The anti-allergenic properties of milk kefir and soymilk kefir and their beneficial effects on the intestinal microflora

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Abstract: Food allergy is now recognized as a worldwide problem, and like other atopic disorders its incidence appears to be increasing. Kefir is reported to possess the ability to reduce intestinal permeation of food antigens; however, no experimental study has clearly evaluated the relationships between kefir consumption, allergen-specific IgE response, and intestinal microflora. The aim of this study was to evaluate the effect of oral consumption of milk kefir and soymilk kefir on *in vivo* IgE and IgG1 production induced by ovalbumin (OVA) in mice. The effects of kefir administration on the murine intestinal microflora were also examined. Oral administration of milk kefir and soymilk kefir for 28 days significantly increased the fecal populations of bifidobacteria and lactobacilli, while it significantly decreased those of *Clostridium perfringens*. Milk kefir and soymilk kefir also significantly decreased the serum OVA-specific IgE and IgG1 levels for both groups, but not those of the IgG2a analogues. Consumption of milk kefir and soymilk kefir suppressed the IgE and IgG1 responses and altered the intestinal microflora in our supplemented group, suggesting that milk kefir and soymilk kefir may be considered among the more promising food components in terms of preventing food allergy and enhancement of mucosal resistance to gastrointestinal pathogen infection.

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Keywords: kefir; intestinal microflora; allergy; IgE; IgG1

INTRODUCTION

Food allergy is characterized by an abnormal immunological reactivity to food proteins in certain genetically predisposed individuals. This response generates a wide variety of symptoms and clinical manifestations expressed in several affected organ systems such as the skin, respiratory tract, and gastrointestinal tract.¹ Food allergy is now recognized as a worldwide problem and, like other atopic disorders, it appears to be increasing. The prevalence of food allergy is greatest in the first few years of life, affecting about 6% of infants under 3 years of age but decreasing over the first decade.² Recent estimates suggest that nearly 4% of adults suffer food allergies, a prevalence much higher than appreciated in the past.³ Further, food allergy remains a leading cause of anaphylaxis treated in emergency departments in a number of countries, and the public has become increasingly aware of the problem. As allergen-specific IgE is directly involved in the mediation of many allergic reactions, development of a method for inhibiting IgE production is a useful approach for preventing allergic diseases. Dietary studies have suggested that long-term consumption of yogurt can reduce some of the clinical symptoms of allergy in

adults with atopic rhinitis or nasal allergies, and can lower serum levels of IgE.^{4,5} In addition, milk whey formula supplemented with *Lactobacillus rhamnosus* may alleviate some aspects of atopy and intestinal inflammation among infants with allergic symptoms.⁶ Although contradictory results have been obtained,⁷ the majority of the reported results indicate that fermented dairy products, including kefir, possess anti-allergy properties.^{8,9}

Kefir is an acidic and mildly alcoholic fermented dairy product that is believed to contain many biologically active components, and it has been postulated that the longevity of Bulgarian peasants is partly due to their frequent consumption of this fermented milk.¹⁰ In Soviet countries, kefir consumption has anecdotally been recommended for clinical treatment of gastrointestinal and metabolic diseases, hypertension, ischemic heart disease, and allergy.¹¹ Traditionally, kefir is produced from milk fermented with a mixed microflora confined to a matrix of discrete kefir grains, which are recovered after fermentation.¹² Various lactic acid bacteria and yeasts have been identified as being present in kefir grains, including *Lactobacillus brevis*, *L. helveticus*, *L. kefir*, *Leuconostoc mesenteroides*, *Kluyveromyces*

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lactis, *K. marxianus*, and *Pichia fermentans*.^{13,14} The microorganisms contained within the kefir grains produce lactic acid, bacteriocins, and several kinds of antibacterials, such products inhibiting the proliferation of both degrading and pathogenic microorganisms in kefir milk.¹³ Furthermore, kefir reportedly possesses the ability to reduce intestinal permeation by food antigens, which contributes to suppression of oral sensitization.⁹

