The anti-allergic activity of *Lactobacillus plantarum* L67 and its application to yogurt

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ABSTRACT

Recently, interest in the beneficial role of probiotics in the protection and management of allergic diseases caused by immune disorders has been increasing. This study investigated the inhibitory effect of *Lactobacillus plantarum* L67 on induced allergic inflammatory response in bisphenol A–treated rat basophilic leukemia 2H3 (RBL-2H3) cells and mouse splenocytes. We also evaluated the applicability of *L. plantarum* L67 as a yogurt starter culture. We measured the ability of *Lactobacillus* strains to induce the production of IL-12 and IFN- γ in cultured splenocytes by ELISA. Bisphenol A (50 μ M)–treated RBL-2H3 cells were cotreated with a glycoprotein (18 kDa) isolated from *L. plantarum* L67 (5–100 μ g/mL) for 30 min. We measured the expression of mitogen-activated protein kinase (ERK and p38), AP-1 (c-Fos and c-Jun), T-bet, and GATA-binding protein 3 (GATA-3) using Western blotting to examine the differentiation of T helper cells. Furthermore, we evaluated the gene expression of IL-1 β , IL-6, and IL-10 using real-time quantitative PCR. Finally, we evaluated the applicability of *L. plantarum* L67 as a yogurt starter by measuring pH, enumeration of bacteria, and sensory scores. Our results showed that L67 protein inhibited the phosphorylation of ERK and p38 mitogen-activated protein kinase through the transcriptional activation of AP-1 in bisphenol A–treated RBL-2H3 cells. During differentiation of T helper cells, the expression of transcription factor GATA-3 was significantly suppressed by L67 protein (100 μ g/mL) treatment, whereas expression of transcription factor T-bet was increased. In addition, the L67 protein significantly attenuated the expression of T helper 2-linked cytokines IL-1 β , IL-6, and IL-10. These results indicate that *L. plantarum* L67, made available as yogurt starters and dietary sup-

plements, has the potential to prevent allergy-related immune disorders.

Key words: allergy, bisphenol A, *Lactobacillus plantarum* L67, starter

INTRODUCTION

Recent studies have demonstrated that probiotics have beneficial effects for human health. Probiotics are live microorganisms that can improve the balance of beneficial intestinal microflora in the host organism (Sornplang and Piyadeatsoontorn, 2016). *Lactobacillus plantarum*, in particular, exhibits numerous beneficial effects as an anti-cancer agent, an anticoagulant, an antiviral, and immune modulator, an anti-inflammatory, an anti-diabetic agent, and an antioxidant; it also exhibits free radical scavenging activity (Andersson et al., 2010; Li et al., 2012; Giardina et al., 2014; Kassayová et al., 2014). In addition, some lactobacilli have been shown to inhibit type I allergic diseases by regulating the immune system (Segawa et al., 2008; Hong et al., 2014; Konieczna et al., 2015). These investigations have revealed that specific probiotic bacteria may be a promising tool for promoting potentially anti-allergic processes through immune regulation.

Glycoproteins isolated from some *Lactobacillus* strains have been known to have beneficial effects, such as controlling immature dendritic cells, regulating T-cell function, and inhibiting the adherence of pathogens in the gut (Rhee and Park, 2001; Johnson-Henry et al., 2007; Konstantinov et al., 2008; Song et al., 2015). The glycoprotein (18 kDa) isolated from *L. plantarum* L67 is known to have anti-inflammatory and anti-allergy effects (Song et al., 2016a,b).

Bisphenol A (BPA) is an estrogenic, endocrine-disrupting chemical that has been used extensively in dentistry, food packaging, and lacquers for food cans and water pipes (Vandenberg et al., 2007). Additionally, BPA has been shown to affect the immune system in both humans and other animals. Recent studies have

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shown that BPA can interfere with the immune system via the production of cytokines and immunoglobulins in macrophages. Exposure to BPA can regulate the expression of T helper (Th) type 2 (Th2)-related cytokines, including IL-6 and IL-10, as well as increase the production of IL-4 and antigen-specific IgE (Yan et al., 2008; Lee and Lim, 2010; O'Brien et al., 2014). Several environmental chemicals, including BPA, are commonly used in everyday life, and these chemicals can affect human endocrine and immune systems (Chalubinski and Kowalski, 2006). Moreover, BPA can cause diseases such as atopic dermatitis, allergic rhinitis, and bronchial asthma (Bornehag et al., 2004; Jaakkola et al., 2004).

