JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Effect of Heat-Inactivated Kefir-Isolated *Lactobacillus kefiranofaciens* M1 on Preventing an Allergic Airway Response in Mice

Wei-Sheng Hong,[†] Yen-Po Chen,[†] Ting-Yeu Dai,[†] I-Nung Huang,[†] and Ming-Ju Chen*[,†,‡]

[†]Department of Animal Science and Technology, and [‡]Center for Biotechnology, National Taiwan University, Taipei 106, Taiwan, Republic of China

ABSTRACT: In this study, we assessed the anti-asthmatic effects of heat-inactivated *Lactobacillus kefiranofaciens* M1 (HI-M1) and its fermented milk using different feeding procedures and at various dosage levels. The possible mechanisms whereby HI-M1 has anti-allergic asthmatic effects were also evaluated. Ovalbumin (OVA)-allergic asthma mice that have been orally administrated the HI-M1 samples showed strong inhibition of production of T helper cell (Th) 2 cytokines, pro-inflammatory cytokines, and Th17 cytokines in splenocytes and bronchoalveolar fluid compared to control mice. An increase in regulatory T cell population in splenocytes in the allergic asthma mice after oral administration of H1-M1 was also observed. In addition, all of the features of the asthmatic phenotype, including specific IgE production, airway inflammation, and development of airway hyperresponsiveness, were depressed in a dose-dependent manner by treatment. These findings support the possibility that oral feeding of H1-M1 may be an effective way of alleviating asthmatic symptoms in humans.

KEYWORDS: Allergy, asthma, *Lactobacillus kefiranofaciens*, kefir, Th17

INTRODUCTION

Allergic asthma, a type-1 hypersensitivity, is characterized by an allergen-induced chronic inflammation of the lungs together with airway hyperresponsiveness (AHR); it is also associated with an enhancement of allergen-induced eosinophilia, goblet cell hyperplasia, allergen-specific immunoglobulin (Ig) E levels, T helper cell (Th) 2 dominant cytokines [interleukin (IL)-4, IL-5, and IL-13] production, and lymphocyte infiltration into the airways.[1] In this context, there is increasing evidence to support a therapeutic role for lactic acid bacteria (LAB) in the treatment of allergy. Clinical trials have demonstrated that *Lactobacillus rhamnosus* GG[2,3] and *Lactobacillus fermentum*[4] may effectively improve atopic dermatitis in children. Furthermore, a double-blind randomized trial showed that oral treatment with *L. rhamnosus* GG reduced the rate and severity of respiratory virus infection in children.[5] A number of hypotheses have been put forward as the possible mechanisms behind the anti-allergic action of LAB. One is idea that certain strains may excite intestinal associated lymphoid tissues and instruct the developing immune system to reverse the abnormal Th2 response, resulting in a more balanced situation.[6,7]

Kefir is an acidic and mildly alcoholic fermented milk that is believed to contain abundant nutrition as well as having functional properties.[8] One study seems to indicate that oral administration of kefir is able to modulate immune responses to improve the allergic reactions, inhibit tumor growth, and induce the tumor cell lysis by apoptosis.[9] Another study of its anti-asthma properties showed that kefir inhibited the production of allergen-specific IgE in serum, suppressed the accumulation of eosinophilia in lung tissue, and reduced mucus hypersecretion by goblet cells in airways.[10] However, confirmation of the possible immunomodulatory activity of kefir and clarification of the underlying mechanisms are still not available.

The difference between kefir and other fermented milk products is that kefir is fermented using starter "kefir grains", which are composed of protein, polysaccharide, and a complex microflora population.[11] Kefir grains, which contain a variety of different species of microorganisms, would seem to constitute the key factor that affects the functional properties of kefir. Despite indications that LAB treatments can modulate some immune responses in the lung,[6,12−14] up to the present, there has been no report investigating the effects of feeding kefir LAB orally in relation to the prevention of asthma. In this context, in our previous studies, 50 LAB strains from the kefir grains were isolated and identified by a combination of polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and 16S rDNA sequencing.[15] Two of these lactobacilli (*Lactobacillus kefiranofaciens* M1 and *Lactobacillus kefiri* M2) isolated from kefir grains were found to influence the production of Th1 and pro-inflammatory cytokines *in vitro*.[16] Additionally, the oral feeding of heat-inactivated *L. kefiranofaciens* M1 (HI-M1) from kefir grains was found to effectively inhibit the IgE production response in ovalbumin (OVA)-sensitized mice.[17] These findings suggest that *L. kefiranofaciens* M1 in kefir may play an important role in the anti-allergic activities of kefir.

The anti-allergic effects have been found to be strain-specific. The distinct differences in immune responses between strains might be due to the different inherent characteristics of the organisms, which include persistence in the gut, colonization, and intrinsic immunogenicity.[12] In the literature, various *Lactobacillus* spp., such as *Lactobacillus casei*,[13] *L. rhamnosus*,[6] *Lactobacillus acidophilus*,[14] and *Lactobacillus reuteri*,[12] have been reported to have anti-asthmatic effects, but no study has demonstrated that *L. kefiranofaciens* has anti-asthmatic effects. Thus, in this present study, we assessed the anti-asthmatic effects of heat-inactivated *L. kefiranofaciens* M1 and its fermented milk using

Received: May 14, 2011
Revised: July 12, 2011
Accepted: July 12, 2011
Published: July 12, 2011

ACS Publications © 2011 American Chemical Society

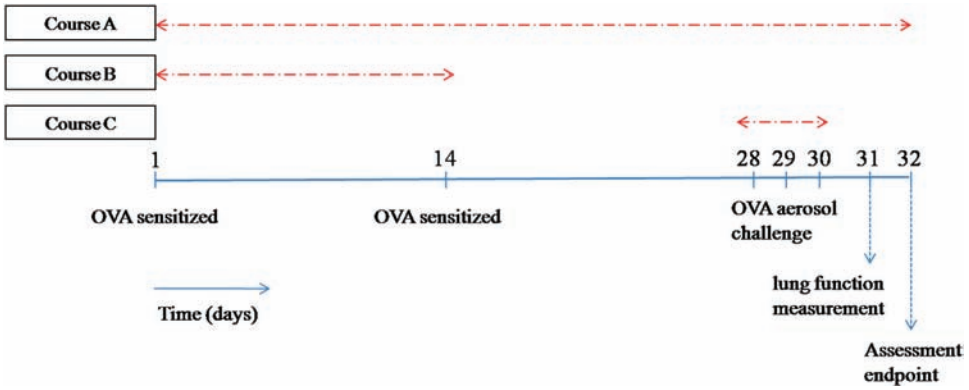

Figure 1. Experimental design of OVA-allergic asthma mice with different oral administration procedures of HI-M1 isolated from kefir grains. OVA-sensitized mice ($n = 6-8$ per group) received 10^7, 10^8, or 10^9 CFU day^{-1} mouse^{-1} of HI-M1 in 200 μL of PBS via a gavaging needle. HI-M1 was orally applied in three feeding procedures: course A, throughout the experimental period (32 days); course B, for 14 subsequent days from days 1 to 14; and course C, for only 3 days on days 28−30 at 1 h before the OVA aerosol challenge. The positive controls were fed with 200 μL of PBS and sensitized and challenged with OVA.

different feeding procedures and various dosages. At the same time, the possible mechanisms by which HI-M1 has an anti-allergic asthmatic effect were also evaluated.

