

Effects of lysed *Enterococcus faecalis* FK-23 on allergen-induced serum antibody responses and active cutaneous anaphylaxis in mice

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Summary

Background Our previous studies have presented evidence that lysed *Enterococcus faecalis* FK-23 (LFK), a lysozyme and heat-treated probiotic product, can inhibit allergen-induced local accumulation of eosinophils in mice.

Objective The purpose of this experimental study was to evaluate the influence of orally administered LFK on the host immune responses.

Methods BALB/c mice were sensitized subcutaneously, and challenged intraperitoneally by cedar pollen allergen. Blood and spleen samples were collected after oral administration of LFK 60 mg/day for 21 days. The serum levels of total and allergen-specific IgE and IgG2a antibodies and the production of IL-4, IL-5 and IFN- γ generated by allergen-stimulated cultured splenocytes were determined. Additionally, the effect of LFK on active cutaneous anaphylaxis (ACA) induced by ovalbumin (OVA) challenge in mice was measured after 28 days LFK treatment.

Results No significant differences in serum immunoglobulin levels, as well as in cytokine production of splenocytes were observed between LFK-treated and control mice ($P > 0.05$). There was, however, an increasing tendency of allergen-specific IgG2a level in mice after LFK treatment for 21 days compared with controls ($P = 0.060$). Furthermore, the serum ratio of specific IgE to IgG2a was found to be significantly decreased in the LFK group ($P = 0.005$). In addition, a significant inhibition of OVA-induced ACA reaction was observed in mice that had been fed for 28 days with LFK compared with control mice ($P = 0.008$).

Conclusion These results suggest that LFK shows an anti-inflammatory effect, which may be part of the mechanism for protection against IgE-mediated allergy.

Keywords allergy, cutaneous anaphylaxis, cytokines, *Enterococcus faecalis*, immunoglobulins, lactic acid bacteria

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Introduction

Intestinal microflora, which plays a crucial role in the programming and development of systemic immunity in early life [1], has received more attention during the last few years as a potential determiner in the development of T-helper type 2 (Th2)-mediated allergic diseases [2]. On the basis of epidemiological data, direct or indirect evidence has shown that alterations of microflora in the gut in childhood are linked to risk of developing atopic sensitization and allergic diseases [3–7]. Moreover, recent clinical trials have demonstrated the beneficial effect of probiotic supplementations made from lactic acid bacteria (LAB) in allergy prevention and therapy [8–13].

Our previous studies have found that oral administration of lysed *Enterococcus faecalis* FK-23 (LFK), an LAB preparation, can inhibit allergen-induced local accumulation of eosinophils in mice [14], and reduce the number of peripheral blood eosinophils and improve tuberculin responses in patients with allergic rhinitis [15]. It is likely that LFK tends to have an effect in preventing and/or shifting the Th2-dominated immune responses; however, the mechanisms of LFK in combating allergy still need clarification. To address this question, we performed this experimental study and evaluated the influence of orally delivered LFK on the host immune responses.

Methods

Experimental animals

Female BALB/c mice were purchased from Charles River Japan Inc. (Shizuoka, Japan). Five-week-old mice were used

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for the experiments. They were fed on pellet diet CE-2 (Clea Inc., Tokyo, Japan) and received tap water which had been filtrated through a PF filter (Organo Inc., Tokyo, Japan). All animals were housed in cages with a 12 h light/dark cycle. Temperature and humidity were controlled at $25.0 \pm 1.0^\circ\text{C}$ and $55.0 \pm 5.0\%$, respectively.

Preparation of lysed *Enterococcus faecalis* FK-23

LFK, which is a product of lysozyme and heat-treated *E. faecalis* FK-23 strain, was prepared as described previously [14].

Allergen sensitization of BALB/c mice

For clarification of the effect of LFK on allergic responses, a mouse model (Fig. 1a) was established as described previously [14]. In brief, BALB/c mice were sensitized with the purified allergen extract (6 mg/mL) from Japanese cedar (*Cryptomeria japonica*) pollen [16]. 0.1 mL of the extract was injected subcutaneously on days 1 and 2, and then 0.2 mL was injected subcutaneously on days 7, 9 and 15. The mice were challenged on day 21 by the intraperitoneal injection of 0.2 mL cedar pollen allergen extract. 60 mg (0.5 mL) of LFK was orally administrated to the experimental mice ($n = 8$) every day during 21 days of the sensitization period. Saline was fed at a dose of 0.5 mL/day to control mice ($n = 8$) for the same duration.

