Anti-Inflammatory Effects of Probiotic *Lactobacillus paracasei* on Ventricles of BALB/C Mice Treated with Ovalbumin

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Abstract

Lactic acid bacteria (LAB) are microorganisms that benefit animals with allergic diseases and intestinal disorders such as inflammatory bowel disease. We propose that LAB can prevent cardiomyocytes inflammation and apoptosis in BALB/c mice using an ovalbumin (OVA)-induced allergy. Thirty-nine male BALB/c mice were divided into five groups: normal control, allergy control and three allergy groups each treated with Kefir I (Kefir I), Kefir II (Kefir II) or GM080 products (GM080). The myocardial architecture and apoptotic molecules in the excised left ventricle from these mice were investigated and post-treatment effects were evaluated. The inflammatory pathway, including toll-like receptor 4 (TLR4), phospholipase-N-Jn-terminal kinase (p-JNK), JNK1/2 and tumor necrosis factor-alpha (TNF-α) and the mitochondria-dependent apoptosis phospholipase-p38 (p-p38), Bcl-2 associated agonist of cell death (Bad), Bcl-2 associated X (Bax) and activated caspase 3, were found to be significantly increased in the hearts of allergy mice. The expression of phospholipase-nuclear factor-κB (p-NFκB), TNF-α, p-p38 and Bad protein products were reduced or retarded in the Kefir I- or II-treated allergy group. The GM080-treated allergy group exhibited significantly lower p-JNK, JNK1/2, phospholipase-Ikappa B (p-IκB), Bax and Bad protein products than the Kefir I and Kefir II allergy groups. These results indicate that LAB can reduce inflammation and prevent apoptosis of cardiomyocytes in the heart of OVA-induced allergy mice.

Key Words: allergy, inflammation, apoptosis, lactic acid bacteria, ovalbumin
Introduction

Lactic acid bacteria (LAB) are a genus of Gram-positive facultative anaerobic or microaerophilic bacteria found in the normal gut microflora of mammals and humans (4). Two major species of LAB probiotic bacteria are the genera Lactobacillus and Bifidobacterium (8). These bacteria have health-promoting effects on improving the health of the intestinal tract in antibiotic-associated (25), traveler’s and pediatric diarrhea (11, 26), or in inflammatory and irritable bowel syndrome (14, 15, 34) and atopic disease patients (21, 34). Recent epidemiological studies and experimental researches have suggested that stimulation of the immune system by LAB can influence the development of tolerance to innocuous allergens (30, 34). Liu et al. (23) has already demonstrated from animal studies that consumption of fermentation of kefir in milk and soy bean can inhibit the allergic reaction and change the intestinal microflora in allergic mice. Therefore, it was suggested that kefir was considered promising in terms of food ingredients and strengthen the prevention of food allergy in gastrointestinal mucosa against the pathogens infection.

Many researches suggested that treatment of cardiovascular disease by consumption of LAB could decrease the blood pressure and levels of cholesterol in humans (24, 31). However, there is little available research in discussing the ways consumption of LAB prevent cardiac inflammation and apoptosis in allergy-prone animals. Tumor necrosis factor-alpha (TNF-α) is a multifunctional pro-inflammatory cytokine and early mediator of the acute-phase response (32, 33). Thus, a high TNF-α level in centenarians is associated with a low ankle-brachial arterial pressure index, and demonstrates peripheral atherosclerosis (6). In addition, parameters of atherosclerosis and thromboembolic complications are also related to TNF-α (12, 13).

In lipopolysaccharide (LPS)-stimulated myocardial, increasing amounts of TNF-α were synthesized by cardiac myocytes locally in chronic heart failure, cardiac sudden death, viral myocarditis and ischemia (7). Synthetic TNF-α would stimulate the production of endogenous second messenger, the sphingosine, which was recommended as sphingolipid involved in TNF-α-mediated cardiomyocytes apoptosis (18). Kapadia et al. (16) have investigated that the elevated levels of TNF-α shown in clinical conditions such as sepsis and ischemic myocardial disorders may contribute to TNF-α-induced cardiac cell death. Apoptosis of cardiomyocytes is also discussed in terms of its potential beneficial role in limiting the area of cardiac cell involvement as a consequence of myocardial infarction, viral infection and primary cardiac tumors (3, 10). Additionally, recent studies (2, 12) have established the molecular mechanism involved in the pathogenesis of LPS-induced cardiac inflammation and dysfunction, especially focusing on the roles of toll-like receptors (TLRs). It was shown that physiological secretion of TNF-α induced apoptosis of mice cardiomyocytes in vivo as quantified by single-cell microgel electrophoresis of nuclei as well as by morphological and biochemical criteria (3).