Allergic inflammation occurs via activation of Th2-related cytokines to induce a Th1/Th2 imbalance. It has been reported that Th1 and Th2 cells are differentiated from T helper cell Th0 by specific transcription factors such as T-box expressed in T-cells (**T-bet**) and GATA-binding protein 3 (**GATA-3**). We know that T-bet is associated with the differentiation of Th1 cells and IFN- γ secretion (Szabo et al., 2003), and that GATA-3 plays a pivotal role in Th2 cell differentiation and inhibits Th1 cell differentiation (Szabo et al., 2000; Zhu et al., 2006).

Recently, we identified a bacterial strain that induced the overexpression of IL-12 and IFN- γ in mouse splenocytes. We isolated a glycoprotein (18 kDa) from this strain. This glycoprotein inhibited allergy-related factors, including histamine and β -hexoaminidase (Song et al., 2016a).

In this study, we found that the glycoprotein (18 kDa) isolated from *L. plantarum* L67 (**L67** protein) inhibits allergy-related inflammatory mediators in BPA-treated rat basophilic leukemia 2H3 (RBL-2H3) cells and primary cultured splenocytes. First, we measured the levels of ERK, p38, and AP-1 by Western blotting. To study the differentiation of T helper cells, we also evaluated GATA-3 and T-bet expression by Western blotting. We evaluated the expression of IL-1 β , IL-6, and IL-10 cytokines by real-time quantitative PCR. Finally, we evaluated the applicability of *L. plantarum* L67 as a yogurt starter culture by measuring pH, enumeration of bacteria, and sensory scores.

MATERIALS AND METHODS

Chemicals and Reagents

We obtained BPA, penicillin G, and streptomycin from Sigma-Aldrich (St. Louis, MO), and we purchased Dulbecco's modified Eagle medium and fetal bovine serum from Gibco BRL (Grand Island, NY). Other

chemicals and reagents were of the highest analytical grade.

Bacterial Strains and Culture

The *Lactobacillus* strains used in this study were isolated from infant feces or kimchi. *Lactobacillus* strains were selected according to their colony characteristics, and they were tested for catalase activity and by Gram staining. These isolates were further screened for their acid and bile salt tolerance capacities according to the method by Park et al. (2002). To identify *Lactobacillus* strains, biochemical properties were first examined using an API 50 CHL kit (BioMerieux, Marcy l'Étoile, France) and 16S rDNA sequencing data as described by Song et al. (2014). Eighteen strains were identified and evaluated in this study (Table 1). *Lactobacillus* strains were grown in de Man, Rogosa, and Sharpe (MRS) broth (Difco, Detroit, MI) at 37°C for 18 h. The bacterial cells were separated by centrifugation at $3,000 \times g$ for 15 min at room temperature, and the precipitated cells were washed twice with sterile saline (0.85% sodium chloride). Skim milk (10%) was added to the sediments, and the suspension was stored at -80°C until use.

IL-12 and IFN- γ Production by Mouse Splenocytes

We evaluated the ability of *Lactobacillus* strains to induce the production of IL-12 and IFN- γ using cultured splenocytes obtained from BALB/c mice. Splenocytes cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin, were seeded at 2.5×10^6 cells/mL on a 96-well culture plate. Spleen cells were cultured with or without 2.5×10^6 cfu/mL lactobacilli at 37°C and 5% CO₂. Supernatants were collected on d 3 to measure IL-12 and IFN- γ production using ELISA. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD). The protocol was approved by the committee on the Ethics of Animal Experiments of Chonnam National University (permit number CNU IACUC-YB-2012-40). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering of animals.

Preparation of Glycoprotein Isolated from *L. plantarum* L67

For preparation of glycoprotein, 10 mL of *L. plantarum* L67 culture was obtained after 18 h of incubation at

Table 1. Genotypic methods applied for identification of lactobacilli

Strain ID	Closest relative (by 16S rDNA gene sequencing)	Identity (%)	Gene bank accession no.
1	<i>Lactobacillus curvatus</i>	99	KJ914899.1
2	<i>Lactobacillus curvatus</i>	99	KC787548.1
3	<i>Lactobacillus curvatus</i>	99	AB600197.1
4	<i>Lactobacillus sakei</i>	99	KC416998.1
5	<i>Lactobacillus brevis</i>	99	JX966418.1
6	<i>Lactobacillus brevis</i>	98	FJ227309.1
7	<i>Lactobacillus brevis</i>	98	FJ532364.1
8	<i>Lactobacillus brevis</i>	99	HM162416.1
9	<i>Lactobacillus brevis</i>	99	AB548886.1
10	<i>Lactobacillus farciminis</i>	99	FJ532365.1
11	<i>Lactobacillus sakei</i>	99	EU626014.1
12	<i>Lactobacillus sakei</i>	99	JQ517277.1
13	<i>Lactobacillus fermentum</i>	98	KM036427.1
14	<i>Lactobacillus acidophilus</i>	99	EU626017.1
15	<i>Lactobacillus acidophilus</i>	99	EU626023.1
L67	<i>Lactobacillus plantarum</i>	100	KR336551
17	<i>Lactobacillus fermentum</i>	99	KM036427.1
18	<i>Lactobacillus plantarum</i>	99	EU626013.1

