J. Microbiol. Biotechnol. (2014), 24(12), 1629–1635
http://dx.doi.org/10.4014/jmb.1406.06062

jmb

Different Immune Regulatory Potential of *Lactobacillus plantarum* and *Lactobacillus sakei* Isolated from *Kimchi*

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Received: June 23, 2014
Revised: July 23, 2014
Accepted: August 4, 2014

First published online
August 11, 2014

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pISSN 1017-7825, eISSN 1738-8872

It is known that lactic acid bacteria (LAB) have many beneficial health effects, including anti-oxidative activity and immune regulation. In this study, the immune regulatory effects of *Lactobacillus sakei* and *Lactobacillus plantarum*, which are found in different types of *kimchi*, were evaluated. *L. sakei* and its lipoteichoic acid (LTA) have greater immune stimulating potential in IL-12, IFN-γ, and TNF-α production as compared with *L. plantarum* in an *in vitro* condition. On the other hand, *L. plantarum* is assumed to repress the Th1 immune response in murine experiments. After being injected with LPS, *L. plantarum*-fed mice maintained a healthier state, and the level of TNF-α in their blood was lower than in other bacterial strain-fed mice and in the LPS-only control mice. Additionally, IL-12 production was significantly decreased and the production of IL-4 was greatly increased in the splenocytes from *L. plantarum*-fed mice. Further experiments revealed that the pre-injection of purified LTA from *L. plantarum* (pLTA), *L. sakei* (sLTA), and *S. aureus* (aLTA) decreased TNF-α and IL-4 production in LPS-injected mice. Mouse IL-12, however, was significantly increased by aLTA pre-injection. In conclusion, the *L. sakei* and *L. plantarum* strains have immune regulation effects, but the effects differ in cytokine production and the regulatory effects of the Th1/Th2 immune response.

Keywords: *Lactobacillus plantarum*, *Lactobacillus sakei*, *kimchi*, cytokine, lipoteichoic acid, immune regulation

Introduction

Numerous reports have demonstrated the beneficial effects of lactic acid bacteria (LAB). Such effects include anti-oxidative effects, antitumor effects, maintenance of the normal flora of the digestive tract, prevention of enteritis, and regulation of immune responses, among others [26]. Currently, the effects of LAB on the immune system have been well studied. Most of the immune responses induced by LAB are innate immune responses that execute immune reactions before the adapted immune response is activated. Innate immune responses transmit various signals that instruct the adapted immune system to activate [22]. Probiotics are defined as "living microorganisms, which on ingestion in certain numbers, exert health benefits beyond inherent basic nutrition" [15]. LAB, including members of the *Lactobacillus* genus, are members of the commensal microorganisms of the gastrointestinal tract of humans and mammals and are generally recognized as probiotics [9, 17].

It is widely believed that intestinal LAB play an important role in promoting the health of the host, including the modulation of immune responses [1, 13, 15]. One potential function of LAB is their involvement in the development and maintenance of homeostasis in the intestine-associated immune system [1, 31]. It has been shown that peptidoglycan and other cell wall components of LAB may play a significant role in stimulating immunocompetent cells in the intestinal tract [1, 2, 7, 15]. Intestinal LAB, including various species of *Lactobacillus*, interact regularly with intestinal cells, which include antigen-presenting cells and

intestinal epithelial cells [16, 27, 32]. It has been reported that lactobacilli may moderate allergic reactions by maintaining the balance between Th1 and Th2 responses [1, 11, 23]. This balance is thought to be maintained by specialized subsets of T-regulatory (Treg) cells that produce suppressive cytokines, such as interleukin (IL)-10 and transforming growth factor (TGF)-β [8, 24].