Soybeans, like milk proteins, are one type of allergen causing food allergic reactions in children; however, they are not implicated as often as milk, eggs, and peanuts.² In fact, soy formulas currently represent a valid nutritional alternative in patients with cow's milk allergy. The Committee on Nutrition of the American Academy of Pediatrics (AAO) has recommended formulas based on intact soy protein isolates for the initial treatment of food allergy in infants, particularly after the age of 6 months.¹⁵ Soybeans are an excellent source of low-cost protein and have been an important nutritional component in the typical diets of many countries. Recent nutritional and medical research has revealed the great potential of soy foods for lowering blood cholesterol levels and heart disease and cancer incidence.^{16,17} Soymilk, obtained by aqueous extraction from whole soybeans, is a well-known food product, which is growing in popularity in many areas of the world.¹⁸ Soymilk may offer nutritional and health benefits since it contains no cholesterol or lactose and only small quantities of saturated fatty acids. Furthermore, there is evidence that consumption of fermented soymilk significantly improves the human intestinal ecosystem by increasing the presence of probiotics.¹⁹

As both kefir and soybeans are beneficial to health, in previous studies we have attempted to subculture kefir grains in soymilk to produce soymilk kefir.^{20,21} We demonstrated that orally administered soymilk kefir not only inhibited tumor growth and induced the apoptotic form of tumor cell lysis, but also increased the total IgA levels in tissue extracts from the wall of the small intestine in mice.²² We also demonstrated that soymilk kefir appears to possess significant antimutagenic and antioxidant activities *in vitro*.²³ To the best of our knowledge, however, the anti-allergy properties of soymilk kefir and its effect on the intestinal bacterial ecosystem have not been previously reported.

The aim of this study, therefore, was to investigate the effect of oral feeding of soymilk kefir on *in vivo* IgE and IgG1 production induced by ovalbumin (OVA) in mice. The effect of soymilk kefir administration on the intestinal bacterial ecosystem in the studied animals was also examined.

MATERIALS AND METHODS

Preparation of soymilk

One kilogram of dry, mature, whole soybeans was soaked in 3 L of distilled water at 25 °C for

24 h. The soak water was then decanted and the beans were washed, and then ground in 6 L of boiling distilled water in a blender (Waring Division, Dynamics Corporation, New Hartford, CT, USA). The resulting suspension was filtered through three layers of cheesecloth, autoclaved for 15 min at 121 °C, and then stored at 4 °C until being used.

Kefir grains

Kefir grains were collected from households in northern Taiwan. In the laboratory, these were inoculated (5% w/v) and propagated in sterilized (121 °C for 15 min) reconstituted milk (10% w/v) or soymilk at 20 °C for 20 h with twice- or thrice-weekly transfers, and kept at 4 °C or –80 °C for short- or long-term storage, respectively.

Milk kefir and soymilk kefir manufacture

Milk kefir and soymilk kefir were manufactured from sterilized (121 °C for 15 min) reconstituted milk (10% w/v) and soymilk inoculated with 5% (w/v) kefir grains, respectively, and incubated at 20 °C for 20 h. At the end of fermentation, milk kefir and soymilk kefir were filtered through three layers of cheesecloth in order to remove the kefir grains, and then lyophilized and stored at 4 °C until being used.

Animals

Male BALB/c mice were obtained from the Laboratory Animal Center of National Taiwan University at 5 weeks of age (weight 22.1 ± 1.6 g). They were housed in plastic cages (*n* = 5 per cage) at controlled temperature (22 ± 1 °C) and relative humidity (55% ± 5%), and maintained on a reverse 12 h light/dark cycle (8 am/8 pm). Laboratory rodent chow (No. 5001, Ralston Purina, St Louis, MO, USA) and water were provided *ad libitum* for 2 weeks before the experiments were begun. All animal experimental procedures followed the Guide for the Care and Use of Laboratory Animals (National Science Council, Taipei, Taiwan).