37°C. Pellets were collected by centrifugation at 12,000 × *g* at 4°C, and washed 3 times with sterile deionized water. The washed cells were resuspended in 10 g/L of SDS solution and boiled for 10 min. The supernatants were harvested after centrifugation at 12,000 × *g* at 4°C for 15 min and dialyzed against distilled water at 4°C. Supernatants were dried using a freeze-dryer and stored at −70°C (Song et al., 2016a). Protein concentrations were measured using the Bradford assay (Bradford, 1976).

Cell Culture

The RBL-2H3 cells were provided by the Korea Cell Line Bank (Seoul, Korea). The cells were incubated in Dulbecco's modified Eagle medium supplemented with fetal bovine serum (10%), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C and 5% CO₂. The medium was changed twice a week. The cells (2.3 × 10⁶ cells/mL) were divided into 6-well plates. Splenocytes were collected from BALB/c mice using aseptic conditions in Hanks' balanced salt solution. Spleens were crushed by passage through a 40-µm cell strainer (Falcon, Corning Inc., Corning, NY). The supernatant of homogeneous cells was precipitated by adding ammonium chloride (0.8% wt/vol). After centrifugation, the cells were washed with PBS and resuspended in RPMI 1640 medium supplemented with fetal bovine serum (10%), penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37°C in 5% CO₂. Then, cells (1 × 10⁶ cells/mL) were divided into 6-well or 96-well flat-bottomed plates. They were then cotreated with 50 µM BPA and L67 protein (5–100 µg/mL) for 30 min.

Preparation of Cell Extracts for Western Blotting

Cells were stimulated with BPA (50 µM) or cotreated with L67 protein (5–100 µg/mL) for 30 min. Cells were then rinsed with PBS after removing the medium. Protein was isolated according to the method of Oh and Lim (2008). Protein concentration was measured using the Lowry method (Lowry et al., 1951), and protein samples were stored at −70°C.

Western Blot Analysis

Proteins were analyzed by 10% polyacrylamide mini-gel electrophoresis at 100 V for 2 h at room temperature using a Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, CA). After protein transfer onto nitrocellulose membranes (Millipore, Bedford, MA), the membranes were incubated overnight at 4°C with rabbit polyclonal antibodies [1:3,000, T-bet, GATA-3, mitogen-activated protein kinase (**MAPK**), AP-1, and α-tubulin] in Tris-buffered saline Tween 20 (**TBS-T**) solution. After 3 washes with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; Cell Signaling, Danvers, MA) in TBS-T solution for 1 h 30 min at room temperature. Protein bands were visualized by chemiluminescence (ELPIS Biotechnology, Taejeon, Korea). Band intensities were measured using Scion imaging software (Scion Image Beta 4.02, MD).

RNA Isolation and Real-Time Quantitative PCR

Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA), according to the manufac-

Table 2. Oligonucleotides used for real-time PCR

Gene	Sequence of PCR primers (5' to 3')
IL-1 β	Forward: TTGACGGACCCCAAAAGATG Reverse: AGAAGGTGCTCATGTCCTCA
IL-6	Forward: GTTCTCTGGGAAATCGTGGA Reverse: TGTACTCCAGGTAGCTATGG
IL-10	Forward: CTGGACAACATACTGCTAACCGAC Reverse: ATTCAATTCATGGCCTTGTAGACACC
GAPDH	Forward: CAAAAGGGTCATCATCTCTG Reverse: CCTGCTTCACCACCTTCTTG

turer's instructions (www.qiagen.com/goto/microRNA-protocols). We prepared cDNA from 1 μ L of total RNA (1 μ g/20 μ L), using the Maxime RT Premix Oligo (dT) real-time quantitative PCR kit (Intron, Seongnam, Korea). The primers used are shown in Table 2. The expression levels of the respective genes were measured by real-time PCR using 2X Prime Q Master Mix (Kapa Biosystems, Boston, MA) and analyzed using a CFX96TM Real-Time system (Bio-Rad). Amplifications were conducted using 50 cycles for IL-1 β , IL-6, IL-10, and GAPDH (30 s at 95°C, 30 s at 56°C, and 60 s at 72°C; Table 2). The sample Δ cycle threshold (Δ Ct) values were calculated as the differences between the Ct values for IL-1 β , IL-6, or IL-10, and the values for GAPDH (reference) in cells treated with BPA alone or cotreated with BPA and L67 protein. The relative gene expression levels between the treatment group and the control were determined using the formula: $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Yogurt Manufacturing