To date, most studies have focused on the immunomodulatory effects of viable LAB; however, we investigated those of heat-killed LAB in this study. Heat-killed LAB have the advantages of allowing a longer product shelf life and easier storage and transport. Studies have demonstrated that LAB can effectively stimulate the production of IL-12 and interferon (IFN)-γ and modulate immune responses in mice and humans [23, 30]. Many bacterial strains have been discovered, but most bacterial strains are isolated from milk and industrial meat products. There are very few probiotic bacterial strains isolated from vegetable matter. The traditional Korean fermented food *kimchi* is rich in vegetable matter and probiotic bacterial strains [20]. Several LAB strains have been isolated from *kimchi*, but there are few reports investigating their functional effects [4, 21]. Our study focused on the immune characteristics of two *Lactobacillus* strains that inhabit *kimchi*, *Lactobacillus sakei* and *L. plantarum*. *L. sakei* was isolated from a commercial Korean *kimchi* during the course of this experiment. *L. plantarum* was discovered during our previous work on the anti-inflammatory potency of *L. plantarum* LTA [18, 19]. This study focused on the influence of *L. plantarum* and *L. sakei* in murine models under *in vivo* conditions.

Materials and Methods

Bacterial Strains and Culture Conditions

L. sakei was isolated from the Korean traditional food *kimchi*, and the strain was confirmed by 16S rRNA/16S-23S rRNA IRS gene sequencing. The following primers (Bioneer, Korea) were used to amplify the 16S rRNA and 16S-23S rRNA IRS genes: 16s forward (AGTTGATCCTGGCTC), 16S reverse (ACCTTGTTA CGACTT) [28]; and IRS forward (TGCGGCTGGATCACCTCC TT), IRS reverse (GGTTCTTTTCACCTTTCCCTC) [6]. The resultant PCR products were sequenced (Bionics, Korea) for phylogenetic analysis. *L. sakei, L. rhamnosus* GG (KCTC 5033), and *L. plantarum* (ACTCBAA-793) were cultured in Man, Rogosa, and Sharpe (MRS) broth (Difco, USA). *Staphylococcus aureus* (KCTC 1621) was cultured in Brain Heart Infusion (BHI) broth (Difco). The cells were harvested by centrifugation at 5,000 ×*g* for 10 min and then washed three times with sterile saline solution to purify lipoteichoic acid (LTA), which was then freeze dried.

Purification of LTA

LTA was prepared from the *Lactobacillus* strains and *Staphylococcus aureus* using the methods detailed by Han *et al*. [12]. Harvested cells were broken up by sonication. The sonicated cells were extracted with *n*-butanol, and LTA was first purified using hydrophobic interaction chromatography with an Octyl-Sepharose CL-4B column. For further purification, DEAE Sepharose ion-exchange column chromatography was performed. Purified LTAs were stored by freeze-drying.

Animals

Female 7- to 8-week-old BALB/c mice were bred and raised under conventional conditions at the Kyung Hee University Biocenter. They were housed in individual cages at 23 ± 3°C, and offered feed and water *ad libitum*. The Institutional Animal Care and Use Committee of Kyung Hee University approved this study.

Material Administration to Mice

Mice were each fed freeze-dried heat-killed bacterial cells (5 mg/day with 1% skim milk), which were dissolved in 200 μl of sterilized water for 2 weeks before the experiments were performed. Mice were injected with 10 mg/kg LPS (Sigma, USA) and their sera were collected 24 h after LPS injection. Mice were injected with 20 μg of ovalbumin (OVA) dissolved 5:5 in phosphate-buffered saline (PBS) and an alum solution (Sigma), 1 week after the sera were collected to detect and measure levels of immunoglobulin. The mice were sacrificed to obtain their splenocytes after 2 weeks of treatment.

Preparation of Spleen Cell Suspensions

Spleens were removed aseptically from the mice and placed individually into 5.0 ml of complete RPMI-1640 medium. Single cell suspensions were prepared by chopping the spleens into small pieces with sterile scissors and then forcing the spleen tissue up and down through a 1.0 ml syringe. The resulting suspension was then transferred to a tube containing 5.0 ml of complete RPMI-1640 and centrifuged at 4°C at 1,500 rpm for 10 min. The cell pellet was resuspended in 0.8 ml of ammonium-chloride-potassium lysis buffer and incubated for 1 min. After washing twice in complete RPMI-1640, the cells were adjusted to a final concentration of 2×10^6 cells/ml in complete RPMI-1640.

