

The Antiallergic Effect of Kefir Lactobacilli

Wei-Sheng Hong, Yen-Po Chen, and Ming-Ju Chen

Abstract: This study demonstrated that oral feeding of heat-inactivated *Lactobacillus (Lb.) kefirifaciens* M1 from kefir grains effectively inhibited immunoglobulin (Ig) E production in response to ovalbumin (OVA) *in vivo*. The pattern of cytokine production by splenocyte cells revealed that the levels of cytokines produced by T helper (Th) 1 cells increased, and those of cytokines produced by Th2 cells decreased in the heat-inactivated M1 feeding group. These findings indicated that *Lactobacillus kefirifaciens* M1 in the kefir played an important role in antiallergic activities. By additional analysis using flow cytometry and microarray, the mechanism of suppression of IgE production by oral feeding of the heat-inactivated M1 probably occurs because of upregulation of the expression of *Cd2*, *Stat4*, and *Ifn γ* leading to skewing the Th1/Th2 balance toward Th1 dominance, elevation of the CD4⁺CD25⁺ regulatory T (Treg) percentage, and reduction of activated CD19⁺ B cells. Downregulation of complement system and components was also involved in suppression of IgE production.

Keywords: antiallergic effect, kefir, *Lactobacillus kefirifaciens*, microarray

Practical Application: Kefir has long been considered good for health. Its health benefits include immunoregulatory effects. However, there is a lack of knowledge concerning the immunoregulatory effects induced by kefir lactic acid bacteria (LAB). Our data clearly demonstrated the antiallergic activity of kefir LAB, *Lactobacillus (Lb.) kefirifaciens* M1. By additional analysis using flow cytometry and microarray, the possible mechanism of suppression of IgE production by oral feeding of the heat-inactivated M1 was also elucidated. Our findings indicated that *Lactobacillus kefirifaciens* M1 may have a great potential for utilization in functional food products.

Introduction

Kefir differs from other fermented products in that it is the product of milk fermentation in the presence of a mixed group of microflora confined to a matrix of discrete “kefir grains,” which are recovered subsequent to fermentation (Marshall and Cole 1985). Kefir has long been considered good for health. Its health benefits include immunoregulatory effects. It has been shown in the literature that oral kefir not only inhibited tumor growth and induced the apoptotic form of tumor cell lysis (Liu and others 2002; de LeBlanc and others 2006) but also suppressed immunoglobulin (Ig) E and IgG responses (Liu and others 2006). In addition, kefir displayed an antiasthma effect. Histological studies have indicated that kefir inhibits eosinophilia in lung tissue and mucus hypersecretion by goblet cells in the airway (Lee and others 2007). However, there is a lack of knowledge concerning the immunoregulatory effects induced by kefir lactic acid bacteria (LAB). Moreover, the mechanisms responsible for these effects have not been fully determined.

Many studies have examined the efficacy of LAB supplementation in the prevention and treatment of allergic disorders. Although the detailed mechanisms of these antiallergic effects exerted by LAB have not yet been clarified, cytokines induced by LAB are considered to play key roles in immunoregulation (Shida and

others 2006). Matsuzaki and others (1998) reported that administration of *Lactobacillus (Lb.) casei* strain Shirota to ovalbumin (OVA) sensitized mice enhanced the production of T helper (Th) 1 cytokines (interferon [IFN]- γ and interleukin [IL]-2) and inhibited the secretion of Th2 cytokines (IL-4, IL-5, and IL-10). Using a food allergy model, Shida and others (2002) also proved that *Lactobacillus casei* strain Shirota administration is able to suppress IgE and IgG1 secretion from antigen-stimulated splenocytes through promoting a dominant Th1 response mediated by IL-12 induction. Fujiwara and others (2005) demonstrated that oral feeding with *Lactobacillus paracasei* strain KW3110 could repress both Th2 cell generation and eosinophil activation. Furthermore, supplementation with *Lactobacillus paracasei* strain KW3110 in a mouse model of atopic dermatitis-like symptoms significantly reduced the development of skin lesions, accompanied by lower plasma IgE levels (Wakabayashi and others 2008). Clinical studies (Aat-touri and Lemonnier 1997; Cross 2002) have shown promising outlets for the use of nonpathogenic lactobacilli as provisioners of pro-Th1 immune signals in antiallergy immunotherapy.

In our previous studies, we isolated and identified microorganisms from kefir grains using a combination of polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) and 16S rDNA sequencing (Chen and others 2008). Fifty LAB strains were isolated and identified in the kefir grains. We further used these LAB strains and their supernatants to investigate the *in vitro* immunomodulating capacity by cytokine profiles through a toll-like receptor (TLR) pathway. Results indicated that 2 lactobacilli (*Lactobacillus kefirifaciens* M1 and *Lactobacillus kefirifaciens* M2) isolated from kefir grains influenced the production of Th1 and proinflammatory cytokines by macrophages and may be beneficial

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for promotion of the Th1/Th2 balance (Hong and others 2009). Thus, in this present study, we examined the antiallergic effects of *Lactobacillus kefirifaciens* M1 and *Lactobacillus kefir* M2 isolated from kefir grains by modulation of the Th1/Th2 balance and inhibition of IgE production in OVA-sensitized Th2-polarized mice. In addition, to clarify the mechanism by which kefir LAB induces suppressive effects on the development of allergic diseases, genome-wide gene expression analysis of murine Peyer's patches after oral feeding of kefir LAB was also conducted.

Materials and Methods

LAB samples

Lactobacillus kefirifaciens M1 (M1) and *Lactobacillus kefir* M2 (M2) were isolated and identified using a combination of PCR-DGGE and 16S rDNA sequencing from kefir grains (Chen and others 2008). Both lactobacilli strains were grown in deMan, Rogosa, and Sharp (MRS) broth (Difco Laboratories, Detroit, Mich., U.S.A.) at 37 °C, and then harvested by washing and resuspending 3 times in phosphate buffer saline solution (Amersco, Solon, Ohio, U.S.A.). The cell suspension was adjusted to 1.5×10^7 , 3×10^7 , and 6×10^7 CFU mL⁻¹. M1 and M2 were inactivated by heating at 85 °C for 40 min.

In vitro cytokine production by murine peritoneal macrophages and splenocytes

Six-wk BALB/c female mice (Natl. Laboratory Animal Center, Taipei, Taiwan) were used in this part of study. The murine peritoneal macrophages were collected using the protocol described by Assreuy and others (1994), Netea and others (2005), and Hong and others (2009).

Splenocytes were also isolated from 6-wk BALB/c female mice (Natl. Laboratory Animal Center). The mice were sacrificed by cervical dislocation. The spleens were removed and placed in individual sterile Petri plates containing 10 mL of Roswell Park Memorial Inst. medium 1640 (Roswell Park Memorial Inst. [RPMI] medium, HyClone, Logan, Utah, U.S.A.), supplemented with 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Sigma). The spleens were cut into pieces with a sterile scalpel and then pressed through a 70-µm cell strainer (BD Falcon, Bedford, Mass., U.S.A.) under aseptic conditions. The cells were suspended in 7 mL complete RPMI and centrifuged (1200 rpm) at room temperature for 5 min. The pellets were resuspended in 10 mL of RPMI with 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Sigma, St. Louis, Mo., U.S.A.), stained

with 0.5% trypan blue (0.5% Trypan-blue, Invitrogen, Carlsbad, Calif., U.S.A.), and counted using a microscope (Optima, Aurora, Colo., U.S.A.). The cell suspension was then adjusted to 1×10^7 cells/mL in RPMI medium (HyClone) supplemented with 10% low endotoxic fetal calf serum and 1% antibiotics.

