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Antiallergic Effect of Milk Fermented with Lactic Acid Bacteria in a Murine Animal Model

SANDY PENG, JIN-YUARN LIN, AND MEEI-YN LIN*

Department of Food Science and Biotechnology, National Chung Hsing University, 250 Kuokuang Road, Taichung 402, Taiwan

The objective of this study was to assess the antiallergic effect of fermented milk prepared, respectively, with *Streptococcus thermophilus* MC, *Lactobacillus acidophilus* B, *Lactobacillus bulgaricus* Lb, *L. bulgaricus* 448, and *Bifidobacterium longum* B6. Female BALB/c mice fed fermented milk were immunized intraperitoneally with ovalbumin (OVA)/complete Freund's adjuvant (CFA) to evaluate the immune response by observing the secretion of cytokines IL-2, IL-4, and IFN-γ and serum antibody IgE. The results showed that supplementation with lactic acid bacteria fermented milk did not significantly change the IL-2 spontaneous and OVA-stimulated secretions of splenocytes. However, both spontaneous and OVA-stimulated secretions of splenocytes from mice fed lactic acid bacteria fermented milk showed significantly ($P < 0.05$) lower levels of IL-4 (Th2 cytokine) than those from OVA/CFA-immunized mice fed non-fermented milk (OVA/CFA−milk group). The spontaneous secretion of IFN-γ (Th1 cytokine) by splenocytes from mice fed *L. bulgaricus* 448 or *L. bulgaricus* Lb fermented milk significantly increased as compared to that from the OVA/CFA−milk group. The results showed that the ratios of IFN-γ to IL-4 of both spontaneous and OVA-stimulated secretions in splenocytes from mice fed lactic acid bacteria fermented milk increased significantly as compared to that of PBS- or OVA/CFA−milk groups. The serum levels of OVA-specific IgE in fermented milk fed groups, especially the group fed S. *thermophilus* MC fermented milk, were significantly lower than those in the OVA/CFA−milk group through a 6 week feeding experiment. The results showed that milk fermented with lactic acid bacteria demonstrated in vivo antiallergic effects on OVA/CFA-immunized mice via increasing the secretion ratio of IFN-γ/IL-4 (Th1/Th2) by splenocytes and decreasing the serum level of OVA-specific IgE.

KEYWORDS: Lactic acid bacteria; IFN-γ; IL-2; IL-4; antiallergic effect

INTRODUCTION

Probiotics are conventionally defined as live microbial food supplements that improve intestinal microbial balance (*1*). It is now understood that lactic acid bacteria including bifidobacteria present in the intestine and in fermented foods play important roles in imparting health benefits. Various strains of lactic acid bacteria have been demonstrated to benefit a number of host physiological responses including immune function (*2*−*4*).

It is believed that exposure to environmental microorganisms during the neonatal and early childhood periods results in T helper cell type 1 (Th1)-biased immunity and prevents the induction of the proallergic Th2 immune response (*5*). However, modern methods of hygiene and sanitation have decreased children's contact with certain microorganisms (*6*). Children who developed allergy during the first 2 years of life showed less colonized normal intestinal microflora including lactic acid bacteria than healthy infants (*7*). The intestinal microflora is an important constituent of the gut mucous barrier against allergens from food sources (*8*).

Dietary studies have suggested that long-term consumption of yogurt and other lactic acid bacteria containing foods can alleviate some of the clinical symptoms of allergy such as atopic rhinitis and nasal allergies in adults and lower IgE levels in serum, particularly among the elderly (*9*−*11*). The mode by which allergies are reduced is uncertain, but the most widely accepted belief is that lactic acid bacteria containing foods are able to promote deviation away from a proallergy phenotype via the mechanism of immunomodulation (*12*). Most allergic diseases reflect an imbalance in lymphocyte-governed immunity toward Th2 immune responses (*12*). Secretion of the cytokines interleukin (IL)-4, IL-5, IL-10, and IL-13 by allergen-primed Th2 cells can provoke IgE accumulation and recruit granular effector cells such as eosinophils, basophils, and mast cells to the site of allergic inflammation (*13*−*15*). Interferons, particularly interferon (IFN)-γ, a potential cytokine in Th1 immune balance, can down-regulate IL-4 expression and reduce B cell's immunoglobulin isotype switching to IgE (*16*, *17*).

