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Full Length Research Paper

A study of the immunomodulatory effect of probiotics

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The aim of this study was to evaluate the immunomodulatory effect of probiotics, namely the production of interferon γ (IFN-γ) and interleukin-4 (IL-4) cytokines, *in vitro* and *in vivo*. Our experimental groups included ten lactic acid bacterial (LAB) strains, complex strains, a LAB cell free fraction and a control group. Our models included human peripheral blood mononuclear cells (PBMCs) as the human model and BALB/c mice as the animal model. The experiment was carried out over a period of 4 weeks during which the food intake and the body weight of our animal model was reported weekly. BALB/ c mice were randomly divided into three groups and injected with 2 µg/ mouse and 6 µg/ mouse ovalbumin (OVA) mixed with complete Freund's adjuvant (CFA) at week zero and two. After week four the serum total immunoglobulin E (IgE) was measured. The results show that probiotic products induced IFN-γ, suppressed IL-4, and increased the IFN-γ/ IL-4 (Th-1/ Th-2) ratio significantly in PBMCs. Probiotic products also decreased significantly the serum total IgE and OVA-specific IgE levels in our animal model. Our study indicates that the multi-species probiotics may therefore have an anti-allergy effect.

Key words: Probiotics, human peripheral blood mononuclear cell, cytokines, immunoglobulin E, anti-allergy effect.

INTRODUCTION

Food allergies to common allergens such as those found in eggs, peanuts and milk, typically produce a heightened IgE response, and they are usually characterized by an imbalance in the Th1/ Th 2 cell ratio (Tanabe, 2008). Evidence from patients suggests that a polarized Th2 response stimulates the production of IL-4 and interleukin 5 (IL-5). It is the elevated level of these cytokines that is thought to be responsible for the symptoms associated with food allergies. It has also been suggested that increasing the Th1 cell number could be a possible means of treatment. For example, interleukin 12 (IL-12), a Th1 maturation factor, stimulates Th-1 cells to produce IFN-γ that in turn prevents a Th2 associated response and suppresses IgE production (Kato et al., 1999).

Probiotics are generally regarded as live and safe microorganisms that carry important health benefits for both humans and animals (Fuller, 1986). Their role is to modulate naturally occurring microflora (Fuller, 1986) which under normal circumstances plays an important role in homeostasis. Some researches suggest that the probiotics may be a useful material for the treatment of allergic disorders (Tanaka and Ishikawa, 2004). When intestinal bacteria are removed by antibiotic medication in weaning mice, allergic symptoms are accompanied by elevated serum levels of total IgE (Oyama et al., 2001). Moreover, normalized intestinal microflora is considered to be a potential target for the management of immune disorders (Morita et al., 2006). Recently, various researchers have indicated that lactic acid producing bacteria (LAB) may possess immune modulating and anti-allergenic properties (Matsuzaki, 1998; Ouwehand,

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2007).

In recent years, numerous other LAB strains have shown probiotic potential in animal studies. Evidence has accumulated that probiotic strains can exhibit the same activities as commensal bacteria, including immunomodulation. Studies have shown that some LAB strains, such as *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus* GG, *Bifidobacterium bifidum*, *Bifidobacterium infantis* and *Bifidobacterium longum*, can increase Th1 cell production (Özdemir, 2010). Research publications indicate that these species may therefore have the potential to help in the modulation of immune response to common allergens.

It is also known that multi-strain and/or multi-species probiotics may be more effective than mono-strain probiotics (Sanders and Huis in't Veld, 1999; Timmerman et al., 2004) and that the sum total effect of synergistic strains with specific properties produces a greater response than when they are administered separately (Timmerman et al., 2004). The aim of this study was to test the regulatory ability of multiple LAB strains on *in vitro* cytokine production of PBMCs, the IgE response in serum and the systemic hypersensitivity type 1 response in the ovalbumin-induced allergy BALB/c animal model.

