ORIGINAL ARTICLE

Effect of Lysed *Enterococcus faecalis* FK-23 on Allergen-Induced Immune Responses and Intestinal Microflora in Antibiotic-Treated Weaning Mice

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Abstract

Background: Recent epidemiological studies have indicated that early life receipt of antibiotics may be associated with an increased risk of developing atopic disorder. Lysed *Enterococcus faecalis* FK-23 (LFK), a probiotic product of *E faecalis,* has been shown to have inhibitory effects on allergen-induced immune responses in mice.
Objective: The purpose of this study was to evaluate the effects of LFK on immune responses and intestinal microflora in antibiotic-treated, and allergen-sensitized weaning mice.
Methods: Three-week-old BALB/c mice were sensitized with cedar pollen allergen to establish the experimental model. The allergen-induced peritoneal accumulation of eosinophils, serum levels of total and allergen-specific immunoglobulin (Ig) E and IgG2a, and the intestinal bacterial flora were determined in the control, antibiotic, LFK and antibiotic-LFK groups (n = 7 in all groups). Orally administered erythromycin, one kind of macrolide antibiotic, was used for the experiments.
Results: There was no significant difference in the allergen-induced peritoneal accumulation of eosinophils and serum specific IgE and IgG2a levels in erythromycin-treated mice compared to a control group. However, the ratio of serum total IgE to IgG2a levels was significantly increased in erythromycin-treated mice relative to that found either in LFK-treated mice or in erythromycin-treated mice with LFK supplementation. The total aerobes, total anaerobes and *Enterococcus* species of intestinal microflora were not significantly different among all groups. *Lactobacillus* species were distinctly eliminated in the mice exposed to erythromycin on day 7 and totally recovered in erythromycin-treated mice with LFK intervention on day 28, but could not be recovered in the erythromycin-treated mice without LFK intervention.
Conclusions: Our results suggest that LFK may improve the intestinal ecosystem disturbed by antibiotic use, and thereby prevent subsequent development of atopy. However, whether different antibiotics have different effects on immune responses needs to be addressed further.

Key words: Antibiotics. Eosinophils. Immunoglobulins. Intestinal microflora. *Enterococcus faecalis. Lactobacillus*.

Resumen

Antecedentes: En estudios epidemiológicos recientes, se ha señalado que la toma de antibióticos en una etapa temprana de la vida podría asociarse a un mayor riesgo de padecer trastornos atópicos. Se ha demostrado que el *Enterococcus faecalis* FK-23 (LFK) lisado, un producto probiótico de *E faecalis* tiene efectos inhibidores sobre las respuestas inmunológicas inducidas por alérgenos en ratones. *Objetivo:* El propósito del estudio fue evaluar los efectos del LKF sobre las respuestas inmunológicas y sobre la microflora intestinal en ratones destetados, sensibilizados con alérgenos y a los que se había administrado antibióticos.

Métodos: Se sensibilizaron ratones BALB/c de tres semanas de edad con alérgeno de polen de cedro para establecer el modelo experimental. La acumulación peritoneal de eosinófilos inducida por alérgenos, los niveles en suero de inmunoglobulina (Ig) E e IgG2a totales y específicos de los alérgenos, así como la flora bacteriana intestinal se determinaron en los grupos control, en el grupo al que se administró antibiótico, en el grupo LFK y en el grupo antibiótico-LFK (n = 7 en todos los grupos). Para los experimentos, se utilizó eritromicina, una clase de antibiótico macrólido, que se administró oralmente.
Resultados: No se detectaron diferencias significativas entre los ratones tratados con eritromicina y el grupo control, en cuanto a acumulación peritoneal de eosinófilos inducida por alérgenos y los niveles séricos de IgE específica e IgG2a. Sin embargo, la proporción de IgE total sérica frente a los niveles de IgG2a aumentaron significativamente en los ratones tratados con eritromicina, frente a los que se administró eritromicina con suplemento de LFK. El total de aerobios, anaerobios y *Enterococcus* de la microflora intestinal no presentó diferencias significativas entre grupos. El *Lactobacillus* se eliminó claramente en los ratones expuestos a eritromicina el día 7 y se recuperó totalmente en los ratones tratados con eritromicina con intervención de LFK el día 28, pero no se pudo detectar en los ratones a los que se administró eritromicina sin intervención de LFK.
Conclusiones: Nuestros resultados sugieren que el LFK puede mejorar el ecosistema intestinal alterado por el uso de antibióticos, evitando con ello la subsiguiente aparición de atopia. No obstante, haría falta estudiar si distintos antibióticos tienen efectos distintos sobre las respuestas inmunológicas.