* Corresponding author (telephone 886-4-2284-0385, ext. 2208; fax 886-4-2287-6211; e-mail mylin@dragon.nchu.edu.tw).

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Table 1. pH and Bacterial Counts of Milk Fermented with Lactic Acid Bacteria[a]

	S. thermophilus MC	*L. acidophilus* B	*L. bulgaricus* Lb	*L. bulgaricus* 448	*B. longum* B6
pH	3.68 ± 0.03 a	3.65 ± 0.02 a	3.62 ± 0.01 a	3.69 ± 0.01 a	3.69 ± 0.02 a
CFU/mL	$(2.2 \pm 1.2) \times 10^9$ a	$(2.2 \pm 1.2) \times 10^9$ a	$(1.8 \pm 1.1) \times 10^9$ a	$(2.9 \pm 2.5) \times 10^9$a	$(2.2 \pm 1.5) \times 10^9$ a

[a] Data are presented as mean ± SD ($n = 3$). Values in the same row with different letters are significantly different ($P < 0.05$) by Duncan's multiple-range tests.

According to the study of Fang et al. (*18*), the immunomodulatory effect of probiotics is a strain-dependent characteristic and species speciality in the host. Probiotic lactic acid bacterial strains should thus be evaluated on their own merits; extrapolation from other species or strains is not appropriate (*19*). Selection of probiotic lactic acid bacterial strains with immunological properties must well-define the effects on cytokine expression and IgE producton that favor the claimed immune response. The objective of this study was to assess the antiallergic effect of fermented milk prepared, respectively, with *Streptococcus salivarius* ssp. *thermophilus* MC, *Lactobacillus acidophilus* B, *Lactobacillus delbrueckii* ssp. *bulgaricus* Lb, *L. delbrueckii* ssp. *bulgaricus* 448, and *Bifidobacterium longum* B6. In this study, normal female BALB/c mice were fed fermented or unfermented milk. After treatment for 6 weeks, BALB/c mice were sacrificed to evaluate immune response by observing the production of cytokines IL-2, IL-4, and IFN-γ and antibody IgE.

MATERIALS AND METHODS

Preparation of Fermented Milk. *Streptococcus salivarius* ssp. *thermophilus* MC (*S. thermophilus* MC), *Lactobacillus acidophilus* B (*L. acidophilus* B), *L. delbrueckii* ssp. *bulgaricus* Lb (*L. bulgaricus* Lb), *Lactobacillus delbrueckii* ssp. *bulgaricus* 448 (*L. bulgaricus* 448), and *Bifidobacterium longum* B6 (*B. longum* B6) were used for the preparation of fermented milk. Strains were obtained from our frozen stock culture collection. For the studies, bacteria were cultured in MRS medium (Difco Laboratories, Detroit, MI) at 37 °C. All strains were serially transferred at least three times prior to use. For the preparation of fermented milk, 1% of inoculation was transferred to 10% reconstituted nonfat dry milk and incubated at 37 °C for 18 h. Cell concentrations and pH of fermented milk were determined, and these were not different among the fermentation groups (**Table 1**).

Animals. Inbred specific pathogen-free 5-week-old female BALB/c mice were purchased from National Laboratory Animal Center (Taipei, Taiwan). The mice were kept in plastic cages and given a standard diet (Fwusow Industry Co., Ltd., Taichung, Taiwan). The animal facility was maintained at 25 ± 2 °C with a 12-h dark/light cycle. After 2 weeks of feeding on a standard diet, the mice were subdivided randomly into seven groups (PBS/CFA−milk, OVA/CFA−milk, OVA/CFA−*S. thermophilus* MC, OVA/CFA−*L. acidophilus* B, OVA/CFA−*L. bulgaricus* Lb, OVA/CFA−*L. bulgaricus* 448, OVA/CFA−*B. longum* B6) and tube-fed with skim milk or fermented milk at 0.5 mL/mouse/day for 6 weeks supplemented with the standard diet.