MATERIALS AND METHODS

Preparation of multi-species probiotic product

The components of the probiotics included *L. acidophilus*, *L. plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus fermentum*, *Lactobacillus paracasei*, *L. rhamnosus* GG, *Lactococcus cremors*, *Streptococcus thermophilus*, *B. longu*m and *Candida kefyr*. LAB free fraction comprising suitable corn starch, fructooligosaccharide, skim milk powder, yeast extract, vitamin B_1 and vitamin B_{12} were also used. The probiotic product were manufactured from SynbioTech Inc (Kaohsiung City, Taiwan) and kept refrigerated at -20°C until tested.

Isolation and stimulation of human peripheral blood mononuclear cells

The method described by Kekkonen et al. (2008) was used to isolate PBMCs. PBMCs in blood were purified by density gradient centrifugation over a Ficoll-Paque gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) from freshly collected, leukocyte-rich buffy coats obtained from healthy blood donors (Taichung Blood Center, Taichung, Taiwan). After washing, the cells were resuspended in RPMI 1640 medium (HyClone, Utah, USA) containing 10% heat-inactivated fetal bovine serum (Gibco, NY., USA) and supplemented with 2 mmol/L L-glutamine (Sigma, MO., USA) and 1% 100× Penicillin-Streptomycin (HyClone, Utah, USA). Freshly isolated PBMCs were diluted in RPMI 1640 medium to a final concentration of 2×10^6 cells/ ml. One ml cells were added to a 24-well culture plate and the experiment in triplicate.

The method described by Niers et al. (2005) was modified. PBMCs were cultured in medium. Live multi-LAB product were added in a cell: bacteria ratio of 1 : 1, 10 : 1 and 100 : 1. The polyclonal T cell stimulator phytohaemagglutinin (PHA) was added in 20 µg/ ml served as a positive control. The plates were incubated at 37°C in 5% CO_2. Culture supernatants were collected after 24 h and stored at -80ºC until IFN-γ and IL-4 measurement.

Experimental BALB/c mice

This animal research was approved by the Institutional Animal Care and Use Committee of HungKuang University, Taichung County, Taiwan (approval No. 95014). 60 male 5 week old, specific pathogen-free BALB/c mice, weighing 19 to 21 g were obtained from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). These were acclimatized to the laboratory conditions over a period of two weeks. The animal room was ventilated and maintained at 25 ± 2°C with a relative humidity of 65 ± 5% and a 12 h light/ dark cycle. At day zero, the mice were randomly allocated into three groups of twenty mice. The three groups were control (water); LAB complex product; and LAB cell free fraction. Before the animal model experiment (day 0), the mice were randomly divided into 3 groups (n = 20 per group). BALB/ c mice were randomly divided into three groups and injected with 2 µg/ mouse and 6 µg/ mouse ovalbumin (OVA) mixed with complete Freund's adjuvant (CFA) by intraperitoneal (IP) injection at week zero and two. They were fed a single 0.2 ml dose of a known concentration of multistrain probiotics (3 g dissolved in 20 ml deionized water, $N \times 10^7$ CFU/ml, N=1~9) by oral administration daily for 4 consecutive weeks. The feed intake and body weight of each mouse was measured weekly. On days 0, 7 and 21, blood samples were obtained from the ophthalmic veins of each mouse for measuring the serum total IgE, anti-OVA-IgE levels. On day 28, blood samples were collected by cardiac puncture and the sera were stored at –80°C until measurement of the antibody levels.

ELISA of total IgE, anti-OVA-IgE, IFN-γ and IL-4

Cytokine levels were measured by ELISA using BD pharmingen antibody pairs (BD Biosciences, San Jose, USA) for IFN-γ and IL-4, according to the manufacturer's instructions. 96 well Immuno-Maxisorp plates (Nunc, Roskilde, Denmark) were coated with monoclonal antibodies for total IgE, IFN-γ, or IL-4 and placed in an incubating buffer overnight at 4°C. Plates were blocked and washed. Sera were added to the plates and incubated for 2 h at room temperature. Plates were then washed again, and biotinylated anti-mouse total IgE, IFN-γ, or IL-4 and horseradish peroxidase (HRP)-conjugated streptavidin were added for the detection of total IgE, IFN-γ, or IL-4, respectively, and incubated 1 h at room temperature. The reactions were developed with the 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate for 30 min at room temperature. The colour reactions were stopped with 2N H_2SO_4 and absorbance measured at 450 nm. Equivalent levels of total IgE, IFN-γ, or IL-4 were calculated by comparison with a reference curve generated with standards of total IgE, IFN-γ, or IL-4, respectively. The results were expressed as the concentration of each cytokine in serum (pg/ ml).