Mitochondrion is the main site of action for members of the apoptosis-regulating protein family exemplified by the Bcl-2 family, such as Bcl-2-associated X (Bax) and Bcl-2-associated agonist of cell death (Bad) (1, 17). Bax prevents the release of cytochrome c and Bad enhances the release of cytochrome c from mitochondria, respectively (36). Upon release of cytochrome c to the cytoplasm, it cleaves the pro-enzyme of caspase-9 into the active form and further activates other caspases to induce the apoptotic programmed cell death (5). Caspase-3 activation is mediated by caspase-9 following the release of cytochrome c from mitochondria (29). It is also associated with a pro-apoptotic member of the Bcl-2 family (Bid) and p38 mitogen-activated protein kinase (p38 MAPK) to initiate changes in Bax conformation followed by mitochondrial translocation (9, 35).

The role of LAB in mitochondrial-dependent cardiac apoptotic pathway is still unclear at cardiac abnormalities in allergy diseases. The aim of this study is to determine the myocardial morphology and key components from histological findings, Western blotting analysis from cardiomyocyte-inflamed tissues and apoptosis signaling protein regulators in an ovalbumin (OVA)-stimulated mouse model.

Materials and Methods

Samples

Kefir I and Kefir II were purchased from SynbioTech Incorporation, Tainan, Taiwan, ROC. Used in this study were Kefir I (L. acidophilus, L. plantarum, Bifidobacterium longum, L. fermentum, L. bulgaricus, Candida kefyr, Streptococcus thermophilus and L. cremoris; ≥1 × 10⁷ CFU/g) and Kefir II (Kefir I with the addition of L. paracasei powder and L. rhamnosus GG powder; ≥1 × 10⁷ CFU/g) and Kefir II (Kefir I with the addition of L. paracasei powder and L. rhamnosus GG powder; ≥1 × 10⁷ CFU/g). The GM080 product [GM080; L. paracasei 33 freeze-dried powder, L. fermentum freeze-dried powder, L. acidophilus freeze-dried powder, baby milk, fructo-oligosaccharides powder, glucose, malto-dextrin, magnesium stearate and yogurt species (≥1 × 10⁹ CFU/g)] was purchased from GenMont Biotech Incorporation, Tainan, Taiwan, ROC.

Animals

Thirty-nine 5-week-old male BALB/c mice were
purchased from BioLASCO Taiwan Co., Ltd. All mice were randomly divided into five groups: the normal control (n = 7) and four allergy groups (n = 8 per group). The normal control group was given distilled water 0.2 ml/mice by orogastric intubation once per day. The four allergy groups were given an allergy control of 0.2 ml distilled water, and different LAB [Kefir I (6.6 × 10^7 CFU/kg BW/day), Kefir II (6.7 × 10^7 CFU/kg BW/day) and GM080 (6.8 × 10^8 CFU/kg BW/day)] products orally once per day. The four allergy groups were given an allergy control of 0.2 ml distilled water, and different LAB [Kefir I (6.6 × 10^7 CFU/kg BW/day), Kefir II (6.7 × 10^7 CFU/kg BW/day) and GM080 (6.8 × 10^8 CFU/kg BW/day)] products orally once per day. The allergy mice were intra-peritoneally injected with 2 µg/mice, 6 µg/mice of OVA mixed with complete Freund’s adjuvant (CFA) on day 0 and 14, respectively. All mice were weighed and decapitated after the 28-day treatment protocol. The mice hearts were then excised and cleaned with distilled H2O. The left, right atriums and ventricles were separated and weighed. Ambient temperature was controlled at 25 ± 1°C, relative humidity at 65 ± 5%. In addition, the animals were maintained on a reverse 12 h light-dark cycle (lights on 0700-1900 h). Mice were provided with standard laboratory chow (MF-18; ORIENTAL YEAST Co., LTD, Taichung, Taiwan, ROC) and water ad libitum. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals. All of the protocols were approved by the Institutional Animal Care and Use Committee of Hungkuang University, Taichung, Taiwan (23).

Extraction of Tissue

Cardiac tissue extracts were obtained by homogenizing the left ventricle samples in a lysis buffer (20 mM Tris, 2 mM EDTA, 50 mM 2-mercaptoethanol, 10% glycerol, pH 7.4, proteinase inhibitor and phosphatase inhibitor cocktail) at a ratio of 100 mg tissue/ml buffer for 1 min. The homogenates were placed on ice for 10 min and centrifuged twice at 12,000 × g for 40 min. The supernatant was collected and stored at -80°C for further experiments (22).