Measurement of immunoglobulins in sera

Following intraperitoneal challenge by cedar pollen allergen, blood samples of mice were collected at day 22 (Fig. 1a). Serum total and allergen-specific IgE and IgG2a were measured by a sandwich ELISA, as described elsewhere [17, 18] with slight modifications. For measurement of total IgE and IgG2a, 96-well plates for ELISA were coated with each isotype specific anti-mouse immunoglobulin antibody (PharMingen, San Diego, CA, USA) by incubation overnight at 4°C , and then further treated with 10% fetal calf serum (FCS) added in phosphate-buffered saline (PBS) for 4 h at room temperature to block any nonspecific binding. Subsequently, the serial dilutions of mouse serum samples were incubated in the well for 1 h at room temperature. Purified

mouse IgE (PharMingen) and IgG2a (Ancell Corporation, Bayport, MN, USA) antibodies were used as the standard, respectively. After being washed three times PBS containing 0.05% Tween 20, biotin-conjugated rat anti-mouse IgE (PharMingen) and peroxidase-conjugated rabbit anti-mouse IgG2a (ICN Biomedicals, Aurora, OH, USA) antibodies were added to the wells, followed by the addition of streptavidin-peroxidase (Zymed Laboratories, San Francisco, CA, USA) for 1 h at room temperature. After washing six times, the plates were developed using a tetramethylbenzidine (TMB) substrate solution. The reactions were terminated by the addition of 1 N phosphoric acid. The plates were read in a microplate reader, MTP-300 (Corona Inc., Ibaragi, Japan) at 450 nm, and the concentration of each antibody was calculated according to the individual standard curve.

For determination of the serum levels of allergen-specific IgE and IgG2a, samples with the 2^{-2} and 2^{-4} dilutions were placed on ELISA plates coated previously with cedar pollen allergen. The experimental procedure then proceeded with the methods as described above, and the antibody levels were expressed as the absorbance at 450 nm.

Measurement of cytokine production by cultured splenocytes

Mouse spleens were removed aseptically at day 22 (Fig. 1a), and a single-cell suspension was prepared by gently squeezing them between two glass slides in PBS. Splenocytes were washed and suspended in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan). $150\ \mu\text{L}$ of cultures (5×10^5 cells/mL) were prepared in 96-well, flat-bottom plates. Cultured splenocytes were then stimulated with $50\ \mu\text{L}$ extract of cedar pollen allergen for 48 h at 37°C in a humidified CO_2 incubator. The levels of IL-4, IL-5 and IFN- γ in harvested culture supernatants were determined by an ELISA kit (BioSource International, Camarillo, CA, USA) in accordance with the manufacture's recommendations.

Active cutaneous anaphylaxis

For determination of the influence of LFK on active cutaneous anaphylaxis (ACA), a different mouse model

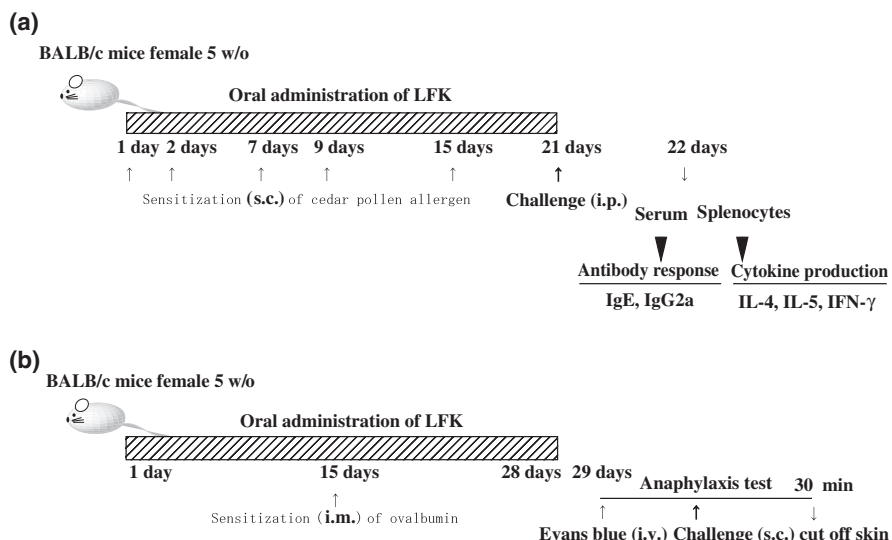


Fig. 1. Experimental procedures in this study. BALB/c mouse models established for testing the effects of lysed *Enterococcus faecalis* FK-23 (LFK) on the serum antibody responses and cytokine production by splenocytes (a), and on the active cutaneous anaphylaxis reaction (b), respectively. s.c., subcutaneous; i.p., intraperitoneal; i.m., intramuscular; i.v., intravenous.