The peritoneal macrophages and splenocytes were stimulated with 10^6 CFU/mL of each strain in 1-mL medium. For the positive control, 50 ng lipopolysaccharide (Sigma) or 20-µg pam3CSK4 (Sigma) was added to 1-mL medium per well. After 24-h incubation at 37 °C, the supernatants were collected and stored at -80 °C until cytokine assays were performed.

In vivo experiments

The experimental procedure for immunization with OVA and administration of heat-inactivated lactobacilli isolated from kefir grains is summarized in Figure 1. On days 0, 13, 20 and 27, 6-wk BALB/c female mice ($n = 5$ to 6 per group) were injected intraperitoneally with 20 µg of OVA (Sigma, Grade V) and 2 mg of Al(OH)₃ (Wako, Chuo-Ku, Osaka, Japan) for a total volume of 0.2 mL. The mice were given tube feedings with 1.5×10^7 , 3×10^7 , or 6×10^7 CFU/mouse/day of heat-inactivated M1 throughout the experimental period. Sera were collected on days 1, 14, 21, and 28 by submandibular bleeding for total and OVA-specific IgE quantification.

On day 28, mice were sacrificed and splenocytes were harvested for culture. Red blood cells (RBCs) in splenocytes were lysed by adding 5 mL RBC lysis buffer (Qiagen, Venlo, The Netherlands) twice. After lysis of RBC, splenocytes were suspended in a concentration of 1×10^7 cells/mL in RPMI medium (Sigma) supplemented with 10% FCS and 1% antibiotics. Cells were cultured with 100 µg/mL OVA at 37 °C for 48 h.

Peyer's patches were also isolated on day 28 after sacrificing the mice. Three to 4 granules from Peyer's patches were collected from each mouse. RNA was isolated using TRIzol reagent (Invitrogen) and stored at -80 °C until microarray analysis.

ELISA for cytokines, total IgE and OVA-specific IgE

Determination of IL-12p40, IL-6, IL-1β, tumor necrosis factor (TNF)-α, and IFN-γ levels in culture supernatants was performed by sandwich enzyme-linked immunosorbent assay (ELISA). Mouse cytokine kits were all obtained from R and D system (Minneapolis, Minn., U.S.A.). The results were expressed as the concentration of each cytokine (pg/mL).

The level of total IgE in the culture supernatant was measured using a mouse IgE ELISA quantitation kit (Bethyl Laboratories,

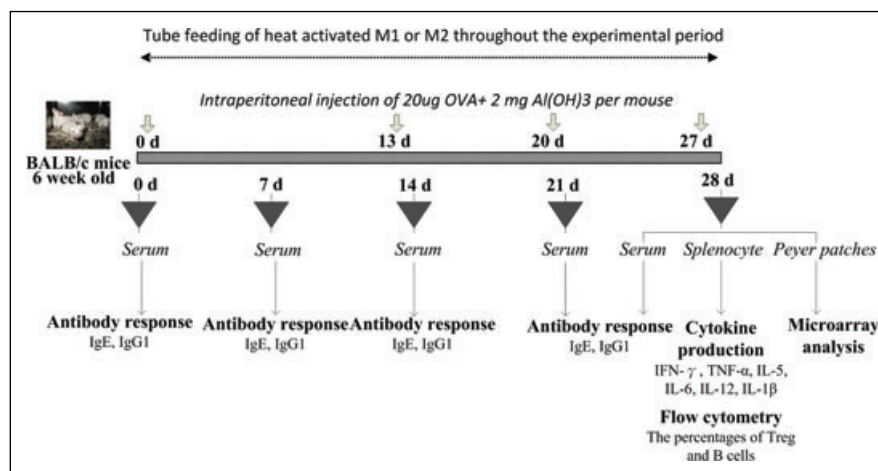


Figure 1—The experimental procedure of immunization with OVA and administration of heat-inactivated lactobacilli isolated from kefir grains.

Montgomery, Tex., U.S.A.). Determination of OVA-specific IgE was performed by an OVA-specific IgE kit (Serotech, Oxford, U.K.). ELISA was performed using the standard protocol as recommended by manufacturer.

Flow cytometry

The percentages of CD4⁺CD25⁺ regulatory T (Treg) cells and B cells on splenocytes from mice fed heat-inactivated M1 were evaluated by flow cytometric analysis. For Treg cells, freshly isolated splenocytes (5×10^6 cell/mL) from mice injected with OVA were double stained with antimouse PE-CD4 antibody (clone GK1.5) and antihuman FITC-CD25 antibody (clone 7D4) for 20 min on ice. For B cells, splenocytes from mice injected with OVA were stained with antimouse FITC-CD19 antibody (clone 6D5). All antibodies were purchased from Beckman Coulter (Fullerton, Calif., U.S.A.). Cells were washed extensively with PBS and analyzed with an EPICS XL flow cytometer (Beckman Coulter).

Microarray analysis

RNA samples from Peyer's patches from each mouse were quantified with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, Del., U.S.A.), and RNA integrity was assessed with an RNA 6000 Nano Labchip kit using the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, Calif., U.S.A.). Only total RNA with an optical density 260/280 ratio >2.0, a Bioanalyzer 28S/18S peak ratio >1.2, and an RNA integrity number superior to 8 was used for microarray analysis. An equimolar pool of total RNA extracted from Peyer's patches of 4 different mice per treatment was made.

RNA samples were labeled using a Quick Amp Labeling Kit (Agilent Technologies) according to the manufacturer's instructions. Briefly, 1.0 μ g of total RNA was amplified and reverse transcribed *in vitro* to cDNA using T7-polymerase, which was subsequently labeled with either cyanine 3 (experimental, RNA from OVA-sanitized mice fed heat-inactivated M1) or cyanine 5 (control, RNA from OVA-sanitized mice fed PBS)-labeled cytidine triphosphate dye. After labeling, the cRNA was hybridized with an Agilent Whole Mouse Genome 4 $\times$ 44k oligo microarray (G4122F-014868, Agilent Technologies), following the manufacturer's protocols. In brief, 825 μ g of Cy-labeled cRNA (pool of equal amounts experiment and control) were fragmented at 60 °C for 30 min and hybridized onto an Agilent Whole Mouse Genome 4 $\times$ 44k oligo microarray in a rotation oven (65 °C for 17 h, 4 rpm in the dark). Following hybridization, the slides were sequentially washed in solutions I, II, and III (Agilent Technologies) and air dried. Slides were then scanned using an Agilent microarray scanner (G2565CA, Agilent Technologies). Spot identification and

quantification were performed with feature extraction software 10.5.1.1 (Agilent Technologies).

Quantitative real-time (RT) PCR

The RNA samples analyzed by microarray analysis were also analyzed by RT PCR. To prepare a cDNA pool from each RNA sample, total RNA (5 μ g) was reverse transcribed using moloney murine leukemia virus reverse transcriptase (Promega, San Luis Obispo, Calif., U.S.A.) and the resulting samples were diluted 40 times by volume with nuclease-free water. Each cDNA pool was stored at -20 °C until further RT (quantitative) PCR analysis.