Electrophoresis and Western Blot

Electrophoresis and Western blotting were performed according to the method of Lee (22). The extracted tissue samples were prepared as described by homogenization with the buffer (Tris-HCl; 20 mM Tris, 2 mM EDTA, 50 mM 2-mercaptoethanol, 10% glycerol, pH 7.4). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with 10% polyacrylamide gels. The samples were then electrophoresed at 85 V for 3.5 h and equilibrated for 15 min in 25 mM Tris-HCl, pH 8.3, containing 192 mM glycine and 20% (v/v) methanol. Electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF) 0.45 µm pore-size membrane (Millipore, Bedford, MA, USA) with a Bio-Rad Scientific Instruments Transphor Unit at 85 V for 2.5 h. PVDF membranes were incubated at 25°C for 1 h in a blocking buffer containing 5% non-fat milk and a TBS buffer (Tris-Base, NaCl, Tween-20, pH 7.4). Antibodies including TNF-α (Cell signaling, MA, USA), TLR4, phospholate-Jun-N-terminal kinase (p-JNK), phospholate-nuclear factor-κB (p-NFκB), phospholate-IκB (p-IκB), phospholate-p38 (p-p38), Bad, Bax, cytochrome c, activated caspase 3 and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted to 1:500 in an antibody binding buffer containing 100 mM Tris-HCl (pH 7.5), 0.9% of (w/v) NaCl, 0.1% (v/v) of Tween-20 overnight at 4°C. The immunoblots were washed three times in a TBS buffer for 10 min during each phase, and then immersed in the second antibody solution containing goat anti-rabbit IgG-HRP (Santa Cruz) for 1 h and diluted 500 fold in the TBS buffer. The immunoblots were then washed in a blotting buffer three times for 10 min at each phase. The immunoblotted proteins were visualized by using an enhanced chemiluminescence ECL Western blotting luminal reagent (Santa Cruz) and quantified using a Fujiﬁlm LAS-3000 chemiluminescence detection system (Tokyo, Japan).

Statistical Analysis

Each experiment was repeated a minimum of three times, and data were expressed as means ± standard deviation (SD). One-way ANOVA with a Tukey-Kramer procedure for multiple comparisons was used to examine the statistical differences between treatments. Differences were considered as significant at P < 0.05.

Results

Cardiac Inflammation-Related Components

In order to investigate the signaling pathways associated with the cardiac inflammation of male BALB/c mice, the key components of TLR4, p-JNK, JNK1/2, p-NFκB, p-IκB and TNF-α were examined (Figs. 1-3). The protein products of p-JNK (Fig. 1C), JNK1/2 (Fig. 1D) and TNF-α (Fig. 3B) extracted from the left ventricles of excited hearts in the allergy control group were all significantly increased compared with the normal control group.

Cardiac Inflammation Associated Signaling Pathways

Using Western blot analysis, TLR4 was expressed in the left ventricular obtained in vivo (Fig. 1). Moreover, the Kefir I group had decreased production levels of TLR4 proteins (Fig. 1B) when compared with
Fig. 1. Representative protein products of TLR4, p-JNK and JNK1/2 extracted from the left ventricles of excised hearts in normal control mice (Conl), allergy control mice (Allergy control) and allergy mice with different lactic acid bacteria spp. (Kefir I, Kefir II and GM080) measured by Western blotting analysis, respectively (A). Bars represent the relative protein quantification of TLR4, p-JNK and JNK1/2 normalized to the α-tubulin level (B-D). The means ± standard deviation (n = 7-8) in each row followed by the same letter are not significantly different at the 5% level.

Fig. 2. Representative protein products of p-NFκB and p-IκB as performed and analyzed in Fig. 1.
the allergy control and Kefir II groups. The levels of p-JNK (Fig. 1C) in the GM080 groups were significantly lower than that in the Kefir I group ($P < 0.05$), but were insignificantly different from those in the normal control group and the Kefir II group ($P > 0.05$). The protein levels of JNK1/2 were significantly decreased in the GM080 groups compared to that of the allergy control, Kefir I and Kefir II groups (Fig. 1D). The p-NF$_\kappa$B levels were markedly lower ($P < 0.05$) in the Kefir I and Kefir II groups than that in the allergy control group (Fig. 2B). Interestingly, its levels of TLR4 (Fig. 1B) and p-NF$_\kappa$B (Fig. 2B) signaling proteins in GM080 remained the same as that in the normal control group. The level of p-I$_\kappa$B in the GM080 group was significantly lower than those in the others allergy groups and the normal control group (Fig. 2C). However, the TNF-$\alpha$ levels in the Kefir I, Kefir II and GM080 groups were significantly lower than that in the allergy control group, but were insignificantly different from that in the normal control group (Fig. 3B).