Yogurt was prepared by heating milk (SNF 11%; raw milk with skim milk powder; Seoul Dairies, Seoul, Korea) to 95°C for 20 min, and then cooling to 40°C. Starter cultures were inoculated aseptically. *Streptococcus thermophilus* and *Bifidobacterium* species were grown in M17 broth (Difco, Detroit, MI) and in BL broth (Teraguchi et al., 1978), respectively. *Lactobacillus acidophilus* and *L. plantarum* L67 strains were grown in MRS broth (Difco). All strains were stored at -80°C in reconstituted skim milk (10% wt/vol)

yeast extract (0.5% wt/vol) medium and were cultured 3 times before use. The tested starters are indicated in Table 3. The pH value during fermentation was measured with a pH meter (FEP20; Mettler Toledo; Greifensee, Switzerland).

Enumeration of Bacteria

We enumerated *L. acidophilus* and *L. plantarum* L67 using MRS-maltose agar (Dave and Shah, 1996) and incubation 37°C for 48 h; *S. thermophilus* using M17 agar at 43°C for 24 h; and *B. longum* according to the method by Teraguchi et al. (1978) using BL-NPNL (nalidixic acid, paromomycin sulfate, neomycin sulfate, and lithium chloride) agar and incubated anaerobically (Bugbox; Ruskinn Technology, Bridgend, UK) at 37°C for 48 h.

Sensory Evaluations

Samples were assessed organoleptically by 6 panelists. Properties were evaluated using sensory rating scales for flavor (1–9 points; no criticism = 9) and for texture and preference (1–5 points; no criticism = 5).

Statistical Analysis

All data are expressed as means with standard errors (SE). The data were processed with SAS software using ANOVA tests and the GLM procedure (SAS version 9.1; SAS Institute Inc., Cary, NC). We analyzed different groups using Duncan's multiple range test (P -value < 0.05).

RESULTS

Detection of Lactobacilli Strains that Induced Significant IL-12 and IFN- γ Secretion from Splenocytes

We measured the ability of 18 *Lactobacillus* strains to improve the Th1/Th2 balance in splenocytes. The secretion of Th1-related cytokines IL-12 and IFN- γ

Table 3. Composition of yogurt starter cultures

Culture	Composition of cultures ¹	Source
Starter A (control)	<i>Lactobacillus acidophilus</i> La5 ($\sim 10^6$), <i>Bifidobacterium animalis</i> ssp. <i>lactis</i> Bb12 ($\sim 10^6$), and <i>Streptococcus thermophilus</i> ($\sim 10^7$)	Chr. Hansen A/S, Hørsholm, Denmark
Starter B (control)	<i>Lactobacillus acidophilus</i> NCFM ($\sim 10^6$), <i>Bifidobacterium longum</i> ($\sim 10^6$), and <i>Streptococcus thermophilus</i> ($\sim 10^7$)	Danisco USA Inc., New Century, KS
Starter C	<i>Lactobacillus plantarum</i> L67 ($\sim 10^6$), <i>Bifidobacterium longum</i> ($\sim 10^6$), and <i>Streptococcus thermophilus</i> ($\sim 10^7$)	Culture Systems Inc., Mishawaka, IN

¹Numbers in parentheses indicate inoculum levels in milk (cfu/mL).

Table 4. Production of IL-12 and IFN- γ from mouse splenocytes induced by various strains of lactobacilli¹