The mice ($n = 10$ per group) were intraperitoneally immunized at 8, 10, and 12 weeks of age with 100 μL of 20 and 60 μg of OVA/mL CFA (Sigma Chemical Co., St. Louis, MO), respectively. Serum samples were collected from experimental mice at 7, 9, 11, and 13 weeks of age.

Cultures of Splenocytes. Splenocytes (1×10^7 cells/mL) from mice stimulated with OVA intraperitoneally were restimulated with OVA at a final concentration of 30 μg/mL in a 24-well culture plate (Nunc, Roskilde, Denmark). The culture media consisted of 90% RPMI-1640 (HyClone, Logan, UT) and 10% fetal calf serum (HyClone), and extra 0.5% complex antibiotics (penicillin−streptomycin−amphotericin) (Arista Biologicals, Allentown, NJ) were added. Splenocytes were incubated at 37 °C for 48 h in a humidified atmosphere supplemented with 5% CO_2.

ELISA Assays of IgE and Cytokines. The OVA-specific IgE titer of serum was determined by ELISA. Immunoplates were coated with 10 μg/mL OVA (200 μL/well) in 0.1 M $NaHCO_3$ (Merck, Darmstadt, Germany) and incubated overnight at 4 °C. Thereafter, the plates were washed three times with 20 mM phosphate-buffered saline (PBS) and 0.05% Tween 20 (USB, Cleveland, OH) and then blocked with 1% bovine serum albumin (BSA)−PBS (200 μL/well) for 2 h at room temperature. The plates were washed again, and samples (100 μL/well) were diluted 50-fold with 1% BSA−PBS. The plates were incubated overnight and then washed five times with PBS and 0.05% Tween 20. After the addition of 100 μL of biotin-conjugated rat anti-mouse IgE monocolonal antibody (BD PharMingen; BD Bioscience, Sparks, MD) and diluted 250-fold with 1% BSA−PBS, the plates were incubated for 1 h at ambient temperature. The plates were washed another six times. Then, 100 μL of streptavidin−HRP (Endogen, Woburn, MA), which was diluted 200× with 1% BSA-PBS, was added to each well and incubated for 20 min at ambient temperature. After six washings, 1× tetramethylbenzidine (TMB; Clinical Science Products Inc., Mansfield, MA) (100 μL/well) was added to the plate. Reaction was stopped by the addition of 50 μL of 2 N H_2SO_4. Absorbance was measured at 405 nm. The OVA-specific IgE titer was expressed as ELISA unit (EI): $EI = (A_{sample} - A_{blank})/(A_{positive\ control} - A_{blank})$, where the absorbance of the positive control represented the level of IgE in serum from OVA/CFA-immunized BALB/c mice.

The cytokines examined in this study were as follows: IFN-γ, IL-2, and IL-4. IFN-γ and IL-2 were measured with ELISA kits (Endogen). IL-4 was also measured with an ELISA kit (R&D, Minneapolis, MN). ELISA kits employ the quantitative sandwich enzyme immunoassay technique. An antibody specific for target cytokine was coated onto the microtiter plate provided by the kit. After the coating procedure, the plates were washed three times with 50 mM Tris and 0.02% Tween 20 and then blocked with 4% BSA−PBS (200 μL/well) for 1 h at room temperature to avoid nonspecific binding. After wash steps, reagent of antibody−enzyme conjugate was added to the plate and washed again. The substrate solution was added to the wells. Absorbance was measured at 450 nm.

Statistical Analysis. Data are presented as mean ± SD ($n = 10$). Statistical comparisons were analyzed by ANOVA, followed by Duncan's multiple-range test. $P < 0.05$ was considered to be significant.