(Fig. 1b) was established as follows. 60 mg (0.5 mL) of LFK was orally administrated to 5-week-old female BALB/c mice ($n = 15$) per day for 28 days. Saline was fed to the age- and sex-matched mice ($n = 15$) at a dose of 0.5 mL every day for the same duration as the control. The experimental mice were immunized intramuscularly with 0.1 mL of ovalbumin (OVA; Sigma Chemical, St Louis, MO, USA) saline solution (3 mg/mL) at day 15. Following an intravenous injection of 0.05 mL of 1% Evans Blue (Sigma Chemical), ACA was elicited in the skin of belly by subcutaneous injection of 0.05 mL of OVA at day 29. Thirty minutes after OVA challenge, mice were killed and a piece of skin shaved from a mouse was placed in 3 mL of mixture solution containing 0.05% sodium sulphate anhydrous and 99.5% acetone (Wako Pure Chemical, Tokyo, Japan). After vigorous shaking, the absorbance of extravasated dye was measured colorimetrically at 620 nm.

Statistical analysis

Data were presented as mean $\pm$ SEM. The SPSS 10.0J (SPSS Inc., Chicago, IL, USA) was used for analysis. Statistical differences between the groups were determined by unpaired Student's *t*-test. Total serum IgE and IgG2a levels were logarithm-transformed to normalize the distribution. Differences were considered significant if the *P*-value for the effect was less than 0.05.

Results

Effect of lysed *Enterococcus faecalis* FK-23 on serum immunoglobulin E and immunoglobulin G2a antibodies

Total and allergen-specific IgE and IgG2a antibodies in sera from the mice that had been fed with LFK for 21 days and the control mice were measured. As shown in Table 1, no significant differences in the serum levels of total as well as allergen-specific IgE and IgG2a were found between two groups ($P > 0.05$), but the specific IgG2a level (absorbance at 450 nm) was observed to be an increasing tendency in LFK-treated mice (0.32 ± 0.03) than that in controls (0.26 ± 0.01 , $P = 0.060$). Interestingly, the ratio of specific IgE to IgG2a was significantly lower in the LFK group (0.68 ± 0.08) than that in the control group (1.09 ± 0.09 , $P = 0.005$).

Effect of lysed *Enterococcus faecalis* FK-23 on interleukin-4, interleukin-5 and interferon- γ production by splenocytes

To further verify the effect of LFK on cytokine production generated by allergen-stimulated cultured splenocytes, IL-4, IL-5 and IFN- γ from the culture supernatants were examined. As shown in Table 2, no significant difference in the production of IL-4, IL-5 or IFN- γ could be found between LFK-treated and control mice ($P > 0.05$). Also, no significant difference was observed in the ratio of IL-4 or IL-5 to IFN- γ between experimental groups ($P > 0.05$).

Effect of lysed *Enterococcus faecalis* FK-23 on active cutaneous anaphylaxis

The reaction of ACA following OVA challenge was compared in control mice and in mice that had been orally

Table 1. Effect of LFK on serum IgE and IgG2a antibodies

	Control ($n = 8$)	LFK-treated ($n = 8$)	<i>P</i> -value
Total IgE (log ng/mL)	3.43 ± 0.09	3.25 ± 0.07	0.133
Total IgG2a (log ng/mL)	5.92 ± 0.07	5.82 ± 0.04	0.228
Total IgE/IgG2a ratio	0.58 ± 0.02	0.56 ± 0.01	0.335
Specific IgE (absorbance at 450 nm)	0.28 ± 0.03	0.22 ± 0.03	0.165
Specific IgG2a (absorbance at 450 nm)	0.26 ± 0.01	0.32 ± 0.03	0.060
Specific IgE/IgG2a ratio	1.09 ± 0.09	0.68 ± 0.08	0.005

Values are presented as mean $\pm$ SEM.
LFK, lysed *Enterococcus faecalis* FK-23.

Table 2. Effect of LFK on IL-4, IL-5 and IFN- γ production by splenocytes

	Control ($n = 8$)	LFK-treated ($n = 8$)	<i>P</i> -value
IL-4 (pg/mL)	21.87 ± 2.87	22.30 ± 3.38	0.924
IL-5 (pg/mL)	242.83 ± 55.10	270.30 ± 57.11	0.736
IFN- γ (pg/mL)	10.02 ± 5.46	18.54 ± 3.22	0.235
IL-4/IFN- γ ratio	9.56 ± 4.22	0.93 ± 0.20	0.096*
IL-5/IFN- γ ratio	126.53 ± 60.72	14.30 ± 6.90	0.124*

Values are presented as mean $\pm$ SEM.
**t*-test.
LFK, lysed *Enterococcus faecalis* FK-23.