Strain ID	IL-12 (pg/mL)	IFN- γ (pg/mL)
Control	0 $\pm$ 0.12	200 $\pm$ 7.8
1	35.0 $\pm$ 1.2	1,900 $\pm$ 75.2
2	38.4 $\pm$ 1.1	1,300 $\pm$ 65.2
3	35.4 $\pm$ 0.3	2,050 $\pm$ 10.3
4	25.6 $\pm$ 0.2	2,000 $\pm$ 10.2
5	55.8 $\pm$ 1.7	1,000 $\pm$ 8.7
6	17.3 $\pm$ 0.4	800 $\pm$ 10.5
7	15.2 $\pm$ 0.5	1,310 $\pm$ 54.3
8	10.9 $\pm$ 0.3	400 $\pm$ 5.6
9	11.6 $\pm$ 0.2	408 $\pm$ 7.8
10	64.3 $\pm$ 0.6	1,200 $\pm$ 21.3
11	48.0 $\pm$ 2.3	550 $\pm$ 6.4
12	68.9 $\pm$ 8.7	1,400 $\pm$ 12.3
13	18.1 $\pm$ 0.4	1,950 $\pm$ 15.3
14	13.3 $\pm$ 0.3	200 $\pm$ 4.3
15	36.0 $\pm$ 0.3	300 $\pm$ 3.2
L67	68.7 $\pm$ 4.3	1,800 $\pm$ 43.2
17	16.2 $\pm$ 0.4	210 $\pm$ 5.6
18	14.3 $\pm$ 0.3	1,850 $\pm$ 20.2

¹Splenocytes were isolated from BALB/c mice and cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin at a density of 2.5×10^6 cells/mL in a 96-well culture plate. Cells were cultured with or without 2.5×10^6 cfu/mL lactobacilli at 37°C and 5% CO₂. Supernatants were collected on d 3 to measure cytokine (IL-12, IFN- γ) production, and the concentrations of IL-12 and IFN- γ in supernatants were determined by ELISA.

from splenocytes was measured by ELISA. As shown in Table 4, the ability of splenocytes to induce IL-12 and IFN- γ production depended on the *Lactobacillus* strain. Of the 18 investigated strains, we selected *L. plantarum* L67, which induced secretion of high levels of both IL-12 and IFN- γ in mouse splenocytes (Table 4).

Effect of L67 Protein on Expression of p38 MAPK and ERK in BPA-Treated RBL-2H3 Cells

To determine whether the L67 protein could suppress phosphorylation of p38 in BPA-treated RBL-2H3 cells, we analyzed p38 phosphorylation by Western blotting. Phosphorylation of p38 was increased 4.5-fold in BPA-treated RBL-2H3 cells compared with the control. However, the increased phosphorylation was reduced with L67 protein treatment to 3.67-, 3.62-, 3.43-, and 0.87-fold relative to control (5, 20, 50, and 100 μ g/mL, respectively; Figure 1). In addition, ERK activity was increased by treatment with BPA for 30 min, and this increased activity was reduced in a dose-dependent manner with L67 protein treatment.

Figure 1. Inhibitory effect of *Lactobacillus plantarum* L67 protein on ERK (Western blot in A; relative band intensity in B) and p38 mitogen-activated protein kinase (MAPK; Western blot in C; relative band intensity in D) phosphorylation in bisphenol A (BPA)-treated RBL2H3 cells. Cells were treated with BPA (50 μ M) for 30 min in the absence or presence of L67 protein (5, 20, 50, and 100 μ g/mL). The relative intensities of the bands were analyzed using Scion imaging software (Scion Image Beta 4.02; Scion, Frederick, MD). All data are mean $\pm$ SE from triplicates. #Significant difference between treatments and controls ($P < 0.05$). *Significant difference between BPA and L67 protein treatments in the presence of BPA ($P < 0.05$). **Significant difference between BPA and L67 protein treatments in the presence of BPA ($P < 0.01$). Con = control.

Figure 2. Inhibitory effect of *Lactobacillus plantarum* L67 protein on AP-1 (c-Fos and c-Jun) expression in bisphenol A (BPA)-treated RBL2H3 cells (Western blot in panel A; relative band intensities of c-Fos and c-Jun in panels B and C). Cells were treated with BPA (50 μM) for 30 min in the absence or presence of L67 protein (5, 20, 50, and 100 μg/mL). The relative intensities of the bands were analyzed using Scion imaging software (Scion Image Beta 4.02; Scion, Frederick, MD). α-Tubulin was used as an internal control. All data are mean ± SE from triplicates. #Significant difference between treatments and controls ($P < 0.05$); ***significant difference between BPA and L67 protein treatments in the presence of BPA ($*P < 0.05$; $**P < 0.01$). Con = control.

Effect of L67 Protein on Activity of AP-1 in BPA-Treated RBL-2H3 Cells

As shown in Figure 2, phosphorylation of AP-1 (c-Fos) and AP-1 (c-Jun) increased by 6.65- and 4.73-fold, respectively, with BPA treatment (50 μM) for 30 min. The increased phosphorylation of AP-1 (c-Fos) was diminished to 4.77-, 4.02-, 2.91-, and 2.79-fold in the presence of L67 protein (5, 20, 50 and 100 μg/mL, respectively). The increased phosphorylation of AP-1 (c-Jun) was inhibited to 3.83-, 3.08-, 2.38-, and 1.17-fold in the presence of L67 protein (5, 20, 50, and 100 μg/mL, respectively).