MATERIALS AND METHODS

LAB Samples. *L. kefiranofaciens* M1 (M1) was isolated and identified from kefir grains.[15] The M1 strains was grown in de Man, Rogosa, and Sharpe (MRS) broth (Difco Laboratories, Detroit, MI) at 37 °C and then harvested by washing and resuspending 3 times in phosphate-buffered saline (PBS, Amersco, Solon, OH). The cell suspension was then adjusted to 5×10^9 colony forming units (CFU)/mL prior to use. Inactivation of *L. kefiranofaciens* M1 was carried out by heating the bacteria at 85 °C for 40 min as described in a previous study.[17]

Animals. Female, 6-week-old BALB/c specific pathogen-free mice (National Laboratory Animal Center, Taipei, Taiwan) were accustomed to their new environment for at least 1 week before the start of the experiment. Mice were maintained in an automatic light/dark cycle (light periods of 12 h). Temperature and humidity were kept constant at 22 °C and 50%, respectively. All experiments were performed in accordance with guidelines for animal care of the National Science Council.

Sensitization and Airway Challenge. The OVA-sensitized mouse model of allergic airway inflammation was used in this study and has been described in detail by Lee et al.[10] Briefly, mice were sensitized by intraperitoneal injection of 20 μg of OVA (grade V, Sigma, St. Louis, MO) adsorbed on 2 mg of alum (Wako, Osaka, Japan) in PBS in a total volume of 200 μL on days 1 and 14. On days 28−30, the mice were challenged with OVA (1% in PBS) or PBS by ultrasonic nebulization (Aerogen Ireland, Ltd., Galway, Ireland) for 20 min per mouse. On day 32, the airway responsiveness of the mice was measured and this was followed by bronchoalveolar lavage (BAL). Blood was then collected by submandibular bleeding before the mice were sacrificed.

***L. kefiranofaciens* M1 Treatment.** The experimental procedure for immunization with OVA and administration of HI-M1 using the strain isolated from kefir grains is summarized in Figure 1. OVA-sensitized mice ($n = 6-8$ per group) received 10^7, 10^8, or 10^9 CFU day^{-1} mouse^{-1} of HI-M1 in 200 μL of PBS via a gavaging needle. HI-M1 was orally applied using three different feeding procedures: (1) throughout the experimental period (32 days, course A), (2) for 14 consecutive days from days 1 to 14 (course B), and (3) for only 3 days on days 28−30 at 1 h before the OVA aerosol challenge (course C). The positive controls were fed with 200 μL of PBS and sensitized and challenged with OVA in the same way as described above. The negative controls were fed with 200 μL of PBS and sensitized and challenged with PBS.

Airway Responsiveness. At 24 h after the final OVA challenge, an *in vivo* lung function measurement was performed by whole-body plethysmography (Buxco WBP, Buxco Electronics, Wilmington, NC) as described by Karimi et al.[18] The slope of the dose−response was calculated by linear regression between the measured airway resistance and the $\log_{10}$-transformed methacholine dose (12.5, 25, and 50 mg/mL; Sigma, St. Louis, MO).

Preparation of Splenocytes. The mice were sacrificed by cervical dislocation, and splenocytes were harvested for culture. The preparation of splenocyte cultures followed the procedure described by Hong et al.[17]

BAL and Lung Histology. After the treated mice were sacrificed, lungs were rinsed with 1 mL of PBS. Bronchoalveolar fluid was obtained by aspirating 3 times via tracheal cannulation. The cells in the BAL fluid were removed by centrifugation at 200*g* for 15 min and then resuspended in PBS (1 mL). The supernatants were stored at −80 °C until their cytokine content was measured.[12]

After the BAL was removed, the lungs of the mice were inflated with 10% formalin (Wako, Osaka, Japan), fixed for 24 h, and embedded in paraffin. The fixed and embedded tissue was then stained with hematoxylin and eosin (Sigma, St. Louis, MO) to allow for histological assessment using light microscopy (Optima, Aurora, CO). Periodic acid−Schiff (PAS) stain (IMEB, Inc., San Marcos, CA) was also applied to measure goblet cell and mucus production.

Measurement of the Levels of Cytokines and OVA-Specific IgE. The levels of various cytokines [IL-4, IL-5, IL-13, IL-12p40, IL-6, IL-1β, and IL-17, tumor necrosis factor-α (TNF-α) and IFN-γ] were assessed using the R&D system (Minneapolis, MN). The results are expressed as the concentration of each cytokine (pg/mL).

Blood samples from the treated mice were collected by submandibular bleeding, coagulated for 1 h at room temperature, and then subsequently centrifuged for 5 min at 17500*g*. OVA-specific IgE was determined using an OVA-specific IgE kit (Serotech, Oxford, U.K.).

Flow Cytometry. Regulatory T cells (Treg) from among splenocytes from the allergic-asthma mice fed with HI-M1 were determined by flow cytometric analysis. Cell suspensions from spleen were resuspended at 5×10^6 cells/mL and double-stained with extracellular PE-CD4 and FITC-CD25 (eBiosciences, San Diego, CA) for 20 min on ice. The cells were then washed extensively with PBS and analyzed with an EPICS XL flow cytometer (Beckman Coulter, Brea, CA).

Statistical Analysis. All results were analyzed using the general linear model procedure available from the Statistical Analysis System