Palabras clave: Antibióticos. Eosinófilos. Inmunoglobulinas. Microflora intestinal. *Enterococcus faecalis*. *Lactobacillus.*

Introduction

Allergic disease is a major health problem worldwide and the most common chronic disease of childhood [1,2]. A number of environmental factors have been invoked to explain the increased prevalence of allergic diseases. These include rising air pollution, increasing allergen exposure, changing dietary habits and changing patterns of microbial exposure in early childhood [3]. Moreover, antibiotic use in early life has been proposed as a risk factor that may have contributed to the subsequent development of asthma and atopy [4-9]. Findings from animal studies indicated that the administration of antibiotics to infant mice leads to alterations of the gut flora and impaired type 1 helper T cell (T_H) immune responses [10,11], and adequate probiotic supplementation after antibiotic treatment may improve the intestinal ecosystem, and thereby prevent the T_H2-shifted immunity induced by neonatal antibiotic use [11].

Lysed *Enterococcus faecalis* FK-23 (LFK), a probiotic product of *E faecalis*, has been shown to have inhibitory effects on allergen-induced local accumulation of eosinophils [12] and active cutaneous anaphylaxis [13] in mouse models. On the other hand, in a clinical pilot study, we observed that the number of peripheral blood eosinophils was significantly reduced after LFK treatment in patients with perennial allergic rhinitis [14]. Therefore, it has been hypothesized that the supplementation of LFK may play a role in preventing and/or shifting the T_H2-dominated immune responses. In this study, we investigated the effects of oral antibiotic use on allergen-induced local accumulation of eosinophils, serum total and allergen-specific immunoglobulin (Ig) E and IgG2a levels and intestinal microflora in weaning mice. We also attempted to determine whether the orally administrated LFK can influence these phenomena in the antibiotic treated mice.

Materials and Methods

Preparation of LFK

LFK was prepared as described previously [12]. In brief, *E faecalis* FK-23 was cultured for 24 hours at 37 °C, in a broth medium containing 2% glucose, 2% yeast extract, 2% meat extract and 4% K_2HPO_4. The cells were harvested by centrifugation, washed three times with distilled water and lysozyme treatment (added 1mg/mL) at 37 °C for 2 hours, and heated at 105 °C for 10 minutes, then lyophilized. The resulting preparation is LFK.

Preparation of Cedar Pollen Allergen

The pollen allergen was purified from Japanese cedar (*Cryptomeria japonica*), as previously described [15]. Briefly, 100 g of cedar pollens were defatted with ether and extracted in 2000 mL of 0.125 M ammonium bicarbonate (pH 8.0) at 20 °C for 48 hours. After extraction, the pollen was separated from the supernatant by centrifugation (10 000 × *g*) and was extracted in 1 200 mL of 0.125 M ammonium bicarbonate over a 24 hour period. The combined supernatants were dialyzed against 5mM ammonium bicarbonate and lyophilized.

Experimental Animals

Female BALB/c mice were purchased from Charles River Japan Inc. (Yokohama, Japan). The three-week-old mice used for experiments were fed on a pellet diet (CE-2, Clea Japan Inc, Tokyo, Japan) and received tap water which had been filtered through a PF filter (Organo Co, Tokyo, Japan). All animals were housed in cages with a 12 hour light/dark cycle. Temperature was controlled at 25.0 ± 1.0 °C and humidity 55.0 ± 5.0%. The experiments were performed in accordance with the guidelines for the care and use of experimental animals established by the Japanese Association for Laboratory Animals Science in 1987.