Inhibitory Effect of L67 Protein on the Expression of GATA-3 and T-bet in BPA-Treated Primary Cultured Mouse Splenocytes

As shown in Figure 3, expression of GATA-3 was increased by 2.6-fold in BPA-treated splenocytes, whereas expression of T-bet was reduced to 0.15-fold compared with the control. The increased expression of GATA-3 was inhibited by L67 protein treatment in a dose-dependent manner, and the decreased expression

of T-bet increased with L67 protein treatment. Expression of GATA-3 was reduced to 1.5-, 1.12-, 1.03-, and 0.6-fold with 5, 20, 50, and 100 μg/mL of L67 protein, respectively. Expression of T-bet was augmented to 0.2-, 0.74-, 0.76-, and 0.8-fold at 5, 20, 50, and 100 μg/mL of L67 protein, respectively.

Inhibitory Effect of L67 Protein on Gene Expression of IL-6, IL-10, and IL-1β in BPA-Treated RBL-2H3 Cells

To study the effects of L67 protein treatment on allergy-related cytokines, we investigated the expression profile of IL-6, IL-10, and IL-1β genes in BPA-treated RBL-2H3 cells. As shown in Figure 4, cells stimulated with BPA showed significantly enhanced expression of IL-6, IL-10, and IL-1β compared the control. However, L67 protein treatment of cells led to inhibition of IL-6, IL-10, and IL-1β induction in a dose-dependent manner compared with BPA treatment alone. BPA-induced IL-6 gene transcription increased by 4.5-fold, but it decreased to 4.2-, 3.6-, 2.8-, 1.9-, and 1.8-fold upon addition of 5, 20, 50, and 100 μg/mL of L67 protein, respectively. Similarly, IL-10 and IL-1β gene

transcription both increased by 1.8-fold in response to BPA treatment but decreased with the addition of L67 protein.

Applicability of *L. plantarum* L67 as a Yogurt Starter Culture

Table 5 shows the decremental change in pH during fermentation of cultures until pH 4.5 (endpoint). Five hours of fermentation was required to reach a pH of 4.5 in all starter cultures. Probiotic strains *L. acidophilus* La5, *L. acidophilus* NCFM, *Bifidobacterium animalis* Bb12, and *L. plantarum* L67 did not have obvious differences in acidification.

The number of viable cells of *S. thermophilus* increased rapidly during fermentation, whereas bifidobacteria and lactobacilli increased steadily by approximately 1 log (Table 6). We observed no differences in fermentation time or total number viable of cells in the starter cultures. As shown in Table 7, organoleptic evaluation indicated that the yogurts with *L. plantarum* L67

showed results that were strikingly similar to the other commercial starters.

DISCUSSION

In the present study, we explored whether L67 protein inhibits the activity of allergy-related mediators, as well as the induction of cytokines in BPA-treated RBL-2H3 cells and in primary cultured splenocytes. Several studies have demonstrated that lactobacilli regulate macrophage activation and modulate the Th1/Th2 balance (Segawa et al., 2008; Won et al., 2011). Several strains of lactobacilli possess Th1-inducing activity, reduce total or ovalbumin-specific IgE production, and change associated cytokine patterns in a strain-dependent manner (Lee et al., 2013). First, we examined 18 strains of lactobacilli for their ability to induce secretion of Th1-related cytokines, such as IL-12 and IFN- γ , from mouse splenocytes. We selected *L. plantarum* L67 for further evaluation, based on its ability to induce high levels of IL-12 and IFN- γ secretion (Table 1).

Figure 3. Inhibitory effect of *Lactobacillus plantarum* L67 protein on GATA-binding protein 3 and T-bet expression in bisphenol A (BPA)-treated, primary cultured mouse splenocytes (Western blot in panel A; relative band intensities of GATA-3 and T-bet in panels B and C). Primary cultured mouse splenocytes were treated with BPA (50 μ M) for 30 min in the absence or presence of L67 protein (5, 20, 50, and 100 μ g/mL). The relative intensities of the bands were analyzed using Scion imaging software (Scion Image Beta 4.02; Frederick, MD). α -Tubulin was used as an internal control. All data are mean $\pm$ SE from triplicates. #Significant difference between treatments and controls ($P < 0.05$); ***significant difference between BPA and L67 protein treatments in the presence of BPA ($*P < 0.05$; $**P < 0.01$). Con = control.