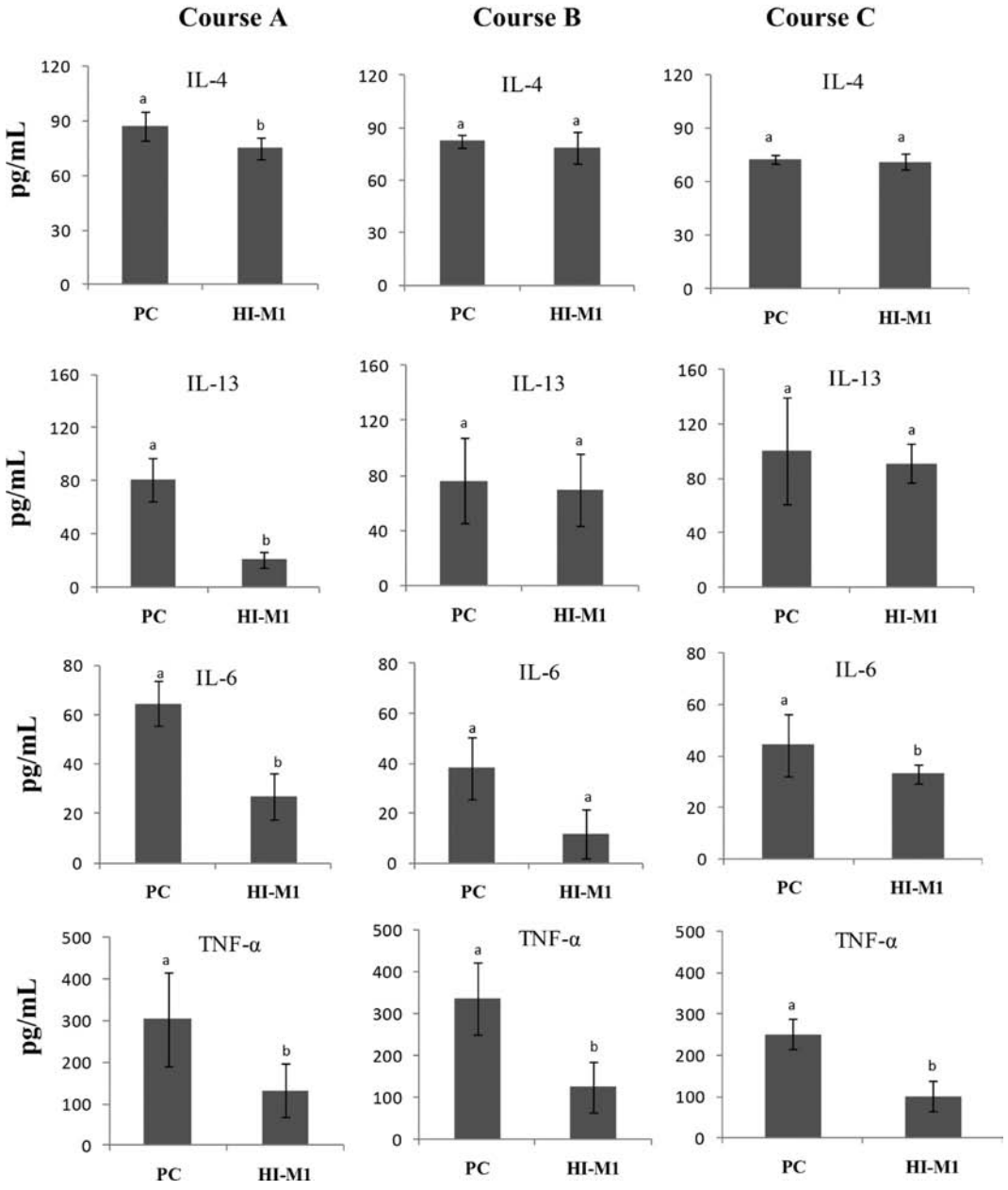

Figure 2. Effects of the different feeding procedures of HI-M1 on Th2 and pro-inflammatory cytokines in splenocytes of OVA-allergic asthmatic mice. HI-M1 (10^8 CFU day^{-1} mouse^{-1}) was orally applied in three feeding procedures: course A, throughout the experimental period (32 days); course B, for 14 subsequent days from days 1 to 14; and course C, for only 3 days on days 28−30 at 1 h before the OVA aerosol challenge. The positive controls (PCs) were fed with 200 μL of PBS and sensitized and challenged with OVA. Values are the mean ± standard error of the mean (SEM). Means for each cytokine without a common letter differ significantly ($p < 0.01$).

software package, version 8.1. Duncan's multiple range test was used to detect differences between the treatment means. Each experiment was conducted in triplicate.

RESULTS

Effect of Different Oral Feeding Procedures. To evaluate the effect of the different M1 feeding procedures (Figure 1) on protecting against allergy airway response, we examined pro-inflammatory cytokine production and Th2 cytokine production in splenocytes *ex vivo* from the OVA-allergic asthma mice. As shown in Figure 2, the mice from the orally administrated 32 day HI-M1 sample (course A) showed a significant inhibition of splenocyte IL-4, IL-13, IL-6, and TNF-α production compared to the mice without feeding with HI-M1 samples ($p < 0.01$) after OVA sensitization and challenge. Among the mice treated with the two other feeding procedures (courses B and C), the secretion of pro-inflammatory cytokines (IL-6 and TNF-α) was significantly downregulated in splenocytes after OVA sensitization and challenge but no significant difference in the levels of the Th2 cytokines (IL-4 and IL-13) compared to the positive control mice (OVA sensitization and challenge) was found. Similar findings were observed for the BAL fluid (Figure 3). The mice that had undergone course A had a significantly lower production of

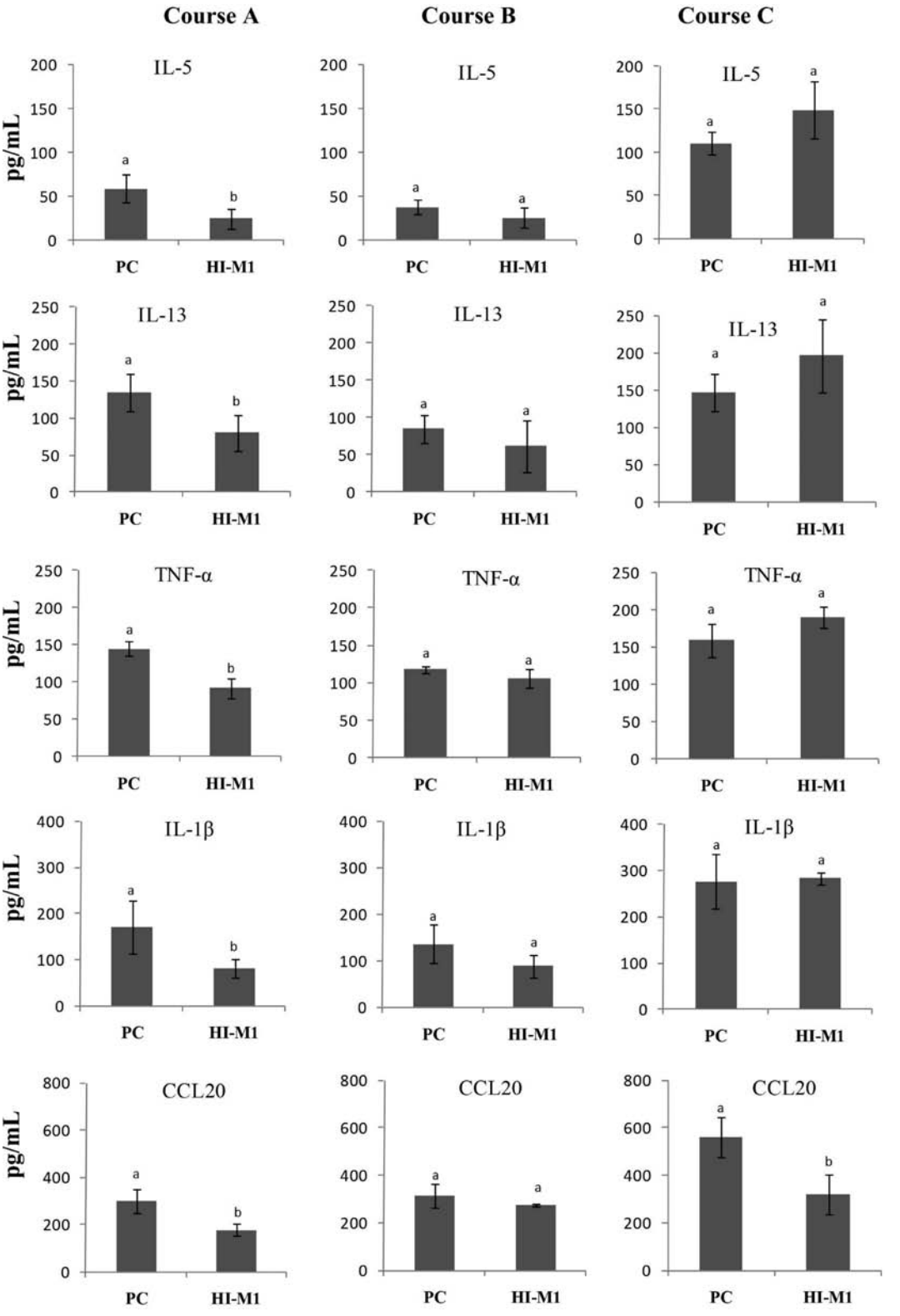

Figure 3. Effect of the different feeding procedures of HI-M1 on Th2 and pro-inflammatory cytokine level in BAL fluid of OVA-sensitized mice after OVA challenge. HI-M1 (10^8 CFU day^{-1} mouse^{-1}) was orally applied in three feeding procedures: course A, throughout the experimental period (32 days); course B, for 14 subsequent days from days 1 to 14; and course C, for only 3 days on days 28−30 at 1 h before the OVA aerosol challenge. The positive controls (PCs) were fed with 200 μL of PBS and sensitized and challenged with OVA. Values are the mean ± SEM. Means for each cytokine without a common letter differ significantly ($p < 0.01$).