Specific oligonucleotide primer pairs (Table 1) were selected and used for RT PCR (Roche Universal ProbeLibrary). RT-PCR reactions were performed on a Roche LightCycler 1.5 using a LightCycler® FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Cat. 03 515 885 001, Castle Hill, Australia). At each RT-PCR run, the data were automatically analyzed by the system and an amplification plot was generated for each cDNA sample. From each of these plots, LightCycler3 Data analysis software automatically calculated the Cp value.

Statistical analysis

All results were analyzed using the general linear model procedure available from the Statistical Analysis system software package version 8.1. Duncan's multiple range test (Montgomery 1991) was used to detect differences between treatment means. Each experiment was conducted in triplicate.

Results

Effect of kefir lactobacilli on Th1 cytokine production by peritoneal macrophages and splenocyte

The ability of 2 kefir lactobacilli to induce Th1 cytokines, IFN- γ , TNF- α , IL-6, IL-12, and IL-1 β , was tested in murine peritoneal macrophages. As indicated in Figure 2, live and heat-inactivated *Lactobacillus kefir* M1 and *Lactobacillus kefir* M2 significantly induced production of IFN-, TNF- α , IL-6, IL-12, and IL-1 β in murine peritoneal macrophages ($P < 0.05$). Except for TNF- α and IL-1 β , the inducing abilities were not significantly different ($P > 0.05$) between the live and heat-inactivated kefir lactobacilli, as well as between the 2 kefir lactobacilli. Heat-inactivated kefir lactobacilli demonstrated better stimulation of TNF- α than live strains. On the other hand, live strains showed higher production of IL-1 β than heat-inactivated strains. The effects of *Lactobacillus kefir* M1 and *Lactobacillus kefir* M2 on cytokine secretion by murine splenocytes were also investigated (Figure 3). The trends of cytokine production by splenocytes were similar to those by peritoneal macrophages (Figure 2). The induction of IFN- γ and IL-12, which are important Th1 cytokines, was

Table 1—Primers information for real-time PCR.

Sense primer	Antisense primer	Gene symbol	Accession number
TTGTCGGTGGTGGCAGTGTATC	GGCTTCCTCGGGCTTCTTGG	<i>C3</i>	NM_009778
TTTGCCGACAGCCTTACTCT	AGAAGGTTCTGGGTGGGATT	<i>C2</i>	NM_013484
TGACCTGGAGAAGCTGACCT	TGGTAGGGACCGAGTCAAAG	<i>C4b</i>	NM_009780
ACCTGGTTCTGGCTGTATGG	GCAGAGTTCCATGCTGTGTA	<i>C1s</i>	NM_144938
CACCCAGACTACCGCCAAGATG	TCCCGAATCCGCTGACATAACC	<i>C1r</i>	NM_023143
TCCCCAGAAGAATCATGAGT	GGGTTTATCGCCTCACACTT	<i>Cd2</i>	NM_013486
CCAAGGAAACCAACTGAGGA	TGTTTTGATTCTGGGTGCTG	<i>Cd3g</i>	NM_009850
TGTGGTTGGTTTGCTACGAG	TGGCTCACTCCGTTGACTAA	<i>Cd28</i>	NM_007642
ATGGCTGAAAACATCCCTGA	GCTTCGGATTGTTGAAATGG	<i>Stat4</i>	NM_011487
CCCATTGGTGGTAAGCAAT	TGGACAGGAGCACATTCTCA	<i>Prf1</i>	NM_011073
TCCCTGAAAGAAAGCAGTGTC	TGTCATTCCGGGTGTAGTCACA	<i>Ifir</i>	NM_008337

significantly enhanced by *Lactobacillus kefiranofaciens* M1 and *Lactobacillus kefir* M2 ($P < 0.05$).

The *in vitro* results depicted that both *Lactobacillus kefiranofaciens* M1 and *Lactobacillus kefir* M2 could induce Th1 cytokines in murine peritoneal macrophages and splenocytes, which might inhibit the Th2 response and IgE production. The ability of heat-inactivated kefir lactobacilli to enhance Th1 cytokines was not significantly different than that of the live strains. Thus, this study analyzed IgE production and cytokine secretion in OVA-sensitized mice fed heat-inactivated kefir lactobacilli. We selected *Lactobacillus kefiranofaciens* M1, which had the strongest IL-12-inducing activity, for the following animal study.

Suppression of the increase in serum total IgE and OVA-specific IgE of OVA-sensitized mice by oral administration of the heat-inactivated *Lactobacillus kefiranofaciens* M1

The total IgE levels in serum are presented in Figure 4. The total IgE production in the serum of OVA-sensitized mice was inhibited by oral feeding of heat-inactivated M1. A dose-response study (Figure 4) showed that the groups that were given concentrations higher than 3×10^7 CFU/mouse/day had significantly suppressed total IgE production in the serum compared with that in OVA control mice. The higher dosage apparently led to a better IgE inhibition effect.