Administration of Antibiotic and LFK

Erythromycin 2 mg/d/mouse (0.2 mL) was orally administered to the mice (n = 14) in a 7-day course. Saline

was fed in a dose of 0.2 mL/mouse per day for the same duration in another group of mice (n = 14). Subsequently, all experimental mice underwent a 21-day allergen sensitization by cedar pollen extract, during which LFK 60 mg (0.5 mL/d) was orally administered to half of the antibiotic-treated mice (antibiotic-LFK group; n = 7) and half of the saline-treated mice (LFK group; n = 7), while saline in a dose of 0.5 mL/day/head was administered to the remaining half of the antibiotic-treated mice (antibiotic group; n = 7) and the saline-treated mice (control group; n = 7).

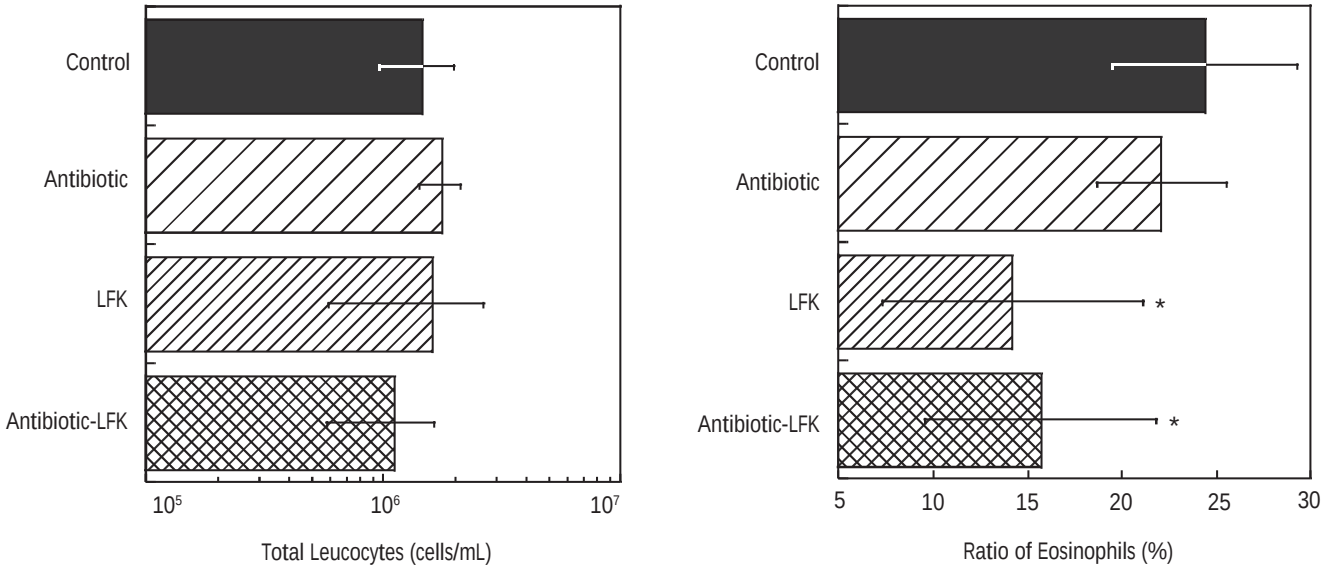
Allergen-Induced Cell Accumulation

The animal model was prepared according to the procedure, as described in literature [16]. BALB/c mice were sensitized with the cedar pollen allergen. 0.1 mL of the allergen dilution was injected subcutaneously on day 0 and 1, and 0.2 ml was injected subcutaneously on day 6, 8 and 14. The mice were challenged on day 20 by the intraperitoneal injection of 0.2 mL of the allergen dilution. Peritoneal cells were harvested 24 hours after challenge with 4 mL of phosphate-buffered saline (PBS) containing 1.0% fetal calf serum and 5U/mL heparin. An appropriate PBS dilution of the infusion was added to Turk's solution, and the total number of blood cells was counted with a hemocytometer under a microscope. For this purpose, 50 µL of the peritoneal cell suspension (5×10^5 cells/mL) was smeared on a microscope slide after centrifugation. A differential cell count was carried out under a microscope after fixation and staining with May-Grunwald Giemsa dye.