Experimental procedures

Before sensitization to antigen (day 0), the mice were randomly divided into five groups (*n* = 10 per group) consisting of animals of similar body weight. Animals in the control group were given distilled water 5 mL kg⁻¹ body weight by orogastric intubation once per day. Animals in the other four experimental groups were given the same amount of reconstituted milk, milk kefir, soymilk, or soymilk kefir (10% w/v in water) orally once per day. All mice were intraperitoneally injected with 20 µg of OVA (Sigma Grade V, Sigma Chemical., St Louis, MO, USA) mixed with 2 mg of Al(OH)₃ in a total volume of 0.2 mL saline on days 0 and 14. In addition to their treatment, all mice were allowed free access to standard rodent chow and drinking water throughout the experimental period.

On days 0, 14, and 28, each mouse was weighed and the fecal samples collected for the determination

of fecal bacterial flora. On days 0, 7, and 21, blood samples were obtained from the ophthalmic veins of the mice for the measurement of serum OVA-specific IgE, IgG1 and IgG2a levels. Experiments were terminated on day 28. The diets were removed 16 h prior to anesthetization of the animals with an intraperitoneal injection of sodium pentobarbital (Nembutal, Abbott Laboratories, Chicago, IL, USA). Blood samples were collected by cardiac puncture, and the sera were kept at -80°C until measurement of the antibody levels.

Determination of fecal bacterial flora

Approximately $1\mu\text{g}$ (wet weight) of feces was suspended in an anaerobic solution, followed by serial 10-fold dilution to produce different concentrations. Certain microorganisms were isolated from fecal samples using the media and methods developed by Molly *et al.*²⁴ From the appropriate dilutions, a 0.1 mL aliquot was spread onto bifidobacteria iodoacetate medium-25 (BIM-25) agar for incubating *Bifidobacterium* spp., MRS bromocresol green agar for *Lactobacillus* spp., or tryptose-sulfite-D-cycloserine (TSC) agar for *Clostridium perfringens*. The plates were incubated under anaerobic conditions at 37°C until the appearance of colonies. The results are expressed as logarithmic colony-forming units (log CFU) per gram of feces wet weight.

Measurement of antibody levels of OVA-specific IgE, IgG1, and IgG2a in the serum

Levels of OVA-specific IgE, IgG1 and IgG2a in test serum samples were measured by sandwich ELISA.²⁵ Ninety-six-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with $100\mu\text{L}$ per well of $3\mu\text{L mL}^{-1}$ OVA in carbonate–bicarbonate buffer (pH 9.6). The unbound materials were removed by washing three times with $150\mu\text{L}$ per well of phosphate-buffered saline containing 0.05% Tween 20 (PBST). The plates were then blocked with 1% gelatin (Sigma Chemical Co., Saint Louis, MO, USA) in PBS ($200\mu\text{L/well}$) at 37°C for 1 h. After the plates were washed another three times with PBST, $100\mu\text{L}$ portions of diluted mouse serum were added to triplicate wells. After incubation overnight at 4°C , the plates were washed three times with

PBST, and then $100\mu\text{L}$ of biotinylated rat anti-mouse IgE monoclonal antibody, biotinylated rat anti-mouse IgG1 monoclonal antibody, or biotinylated rat anti-mouse IgG2a monoclonal antibody (BD Pharmingen, San Diego, CA, USA) were added. Following another hour of incubation at 37°C , the plates were washed three times with PBST, and $100\mu\text{L}$ of alkaline phosphatase-conjugated avidin (Sigma) was added to each well. After the plates were washed three more times with PBST, $100\mu\text{L}$ of *p*-nitrophenyl phosphate (1 mg mL^{-1}) dissolved in a diethanolamine buffer (pH 9.8) was added. After incubation in the dark at room temperature for 30 min, color development was stopped by addition of $100\mu\text{L}$ of 3 mol L^{-1} NaOH. The absorbance of each well at a wavelength of 405 nm was measured using a Multiskan Ascent ELISA reader (LabSystems, Helsinki, Finland). The level of OVA-specific antibody was estimated by comparison with hyperimmunized mouse serum. Hyperimmunized mouse serum was obtained from BALB/c mice sensitized by three intraperitoneal injections of OVA adsorbed onto alum, and the OVA-specific IgE, IgG1, and IgG2a levels of this standard serum were taken to be 10 000, 100 000, and 50 000 units mL^{-1} , respectively.