pro-inflammatory cytokines (TNF-α, IL-1β, and CCL20) and Th2 cytokines (IL-5 and IL-13) than control mice ($p < 0.01$) after OVA sensitization and challenge. In contrast, the mice treated with the two other feeding procedures showed no

dx.doi.org/10.1021/jf201913x |J. Agric. Food Chem. 2011, 59, 9022–9031

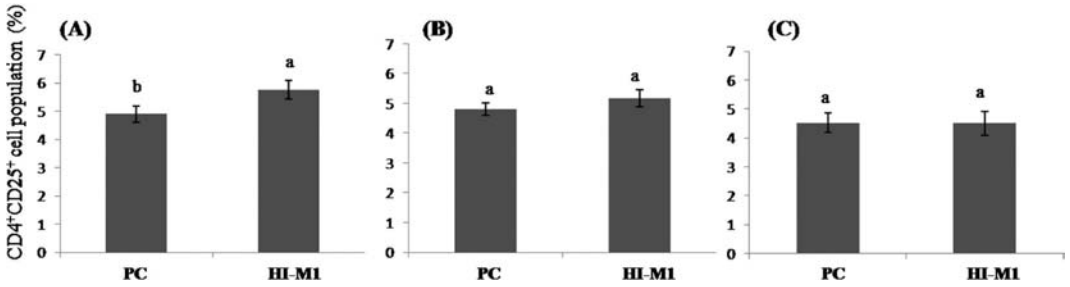

Figure 4. Analysis of the $CD4^+CD25^+$ cell population in splenocytes in different feeding procedures of HI-M1 in OVA-sensitized mice after OVA challenge. HI-M1 (10^8 CFU day^{-1} $mouse^{-1}$) was orally applied in three feeding procedures: (A) throughout the experimental period (32 days, course A), (B) for 14 subsequent days from days 1 to 14 (course B), and (C) for only 3 days on days 28−30 at 1 h before the OVA aerosol challenge (course C). The positive controls (PCs) were fed with 200 μL of PBS and sensitized and challenged with OVA. Values are the mean ± SEM. Means for each value without a common letter differ significantly ($p < 0.01$).

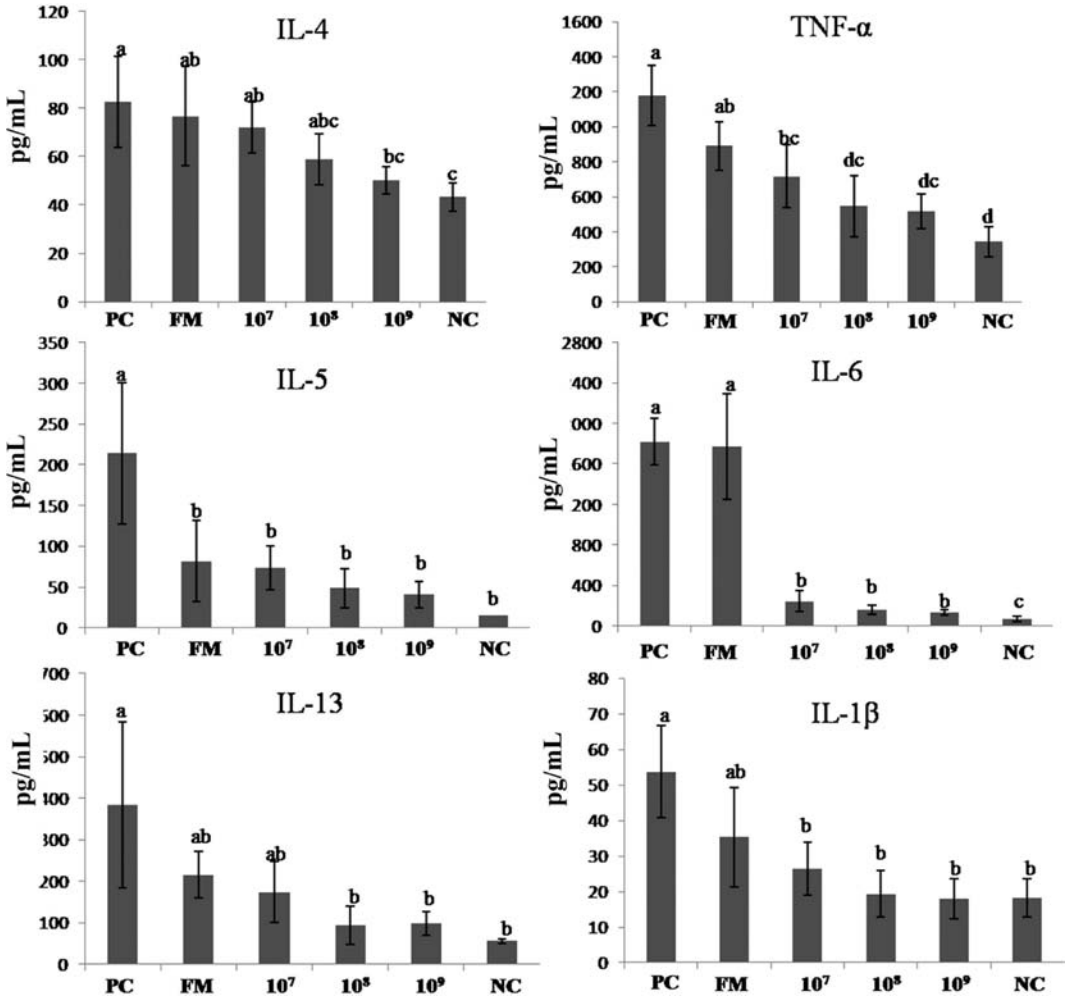

Figure 5. Effect of oral administration of different dosages of HI-M1 on Th2 and pro-inflammatory cytokine secretion in splenocytes of OVA-sensitized mice after OVA challenge. The fermented milk (FM) group was fed 500 μL of *L. kefiranofaciens* M1 fermented milk to OVA-sensitized and -challenged mice. The positive controls (PCs) were fed with 200 μL of PBS and sensitized and challenged with OVA. The negative controls (NCs) were fed with 200 μL of PBS and sensitized and challenged with PBS. Values are the mean ± SEM of 6−8 mice per group. Means for each cytokine without a common letter differ significantly ($p < 0.01$).

significant difference in either pro-inflammatory or Th2 cytokines compared to the positive control mice.

In addition, the effect of the different feeding procedures on the induction of Treg cells in splenocytes of OVA-allergic asthma mice was also evaluated. The results indicated that the percentage of Treg cells was significantly increased ($p < 0.01$) in splenocytes among mice that had undergone the course A compared to the positive control mice after OVA inhalation (Figure 4); in contrast,