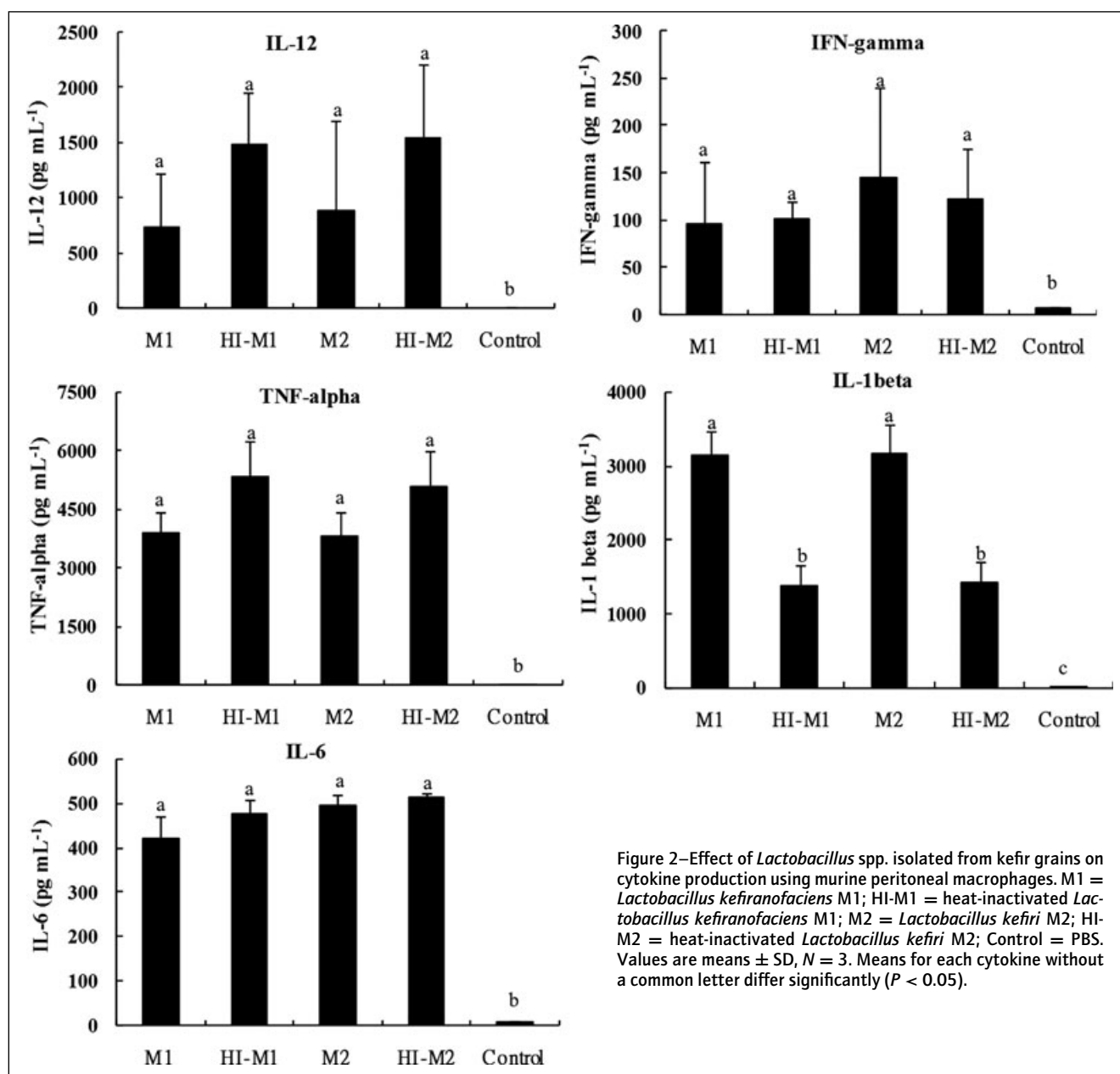


Figure 2—Effect of *Lactobacillus* spp. isolated from kefir grains on cytokine production using murine peritoneal macrophages. M1 = *Lactobacillus kefiranofaciens* M1; HI-M1 = heat-inactivated *Lactobacillus kefiranofaciens* M1; M2 = *Lactobacillus kefir* M2; HI-M2 = heat-inactivated *Lactobacillus kefir* M2; Control = PBS. Values are means $\pm$ SD, $N = 3$. Means for each cytokine without a common letter differ significantly ($P < 0.05$).

Time-course response of oral administration of heat-inactivated M1 on IgE and OVA-specific IgE

The effects of heat-inactivated M1 on IgE and OVA-specific IgE were studied from day 0 to day 28 (Figure 5). To evaluate the immunomodulative effect of oral administration of heat-inactivated M1, BALB/c mice were injected with OVA at 0, 2, 3, and 4 wk. This immunization schedule resulted in a marked increase in

serum IgE levels (Figure 5, control). Production of IgE was significantly lower ($P < 0.05$) in the M1 group than in the control group from the 1st to 4th OVA injection (Figure 5A). The production of OVA-specific IgE was also significantly lower ($P < 0.05$) in the M1 group after the 3rd OVA injection (Figure 5B). The *in vivo* results suggest that heat-inactivated *Lactobacillus kefir* M1 has the potential to inhibit IgE production.

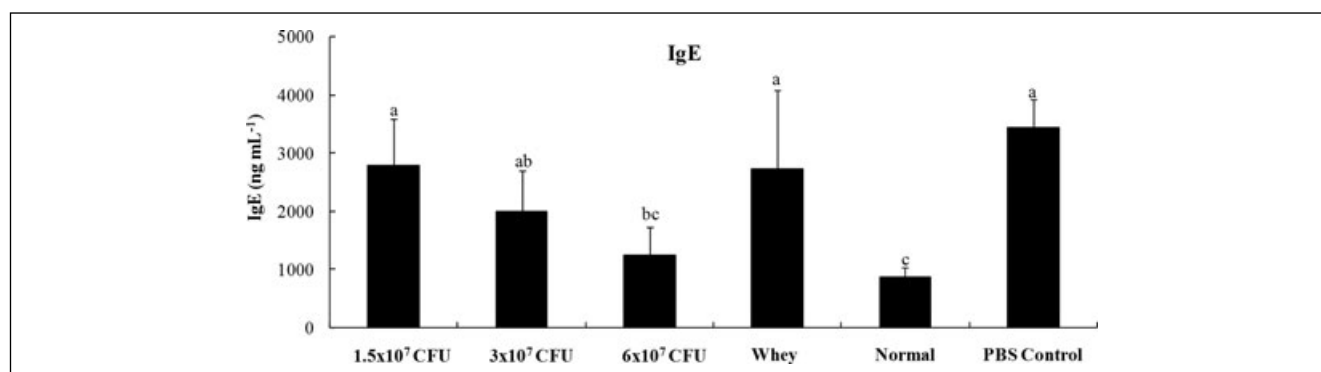
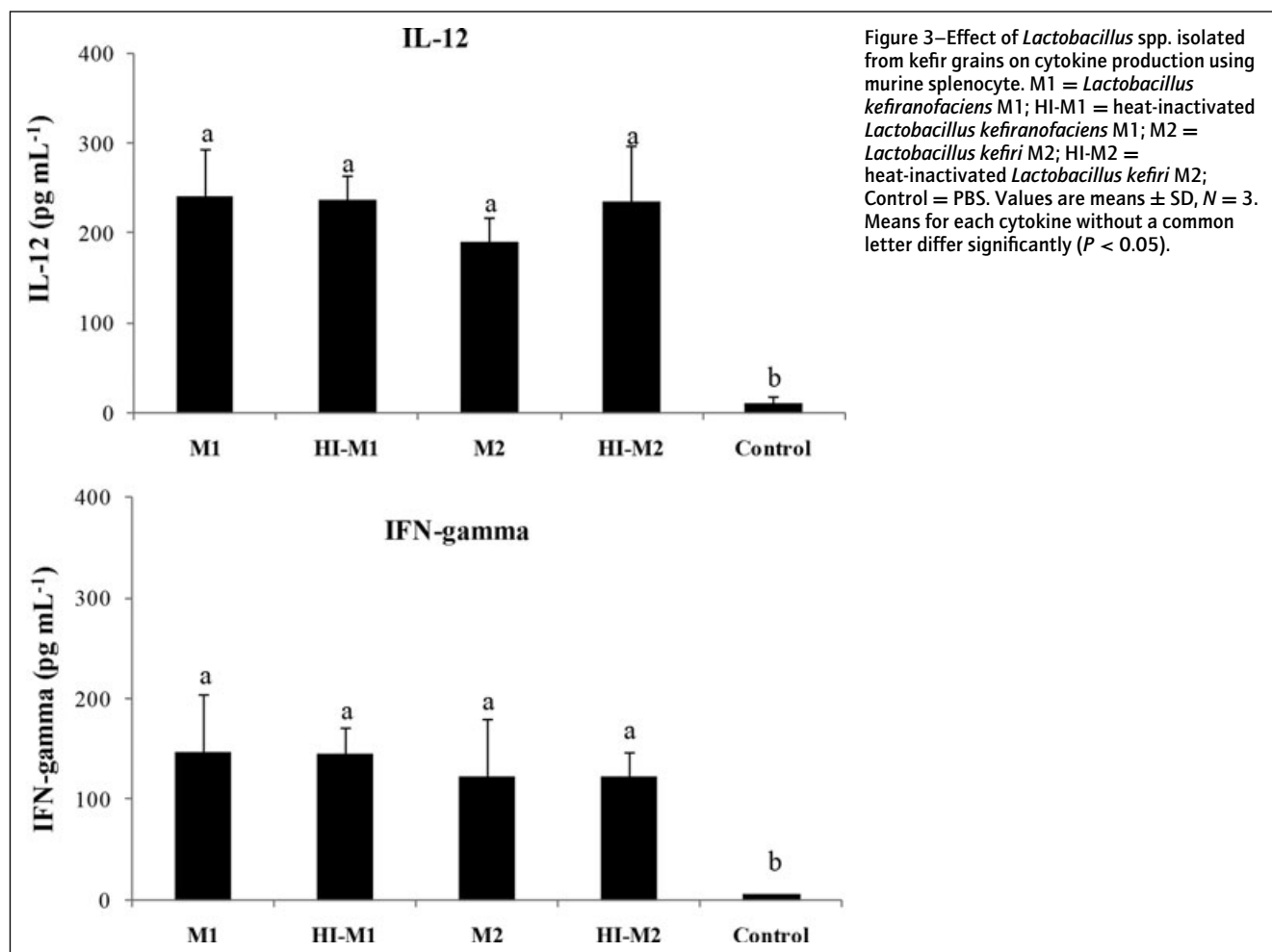