Statistical analysis

All results were analyzed using the general linear-model procedure available from the Statistical Analysis System software package version 8.1.²⁶ Duncan's multiple range test²⁷ was used to detect differences between treatment means. Each assay was conducted in triplicate.

RESULTS AND DISCUSSION

Food intake and body weight gain

Table 1 presents the changes in average body weight throughout the course of the experiment for each group. No significant differences were observed for body weight gain between the different groups throughout the experiment. The basic dietary consumption of each group was not significantly different (results not shown). Therefore, it could be assumed that additional supplement of milk, soymilk, or kefir did not affect the growth of mice.

Table 1. Body weight of mice after oral administration of different samples

Group	Body weight (g) ^a			
	0-day ^b	7-day	14-day	28-day
Control	27.37 ± 1.76	27.80 ± 1.56	28.13 ± 1.01	28.33 ± 1.90
Milk	27.20 ± 1.32	27.22 ± 1.34	27.33 ± 1.36	28.35 ± 1.18
Milk kefir	27.43 ± 1.88	27.61 ± 2.01	28.37 ± 2.09	29.18 ± 2.47
Soymilk	27.12 ± 1.47	27.60 ± 1.28	28.27 ± 1.81	29.30 ± 1.64
Soymilk kefir	27.25 ± 1.81	27.66 ± 1.94	28.40 ± 1.74	29.23 ± 1.74

^a Values are mean $\pm$ SD of 10 mice.

^b Days after oral administration of different samples.

Effect of milk kefir and soymilk kefir on fecal bacterial flora

The results of the fecal microbial analyses are presented in Table 2. Consumption of milk kefir and soymilk kefir for 28 days significantly increased the fecal populations of bifidobacteria and lactobacilli ($P < 0.05$), while significantly decreased the fecal populations of *Clostridium perfringens* ($P < 0.05$).

The effects of diet on the intestinal microflora have gained increasing interest because of the evidence that a balanced intestinal micro-ecology is a health-promoting factor. Both bifidobacteria and lactobacilli are beneficial bacterial populations. Increased concentrations of these organisms have been associated with decreased fecal concentrations of potentially pathogenic bacteria and decreased levels of carcinogenic and putrefactive compounds in the digesta. A reduction in the number of clostridia in the intestine is beneficial because this organism is pathogenic and can exert harmful effects on the host.^{28,29} The contribution of fermented milk for improvement of the intestinal microflora has been widely recognized. Lactic acid bacteria in fermented products enhance resistance against intestinal pathogens via antimicrobial mechanisms. These include competitive colonization and production of organic acids, such as lactic and acetic acids, bacteriocins and other primary metabolites.³⁰ By competitive colonization, lactic acid bacteria inhibit the adhesion of gastrointestinal pathogens to the intestinal mucosa. Santos *et al.*³¹ found that some strains of *Lactobacillus* spp. isolated from kefir grains possess the ability to adhere to human enterocyte-like Caco-2 cells and inhibit attachment of *Salmonella typhimurium*. Thus, we suggest that the decreases in *Clostridium perfringens* populations achieved in the milk kefir and soymilk kefir groups may be due to the adherence ability of the *Lactobacillus* present in kefir grains.

Production of organic acids, such as lactic and acetic acids, by lactic acid bacteria lowers intestinal pH and thereby inhibits the growth of pathogens. These organic acids also increase peristalsis, thereby indirectly removing pathogens by accelerating their rate of transit through the intestine.³⁰ Romond *et al.*³² observed that consumption of cell-free whey from milk fermented with bifidobacteria was capable of modifying the human intestinal ecosystem. After consumption of the cell-free fermented whey for 7 days, fecal excretions of *Bacteroides fragilis*, *Clostridium perfringens*, and clostridial spores decreased, while counts of bifidobacteria increased. Angulo *et al.*¹³ have reported that the microorganisms contained within the kefir grains might produce lactic acid, bacteriocins, and several kinds of antibacterials, such products inhibiting the proliferation of both degrading and pathogenic microorganisms in kefir milk. In the present study, we found that the fecal populations of *Clostridium perfringens* in the milk kefir and soymilk kefir groups were significantly lower than those in the unfermented milk

and soymilk groups, and suggest that certain metabolites might be produced during kefir fermentation and that they could possibly decrease the intestinal populations of *Clostridium perfringens*.