Measurement of Serum IgE and IgG2a

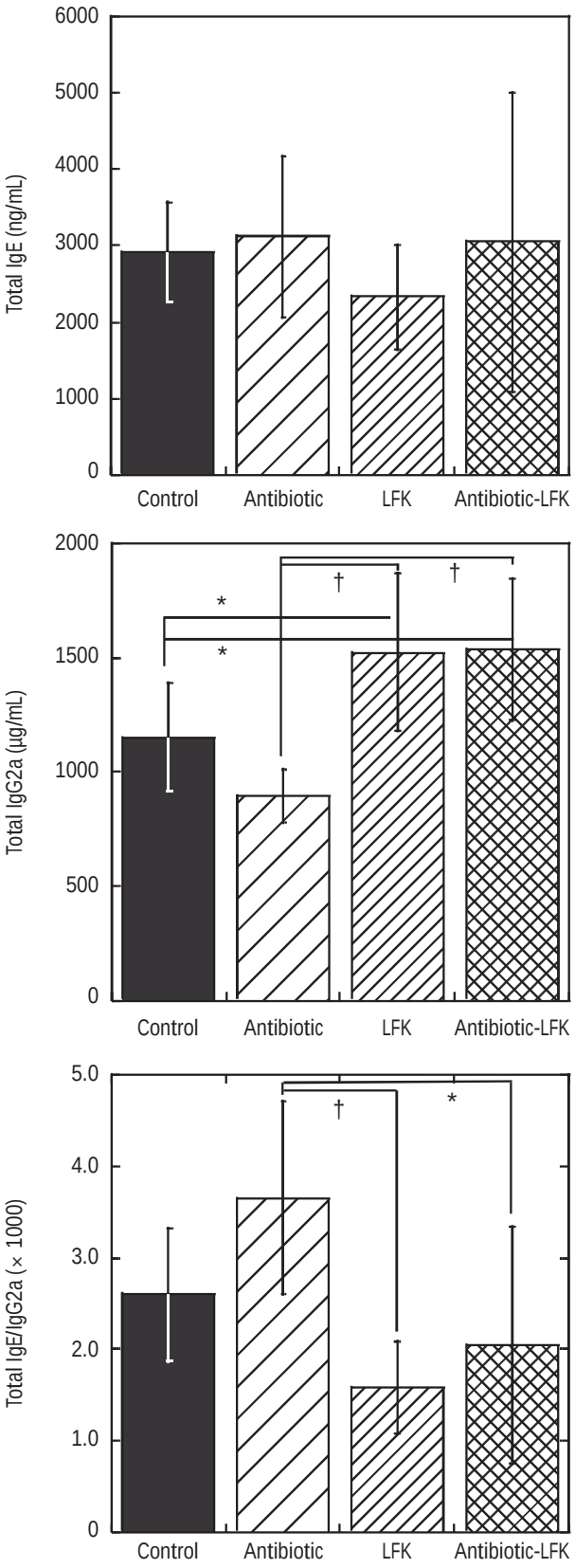
The serum total IgE and IgG2a levels were determined by a sandwich enzyme-linked immunosorbent assay (ELISA). 96-well plates were coated with each isotype specific anti-mouse immunoglobulin antibodies (PharMingen, San Diego, California, USA) by incubation overnight at 4°C, and then further treated with 10%-FCS added in PBS for 4 hours at room temperature to block any nonspecific binding. Next, the serial dilutions of the serum samples were incubated in the well for 1 hour at room temperature. Purified mouse IgE (PharMingen) and IgG2a (Ancell Co, Bayport, Minnesota, USA) antibodies were used as the standard. After being washed three times with PBS containing 0.05% Tween 20, biotin-conjugated anti-mouse IgE (PharMingen) and peroxidase-conjugated anti-mouse IgG2a (ICN Pharmaceuticals Inc, Aurora, Ohio, USA) antibodies were added to the wells, followed by the addition of Streptavidin-peroxidase (ZYMED, San Francisco, California, USA). After washing six times, the plates were developed using a TMB-substrate solution (KPL Inc, Gaithersburg, Maryland, USA). The reactions were terminated by the addition of phosphate acid. The plates were read in a microplate reader (MTP-300; Corona Electric, Hitachinaka, Japan) at 450nm, and amounts of each antibody were 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 dilution 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.