The mechanisms leading to a predominant Th2 response during T-cell differentiation are still not fully understood. Among the many factors associated with Th2 differentiation, MAPK is a key mediator of the expression of Th2-related cytokines such as IL-4 and IL-10 (Yamashita et al., 2005; Watanabe et al., 2005). Therefore, the MAPK signaling pathway is an important therapeutic target in allergic disorders induced by a predominantly Th2 immune response (Abdel-Raheem et al., 2005). We measured the activity of ERK and p38 to determine whether L67 protein suppressed MAPK signaling and, consequently, the expression of IL-1 β , IL-6, and IL-10. Our results showed that L67 protein inhibited BPA-induced ERK and p38 MAPK phosphorylation in RBL-2H3 cells. Further, it has been reported that various probiotics can inhibit inflammatory markers such as MAPK and AP-1 induced by lipopolysaccharides or other inflammatory derivatives (Kumar et al., 2014; Li et al., 2014; Jeong et al., 2015). In our study, L67 protein inhibited inflammatory response markers induced by BPA.

Activation of ERK or JNK leads to nuclear translocation of AP-1 and increased expression of pro-inflammatory cytokine genes (Banerjee and Gerondakis, 2007). *Lactobacillus reuteri* suppresses activation of the AP-1 translocation factor, which regulates the expression of pro-inflammatory cytokine genes in response to activation of toll-like receptor (Lin et al., 2008). In our study, L67 protein also inhibited activation of AP-1 (c-Fos and c-Jun).

Several factors are involved in cell development from Th0 to Th1 and Th2 cells. In particular, GATA-3 is well known as a key transcriptional factor in Th2 cell differentiation (Lin et al., 2008). Furthermore, activation of GATA-3 induces the production of Th2 cytokines such as IL-4 and IL-10 and represses the production of Th1-related cytokines. Patients with specific allergies also have increased expression of GATA-3 (Nakamura et al., 2000; Zhu et al., 2006). In this study, GATA-3 was upregulated by BPA, and expression of T-bet was reduced. The increased expression of GATA-3 induced by BPA treatment was suppressed by cotreatment with L67 protein; conversely, L67 protein treatment induced the expression of T-bet in BPA-treated splenocytes.

These results indicate that L67 protein helps to maintain the balance between Th1 and Th2 cells by regulating the expression of T-bet and GATA-3. Differentiated Th1 cells produce IFN- γ and lymphotoxin, which are

Figure 4. Inhibitory effect of *Lactobacillus plantarum* L67 protein on IL-1 β , IL-6, and IL-10 expression in bisphenol A (BPA)-treated RBL2H3 cells. Cells were treated with BPA (50 μ M) for 30 min in the absence or presence of *L. plantarum* L67 protein (5, 20, 50, and 100 μ g/mL). The expression of IL-1 β , IL-6, and IL-10 mRNA was evaluated by real-time quantitative PCR using isolated total RNA. The mRNA levels (transcript fold level normalized to that of *GAPDH*) were calculated relative to the transcript levels detected in the untreated control. All data are mean $\pm$ SD from triplicates. #Significant difference between treatments and controls ($P < 0.05$); ***significant difference between BPA and L67 protein treatments in the presence of BPA (* $P < 0.05$; ** $P < 0.01$).

Table 5. Changes in pH values during yogurt fermentation with 3 different starter cultures (mean $\pm$ SE)

Time	Starter A	Starter B	Starter C
0 h	6.55 $\pm$ 0.005	6.52 $\pm$ 0.004	6.61 $\pm$ 0.007
2 h	6.01 $\pm$ 0.006	5.98 $\pm$ 0.006	6.08 $\pm$ 0.006
4 h	4.75 $\pm$ 0.004	4.68 $\pm$ 0.006	4.72 $\pm$ 0.005
Endpoint	4.44 $\pm$ 0.007	4.46 $\pm$ 0.007	4.45 $\pm$ 0.005
	(5 h 10 min)	(5 h 5 min)	(5 h 10 min)

Table 6. Number of viable cells (cfu/mL) of lactic acid bacteria during yogurt fermentation with 3 different starter cultures