**Cardiac Mitochondria-Dependent Apoptotic Pathways with Different LAB Treatments Expression of TLR4 and p-p38 Proteins**

In order to investigate the signaling pathways associated with cardiac apoptosis induced by the allergy and the normal control groups, we examined the levels of the components of p-p38 present in each group (Fig. 4). TLR4 not only increased the production of p-p38 mitogen-activated protein kinase, it additionally activated the signaling proteins Bax, Bad, cytochrome c and caspase 3, which as a result induced cell apoptosis. In this study, an increase in p-p38 protein was triggered in the allergy control group, but this reaction could be reversed through the application of the aforementioned Kefir I, Kefir II and GM080 products (Fig. 4B).

**Bcl-2 Family Protein in Mitochondrial-Dependent Apoptotic Pathway**

In order to further understand the cardiac Bcl-2 family in the mitochondria-dependent apoptotic pathway, we examined the protein levels of the Bcl-2 family (Bad and Bax) in the cardiomyocytes of normal and allergy groups (Fig. 5A). The results showed that when compared with the allergy control group, the Bad protein products were significantly lower ($P < 0.05$) in the GM080 groups, but this reaction could be reversed through treatment with the aforementioned GM080 products (Fig. 5B). The level of Bax protein product was higher in the allergy control mice than in the normal control group, and all allergy groups were treated with LAB (Kefir I, Kefir II and GM080). Additionally, this level was lower in the GM080 group (Fig. 5C) than in the Kefir I, Kefir II and normal control group ($P < 0.05$).
Fig. 4. Representative protein products of p-p38 and p38 as performed and analyzed in Fig. 1.

Fig. 5. Representative protein products of Bad and Bax as performed and analyzed in Fig. 1.
Release of Cytochrome c

Additionally, to further understand the levels of release of cytochrome c from the mitochondria into the cytosol of the mitochondria-dependent apoptotic pathway, we examined the cytosolic cytochrome c in the hearts of the normal control and allergy control groups (Fig. 6A). In comparison to the cytosolic cytochrome c of all LAB-treated allergy groups (Fig. 6B), the levels were higher in the allergy control group, and were significantly lower in the Kefir II and GM080 groups ($P < 0.05$).

Levels of Caspase 3 in the Cardiac Tissues

The levels of activated-caspase 3 were expressed in the left ventricular obtained in vivo (Fig. 6A). Activated-caspase 3 levels (Fig. 6C) were significantly higher in the allergy control and Kefir I groups than in the GM080 and normal control group ($P < 0.05$). However, the GM080 group of caspase 3 protein expression was not significantly changed compared with the Kefir II group ($P > 0.05$).

Discussion

This is the first study to show a benefit following administration of probiotics in BALB/c mouse with OVA-induced allergy and provides further evidence for a role of probiotics in the management of this condition. Cardiomyocytes express most known toll-like receptors (2, 12). Of these, TLR4 signal via NF$\kappa$B resulting in decreased contractility and a concerted inflammatory response (2). The integrated finding “Kefir I treatment reversed OVA-induced cardiac TLR4 protein signaling inflammation associated signaling pathways” was first clarified in this study (Fig. 1B). In reference to the cardiomyocyte inflammation induced in the allergy group, we examined the levels of the components of TLR4, p-JNK, JNK1/2, p-NF$\kappa$B, p-I$\kappa$B and TNF-\(\alpha\) in Figs. 1-3. The proteins p-JNK (Fig. 1C), JNK1/2 (Fig. 1D) and TNF-\(\alpha\) (Fig. 3B) extracted from the left ventricles of excised hearts in the allergy control group were significantly increased in levels when compared with that of the normal control group.