* $P < .01$ was considered to be significantly different from the control group.

Figure 1. Effect of lysed *Enterococcus faecalis* FK-23 (LFK) on peritoneal accumulation of total leukocytes in erythromycin-treated mice.



* $P < .01$
† $P < .0001$ were considered to be significantly different from the control group.

Determination of Intestinal Microflora

The intestinal bacterial flora was determined according to the methods described in detail elsewhere [17,18]. Briefly, approximately 100 mg (wet weight) of feces was suspended in anaerobic diluents, and then serial 10-fold dilutions from 10-1 to 10-8 were prepared. From the appropriate dilutions, 0.1 mL aliquot was then spread on two nonselective agar plates (TS, BL) and six selective agar plates (DHL, SF, LBS, BS, *Bacteroides* species and CW with kanamycin (Nissui Inc, Tokyo, Japan). After incubation for 24 hours (aerobes) or 48 hours (anaerobes), the bacterial groups were recognized based on both colonial and cellular morphology, Gram staining, spore formation, and aerobic and anaerobic growth.

Statistical Analysis

Data were expressed as means ± sd. SPSS 10.0J was used for analysis. For all data, an initial one-way analysis of variance followed by Fisher's PLSD comparison test was used to examine differences between groups.

Results

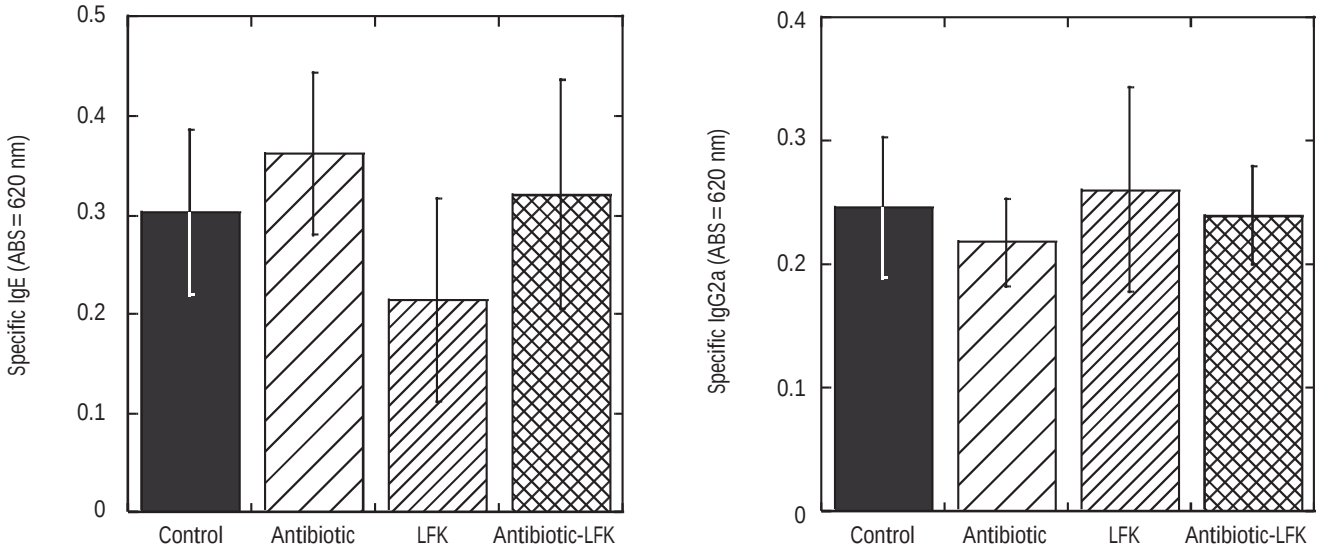
Peritoneal Accumulation of Eosinophils

As shown in Figure1, the total number of accumulated cells in the control, antibiotic, LFK and antibiotic-LFK groups was 1.46 × 106, 1.76 × 106, 1.61 × 106 and 1.11 × 106 cells/mL, respectively. No significant difference was observed between any two groups ($P > .05$). The ratio of eosinophils in the control, antibiotic, LFK and antibiotic-LFK groups was 24.4%, 25.1%, 14.2% and 15.7%, respectively. A significant difference was found between the control and LFK groups ($P = .007$), as well as between the control and antibiotic-LFK groups ($P = .008$).