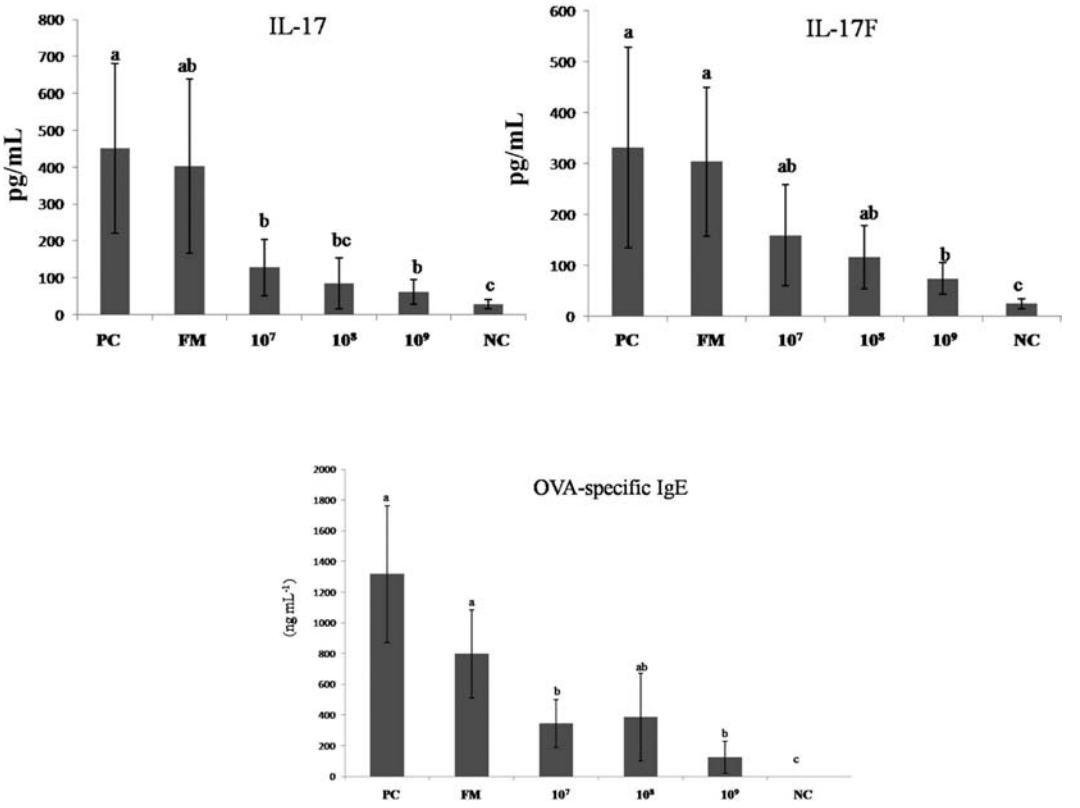

Figure 6. Effect of different dosages of HI-M1 on Th17 cytokine secretion in BAL fluid and OVA-specific IgE productions in serum of OVA-sensitized mice after OVA challenge. The fermented milk (FM) group was fed 500 μL of *L. kefiranofaciens* M1 fermented milk to OVA-sensitized and -challenged mice. The positive controls (PCs) were fed with 200 μL of PBS and sensitized and challenged with OVA. The negative controls (NCs) were fed with 200 μL of PBS and sensitized and challenged with PBS. Values are the mean ± SEM of 6−8 mice per group. Means for each cytokine without a common letter differ significantly ($p < 0.01$).

the two other feeding procedures showed no difference in the Treg cell numbers (Figure 4). As a result of the above experiments, mice from the orally administrated 32 day HI-M1 sample (course A), which demonstrated a significant inhibition in pro-inflammatory and Th2 cytokines as well as an increase in the population of Treg cells, was selected for the following investigation.

Effect of H1-M1 Dosages on Th2, Pro-inflammatory, and Th17 Cytokines. To assess the importance of the dosages of HI-M1 on protection against an allergic airway response, Th2 and pro-inflammatory cytokines were determined in splenocytes *ex vivo* of the OVA-allergic asthma mice using varying doses of H1-M1. The results indicated that the increase in Th2 cytokines (IL-4, IL-5, and IL-13) and pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) were significantly attenuated in splenocytes ($p < 0.01$) among OVA-allergic asthma mice pretreated with 10^8 and 10^9 CFU day^{-1} mouse^{-1} of HI-M1 (Figure 5). On the other hand, the mice administrated M1 fermented milk sample showed no such inhibitory effect on Th2 and pro-inflammatory cytokines compared to the positive control groups. Th17 cytokine levels were also investigated in the BAL fluid (Figure 6). Treatment with 10^7, 10^8, and 10^9 CFU day^{-1} mouse^{-1} of HI-M1 significantly inhibited the increase in IL-17 in BAL fluid of the allergic asthmatic mice, but no suppressing effect on IL-17F in BAL fluid was observed until the mice were treated with the highest dose (10^9 CFU day^{-1} mouse^{-1}) of HI-M1.

Effect of M1 Dosage on OVA-Specific IgE Production. When a dose−response study of the oral administration of HI-M1 on OVA-specific IgE production in the serum of the OVA-allergic asthma mice was conducted, the OVA-specific IgE levels in serum were dramatically elevated in the positive control mice compared to the negative controls (Figure 6). Mice orally fed 10^7 CFU day^{-1} mouse^{-1} of HI-M1 or higher showed significantly reduced OVA-specific IgE levels ($p < 0.01$). However, no such inhibitory effect on OVA-specific IgE in serum was found among mice administrated M1 fermented milk compared to the positive control mice.

Effect of M1 Dosage on Histological Changes. To observe the anti-inflammatory effect of the HI-M1 on the airways of the OVA-allergic asthma mice, histological studies were performed. OVA aerosol challenge induced marked infiltration of inflammatory cells into the peribronchiole and perivascular connective tissue (Figure 7B). The majority of the infiltrated inflammatory cells were monocytes and eosinophils. The increase in eosinophils in the lung parenchyma of the OVA-allergic asthma mice was significantly reduced by treatment with 10^8 and 10^9 CFU day^{-1} mouse^{-1} of HI-M1 (panels E and F of Figure 7). This inhibition of inflammatory cell infiltration into the airways together with epithelial cell disruption were not observed in the lungs when the mice were treated with the M1 fermented milk or 10^7 CFU day^{-1} mouse^{-1} of HI-M1 (panels C and D of Figure 7).

PAS stain was also applied to evaluate goblet cell hyperplasia. The results indicated that overproduction of mucus and the presence of goblet cell hyperplasia could be seen in the bronchial airways of OVA-allergic asthma mice but not in those of normal mice (panels A and B of Figure 8). The mice that had been orally treated with HI-M1 showed significant suppression of the

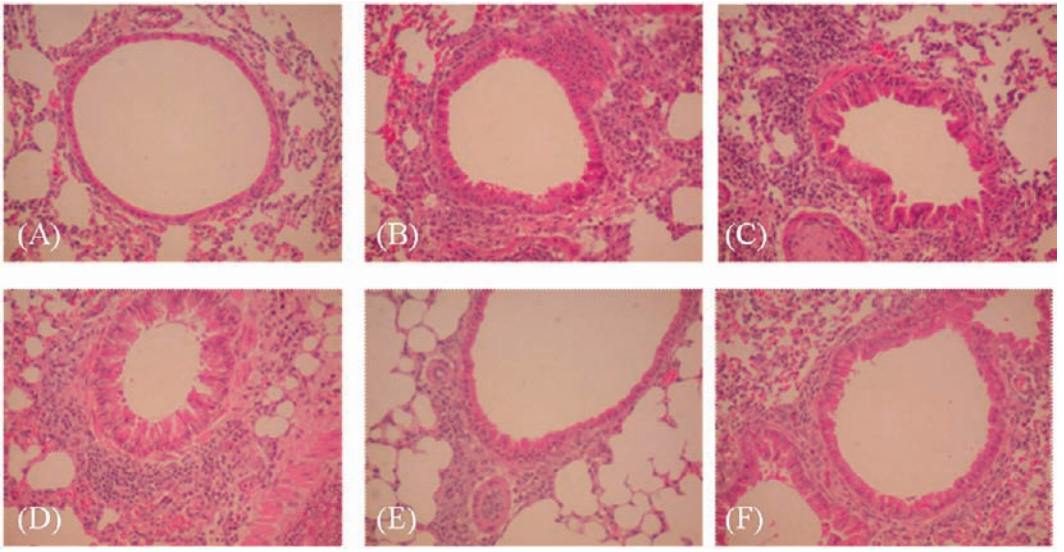

Figure 7. Effect of HI *L. kefiranofaciens* M1 on lung tissue inflammatory cells infiltrated and airway remodeling in OVA-sensitized mice after OVA challenge. Representative sections of lung tissue from (A) negative control, (B) positive control, (C) *L. kefiranofaciens* M1 fermented-milk-treated group, (D) 10^7 CFU day^{-1} mouse^{-1} of HI-M1-treated group, (E) 10^8 CFU day^{-1} mouse^{-1} of HI-M1-treated group, and (F) 10^9 CFU day^{-1} mouse^{-1} of HI-M1-treated group.