RESULTS

Effect of Fermented Milk on the OVA-Specific IgE Production. It is known that IgE-mediated mast cell degranulation plays a critical role in the immediate food-induced anaphylactic reaction. The levels of OVA-specific IgE, a key to triggering the release of mediators that cause allergic reactions, were measured after supplementation with lactic acid bacteria fermented milk for 6 weeks. As shown in **Figure 1**, the OVA-specific IgE titer in serum collected from mice fed *L. bulgaricus* 448 or *B. longum* B6 fermented milk increased significantly after the first immunization. All treatments except the PBS/CFA−milk group showed elevated OVA-specific IgE levels in serum after the second immunization. The OVA-specific IgE concentrations were dramatically increased in the OVA/CFA−milk group at weeks 4 and 6 during the experimental period. At the end of the experiment, the OVA-specific IgE levels in all fermented milk groups were not significantly different from each other. However, the OVA-specific IgE levels in fermented milk groups were significantly lower than that in the OVA/CFA−milk group. According to the results, OVA-specific IgE titers in the serum of groups fed fermented milk,

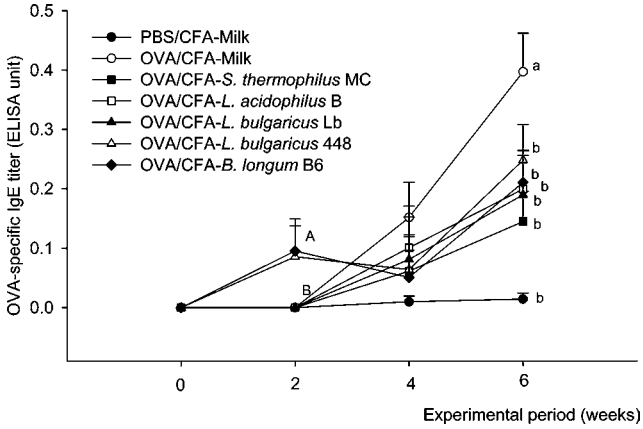

Figure 1. Serum OVA-specific IgE titers of female OVA/CFA-immunized BALB/c mice fed milk fermented with lactic acid bacteria.

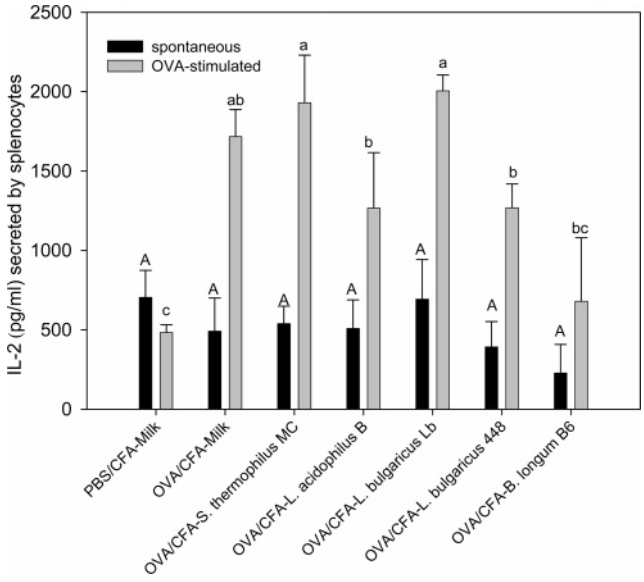

Figure 2. IL-2 secretion of splenocytes from female OVA/CFA-immunized BALB/c mice fed milk fermented with lactic acid bacteria.

especially the group fed *S. thermophilus* MC fermented milk, were significantly lower than those in the OVA/CFA−milk group.

Cytokine Secretions of Splenocytes. To clarify the mechanisms involved in immunoregulation in mice fed fermented milk, the cytokine secretions of splenocytes from mice sensitized with OVA were examined.

Although supplementation with lactic acid bacteria fermented milk did not significantly change the IL-2 levels of spontaneous and OVA-stimulated secretions in splenocytes, as shown in **Figure 2**, the increased levels of IL-2 of OVA-stimulated splenocytes from OVA/CFA-immunized mice indicated that mice were substantially immunized by allergen OVA as compared to PBS/CFA-immunized ones.