To measure anti-OVA-IgE the procedure described by Peng et al. (2007) was used. Immuno-Maxisorp plates were coated with 100 µl of 10 µg/ ml of OVA in coating buffer and ELISA performed as described above with absorbance measured at 405nm. The anti-OVA-IgE titre was expressed as ELISA units (EI).

$$EI = (A_{sample} - A_{blank}) / (A_{positive\ control} - A_{blank}).$$

The absorbance of the positive control represented the level of IgE in serum from OVA immunized BALB/c mice.

Statistical analysis

Data were expressed as the mean ± SD and the statistical

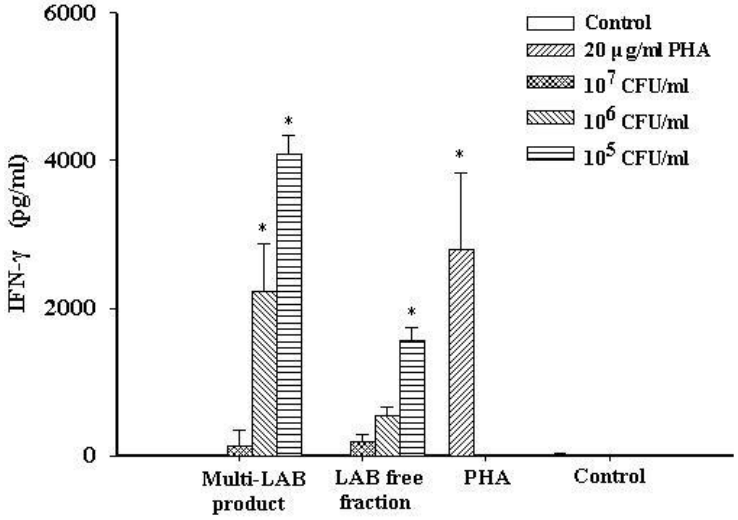

Figure 1. IFN-r production from PBMCs after stimulation 24 hours with different concentrations of multi-LAB product. * means significantly different from control ($P < 0.05$).

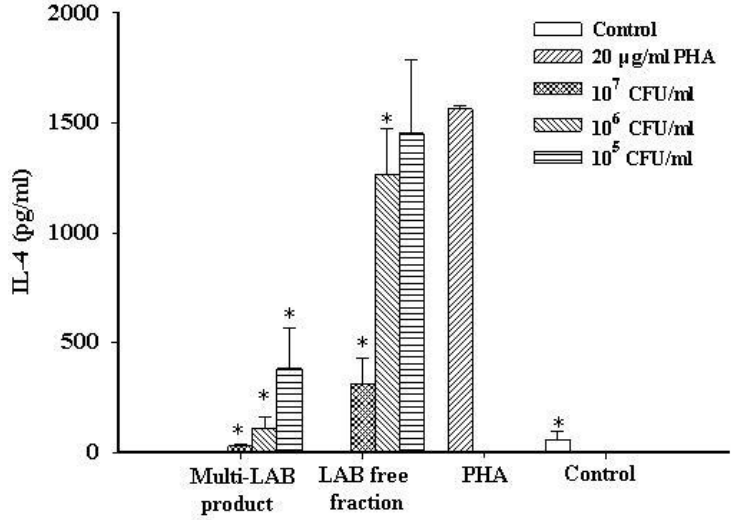

Figure 2. IL-4 production from PBMCs after stimulation 24 hours with different concentrations of multi-LAB product. * means significantly different from PHA ($P < 0.05$).

significance was conducted by one-way analysis of variance (ANOVA) following Duncan's New Multiple Range Test using Statistical Analysis System SAS Enterprise Guide 2.1.40. Statistical significance is considered as $P < 0.05$.