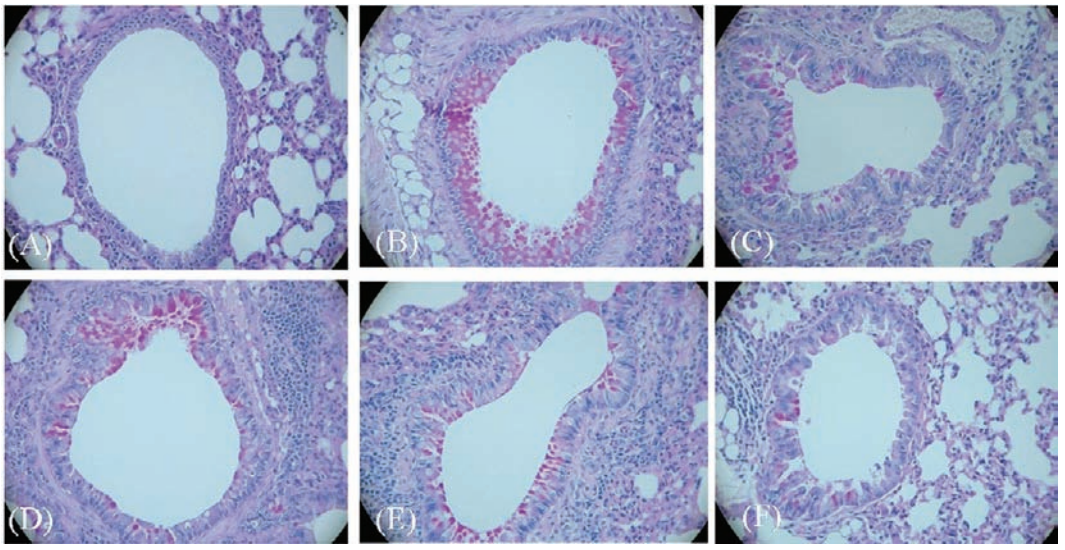

Figure 8. Effect of HI *L. kefiranofaciens* M1 on mucus production in OVA-sensitized mice after OVA challenge. Representative sections of lung tissue from (A) negative control, (B) positive control, (C) *L. kefiranofaciens* M1 fermented-milk-treated group, (D) 10^7 CFU day^{-1} mouse^{-1} of HI-M1-treated group, (E) 10^8 CFU day^{-1} mouse^{-1} of HI-M1-treated group, and (F) 10^9 CFU day^{-1} mouse^{-1} of HI-M1-treated group.

increase in mucus and goblet cell hyperplasia (panels D−F of Figure 8). In particular, there was a major reduction in the number of PAS-stained goblet cells in the 10^9 CFU day^{-1} mouse^{-1} treated asthma group compared to the positive controls (Figure 8F).

Effect of HI-M1 on the Airway Response to Methacholine. Finally, we investigated the inhibitory effect of HI-M1 on the response to methacholine (Figure 9). The OVA-allergic asthma mice showed a significant increase in enhanced pause (Penh) in response to doses of aerosolized methacholine compared to the normal negative controls ($p < 0.01$). The development of this increased airway response was significantly attenuated when the mice were pretreated with 10^9 CFU day^{-1} mouse^{-1} of HI-M1.

DISCUSSION

In this present study, we assessed the anti-allergic asthma effects of HI-M1 using a well-established OVA-induced asthma model. The mice from the orally administrated H1-M1 sample showed strong inhibition of the production of Th2 (IL-4, IL-5, and IL-13), pro-inflammatory (IL-6, IL-1β, TNF-α, and CCL20), and Th17 cytokines in splenocytes and BAL from the OVA-allergic asthma mice, and this was associated with the intervention (Figures 2 and 3) in a dose-dependent manner (Figures 5 and 6). The increase in the Treg cell population in splenocytes in the orally administrated HI-M1 group of allergic asthma mice was also observed (Figure 4). The pathophysiological features of allergic asthma were thought to result from an aberrant

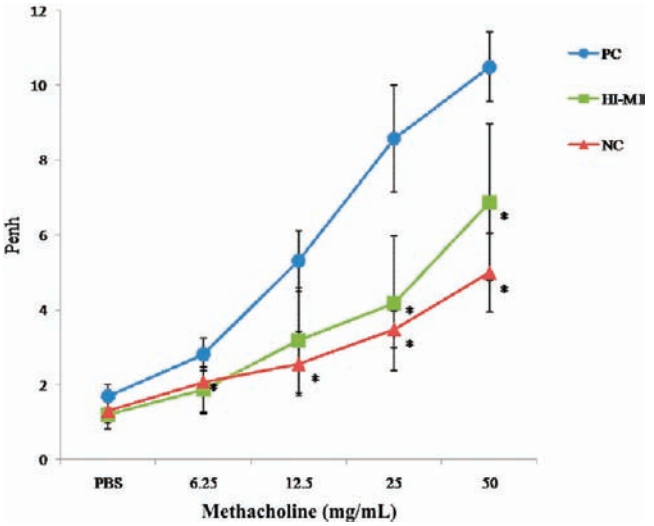

Figure 9. Effect of HI-M1 on the airway response to aerosolized methacholine measured 24 h after the last OVA challenge in OVA-sensitized mice as expressed by Penh. The positive controls (PCs) were fed with 200 μL of PBS and sensitized and challenged with OVA. The negative controls (NCs) were fed with 200 μL of PBS and sensitized and challenged with PBS. Values are the mean ± SEM of 8−10 mice per group. (∗) Significant difference from the PC ($p < 0.01$).

expansion of $CD4^+$ T cells that produce type-2 cytokines.[1] Animal models and clinical studies in humans have revealed a significant position for Th2 cells secreting IL-4, IL-5, and IL-13 in the pathogenesis of allergic asthma.[19] Upregulated expression of lymphocytes IL-4 mRNA together with IL-5 mRNA in BAL cells has been observed following allergen challenge.[20] IL-4 and IL-5 have been found to be crucially involved in the local infiltration and activation of eosinophils. On the other hand, the Th2 cytokine IL-13, which shares a receptor component and signaling pathways with IL-4, is important to the induction of airway hyperreactivity and allergic inflammation.[1,21] IL-13 and IL-4 independently also show a synergistic effect with TNF-α on eosinophil activation.[22] Additionally, previous studies have observed an increased release of TNF-α, IL-1β, and IL-6[23] in BAL of asthmatic patients. The increased levels of these pro-inflammatory cytokines seem to act on epithelial cells and attenuate the excessive host inflammatory responses associated with allergic asthma.[24]

IL-17, produced by Th17 lymphocytes, is able to excite the expression and release of a number of pro-inflammatory mediators, including IL-6 and CCL2, in bronchial epithelial cells.[25] The production of IL-17 has also been shown to direct an influx of neutrophils; that is because IL-17 stimulates the secretion of CXC chemokines, as well as pro-inflammatory cytokines.[26] There is also evidence for increased levels of IL-17 in the airways of asthmatic patients. Laan et al.[27] reported that free soluble IL-17 is produced during the severe inflammation that is characterized by high neutrophil numbers in human airways. Alcorn et al.[28] indicated that OVA sensitization and subsequent OVA challenge resulted in increases in IL-17A and IL-17F, members of the IL-17 cytokine family, both mRNA and protein, in lung homogenates. Overexpression of IL-17F in lungs has been found to induce airway neutrophilia.[29] Th 17 cytokine levels could be enhanced by IL-6. IL-6 suppresses the expression of Treg differentiation, which favors expression by Th17 cells.[25]

$CD4^+CD25^+$ Treg cells suppress the proliferation and cytokine production by $CD4^+CD25^-$ T cells, as well as $CD8^+$ T cells and established Th1 cells.[30] There is accumulating evidence linking the immunomodulatory function of LAB components to various immune suppression mechanisms; these are coordinated by Treg cells that attenuate both the Th1 and Th2 response.[18] Feleszko et al.[6] reported that the young individuals treated with *L. rhamnosus* GG showed a soothed allergic airway response compared to adult animals, which was related to an increase in Treg cell expression. Karimi et al.[18] also indicated that *L. reuteri*-induced attenuation of the allergic airway response in mice was mediated in a non-antigen-specific manner by Treg cells. Our findings of a decrease in IL-17 cytokines and an increase in Treg cells among the orally administrated HI-M1 sample of allergic asthma mice might involve the suppression of Th2 and pro-inflammatory cytokines. The observed allergen-specific systemic immune-regulatory responses were probably associated with beneficial effects relative to the target organ.