To further determine the cytokine secretion pattern of oral administration with milk fermented with lactic acid bacteria, we measured IFN-γ and IL-4 in the supernatant of splenocyte cultures. The production of IFN-γ from splenocyte cultures is presented in **Figure 3**. Mice fed *L. bulgaricus* 448 or *L. bulgaricus* Lb fermented milk had significantly increased spontaneous secretion of IFN-γ as compared to that from the OVA/CFA−milk group ($P < 0.05$). The OVA-immunized mice fed *S. thermophilus* MC, *L. bulgaricus* Lb, and *B. longum* B6 fermented milk also demonstrated significantly increased OVA-

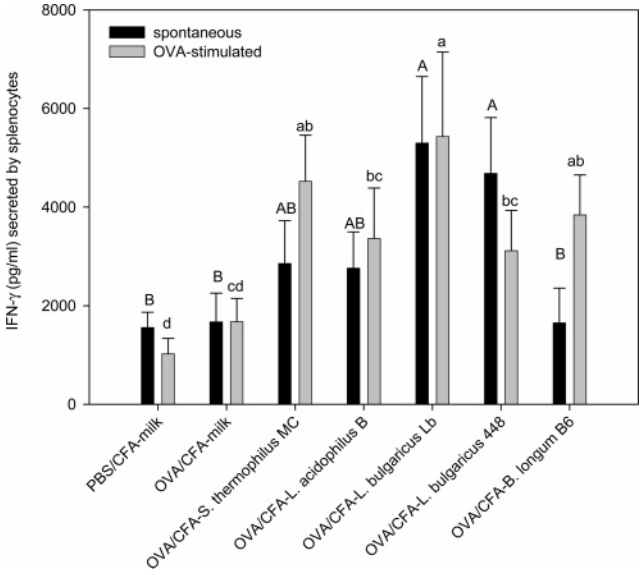

Figure 3. IFN-γ secretion of splenocytes from female OVA/CFA-immunized BALB/c mice fed milk fermented with lactic acid bacteria.

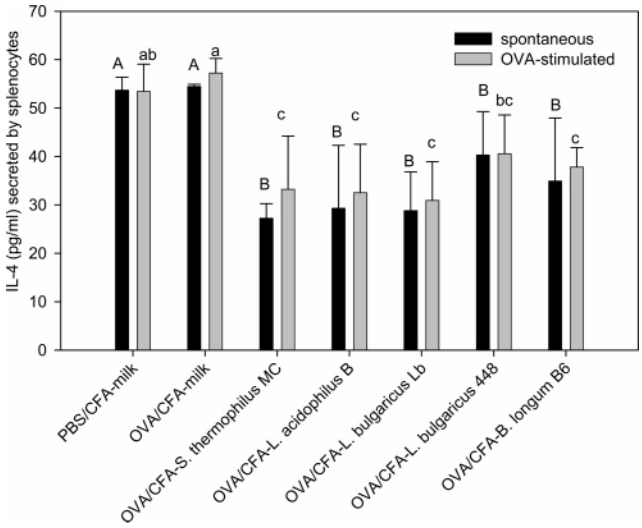

Figure 4. IL-4 secretion of splenocytes from female OVA/CFA-immunized BALB/c mice fed milk fermented with lactic acid bacteria.

stimulated IFN-γ production compared with mice fed nonfermented milk.

The IL-4 secretion of splenocytes is shown in **Figure 4**. Both spontaneous and OVA-stimulated secretions of splenocytes from mice fed lactic acid bacteria fermented milk showed significantly lower levels of IL-4 than those from mice fed nonfermented milk.

To clarify the secretion pattern of Th1 and Th2 cytokines affected by fermented milk administrated in vivo, the ratios of IFN-γ to IL-4 of both spontaneous and OVA-stimulated secretions produced by splenocytes were further calculated and are presented in **Table 2**. The results showed that the ratios of IFN-γ to IL-4 of both spontaneous and OVA-stimulated secretions in splenocytes from mice fed lactic acid bacteria fermented milk increased significantly as compared to that of PBS- or OVA/CFA−milk groups.