RESULTS

Cytokine release by PBMCs *in vitro*

The effect of multistrain probiotic bacteria directly on the different cell populations as well as possible interfering effects of bacteria on cytokine production was evaluated. Different concentrations of multi-LAB product (1×10^5, 1×10^6 and 1×10^7 CFU/ ml, equivalent to 0.01, 0.1 and 1 bacteria per PBMC, respectively) were used to examine the effect of dose on IFN-γ production. We co-cultured PBMCs with the multi-LAB product, LAB free fraction and only PHA to observe typical patterns of cytokine release. When PBMCs cell: bacteria ratios were 10:1 and 100:1, the multi-LAB product group stimulated the production of IFN-γ and exhibited the better effect (Figure 1), and also repressed the production of IL-4 (Figure 2).

Table 1. Effects of different concentrations of multi-LAB product on the *in vitro* IFN-γ/ IL-4 ratio of PBMCs were used in the experiment.

Group	IFN-γ/ IL-4		
	10^7 CFU/ml	10^6 CFU/ml	10^5 CFU/ml
PHA treated PBMC	1.92±0.41[a]	1.92±0.41[c]	1.92±0.41[b]
Multi-LAB product	01.86±9.93[a]	20.36±2.61[a]	11.80±4.15[ao]
LAB free fraction	00.29±0.27[a]	00.36±0.03[c]	00.04±0.27[bc]

[a,b,c]Different letters within a column indicate significant difference ($P < 0.05$).

Table 2. Effect of multi-LAB product on feed intake of BALB/c mice.

Group	1 Week	2 Week	3 Week	4 Week
Control	3.34 ± 0.26[a]	3.58 ± 0.14[ao]	3.17 ± 0.73[ab]	4.02 ± 1.36[a]
Multi– LAB product	3.37 ± 0.52[a]	3.37 ± 0.22[a]	2.70 ± 0.24[bo]	3.52 ± 0.34[a]
LAB free fraction	3.44 ± 0.26[a]	3.32 ± 0.37[a]	3.54 ± 0.40[ao]	3.70 ± 0.20[a]

[a,b]Different letters within a column indicate significant difference ($P < 0.05$).

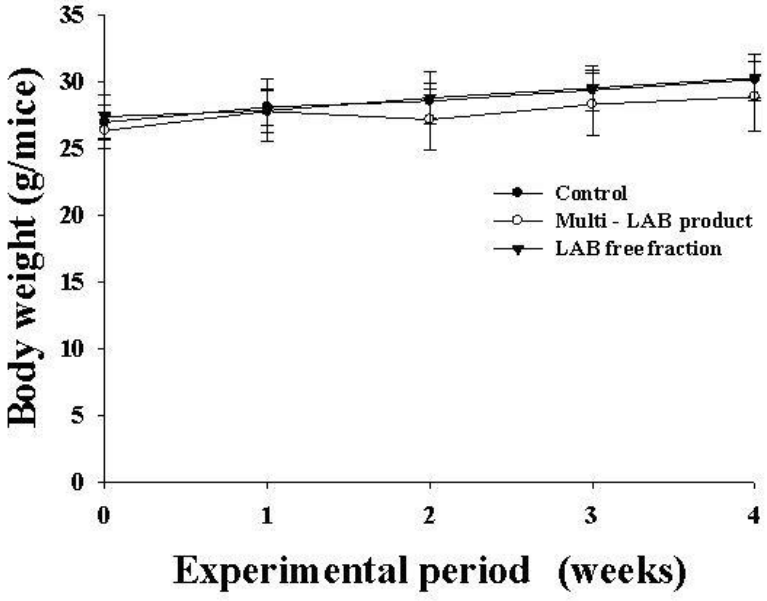

Figure 3. Body weight of mice fed multi-LAB product, LAB free fraction and control up to 4 weeks.