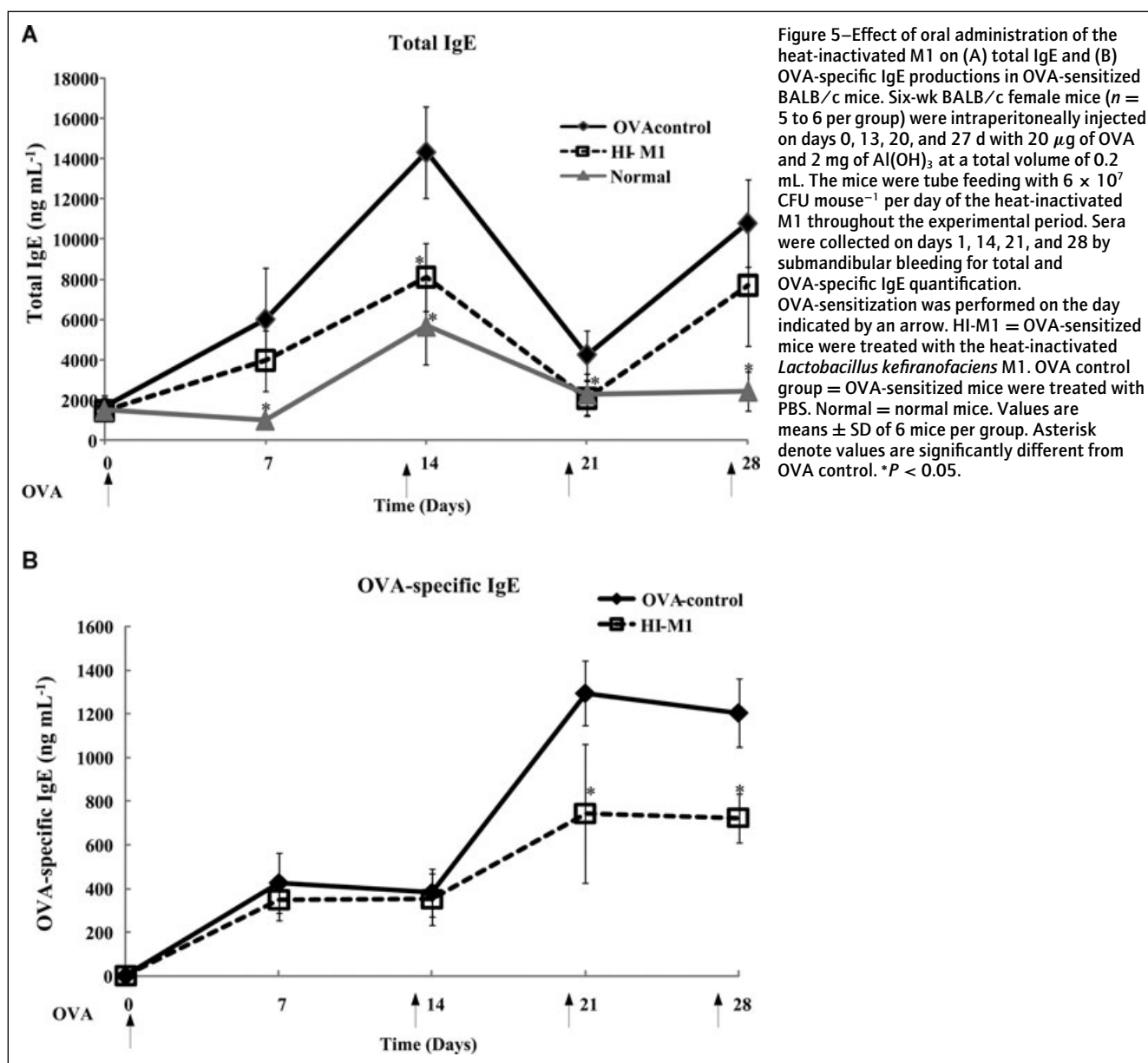
Figure 4—Effect of the heat-inactivated M1 isolated from kefir grains on sera IgE production using allergy mice. Six-wk BALB/c female mice ($n = 5$ to 6 per group) were intraperitoneally injected on days 0, 13, 20, and 27 d with 20 μ g of OVA and 2 mg of $Al(OH)_3$ at a total volume of 0.2 mL. The mice were tube feeding with 1.5×10^7 , 3×10^7 , or 6×10^7 CFU mouse⁻¹ per day of the heat-inactivated M1 throughout the experimental period. Sera were collected on day 28 by submandibular bleeding for total IgE quantification. Whey = the mice were tube feeding with 500 μ L mouse⁻¹ per day of kefir whey. Control = PBS. Values are means $\pm$ SD of 6 mice per group. Means for each antibody without a common letter differ significantly ($P < 0.05$).

Effect of cytokine productions by splenocytes

To clarify the mechanisms involved in the inhibition of IgE production in OVA-sensitized mice, we examined cytokine production in splenocytes from OVA-sensitized mice *ex vivo*. As shown in Figure 6, the production of IL-12 by the splenocytes from mice fed heat-inactivated *Lactobacillus kefiranofaciens* M1 was higher ($P < 0.05$) than that from OVA control mice. In contrast, the production of IL-5 by splenocytes in the group fed heat-inactivated *Lactobacillus kefiranofaciens* M1 was significantly lower ($P < 0.05$) than that from controls. There were no significant differences between the 2 groups in IFN- γ production. These data demonstrate that oral administration of heat-inactivated *Lactobacillus kefiranofaciens* M1 for 28 d can regulate the Th1/Th2 balance by inhibition of IL-5 and augmentation of IL-12 in splenocytes from OVA-sensitized mice. The functional increase in Th1 cells and the decrease in Th2 cells were probably critical in the inhibition of IgE production in mice.