The TLR4 (2), p-NF$\kappa$B (2) and TNF-\(\alpha\) (10) signaling protein were increased by cardiac cells after exposure to endotoxin, and can induced death of cardiomyocytes. In our finding, the GM080-treated p-JNK, JNK 1/2, p-I$\kappa$B and TNF-\(\alpha\) signaling protein
Wang, Tseng, Chang, Lin, Tsai, Tsai, Lu, Lai, Huang and Tsai were significantly reduced, and also decreased slightly in the expression of TLR4 and p-NFκB. The inflammation-related signaling (TLR4)-p-JNK-JNK1/2-(p-NFκB)-p-1κB-TNF-α pathway was all significantly deactivated in the hearts of allergic BALB/c mice with GM080-treatment. Therefore, the inflammation-associated signaling of cardiomyocytes was presumably reversed by the treatment of GM080 products, which may be useful for allergy-induced cardiomyocytes inflammation therapy.

The ‘intrinsic’ mitochondria-dependent apoptotic pathway is mediated by endogenous factors, especially in mitochondrion (15, 27). The mitochondrion is the main site of action for members of the apoptosis-regulating protein family exemplified by Bcl-2 family (19). Shifting the balance of Bcl-2 family members toward pro-apoptotic effects enhances the release of cytochrome c from the mitochondria (36, 37). The released cytochrome c forms a complex with procaspase 9 and its cofactor Apaf-1. The activated caspase 9 can further activate caspase 3 and executes the apoptotic program (5, 20).

In this study, we found that while the level of TLR4 was increased in the OVA-induced allergy group, p-p38 mitogen-activated protein kinase was enhanced as well. It further activated the proteins Bax, Bad, cytochrome c, caspase 3, and, thus, induced cell apoptosis (Fig. 7). We have provided evidence that p-p38 was compensated for and its level was, therefore, increased in the allergy group, which could be reversed by all LAB treatments (Fig. 4). However, the mitochondria-dependent apoptotic pathway was increased in cardiac tissues in OVA-induced allergy compared with the normal control group as a result of a series of experiments, including the significantly increase of Bad and Bax (Figs. 5B and 5C), the slightly increase of cytosolic cytochrome c (Fig. 6B) and activated-caspase 3 (Fig. 6C).

All the key components of mitochondria-dependent apoptotic pathways from the upstream cascade to the downstream cascade consistently show evidence in favor of pro-apoptotic effects in allergy (24, 28). Therefore, our findings strongly suggest that as cardiomyocyte mitochondria-dependent apoptotic pathways in allergy become more active, they might provide a greater potential to develop cardiac apoptosis that may eventually develop into heart failure. Most interestingly, all these changes can be reversed by the LAB treatment.

The relationship of p38 MAPK was activated with Bax translocation and the subsequent release of cytochrome c from mitochondria during cyanide-induced apoptosis (35). In this study, our results showed that the partially inhibition of the release of cytochrome c after LAB treatment was attributed to the blockade of activation of p38 MAPK (Fig. 7). The apoptotic pathway is mediated by mitochondrial release of cytochrome c and subsequent activation of the caspase cascade (36). In the current study, the GM080-treated Bad, Bax, cytochrome c and caspase 3 signaling protein were significantly reduced, and were also decreased slightly in the expression of p-p38 signaling protein. Therefore, the apoptosis-related signaling (p-p38)-Bad-Bax-cytochrome c-caspase 3...
pathway was all significantly deactivated in the hearts of allergic BALB/c mice with GM080-treatment.

The experimental results revealed that allergy-induced activation of TLR4 subsequently led to activation of JNK1/2, which further resulted in both the increases of expression of the p-I\textsubscript{B} and TNF-\textalpha signaling protein. Activated p-NF\textkappaB triggered the downstream target TNF-\textalpha expression resulting in the inflammatory responses (2). In addition, we also observed the activation of p38 MAPK, which might have further induced the up-regulation of pro-apoptotic p-p38, Bad, Bax and caspase 3 activated in cardiomyocytes of allergy mice, which executed the apoptosis process in cardiomyocytes. When evaluating BALB/c mice treated with Kefir I, Kefir II and GM080, we observed that the inflammation-related signaling (TLR4)-p-JNK-JNK1/2-(p-NF\textkappaB)-p-I\textsubscript{B}-TNF-\textalpha pathway and the (p-p38)-Bad/Bax-cytocchrome c-caspase 3 pathway were all significantly deactivated in the hearts of allergic BALB/c mice.

In conclusion, this study showed that expression of the signaling proteins of inflammation and apoptosis for cardiomyocytes of BALB/c mice would increase for OVA treatment, but decrease for GM080 treatment. Therefore, GM080 should presumably improve the inflammation and apoptosis of cardiomyocytes. As a result, further clinical research on the direct relationship between LAB and cardiomyocytes inflammation is necessary in order to understand the direct effects of LAB against cardiomyocyte apoptosis.

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**References**