In vitro studies, using IFN-γ as a Th1 parameter and IL-4 secretion as a Th2 parameter revealed a wide variety of IFN-γ-inducing (Figure 1) and IL-4-repressing (Figure 2) activities. The ratio of IFN-γ to IL-4 secretion by PBMCs increased significantly when the multi-LAB product was added, in comparison to the PHA or LAB free fraction (Table 1). The highest ratio value of IFN-γ to IL-4 was observed in the multi-LAB product group at the LAB concentration of 10^6 CFU/ ml.

Feed intake and changes of body weights

There was no significant difference in the daily food intake (Table 2) during the first, second and fourth week among the animals treated with multi-LAB product, LAB free fraction or the control. During the third week, the food intake of the multi-LAB strain group decreased slightly. As is shown in Figure 3 weekly body weights steadily increase with time. The mean body weight of the mice

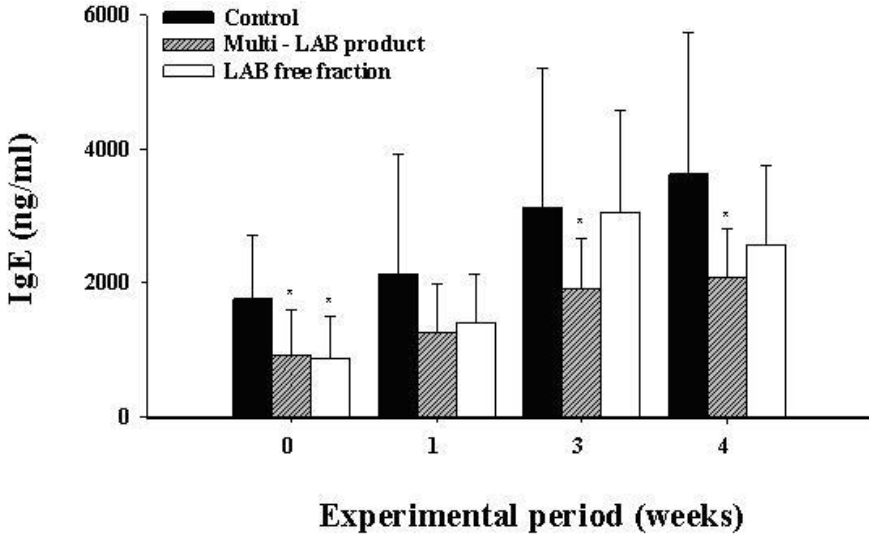

Figure 4. Effect of multi-LAB product on the serum total IgE levels in OVA- sensitized BALB/c mice. *means significantly different from control ($P < 0.05$).

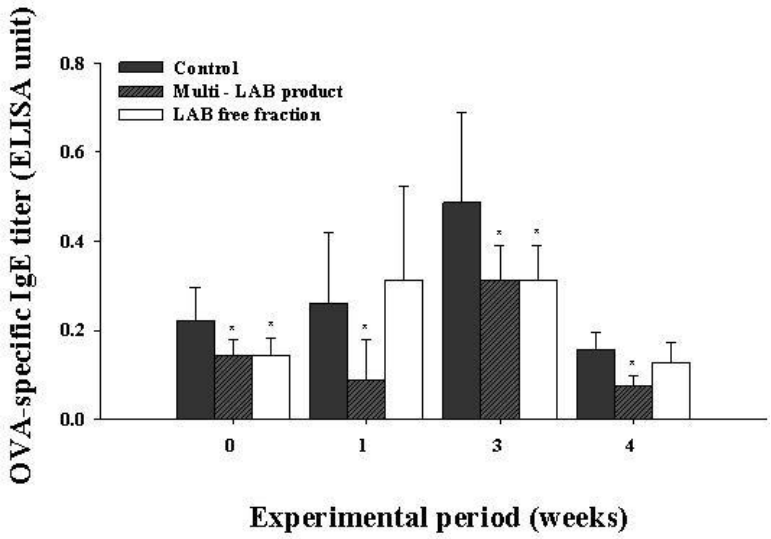

Figure 5. Effect of multi-LAB product on the serum OVA-specific IgE levels in OVA- sensitized BALB/c mice.* means significantly different from control ($P < 0.05$).

increased continuously during the four weeks and there was no significant difference in mean body weight between the three experimental groups.