Effects of the heat-inactivated *Lactobacillus kefiranofaciens* M1 on CD4⁺CD25⁺ regulatory T cells and CD19⁺ B cell of splenocytes from OVA-sensitized mouse

To evaluate the effects of heat-inactivated *Lactobacillus kefiranofaciens* M1 on CD4⁺CD25⁺ regulatory T cells and B cells, CD4, CD25, and CD19 expression in murine splenocytes was measured by flow cytometry after 28-d of oral feeding of heat-inactivated M1 in OVA-sensitized mice. The percentage of CD4⁺CD25⁺ regulatory T cells was significantly increased in OVA-sensitized mice given heat-inactivated M1 ($5.3 \pm 0.9\%$ per 10^4 splenocytes) compared with that in the OVA control ($4.0 \pm 0.2\%$) (Figure 7A), whereas the expression level of CD19 was downregulated in mice administered heat-inactivated M1 (Figure 7B) compared with the OVA control group. These data suggest that oral feeding of heat-inactivated M1 can enhance CD4⁺CD25⁺ regulatory T cells to suppress Th 2 cytokines. In addition, as a B cell lineage marker,



the CD19 antigen is expressed from the early pro-B cell stage to the B cell lymphoblast stage. Treatment with heat-inactivated *Lactobacillus kefiranofaciens* M1 may have significantly inhibited IgE production in serum because of a decrease in maturation of B lymphocytes.

Effects of the heat-inactivated *Lactobacillus kefiranofaciens* M1 on differential gene expression in Peyer's patch cells of OVA-sensitized mice

Peyer's patches in the intestinal mucosa are essential for host defense and immune regulation in the enteric system. To better understand molecular mechanisms that might be involved in the antiallergic effects of *Lactobacillus kefiranofaciens* M1, we investigated the specific and differential gene expression in Peyer's patch cells of OVA-sensitized mice fed heat-inactivated M1 for 28 d. cDNA microarray profiles showed that the expression of 1568 genes and expressed sequence tags (ESTs) changed by at least 2.0-fold in Peyer's patch cells in OVA-sensitized mice fed heat-inactivated M1 compared with no M1 control pair-wise comparisons. Among the 1568 genes and ESTs, 1034 were downregulated and 534 were upregulated (data not shown).

These genes were further categorized using the Biocarta pathway database. The results (Table 2) revealed that the gene expression involved in immune response and inflammation (such as *Ifir*, *Cd3*, *Cd28*, *Stat4*, and *Ccr7*) as well as cell adhesion (*Cd2*) was upregulated in the group fed heat-inactivated M1. Other genes involved in the classic complement pathway and lectin-induced pathway (such as *C1r*, *C1s*, *C2*, and *C3*) were downregulated. The differential expression of those genes was later confirmed using RT-PCR. RT-PCR results correlated with the microarray results (data not shown).

Discussion

In the present study, we demonstrated that kefir lactobacilli, *Lactobacillus kefiranofaciens* M1 (M1) and *Lactobacillus kefir* M2 (M2), stimulated the production of IFN- γ and IL-12 *in vitro* in both peri-

toneal macrophages and splenocytes. Several earlier studies have reported *in vitro* cytokine response patterns of the innate immune defense system activated by probiotic lactobacilli (Shida and others 1998; Fujiwara and others 2004). The activation of the Th1 response by probiotic lactobacilli is mediated by cytokine production, including IL-12 and IFN- γ . IL-12 is well known to play a critical role by both inducing a Th1 dominant immune response and enhancing cellular immunity (Shida and others 2006). Shida and others (1998) clearly documented that *Lactobacillus casei* Shirota can promote Th1 cells from naïve CD4⁺ T cells through secretion of IL-12 by macrophages. IFN- γ , secreted from Th1 cells, is an antagonist of IL-4 and inhibits IgE production (Rousset and others 1991; Matsuzaki and others 1998; Segawa and others 2008). IL-12 and IFN- γ are important Th1 cytokines that can activate phagocytic cells in response to an encounter with pathogenic bacteria, and suppress the Th2-polarizing response. In this sense, the live and heat-inactivated M1 and M2 investigated in this study probably promoted Th1 immunoregulation activity against the Th2-polarizing response.

The antiallergic properties of heat-inactivated kefir lactobacilli were also investigated in this study. The ability of heat-inactivated kefir lactobacilli to enhance Th1 cytokines showed no significant differences compared with live analogs (Figure 2 and 3). It had been believed that the immunogenicity of LAB depends on the survival of the bacteria in the gastrointestinal tract, and their ability to adhere to the mucosal surface (Nonaka and others 2008). However, several studies have suggested that not only live but also heat-inactivated LAB exhibit immunostimulatory effects (Murosaki and others 1998; Sashihara and others 2006; Segawa and others 2008). The components of the cell wall might play an important role in the immunostimulatory effects of heat-inactivated LAB. Our previous study indicated that peptidoglycans in kefir LAB were the possible immunomodulin. Another of our other studies illustrated that kefir LAB influences the secretion of proinflammatory cytokines through TLR 2 (Hong and others 2009). TLR2-mediated signals, responding to certain components in the