Oral administration of soymilk also provides some beneficial effects in terms of increasing the bifidobacteria population (Table 2). This result, which is consistent with the findings of Cheng *et al.*,¹⁹ may be due to the presence of certain types of oligosaccharides, such as raffinose and stachyose, in soybeans that can be utilized by the bifidobacteria and lactobacilli as energy sources.³³

Effects of milk kefir and soymilk kefir on serum OVA-specific IgE, IgG1, and IgG2a levels

The serum levels of OVA-specific IgG1, IgG2a, and IgE for each group are presented in Fig. 1. A diet

Figure 1. Effects of milk, soymilk, milk kefir and soymilk kefir on the serum OVA-specific IgG1, OVA-specific IgG2a, and OVA-specific IgE levels in OVA-sensitized BALB/c mice. C, M, MK, S, and SK in the figure indicate control, milk, milk kefir, soymilk, and soymilk kefir groups, respectively. All data are expressed as mean $\pm$ SD ($n = 10$). * $P < 0.05$ was considered significantly different from the corresponding value in the control group.

Table 2. Mean counts of *Bifidobacterium*, *Lactobacillus*, and *Clostridium* in feces of mice after oral administration of different samples

Group	Bacterial counts (log CFU g ⁻¹ feces) ^a								
	<i>Bifidobacterium</i> spp.			<i>Lactobacillus</i> spp.			<i>Clostridium</i> spp.		
	Day 0 ^b	Day 14	Day 28	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
Control	8.85 ± 0.55a	8.38 ± 0.39b	8.49 ± 0.27d	8.02 ± 0.48 a	8.16 ± 0.30c	8.26 ± 0.11c	6.02 ± 0.40 a	6.04 ± 0.30 a	6.13 ± 0.22a
Milk	8.96 ± 0.12a	8.59 ± 0.29b	8.54 ± 0.18d	8.25 ± 0.13 a	8.26 ± 0.24bc	8.36 ± 0.23c	6.11 ± 0.42 a	6.02 ± 0.16 a	6.02 ± 0.15a
Milk kefir	8.99 ± 0.06a	8.74 ± 0.23ab	9.04 ± 0.11c	8.24 ± 0.17 a	8.44 ± 0.17ab	8.62 ± 0.11ab	6.04 ± 0.45 a	5.90 ± 0.32 a	5.02 ± 0.25b
Soy milk	8.69 ± 0.12a	8.85 ± 0.23ab	9.30 ± 0.28b	8.02 ± 0.45 a	8.45 ± 0.26ab	8.56 ± 0.16b	6.09 ± 0.30 a	6.11 ± 0.31 a	5.91 ± 0.29a
Soy milk kefir	8.74 ± 0.15a	9.18 ± 0.71a	9.58 ± 0.11a	8.31 ± 0.32 a	8.63 ± 0.25a	8.72 ± 0.13a	6.06 ± 0.44 a	5.84 ± 0.43a	4.99 ± 0.22b

^a Values are mean ± SD of 10 mice.^b Days after oral administration of different samples.Values in the same column not followed by the same letter are significantly different ($P < 0.05$).

of milk kefir or soymilk kefir for 28 days significantly decreased the serum OVA-specific IgE and IgG1 levels ($P < 0.05$). However, no significant differences were observed for the serum OVA-specific IgG2a levels between all groups ($P > 0.05$; Fig. 1).