DISCUSSION

In the present study, all groups fed the lactic acid bacteria fermented milk show markedly reduced OVA-specific IgE levels (**Figure 1**). Matsuzaki et al. (19) have demonstrated that oral

Table 2. Ratio of IFN-γ/IL-4 Cytokine Secretion of Splenocyte from Female OVA/CFA-Immunized BALB/c Mice Fed Milk Fermented with Lactic Acid Bacteria[a]

group	IFN-γ/IL-4 (pg/pg)	
	spontaneous	OVA-immunized
PBS/CFA-milk	27.8 ± 11.5 e	18.3 ± 12.0 d
OVA/CFA-milk	30.3 ± 14.0 e	28.8 ± 13.4 d
OVA/CFA–*S. thermophilus* MC	102.1 ± 20.4 c	144.8 ± 36.5 b
OVA/CFA–*L. acidophilus* B	91.6 ± 4.2 c	106.9 ± 13.8 c
OVA/CFA–*L. bulgaricus* Lb	187.6 ± 12.4 a	175.5 ± 1.4 a
OVA/CFA–*L. bulgaricus* 448	123.4 ± 23.1 b	75.3 ± 11.3 c
OVA/CFA–*B. longum* B6	58.7 ± 14.7 d	100.9 ± 10.8 c

[a] Data are shown as mean ± standard deviation ($n = 10$). Entries bearing different letters in the same column are significantly different ($P < 0.05$) by Duncan's multiple-range test.

administration of heat-killed *Lactobacillus casei* strain Shirota to mice significantly inhibited IgE production in serum. The reduction of serum allergen-specific IgE might alleviate the allergic diseases mediated by mast cells (*13*). Our results suggest that supplementation with lactic acid bacteria fermented milk has an antiallergic effect via the reduction of allergen-specific IgE production.

The inhibitory effect of *L. casei* strain Shirota on IgE production might come from the regulation between Th1 and Th2 cells. Our results show that administration of fermented milk not only inhibits OVA-specific IgE production (**Figure 1**) but also increases the secretion of Th1 cytokines such as IFN-γ (**Figure 3**). IFN-γ induces the up-regulation of MHC class I molecules that present peptides to $CD8^+$ T cells (*20*). $CD8^+$ T cells recognize peptides presented by MHC class I molecules and require IL-2 to proliferate (*21*, *22*). The results in this study suggest that supplementation with fermented milk containing live lactic acid bacteria may enhance the $CD8^+$ T cell activity in the innate immunity, although the immunomodulatory mechanisms remain to be clarified.

$CD4^+$ Th2 cells play a pivotal role in initiating and orchestrating ongoing immunologically mediated allergic diseases. On the contrary, $CD4^+$ Th1 cells that generate high levels of IFN-γ are able to inhibit the development of Th2 cells and IgE production (*23*). The balance of cytokines produced by Th1 and Th2 cells has been reported to be an important factor in the regulation of IgE production (*24*, *25*). In this study, mice fed lactic acid bacteria fermented milk had much lower levels of IL-4, a typical Th2 cytokine (**Figure 4**). The ratios of IFN-γ to IL-4 of both spontaneous and OVA-stimulated secretions in splenocytes from mice fed lactic acid bacteria fermented milk increased significantly as compared to that of PBS- or OVA/CFA−milk groups (**Table 2**). The group fed *L. bulgaricus* Lb fermented milk had the highest ratios. The ratio of IFN-γ (Th1 cytokine) to IL-4 (Th2 cytokine) in splenocytes increases, indicating that mice fed fermented milk tend toward the Th1 immune balance. In vitro studies have revealed T lymphocytes are crucial in the production of IFN-γ when *L. acidophilus* is cocultured with murine splenic leukocytes (*26*). The induction of IFN-γ by lymphoid cells contacting lactic acid bacteria is dependent on the pro-interferon cytokine IL-12 produced by accessory cells (*27*). The results from this study further suggest that milk fermented with lactic acid bacteria has in vivo antiallergic effects on OVA/CFA-immunized mice via increasing the secretion ratio of IFN-γ/IL-4 (Th1/Th2) by splenocytes and decreasing the serum level of OVA-specific IgE. Nevertheless, because the immunomodulatory effect of lactic acid bacteria is a strain-dependent characteristic and species speciality, as shown in our results lactic acid bacteria demonstrate different immunoregulatory effects, and probiotic lactic acid bacterial strains should thus be evaluated on their own merits. Oral administration of milk fermented with lactic acid bacteria exhibits a Th1-skewed immunoregulatory effect on the development of allergen-specific immune response and may meet the purpose of immunoprophylaxis for food allergy and other allergic diseases.

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