Total IgE and IgG2a in Sera

As shown in Figure 2, serum total IgE levels in the control, antibiotic, LFK and antibiotic-LFK groups were 2916, 3116, 2339 and 3047 ng/mL, respectively. Total IgE levels in the LFK group were lower than that in the other groups but not significantly so ($P > .05$). Serum total IgG2a levels in the control, antibiotic, LFK and antibiotic-LFK groups were 1154, 895, 1523 and 1535 ng/mL, respectively. There was a significant increase in the IgG2a levels in LFK group in comparison with the control group ($P = .007$) and the antibiotic group ($P < .0001$). Interestingly, the ratio of total IgE to IgG2a in the antibiotic group was significantly higher than that in the LFK group ($P = .0008$) as well as the antibiotic-LFK group

Figure 2. Effect of lysed *Enterococcus faecalis* FK-23 (LFK) on serum levels of total immunoglobulin (Ig) E and IgG2a in erythromycin-treated mice.



ABS indicates absorbance.

Figure 3. Effect of lysed Enterococcus faecalis FK-23 (LFK) on serum levels of allergen-specific immunoglobulin (Ig) E and IgG2a in erythromycin-treated mice.

Table 1. Distribution of the fecal Microbiota (log cfu/g)*

Flora	Group	Day 0	Day 7	Day 28
Total aerobes	control	8.36	7.90	8.15
	antibiotic	8.36	6.97	8.56
	LFK	8.36	7.90	7.89
	antibiotic-LFK	8.36	6.97	7.98
Total anaerobes	control	8.56	8.58	8.86
	antibiotic	8.56	6.76	8.84
	LFK	8.56	8.58	8.82
	antibiotic-LFK	8.56	6.76	8.66
Lactobacillus Species	control	7.08	7.00	7.92
	antibiotic	7.08	< 3.00	6.11
	LFK	7.08	7.00	7.76
	antibiotic-LFK	7.08	< 3.00	7.60
Enterococcus Species	control	6.86	6.36	6.08
	antibiotic	6.86	6.68	6.15
	LFK	6.86	6.36	6.11
	antibiotic-LFK	6.86	6.68	6.08

* Data are expressed as mean (n = 5). cfu indicates colony forming units; LFK, lysed *Enterococcus faecalis* FK-23.

($P = .0046$), but no significant difference was observed between the control group and any one of other three groups ($P > .05$).

Allergen-Specific IgE and IgG2a in Sera

Serum levels of cedar pollen allergen-specific IgE in the control, antibiotic, LFK and antibiotic-LFK groups were 0.303, 0.362, 0.214 and 0.321, respectively (Figure 3). The LFK group had lower specific IgE levels compared to the control and antibiotic groups, but no significant difference was found among all the groups ($P = .078$). Specific IgG2a levels in the control, antibiotic, LFK and antibiotic-LFK groups were 0.247, 0.218, 0.261 and 0.240, respectively ($P > .05$). There was also no significant difference in the ratio of specific IgE to IgG2a between all the groups ($P > .05$; data not shown).

Intestinal Microflora

We examined the effects of antibiotic and LFK on the composition of intestinal day 0,7 and 28. Enterobacteria, *Clostridium* and *Bacteroides* species were almost identical among all the groups, but *Bifidobacterium* species were not detected. As shown in Table 1, levels of total aerobes, total anaerobes and *Enterococcus* in intestinal flora were not significantly different among all the groups. *Enterococcus* on day 0, 7 and 28 was 6.86, 6.36 and 6.08 log colony forming units (cfu)/g (wet fecal) in the control group; 6.86, 6.68 and 6.15 log cfu/g in the antibiotic group; 6.86, 6.36 and 6.11 log cfu/g in the LFK group; and 6.86, 6.68 and 6.08 cfu/g in antibiotic-LFK group, respectively.