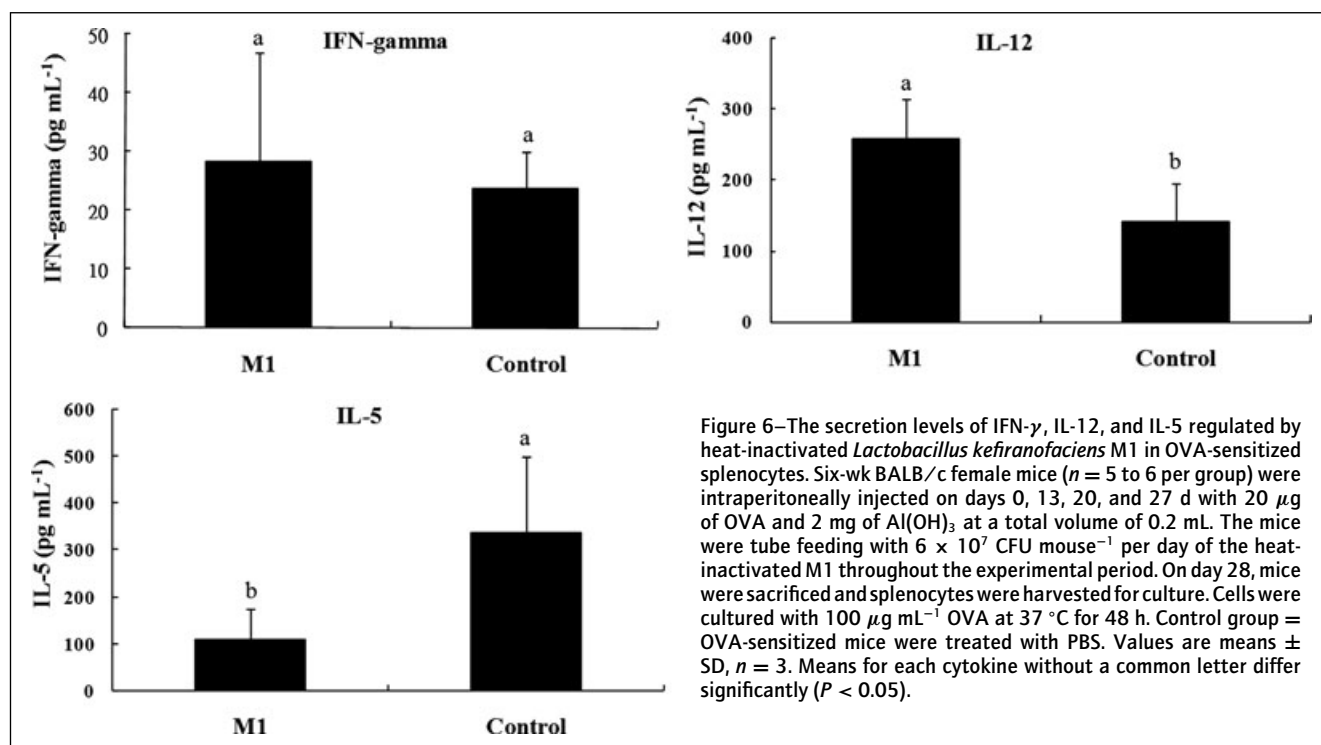


Figure 6—The secretion levels of IFN- γ , IL-12, and IL-5 regulated by heat-inactivated *Lactobacillus kefiranofaciens* M1 in OVA-sensitized splenocytes. Six-wk BALB/c female mice ($n = 5$ to 6 per group) were intraperitoneally injected on days 0, 13, 20, and 27 d with 20 μ g of OVA and 2 mg of Al(OH)₃ at a total volume of 0.2 mL. The mice were tube feeding with 6×10^7 CFU mouse⁻¹ per day of the heat-inactivated M1 throughout the experimental period. On day 28, mice were sacrificed and splenocytes were harvested for culture. Cells were cultured with 100 μ g mL⁻¹ OVA at 37 °C for 48 h. Control group = OVA-sensitized mice were treated with PBS. Values are means $\pm$ SD, $n = 3$. Means for each cytokine without a common letter differ significantly ($P < 0.05$).

cell walls of gram-positive bacteria, are transferred to chromosomes and then trigger the transcription of nuclear factor- κ B mRNA to induce inflammatory cytokines (Lien and Ingalls 2002). Sashihara and others (2006) showed that the stimulatory activity of IL-12 production was positively correlated with the amount of peptidoglycans in the cells. We selected *Lactobacillus kefirifaciens* M1, which had the strongest IL-12-inducing activity, for subsequent studies.

The OVA-sensitized animal test demonstrated that oral administration of heat-inactivated M1 generally tended to reduce serum total IgE (Figure 4) and OVA-specific IgE (Figure 5) in OVA-sensitized BALB/c mice compared with controls. This repression was dose-dependent (Figure 4). The IgE value in the sera of mice given heat-inactivated M1 was significantly lower than that from control mice and mice given other treatments. Although the cause has not yet been clarified, the optimal dosage in maximizing in-

hibition of IgE by different probiotics appears to be important. Segawa and others (2008) indicated that intraperitoneal administration of *Lactobacillus brevis* SBC8803 at a dose of 200 μ g/mouse or administration of a diet with 0.5% SBC8803 was optimal in inhibiting IgE production in OVA-sensitized mice. Matsuzaki and others (1998) concluded that excessive or low doses of *Lactobacillus casei* Shirtoa do not promote the immune balance in allergic mice.

Regarding the effects of heat-inactivated M1 administration in the splenocytes of OVA-immunized mice, M1 induced high IL-12 production and inhibited IL-5 production from splenocytes in OVA-sensitized mice (Figure 6). The IL-12/IL-5 value represents the Th1/Th2 balance. Therefore, suppression of total IgE and OVA-specific IgE production by oral heat-inactivated M1 probably occurred because of improvement in the Th1/Th2 balance toward Th1 dominance. However, IFN- γ levels were not affected

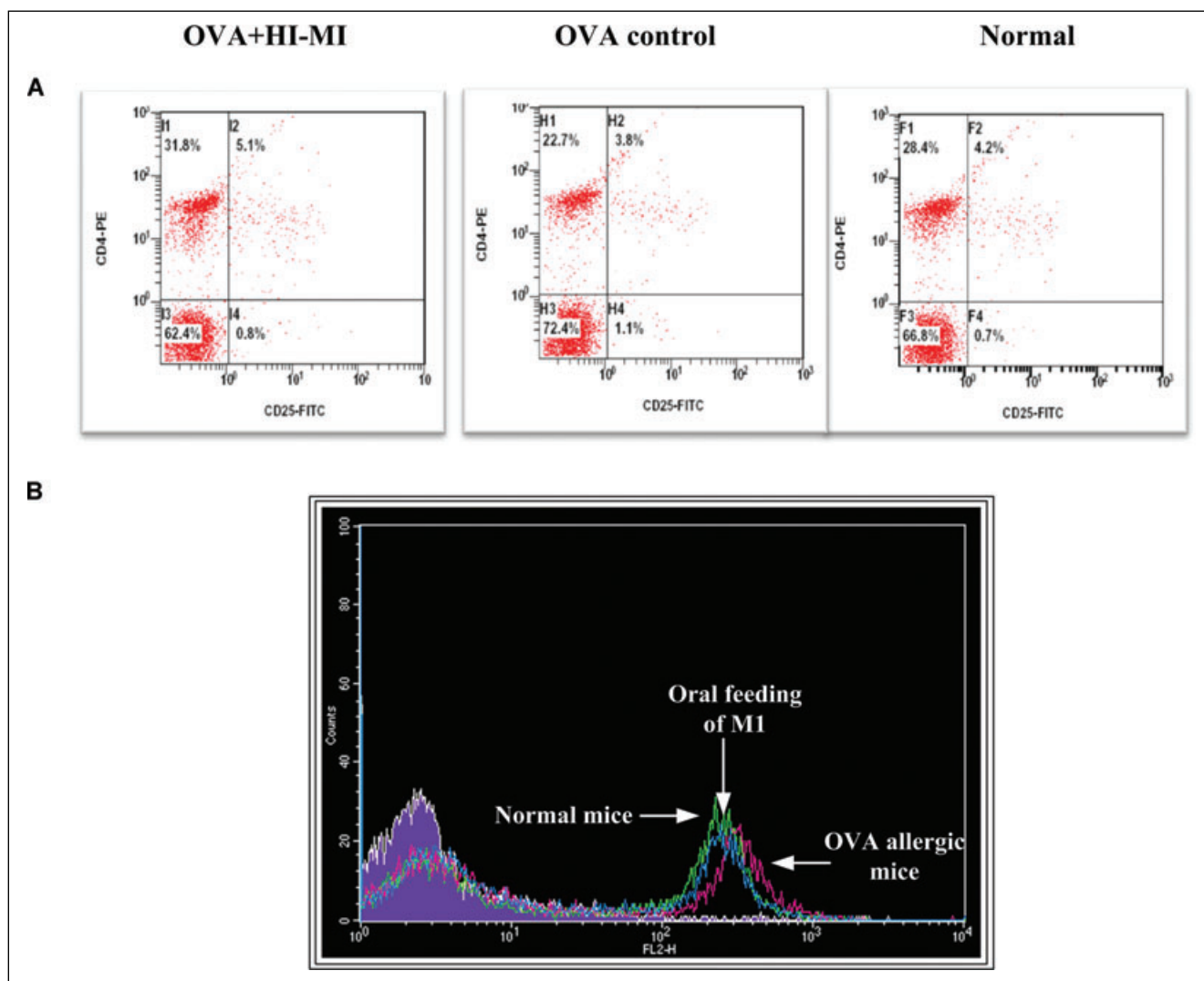


Figure 7—Analysis of the (A) CD4⁺CD25⁺ cells in splenocytes in normal and OVA-sensitized mice. Six-wk BALB/c female mice ($n = 5$ to 6 per group) were intraperitoneally injected on days 0, 13, 20, and 27 d with 20 μ g of OVA and 2 mg of Al(OH)₃ at a total volume of 0.2 mL. The mice were tube feeding with 6×10^7 CFU mouse⁻¹ per day of the heat-inactivated M1 throughout the experimental period. On day 28, mice were sacrificed and splenocytes were harvested. OVA+ HI-M1 = OVA-sensitized mice were treated with the heat-inactivated *Lactobacillus kefirifaciens* M1; OVA control group = OVA-sensitized mice were treated with PBS; Normal = normal mice. (B) Analysis of the expression of CD19 of splenocytes in normal and OVA-sensitized mice. Purple area = nonspecific binding; green line = normal mice; blue line = allergic mice were fed with 6×10^7 CFU day⁻¹ heat-inactivated *Lactobacillus kefirifaciens* M1; pink line = OVA-sensitized mice were treated with PBS. Typical profiles of the sorted fractions are shown.

by heat-inactivated M1. The reason is unknown, but differences between *in vitro* and *ex vivo* reactions may play a role (Sashihara and others 2006).