On the basis of the above results, we further studied the effect of orally feeding HI-M1 on the asthmatic phenotype of the allergic asthma mice. Allergic asthma is characterized by AHR to a variety of allergens, chronic pulmonary eosinophilia, an elevated serum IgE, and excessive airway mucus production.[1] Oral administration of HI-M1 leads to a suppression of all of these features of the asthmatic phenotype, including specific IgE production, airway inflammation, and development of AHR; this occurred in a dose-dependent manner (Figures 6−8). Allergen-induced IgE synthesis is likely to trigger eosinophils, basophils, and mast cells, allowing for the differentiation of Th cells into Th2 cells, which secrete IL-4, IL-5, and IL13. The number of mast cells infiltrated in the airway smooth muscle bundles has been shown to correlate significantly with bronchial hyperresponsiveness in asthmatics, implying their importance for the pathophysiology of asthma.[31]

The suppression of all symptoms of the asthma in the present study was associated with reduced levels of the pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β), the Th2 cytokines (IL-4, IL-5, and IL13), and two Th17 cytokines (IL-17 and IL-17F) in the splenocytes and BAL fluid (Figures 5 and 6). Treg cells also play an important role in attenuating the allergic airway response following oral treatment with HI-M1. Regulatory immune effects of probiotics have been reported in other papers. Lim et al.[13] indicated that, following airway allergen challenge, the mice fed heat-killed *L. casei* showed histological evidence of attenuation of lung inflammation as well as a reduction in the total cell count and Th2 count together with lower levels of pro-inflammatory cytokines in BAL fluid. Forsythe et al.[12] also reported that oral treatment with live *L. reuteri* resulted in the attenuation of many major characteristics of the asthmatic response in a mouse model of allergic airway inflammation.

It is clear that certain functional properties of LAB are dependent upon live organisms, whereas other immunostimulatory effects can be mediated by the heat-inactivated bacteria.[32,33] The effectiveness of heat-activated bacteria in our study corroborated the findings of our previous study,[16,34] in which a cell-wall component, peptidoglycan (PGN), was found to be a likely contributor to the anti-asthmatic effects of *L. kefiranofaciens*. Chapat et al.[35] also reported that bacteria cell-wall components are effective in inhibiting contact dermatitis in mice. The amount of PGN present in the cells might be one reason for the strain-dependent nature of the effect on cytokine production stimulation.[36]

Duration of exposure and dosage, both of which were evaluated in this study, showed a significant effect on the anti-allergic asthma effects. Although all three lengths of feeding courses and dosages have been used for diverse LAB strains in anti-allergic studies,[12] the increase in Treg cells and suppression of Th2 and pro-inflammatory cytokines by *L. kefiranofaciens* M1 in allergic asthma mice was only observed when the M1 strain were administered for an extended feeding period (32 days) (Figures 2−4) and at higher dosages (Figures 5−8). This suggests that the consecutive administration of 10^8 CFU day^{-1} mouse^{-1} or higher of *L. kefiranofaciens* M1 during sensitization and prior to airway challenge for an extended period (32 days) is crucial to the attenuation of the allergic airway response. Several trials that studied the effect of probiotics on allergy prevention have also revealed that the choice of probiotic strains as well as timing of the intervention and the level of exposure are important variables.[37] Additionally, it is worth noting that the M1 fermented milk sample itself did not show the anti-asthmatic effects compared to the positive control (Figures 5−8). The low M1 dose (10^6 CFU day^{-1} mouse^{-1}) present in this fermented-milk-treated group may explain why it is ineffective. In addition, previous studies also indicated that other molecules in fermented milk, such as certain milk-derived proteins and their digests,[38,39] and the microbiota composition[40] could be the factors affecting the anti-asthmatic responses.

When the results presented here are taken together, they clearly demonstrated that oral treatment of heat-inactivated *L. kefiranofaciens* M1 is able to reduce the AHR to methacholine and depress the lung inflammation in OVA-allergic asthma mice in a dose- and time-dependent manner. The prevention of the development of allergen-induced sensitization and airway diseases by HI-M1 occurred via an elevation of Treg activity, a suppression of Th2, pro-inflammatory, and Th17 cytokines in BAL/splenocytes, and an inhibition of specific IgE production in serum. These findings provide strong support for the hypothesis that the intake of kefir lactobacilli, *L. kefiranofaciens* M1, may be an effective approach to the alleviation of asthmatic symptoms. Both exposure time and dosage are two important factors that affect the anti-allergic asthmatic effectiveness of *L. kefiranofaciens* M1. To the best of our knowledge, this is the first paper that completely explores the anti-allergic asthma effects of heat-inactivated *Lactobacillus* spp. isolated from kefir grain.

AUTHOR INFORMATION

Corresponding Author

*Telephone: 886-2-33664169. Fax: 886-2-27324070. E-mail: cmj@ntu.edu.tw.

Funding Sources

This work was supported by the National Science Council of Taiwan.

REFERENCES

(1) Wills-Karp, M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* **1999**, *17*, 255–281.

(2) Kalliomäki, M.; Salminen, S.; Arvilommi, H.; Kero, P.; Koskinen, P.; Isolauri, E. Probiotics in primary prevention of atopic disease: A randomized placebo-controlled trial. *Lancet* **2001**, *357*, 1076–1079.

(3) Kalliomäki, M.; Salminen, S.; Poussa, T.; Arvilommi, H.; Isolauri, E. Probiotics and prevention of atopic disease: A 4-year follow-up of a randomised placebo-controlled trial. *Lancet* **2003**, *361*, 1869–1871.

(4) Weston, S.; Halbert, A.; Richmond, P.; Prescott, S. L. Effects of probiotics on atopic dermatitis: A randomised controlled trial. *Arch. Dis. Child.* **2005**, *90*, 892–897.

(5) Hattaka, K.; Savilhati, E.; Pönkä, A.; Meurman, J. H.; Poussa, T.; Näse, L.; Saxelin, M.; Korpela, R. Effect of long term consumption of probiotic milk on infections in children attending day care centres: Double blind, randomised trial. *Br. Med. J.* **2001**, *322*, 1318–1327.

(6) Feleszko, W.; Jaworska, J.; Rha, R. D.; Steinhausen, S.; Avagyan, A.; Jaudszus, A.; Ahrens, B.; Groneberg, D. A.; Wahn, U.; Hamelmann, E. Probiotic induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma. *Clin. Exp. Allergy* **2006**, *37*, 498–505.

(7) Noverr, M. C.; Huffnagle, G. B. Does the microbiota regulate immune responses outside the gut. *Trends Microbiol.* **2004**, *12*, 562–568.

(8) Farnworth, E. R.; Mainville, I. Kefir: A fermented milk product. In *Handbook of Fermented Functional Foods*; Farnworth, E. R., Ed.; CRC Press: Boca Raton, FL, 2003; pp 77−112.

(9) Liu, J. R.; Wang, S. Y.; Lin, Y. Y.; Lin, C. W. Antitumor activity of milk-kefir and soymilk-kefir in tumor-bearing mice. *Nutr. Cancer* **2002**, *44*, 182–187.

(10) Lee, M. Y.; Ahn, K. S.; Kwon, O. K.; Kim, M. J.; Kim, M. K.; Lee, I. Y.; Oh, S. R.; Lee, H. K. Anti-inflammatory and anti-allergic effect of kefir in a mouse asthma model. *Immunolbiology* **2007**, *212*, 647–654.

(11) Liu, J. R.; Wang, S. Y.; Chen, M. J.; Yueh, P. Y.; Lin, C. W. The anti-allergenic properties of milk kefir and soymilk kefir and their beneficial effects on the intestinal microflora. *J. Sci. Food Agric.* **2006**, *86*, 2527–2533.

