nasal symptoms in our murine model of allergic rhinitis are highly dependent on the IgE antibody-high affinity receptor for the IgE (FcεRI) signaling axis, as demonstrated by a previous study in which sneezing behavior is severely impaired in FcεRI-deficient mice.⁷ The anti-rhinitis effect of FESLAB (Fig. 1) and its ineffectiveness in suppressing the OVA-specific IgE response (Fig. 2A) suggest that FESLAB might have down-modulated the FcεRI-mediated type I allergic reaction to fulfill its anti-inflammatory activity. We next tested this possibility by seeing whether FESLAB suppressed IgE-induced mediator release of the RBL2H3 rat basophilic leukemia cells. We found that pre-stimulation with the FESLAB samples significantly inhibited the DNP-BSA antigen (Ag)-driven β-hexosa-

minidase release from anti-DNP IgE mAb-sensitized RBL2H3 cells in comparison to those stimulated with the control unfermented sake lees or sterile water (at 1:0 and 1:2 dilutions, shown in Fig. 3). These data provide *in vitro* evidence that FESLAB actually possesses the ability to negatively regulate the FcεRI-mediated type I allergic reaction.

Dietary FESLAB significantly prevented the development of allergen-driven nasal symptoms without affecting either the plasma IgE level or the allergen-specific IgE, IgG1, and IgG2a responses (Figs. 1 and 2). These results suggest that the anti-rhinitis action of FESLAB was not accompanied by any modulation of the Th1/Th2 cytokine response. Our cytokine analysis revealed that the FESLAB administration had no effect on IL-4 and IFN-γ secretion from cervical lymph node cells upon re-stimulation with OVA (data not shown), this being in agreement with the OVA-specific antibody response upon feeding with FESLAB. One possible mechanism underlying the anti-rhinitis effect of FESLAB could be attributable to its inhibitory effect on the IgE-mediated type I allergic reaction, as we present the *in vitro* evidence in Fig. 3. However, the *in vivo* relevance of this assumption as well as other possible mechanisms (e.g., suppression of the nasal infiltration of proinflammatory immune cells, or the involvement of inducible regulatory T cells in the prevention of rhinitis) should be addressed in future studies.

The molecular nature of the anti-allergic components in our FESLAB preparation is currently unknown, but we can suggest several candidates: i) bacterial components from *L. paracasei* NPSRIk-4 and/or *L. brevis* NPSRIv-8 cells; ii) fermentation products in FESLAB; and iii) synergistic effects of those components including pre-existing materials from unfermented sake lees.

The first candidate is the most plausible, since recent studies have demonstrated the atopy-preventive effects

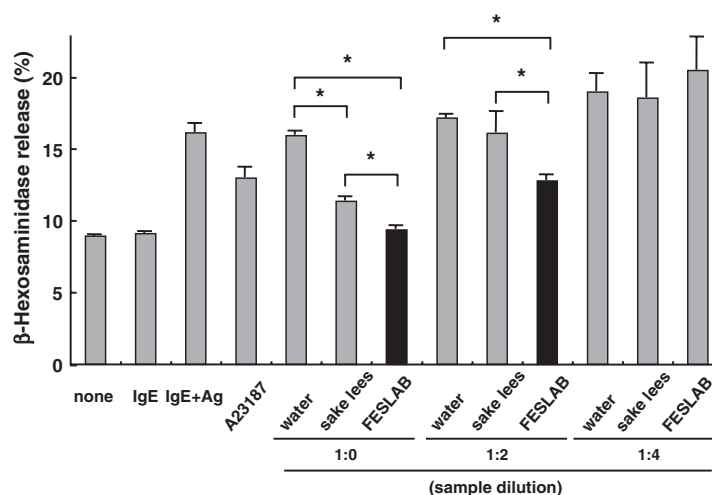


Fig. 3. *In Vitro* Stimulation with FESLAB Suppresses IgE-Mediated Basophil Degranulation.

RBL2H3 rat basophilic leukemia cells sensitized with the anti-DNP-IgE mAb were pre-stimulated with serially diluted FESLAB (from a dilution of 1:0 to 1:4), and degranulation was triggered by adding the DNP-BSA antigen (Ag) as a model allergen. A23187 (a calcium ionophore) was used as a positive control stimulant of mediator release. Sterile water and unfermented sake lees were used as control additives. All diluted samples were fully buffered upon supplementation with a 10× concentrate of the siraganian buffer. Each data value is expressed as the mean $\pm$ SD of triplicate cultures. * $p < 0.05$.

of *L. paracasei*^{11,12)} and *L. brevis*.^{13,14)} However, these studies have also indicated that the oral intake of those lactic acid bacteria prevented *in vivo* IgE production, which is in marked contrast to our results that neither the allergen-specific IgE response nor the total plasma IgE concentration were affected by the oral intake of FESLAB (Fig. 2A). This evidence suggests the mode of anti-allergic action of FESLAB being distinct from those described in the literatures. We also tested the effect of our *Lactobacilli* starter on the degranulation of RBL2H3 cells, but no inhibitory action was apparent upon stimulation with *L. paracasei* NPSRIk-4 and/or *L. brevis* NPSRIv-8 cells (data not shown). This rules out the possibility that the *in vitro* anti-allergic effect seen in Fig. 3 was not caused by the *Lactobacilli* starter by itself, although further investigation is needed to see whether an oral administration of the NPSRIk-4 and/or NPSRIv-8 cells shows the anti-rhinitis potency comparable to that seen in the FESLAB-fed mice. In respect of the second possibility for the role of the fermentation products, we have no definitive evidence that those active compounds are actually accumulated in the course of FESLAB fermentation. We now assume that GABA might be a candidate metabolite, since GABA has recently been reported as a potent atopy-preventing foodstuff,¹⁵⁾ and the *L. brevis* NPSRIv-8 strain is a GABA producer. Indeed, we have observed a GABA accumulation in our FESLAB fermentation of 19 $\mu\text{g/mL}/24\text{h}$, and found respective GABA concentrations in the FESLAB and unfermented sake lees of 77 $\mu\text{g/mL}$ and 58 $\mu\text{g/mL}$ (Kaneoke, M. and Watanabe, K-I., unpublished data). Our preliminary experiment indicates that exogenous GABA stimulation (serial concentrations up to 100 $\mu\text{g/mL}$) had no effect on the IgE-mediated degranulation of RBL2H3 cells (data not shown), suggesting that the *in vitro* degranulation-inhibitory factor in the FESLAB was distinct from GABA *per se*. However, the potential *in vivo* anti-rhinitis role of dietary GABA needs to be addressed in future studies. The oral administration of a mixture of

starter *Lactobacilli* and unfermented sake lees would also be another critical test to see whether the fermentation products were actually involved in the anti-rhinitis action of FESLAB.

In summary, we have shown here that FESLAB suppressed allergic rhinitis-like symptoms as well as the type I allergic reaction, suggesting that this sake lees-derived foodstuff could be beneficial in preventing a nasal allergy such as Japanese cedar pollinosis. Our preliminary study has revealed that the oral administration of FESLAB also delayed the onset of pathogenesis and scratching behavior in atopic dermatitis-prone NC/Nga mice (data not shown). These results further confirm the anti-inflammatory potency of dietary FESLAB.

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