Item	Initial	Final
Starter A		
<i>Lactobacillus acidophilus</i> La5	$1.43 \times 10^6 \pm 0.83$	$1.21 \times 10^7 \pm 1.12$
<i>Bifidobacterium animalis</i> ssp. <i>lactis</i> Bb12	$1.35 \times 10^6 \pm 0.71$	$9.10 \times 10^7 \pm 2.15$
<i>Streptococcus thermophilus</i>	$2.37 \times 10^7 \pm 0.79$	$1.63 \times 10^9 \pm 0.62$
Starter B		
<i>Lactobacillus acidophilus</i> NCFM	$2.54 \times 10^6 \pm 1.56$	$1.01 \times 10^7 \pm 0.61$
<i>Bifidobacterium longum</i>	$1.83 \times 10^6 \pm 0.81$	$1.31 \times 10^7 \pm 0.78$
<i>Streptococcus thermophilus</i>	$1.66 \times 10^7 \pm 0.44$	$1.93 \times 10^9 \pm 1.14$
Starter C		
<i>Lactobacillus plantarum</i> L67	$1.02 \times 10^6 \pm 1.27$	$2.17 \times 10^7 \pm 0.99$
<i>Bifidobacterium longum</i>	$1.12 \times 10^6 \pm 0.44$	$2.01 \times 10^7 \pm 1.04$
<i>Streptococcus thermophilus</i>	$1.53 \times 10^6 \pm 0.62$	$1.88 \times 10^9 \pm 0.95$

necessary for immune resistance against intracellular pathogens (Glimcher and Murphy, 2000). In contrast, Th2 cells release IL-1 β , IL-6, and IL-10, which are associated with atopic and allergic reactions (Tillie-Leblond et al., 1999; Herrick et al., 2000). We know that IL-1 β is a key stimulator in the class switching process that replaces IgE to regulate Th2 and B cells, and that IL-10 functions as a vital inhibitor of IL-12 and IFN- γ (Th1 cytokines), which inhibit the differentiation of Th1 cells (Ritter et al., 2014; Murphy et al., 2000). Because BPA induces the Th2 response, we examined gene expression of IL-1 β , IL-6, and IL-10 in RBL-2H3 cells and found that treatment with BPA stimulated the expression of IL-1 β , IL-6, and IL-10. This indicated that BPA promotes the differentiation of Th0 cells to Th2 cells via IL-1 β , IL-6, and IL-10. However, the increased expression of IL-1 β , IL-6, and IL-10 induced by BPA treatment was significantly inhibited by L67 protein treatment.

We also evaluated the applicability of *L. plantarum* L67 as a yogurt starter culture. *L. plantarum* L67 did not have obvious effects on acidification. We also observed no differences in fermentation time or total number of viable cells in the starter cultures. The organoleptic evaluation indicated that the yogurts with *L. plantarum* L67 showed strikingly similar results to the other commercial starters. During milk fermentation,

lactic acid bacteria can convert lactose into lactic acid, which contributes to the yogurt's taste and texture (Tamime and Deeth, 1980). Hekmat and Reid (2006) reported that the addition of probiotics does not significantly alter the sensory properties of yogurt, because most probiotic strains are not major milk-fermenting microorganisms (Cruz et al., 2010). On the other hand, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* are reported to be exclusively responsible for the production of flavor compounds such as acetaldehyde, diacetyl, and acetoin (Cheng, 2010). These results suggest that *L. plantarum* L67 is capable of being used in mixed-strain cultures in yogurt manufacturing.

The current study shows that L67 protein inhibits the phosphorylation of ERK and p38 MAPK, as well as the activation of AP-1 (c-Fos and c-Jun). With inflammation-related factors (Th2-related cytokines), L67 protein inhibits BPA-induced IL-1 β , IL-6, and IL-10 activity. Bisphenol A stimulates MAPK signaling, which induces increased GATA-3 expression, and GATA-3 induces the differentiation of Th2 cells and secretion of Th2-related cytokines. Our findings showed that L67 protein may regulate the activation of GATA-3 and T-bet through the induction of MAPK signaling, and it relieves the Th1/Th2 cytokine profile imbalance. Taken together, these findings demonstrate that the *L. plantarum* L67 strain is a potential food supplement and starter culture, that could help in the prevention of Th2-related immune disorders.

Table 7. Mean values for sensory evaluation¹ of yogurt with 3 different starter cultures (mean $\pm$ SE)

Starter	Flavor score	Texture score	Preference score
A	7.12 ± 0.051	3.21 ± 0.048	3.43 ± 0.053
B	7.45 ± 0.053	3.19 ± 0.042	3.51 ± 0.056
C	7.36 ± 0.049	3.25 ± 0.051	3.57 ± 0.061

¹Sensory rating scales: flavor: 1–9 points, no criticism = 9; texture: 1–5 points; no criticism = 5; and preference: 1–5 points; no criticism = 5. None of the differences among starters were significant.

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