(12) Forsythe, P.; Inman, M. D.; Bienenstock, J. Oral treatment with live *Lactobacillus reuteri* inhibits the allergic airway response in mice. *Am. J. Respir. Crit. Care Med.* **2007**, *175*, 561–569.

(13) Lim, L. H.; Li, H. Y.; Huang, C. H.; Lee, B. W.; Lee, Y. K.; Chua, K. Y. The effects of heat-killed wild-type *Lactobacillus casei* Shirota on allergic immune responses in an allergy mouse model. *Int. Arch. Allergy Immunol.* **2009**, *148*, 297–304.

(14) Wheeler, J. G.; Shema, S. J.; Bogle, M. L.; Shirrell, M. A.; Burks, A. W.; Pittler, A.; Helm, R. M. Immune and clinical impact of *Lactobacillus acidophilus* on asthma. *Ann. Allergy, Asthma, Immunol.* **1997**, *79*, 229–233.

(15) Chen, H. C.; Wang, S. Y.; Chen, M. J. Microbiological study of lactic acid bacteria in kefir grains by culture-dependent and culture-independent methods. *Food Microbiol.* **2008**, *25*, 492–502.

(16) Hong, W. S.; Chen, H. C.; Chen, Y. P.; Chen, M. J. Effects of kefir supernatant and lactic acid bacteria isolated from kefir grain on cytokine production by macrophages. *Int. Dairy J.* **2009**, *19*, 244–251.

(17) Hong, W. S.; Chen, Y. P.; Chen, M. J. Anti-allergy effects of lactobacilli isolated from kefir grains. *J. Food Sci.* **2010**, *75*, H244–H253.

(18) Karimi, K.; Inman, M. D.; Bienenstock, J.; Forsythe, P. *Lactobacillus reuteri* induced regulatory T cells protect against an allergic airway response in mice. *Am. J. Respir. Crit. Care Med.* **2009**, *179*, 186–193.

(19) Yssel, H.; Groux, H. Characterization of T cell subpopulations involved in the pathogenesis of asthma and allergic diseases. *Int. Arch. Allergy Immunol.* **2000**, *121*, 10–18.

(20) Robinson, D. S.; Bentley, A. M.; Hartnell, A.; Kay, A. B.; Durham, S. R. Activated memory T helper cells in bronchoalveolar lavage fluid from patients with atopic asthma: Relation to asthma symptoms, lung function, and bronchial responsiveness. *J. Allergy Clin. Immunol.* **1993**, *92*, 313–324.

(21) Wong, C. K.; Ho, C. Y.; Ko, F. W. S.; Chan, C. H. S.; Ho, A. S. S.; Hui, D. S. C.; Lam, C. W. K. Pro-inflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-γ, IL-4, IL-10 and IL-13) in patients with allergic asthma. *Clin. Exp. Immunol.* **2001**, *125*, 177–183.

(22) Luttmann, W.; Matthiesen, T.; Matthys, H.; Virchow, J. C. Synergistic effects of interleukin-4 or interleukin-13 and tumor necrosis factor-α on eosinophil activation *in vitro*. *Am. J. Respir. Cell Mol. Biol.* **1999**, *20*, 474–480.

(23) Gosset, P.; Tsicopoulos, A.; Wallaert, B.; Joseph, M.; Capron, A.; Tonnel, A. B. Tumor necrosis factor α and interleukin-6 production

by human mononuclear phagocytes from allergic asthmatics after IgE dependent stimulation. *Am. Rev. Respir. Dis.* **1992**, *146*, 768–774.

(24) Haworth, C.; Brennan, F. M.; Chantry, D.; Turner, M.; Maini, R. N.; Feldmann, M. Expression of granulocyte-macrophage colony-stimulating factor in rheumatoid arthritis: Regulation by tumor necrosis factor-α. *Eur. J. Immunol.* **1991**, *21*, 2575–2579.

(25) Traves, S. L.; Donnelly, L. E. Th17 cells in airway diseases. *Curr. Mol. Med.* **2008**, *8*, 416–426.

(26) Ferretti, S.; Bonneau, O.; Dubois, G. R.; Jones, C. E.; Trifilieff, A. IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. *J. Immunol.* **2003**, *170*, 2106–2112.

(27) Laan, M.; Palmberg, L.; Larsson, K.; Lindén, A. Free, soluble interleukin-17 protein during severe inflammation in human airways. *Eur. Respir. J.* **2002**, *19*, 534–537.

(28) Alcorn, J. F.; Crowe, C. R.; Kolls, J. K. Th17 cells in asthma and COPD. *Annu. Rev. Physiol.* **2010**, *72*, 495–516.

(29) Oboki, K.; Ohno, T.; Saito, H.; Nakae, S. IL-17 and allergy. *Allergol. Int.* **2008**, *57*, 121–134.

(30) Joetham, A.; Takeda, K.; Taube, C.; Miyahara, N.; Matsubara, S.; Koya, T.; Rha, Y. H.; Dakhama, A.; Gelfand, E. W. Naturally-occurring lung CD24 CD25 T cell regulation of airway allergic responses is dependent on IL-10 induction of TGF-*β*. *J. Immunol.* **2007**, *178*, 1433–1442.

(31) Nauta, A. J.; Engels, F.; Knippels, L. M.; Garssen, J.; Nijkamp, F. P.; Redegeld, F. A. Mechanisms of allergy and asthma. *Eur. J. Pharmacol.* **2008**, *585*, 354–360.

(32) Sashihara, T.; Sueki, N.; Ikegami, S. An analysis of the effectiveness of heat-killed lactic acid bacteria in alleviating allergic diseases. *J. Dairy Sci.* **2006**, *89*, 2846–2855.

(33) Segawa, S.; Nakakita, Y.; Takata, Y.; Wakita, Y.; Kaneko, T.; Kaneda, H.; Watari, J.; Yasui, H. Effect of oral administration of heat-killed *Lactobacillus brevis* SBC8803 on total and ovalbumin-specific immunoglobulin E production through the improvement of Th1/Th2 balance. *Int. J. Food Microbiol.* **2008**, *121*, 1–10.

(34) Huang, S. M.; Chen, Y. P.; Hong, W. S.; Chen, M. J. Immunomodulatory properties of the milk whey products obtained by enzymatic and microbial hydrolysis. *Int. J. Food Sci. Technol.* **2010**, *45*, 1061–1067.

(35) Chapat, L.; Chemin, K.; Dubois, B.; Bourdet-Sicard, R.; Kaiserlian, D. *Lactobacillus casei* reduces $CD8^+$T cell-mediated skin inflammation. *Eur. J. Immunol.* **2004**, *34*, 2520–2528.

(36) Shida, K.; Kiyoshima-Shibata, J.; Nagaoka, M.; Watanabe, K.; Nanno, M. Induction of interleukin-12 by *Lactobacillus* strains having a rigid cell wall resistant to intracellular digestion. *J. Dairy Sci.* **2006**, *89*, 3306–3317.

(37) Lee, J.; Seto, D.; Bielory, L. Meta-analysis of clinical trials of probiotics for prevention and treatment of pediatric atopic dermatitis. *J. Allergy Clin. Immunol.* **2008**, *121*, 116–121.

(38) Moller, N. P.; Scholz-Ahrens, K. E.; Roos, N.; Schrezenmeir, J. Bioactive peptides and proteins from foods: Indication for health effects. *Eur. J. Nutr.* **2008**, *47*, 171–182.

(39) Hartmann, R.; Wal, J.-M.; Bernard, H.; Pentzien., A.-K. Cytotoxic and allergenic potential of bioactive proteins and peptides. *Curr. Pharm. Des.* **2007**, *13*, 897–920.

(40) McLoughlin, R. M.; Mills., K. H. G. Influence of gastrointestinal commensal bacteria on the immune responses that mediate allergy and asthma. *J. Allergy Clin. Immunol.* **2011**, *127*, 1087–1094.