Meanwhile, *Lactobacillus* on day 0, 7 and 28 was 7.08, 7.00 and 7.92 log cfu/g (wet fecal) in the control; 7.08, under 3.00 and 6.11 log cfu/g in the antibiotic group; 7.08, 7.00 and 7.76 log cfu/g in the LFK group; and 7.08, under 3.00 and 7.60 log cfu/g in the antibiotic-LFK group, respectively. Therefore, *Lactobacillus* was distinctly eliminated in the mice exposed to erythromycin on day 7 and was not able to recover in the antibiotic group, but totally recovered in the antibiotic-LFK group on day 28.

Discussion

It has been suggested that the rise in prevalence of allergic disease in westernized countries is due in part to a decrease in exposure to infections and an increase in the use of antibiotics early in life [4,19]. Recently, considerable evidence has accumulated showing that exposure to at least one course of antibiotics during the first year of life seems to be a risk factor for childhood asthma [20]. Thus, a causal link between the receipt of antibiotics early in life and subsequent atopic disorder could be speculated.

Antibiotics disturb the microbiota of the gastrointestinal tract, possibly perturbing the developing immune system [10]. Interestingly, probiotic supplementation could modulate the response of the intestinal flora to the effects of antibiotic therapy [21]. Findings from animal studies showed that kanamycin-induced elevation of the serum total IgE levels in BALB/c weaning mice was reversed by supplementation with *E faecalis* [11]. Moreover, the ratio of IgE to IgG2a in the mice supplemented with *E faecalis* significantly decreased in comparison with that in the kanamycin-treated mice without any bacterial supplementation. It has been proposed that adequate probiotic intervention after antibiotic treatment may improve the intestinal ecosystem, and thereby prevent the Th2-shifted immunity induced by neonatal antibiotic use.

It was reported that macrolide and cephalosporin antibiotics were more predictive of subsequent atopy than others in a study of English children, based on family doctor records [4]. In the present study, the immunological changes after oral administration of erythromycin (one kind of macrolide antibiotic) in BALB/c weaning mice were investigated. The results showed that there was no significant difference in the allergen-induced peritoneal accumulation of eosinophils and serum specific IgE and IgG2a levels in erythromycin-treated mice compared to a control group. However, the ratio of serum total IgE to IgG2a levels was significantly increased in erythromycin-treated mice relative to that in either LFK-treated mice or erythromycin-treated mice with LFK supplementation. This suggests that the administration of LFK might be beneficial in preventing the subsequent development of atopy after early life receipt of antibiotics.

It is well known that erythromycin is difficult to absorb from the intestine into the body, and oral administration can induce damage to the gut flora [22]. Results of the present study showed that the total aerobes, total anaerobes and *Lactobacillus* were eliminated in the mice after a 7-day course of erythromycin. In particular, *Lactobacillus* could not be detected on day 7. In erythromycin-treated mice without LFK intervention, total aerobes and anaerobes were recovered the same as in the control group on day 28, while *Lactobacillus* was not recovered completely. In contrast, *Lactobacillus* was totally recovered in the mice treated by erythromycin with LFK intervention. These findings suggest the ability of LFK, a probiotic product of lysozyme- and heat-treated *E faecalis*, to repair intestinal ecosystem impairment induced by antibiotic use.

It has been reported that *Lactobacillus* was decreased in the intestine of allergic children [23], and that the probiotic Lactobacillus GG was effective in the prevention of early atopic eczema in children at high risk [24] and that this preventive effect could extend beyond infancy [25]. However, it is rare for orally administered probiotics to become established in the host intestine because most lactic acid bacteria are excreted in a few days [26]. Therefore, increasing original lactic acid bacteria in the host intestine by probiotic supplementation, rather than by oral administration of live cells may be more essential for restoration of the intestinal ecosystem. Likewise, the effects of probiotics on the immune response may also be indirect and mediated by their effects on the intestinal epithelium, such as permeability and morphology [27].

In conclusion, our data suggest that LFK intervention may improve the intestinal ecosystem disturbed by antibiotic use, and thereby prevent subsequent development of atopy. However, the question of whether different antibiotics have different effects on immune responses needs to be addressed. Further investigations including different experimental designs are also required.

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