Murine helper T cells are mainly classified into two categories, according to the productive pattern of cytokines. Type 1 helper CD4⁺ T cells (Th1) inhibit IgE production by secreting interferon (IFN)- γ . In contrast, Type 2 helper CD4⁺ cells (Th2) are characterized by cytokines, such as IL-4, IL-5, IL-6, and IL-10, which promote the production of IgE.³⁴ The balance between these two cell types is considered important for maintenance of host homeostasis. Once this becomes disturbed, various immunological diseases, such as allergies and infections, can occur from the evasion of host defense mechanisms. Thus, the regulation of these two types of cells seems to be important for the preservation of the host immune response, including IgE and cytokine production.³⁵ Several studies have shown that a polarized Th2 response is often observed in patients with allergy.³⁶ Therefore, enhancing a Th1-type immune response is expected to be beneficial for the treatment of allergic disease. Much attention has recently been focused on the lactic acid bacteria that are capable of regulating the host immune response. In an *in vitro* study, it was demonstrated that the *L. casei* strain Shirota possesses the ability to stimulate macrophages secretion of IL-12, shift the cytokine production pattern from Th2 to Th1 predominance, and then suppress IgE production.³⁷ In addition, a murine model of food allergy has also been used to demonstrate that intraperitoneal administration of *L. plantarum* can down-regulate casein-specific IgE antibody levels *in vivo*, as well as the *in vitro* capacity of splenic lymphocytes to secrete IL-4.³⁸ In the present study, secretion of OVA-specific IgE and IgG1, which is highly dependent on Th2-type cytokines, was markedly inhibited by oral administration of milk kefir and soymilk kefir, whereas IgG2a secretion was not inhibited. Therefore, the immune system might be driven towards the Th1-type responses. These results suggest that milk kefir and soymilk kefir may be beneficial for the prevention of allergic disorders.

The epidemiological data and results of animal experiment suggest that a disorder of the intestinal microflora is closely related to allergy development.³⁹ When intestinal bacteria are transiently removed by antibiotic medication in weaning mice, allergic symptoms are accompanied by elevated serum levels of total IgE.⁴⁰ This has led to the hypothesis that infections with microorganisms that have the ability to induce a dominant Th1 response are beneficial for allergy prevention. In fact, it has been reported that *Mycobacterium bovis* BCG infection promotes a dominant Th1 response and can inhibit IgE response in a murine model of allergic asthma.⁴¹ For humans, however, the risk associated with use of pathogenic microorganisms for prevention of allergic

diseases is excessive. By contrast, non-pathogenic lactic acid bacteria are generally regarded as safe (GRAS) since lactic acid bacteria are consumed in dairy products and other foods.⁴² In this study, we found not only that oral administration of milk kefir and soymilk kefir significantly improved the intestinal microflora by increasing the amount of probiotics, but also that it suppressed IgE and IgG1 responses in a murine allergy model. These results suggest that milk kefir and soymilk kefir may be considered among the more promising food components in terms of allergy prevention and enhancement of mucosal resistance to gastrointestinal infection.

Soybeans are recognized as a good source of several required food nutrients, including many kinds of polyphenols, the main types of which are isoflavone analogues such as daidzin, daidzein, genistin, and genistein. Phenolic compounds found in soy and soy products are considered as potential phytoestrogens because of their ability to interact with estrogen receptors.⁴³ Estrogens have been shown to stimulate the immune system.⁴⁴ Thus, dietary isoflavone intake is likely to affect the host's immune system. Based on this hypothesis, Miyake *et al.*⁴⁵ examined the relationship between dietary isoflavone intake and the prevalence of allergic rhinitis in pregnant women, concluding that a high intake of soy and isoflavones may be associated with reduced prevalence thereof. Several workers have found that at least a partial cleavage of chain takes place in the contained soybean glucosides during the process of soybean fermentation, resulting in a higher level of genistein in the fermented products as compared with the unfermented analogue.⁴⁶ However, in a previous study we observed that the genistein concentrations in soymilk and soymilk kefir did not differ significantly comparing the natural and fermented soybean products.²² Therefore, we suggest that the anti-allergenic property of soymilk kefir demonstrated in this study is not associated with the isoflavones in soymilk.

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