Flow cytometry study indicated that heat-inactivated M1 could increase the relative proportion of CD4⁺CD25⁺ Treg cells and downregulate the relative proportions of CD19⁺ B cells. CD4⁺CD25⁺ Treg cells are thought to play a specialized role in controlling both innate and adaptive immune responses, preventing allergy (Maloy and others 2003; Sakaguchi 2004; Shevach 2004). Ling and others (2004) indicated that the suppression of allergen-driven T cell activation by Treg cells is reduced in atopic versus nonatopic individuals during the pollen season. Stassen and others (2004) also proved that freshly isolated Treg cells strongly inhibit the IL-4-induced development of Th2 cells. Karlsson and others (2004) strongly suggested that CD4⁺CD25⁺ Treg cells reactive to dietary antigens can be induced via the gut. On the other hand, CD19 is a B cell-restricted signaling molecule known to transduce signals initiated through the B cell receptor (Rickert and others 1995). Jarvinen and others (1999) reported that large numbers of activated CD19⁺ B cells could be considered early markers of food allergy. Accordingly, repression of the serum total IgE (Figure 4) and OVA-specific IgE (Figure 5) in OVA-sensitized BALC/c mice might be associated with upregulation of the proportion of CD4⁺CD25⁺ Treg cells and downregulation of the number of activated CD19⁺ B cells.

In OVA-sensitized mice fed heat-inactivated M1, there was a 5.4-fold downregulation of complement components 3 (C3) in Peyer's patches (Table 2). Other genes that are part of the complement components C1, C2, and C4 were also downregulated in mice with the same treatment. Recent data from animal models and humans provide evidence that the complement system is strongly activated during disease, and complement cleavage fragments are important in disease pathogenesis (Hawlish and others 2004). Fearon and Carroll (2000) indicated that the complement system is an important regulator of B cell activation, providing an important mechanism for pathogen recognition by B lymphocytes. Activation of the complement system occurs by 3 different pathways (Walport 2001a, 2001b). All 3 pathways lead to C3 cleavage resulting in 2 activation products, C3a and C3b. Drouin and others (2001) demonstrated that C3-deficient mice have significantly altered allergic lung responses and indicated a role for the com-

plement system in promoting Th2 effector functions in asthma. On the other hand, IFN- γ (IFN γ) in Peyer's patches from OVA-sensitized mice fed heat-inactivated M1 was upregulated by 10.4-fold (Table 2). Several genes associated with Th1 cytokine production were also upregulated. The ability of T lymphocytes to recognize specific antigens in the context of class I and class II major histocompatibility complex (MHC) molecules is determined by either the unique clonotypic T-cell receptor (TCR)-CD3 antigen complex or the CD2 antigen (Brown and others 1989). Ocklind (1988) indicated that concurrent stimulation of CD3 and CD2 is essential for IL-2 production and proliferation. STAT4 is a transcription factor that transduces IL-12, IL-23, and type 1 IFN cytokine signals in T cells and monocytes, leading to T-helper type 1 differentiation, monocyte activation, and IFN- γ production (Korman and others 2008). CD28 is a 90-kDa homodimeric glycoprotein present on the surface of a large subset of T cells that appears to play an important role in the modulation of T cell activation. CD28 expression by IL12-producing macrophages can lead to generation of Th1 T-cell responses (O'Garra and Murphy 1996), anticipating that CD28 binding to CD80 represents a costimulatory signal for T lymphocytes. Upregulation of the expression of *Cd2*, *Cd3*, *Cd28*, and *Stat4*, leading to Th1 cytokine production, including IFN- γ , paralleled the results of our *in vitro* and *in vivo* study. Kang and others (2009) indicated that strong inducers of IFN- γ were reported to directly inhibit IgE generation in the presence of IL-4.

Conclusion

The data presented clearly indicate the anti-allergic activity of kefir LAB, *Lactobacillus kefirianofaciens* M1. Suppression of IgE production by oral feeding of heat-inactivated M1 probably occurs because of upregulation of the expression of *Cd2*, *Cd3*, *Cd28*, *Stat4*, and *Ifnr*, leading to skewing of the Th1/Th2 balance toward Th1 dominance, elevation of the percentage of CD4⁺CD25⁺ Tregs and reduction of activated CD19⁺ B cells. Downregulation of the complement system and components (particularly C3), resulting in reduction of activated CD19⁺ B cells, is also involved in suppression of IgE production. To the best of our knowledge, this is the first complete study of the mechanisms of the antiallergic effects of *Lactobacillus* species isolated from kefir grain through *in vitro*, *in vivo*, *ex vivo*, flow cytometry, and microarray studies. Future studies

Table 2—Effect of the heat-inactivated M1 on gene expression in Peyer's patch cells of OVA-sensitized mice.

Gene name	Symbol	Accession number	Fold change
Downregulation genes:			
Complement component 3	<i>C3</i>	NM_009778	−5.42
Complement component 4b	<i>C4</i>	NM_009780	−3.53
Complement component 2	<i>C2</i>	NM_013484	−3.03
Complement component 1, s subcomponent	<i>C1s</i>	NM_144938	−2.82
Complement component 1, r subcomponent	<i>C1r</i>	NM_023143	−2.42
Complement component 1, q subcomponent, alpha polypeptide	<i>C1qa</i>	NM_007572	−2.15
Complement component 1, q subcomponent, beta polypeptide	<i>C1qb</i>	NM_009777	−2.12
Complement component 1, q subcomponent, C chain	<i>C1qc</i>	NM_007574	−2.07
Upregulation genes:			
Interferon gamma	<i>Ifnr</i>	NM_008337	10.4
Chemokine (C-C motif) receptor 7	<i>Ccr7</i>	NM_007719	3.68
CD28 antigen	<i>Cd28</i>	NM_007642	2.82
Chemokine (C-C motif) ligand 4	<i>Ccl4</i>	NM_013652	2.67
Perforin 1 (pore forming protein)	<i>Pf1</i>	NM_011073	2.47
Chemokine (C-X-C motif) receptor 4	<i>Cxcr4</i>	NM_009911	2.41
CD3 antigen, delta polypeptide	<i>Cd3d</i>	NM_013487	2.21
CD2 antigen	<i>Cd2</i>	NM_013486	2.11
CD3 antigen, gamma polypeptide	<i>Cd3g</i>	NM_009850	2.08
Signal transducer and activator of transcription 4	<i>Stat4</i>	NM_011487	2.05

of different type1 hypersensitivity diseases are necessary to address the antiallergic effects of lactobacilli isolated from kefir grains.

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