Effect of LAB mixture on total and anti-OVA IgE production in serum *in vivo*

In order to monitor the effects of the supplementary LAB mixture, weekly serum samples were obtained from each group of mice following OVA sensitization. The levels of total OVA IgE were measured after oral administration of LAB mixture product, cell free fraction or control during week zero, one, two and four (Figure 4). The total IgE level in sera increased significantly in the control group in contrast to the LAB mixture product group. The anti-OVA-specific IgE level in sera was also significantly higher in the control group compared to the LAB mixture product group after the third week (Figure 5).

DISCUSSION

In this study, we have shown that multi-species probiotic

product induces cytokine expression in PBMCs *in vitro*. This is in accordance with findings that LAB-stimulated PBMCs increase their production of IFN-γ and reduce their production of IL-4 (Pochard et al., 2002; Foligne et al., 2007; Ghadimi et al., 2008; Kekkonen et al., 2008). PBMCs are source of monocytes and therefore provide an adequate model for the study of the immuno-modulatory properties of LAB. A PBMC-based *in vitro* assay may serve as a useful tool for predicting outcomes of, or replacing the *in vivo* murine models used to study the immunomodulatory properties of LAB (Foligne et al., 2007).

IFN-γ has no effector molecule; the proinflammatory cytokine IFN-γ inhibits the production of Th2 cytokines in the immune response (Miettinen et al., 1998). IL-4 has an antagonistic effect on IFN-γ and is associated with activated Th2 lymphocytes (Ghadimi et al., 2008). IL-4 plays a significant role in controlling both cell growth and modulation of the immune response (Chang et al., 2000). This cytokine has antagonist functions to IFN-γ and appears to possess certain anti-inflammatory properties. This IFN-γ/ IL-4 value is representative of the Th1/ Th2 balance. In our study, we have shown that the ratio of IFN-γ to IL-4 secretion by PBMCs in response to the multi-species probiotic product increased significantly. Several experimental studies support the finding that probiotics can increase the secretion of the Th1-cytokine IFN-γ in PBMCs and reduce the secretion of Th2-cytokine IL-4 when stimulated by pretreatment with an adequate allergen (Ghadimi et al., 2008; Miettinen et al., 1998). However, it is known that the shift from Th2 to Th1-cytokine production is indicative of a positive immune response and that the modulation of T cell associated cytokine production tends towards a lower risk of allergy (Ghadimi et al., 2008).

The available data from different researches indicate that numerous LAB strains, that is, *L. casei* strain 911, *B. bifidum* strain BGN4, and *B. bifidum* strain G9-1, are capable of inhibiting total and OVA-specific IgE production when administered orally concomitant with sensitization (Kim et al., 2005; Ohno et al., 2005). Liu et al. (2006) have demonstrated that oral administration of milk kefir and soymilk kefir for 28 days significantly suppresses OVA-specific IgE response in a murine allergy model. In our animal study, the group fed multi-LAB product showed significantly reduced total and OVA-specific IgE levels. Our results suggest that supplementation with multi-LAB strains has an anti-allergic effect due to the reduction of allergen-total/ specific IgE production. Therefore, suppression of total IgE and OVA-specific IgE production via the oral administration of multi-LAB product is probably due to the improvement of the Th1/ Th2 balance toward Th1 dominance.

In our study, the cell-free fraction containing corn starch, fructooligosaccharide, skim milk powder, yeast extract, vitamin B_1 and vitamin B_{12}, also had some anti-allergy effects. Some researches demonstrated that the prebiotic fructooligsaccharide or oligosaccharides could reduce the incidence of allergic reactions (Fujitani et al., 2007; Arslanoglu et al., 2008). From these results we inferred that the multi-LAB product containing prebiotics and probiotics could synergistic enhance the heightened immune response observed in food allergies. In conclusion, these results indicate there is a possibility that the administration of probiotics derived from multi-species LAB may be effective in the prevention and treatment of symptoms associated with type I hypersensitivity disorders.

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