Table 3. Effect of LFK on active cutaneous anaphylaxis (ACA)

	Control ($n = 15$)	LFK-treated ($n = 15$)	<i>P</i> -value
ACA (absorbance at 620 nm)	0.50 ± 0.05	0.33 ± 0.04	0.008

Values are presented as mean $\pm$ SEM.
LFK, lysed *Enterococcus faecalis* FK-23.

administrated with LFK for 28 days. As shown in Table 3, OVA-induced vascular permeability (absorbance at 620 nm) was significantly lower in LFK-treated mice (0.33 ± 0.04) than that in controls (0.50 ± 0.05 , $P = 0.008$).

Discussion

Recent clinical trials have highlighted a role of probiotic therapy in the prevention or management of allergic diseases, especially atopic dermatitis/eczema [8–12], indicating the competence of probiotics in anti-allergy immunoregulation. The term probiotics refers to preparations of microorganisms or components of microbial cells that have a beneficial effect on the health and well-being of the host when ingested [19, 20]. The most commonly used probiotics are *Lactobacillus* and *Bifidobacterium* species, but *Enterococcus* strains and *Escherichia coli* have also been proposed as such in the light of the above definition [20].

LFK is a product of *E. faecalis* FK-23 strain components without viable cells. Using a BALB/c mouse model in this study, we found no significant differences in the serum levels of total as well as allergen-specific IgE and IgG2a antibodies

between the mice that had been fed with LFK for 3 weeks and the control mice. However, after LFK treatment an increasing tendency of specific IgG2a level was shown ($P = 0.060$), and with the results we found significantly reducing specific IgE/IgG2a ratio in LFK-treated mice compared with controls ($P = 0.005$). Given that the serum ratio of IgE to IgG2a is used as a surrogate marker to monitor the Th1 (T-helper type 1)/Th2 balance, our data suggested that oral administration of LFK might have an effect on immune balance between Th1 and Th2 immunities. This hypothesis is supported by Sudo et al. [18] showing that an antibiotic-induced Th2-skewed response in mice was reversed by supplementation with a living strain of *E. faecalis*. In addition, experimental studies have indicated that after oral feeding of heat-killed *Lactobacillus casei* strain, the antigen-specific IgE and Th2 cytokine-dependent inflammatory responses were inhibited [21, 22]. It is possible that probiotic LAB may change the imbalance between Th1 and Th2 responses through down-regulation of T cell activation and IL-4-induced IgE synthesis by indirect mechanisms as suggested by Pessi et al. [23, 24].

The Th1/Th2 decision is crucial to effective immunity and it is likely that many interlocking factors (e.g. antigen-presenting cells and cytokines) contribute to its decision [25]. It is known that the Th2-type cytokines such as IL-4 and IL-5 are essentially associated with IgE-mediated allergy because of their actions on some specific cells known to be involved in the allergic inflammatory reactions. In contrast, the Th1-type cytokine, IFN- γ , has an inhibitory effect on IgE and IgG1 secretion, whereas an enhancing effect on IgG2a secretion *in vivo*. It has been reported that heat-killed *Lactobacillus* strains can increase *in vitro* production of IFN- γ , but reduce the levels of allergen-driven IL-4 and IL-5 either in mouse models [21, 22] or in allergic patients [26], suggesting that some strains of LAB may exhibit an anti-Th2 activity. Conversely, a recent clinical study by Rosenfeldt et al. [12] found no significant changes in the production of either Th1 or Th2 cytokines (IFN- γ , IL-2, IL-4 and IL-10) from peripheral blood mononuclear cells after 6 weeks' administration of probiotic *Lactobacillus* strains to children with atopic dermatitis, but a decrease of serum eosinophil cationic protein levels was observed. In the present study, we also found no significant difference in the production of IL-4, IL-5 or IFN- γ generated by allergen-stimulated cultured splenocytes between the mice orally applied for 3 weeks with LFK and the control mice. Further clarification is needed on whether a longer period of administration of probiotic LAB is required to produce remarkable changes in the cytokine profiles.

Interestingly, in this study we could show that BALB/c mice fed with LFK for 4 weeks effectively reduced the ACA reaction induced by OVA challenge compared with controls ($P = 0.008$). Our finding, together with the published data [27] that certain *Lactobacillus* strain can diminish systemic anaphylaxis in a food allergy mouse model, suggested a possible inhibitory activity of probiotic LAB on IgE (perhaps including IgG1)-mediated mast cell degranulation that drives systemic or topical allergic reactions.

In summary, according to the results presented here and our previous observation that oral LFK can inhibit allergen-induced local eosinophilia in mice [14], we speculate that the anti-inflammatory effects of LFK may be part of the

mechanism in limiting IgE-mediated allergy. It also prompts further investigation of other mechanisms involved and the beneficial effect of LFK on allergic diseases *in vivo*.

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