Enzyme-Linked Immunosorbent Assay (ELISA)

TNF-α, IL-12, and IL-4 were measured using ELISA kits (R&D Systems, MN, USA) and immunoglobulins were also detected using ELISA kits (BD, USA). To measure OVA-specific IgE, we used the method detailed by Sato *et al*. [29]. Standard serum for comparison was obtained from mice immunized three times with 20 μg of OVA and the alum solution. Standard serum was considered to contain 100% of each immunoglobulin.

Statistical Analysis

All experiments were performed at least three times. The data

shown are representative results of the means ± SD of triplicate experiments. Differences were considered statistically significant when the p value was <0.05.

Results

Cytokine Production in Splenocytes Due to *Lactobacillus* Strains

To analyze the role of *Lactobacillus* strains isolated from *kimchi*, mouse splenocytes were treated with heat-killed *L. sakei* and *L. plantarum*. We observed that both *L. sakei* and *L. plantarum* induced the secretion of proinflammatory cytokines. Mouse IL-12 was significantly increased by *L. sakei* (1×10^6 and 1×10^7 cells), whereas *L. plantarum* moderately increased these levels (Fig. 1A). Both *L. sakei* and *L. plantarum* also increased mouse IFN-γ, and the induction of IFN-γ by *L. sakei* was much higher than that by *L. plantarum* (Fig. 1B). Mouse TNF-α was increased by both *L. sakei* and *L. plantarum* up to 1×10^7 cells, but it was slightly decreased by 1×10^8 *L. plantarum* (Fig. 1C). These data indicate that the *Lactobacillus* strains isolated from *kimchi* have a different immune regulatory effect on proinflammatory cytokine expression.

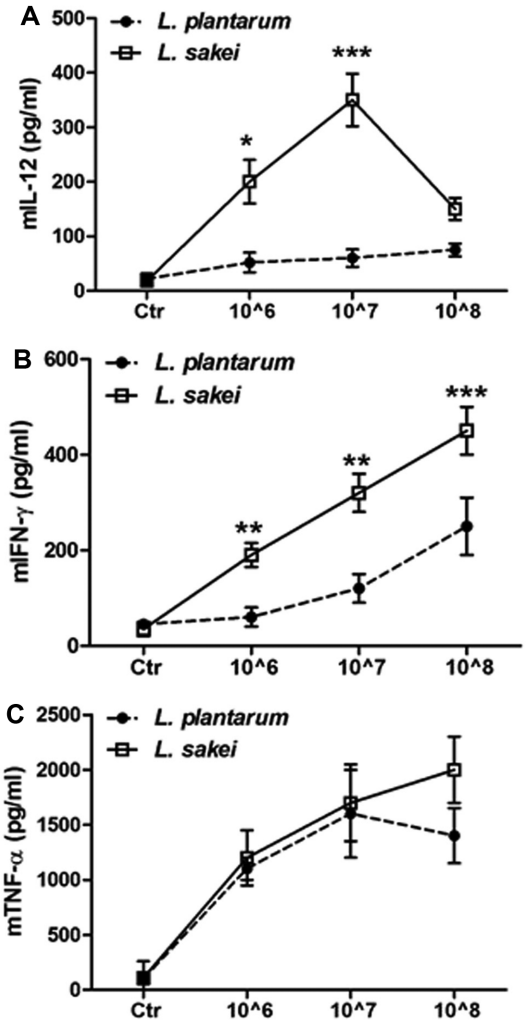

Fig. 1. Potential for different cytokine production by bacterial strains in mouse splenocytes.

Mouse splenocytes were obtained from ovalbumin (20 μg/mouse in alum)-immunized mice (two times in 14 days). Splenocytes were treated with the indicated amount of heat-killed bacteria. Culture supernatants were collected, and IL-12 (**A**), IFN-γ (**B**), and TNF-α (**C**) levels were determined using ELISA. The data are shown as the mean ± SD for the three groups. Ctr indicates the untreated control. Statistical p values were determined using a two-tailed t-test: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with untreated cells.

Immune Response of Bacterial Strains *In Vivo*

To investigate the effects of the bacterial strains *in vivo*, we fed freeze-dried bacteria to mice. Mice were orally administered with freeze-dried heat-killed bacteria for 14 days, and LPS was intraperitoneally injected into the mice of each group. Blood was collected 24 h after LPS injection, and the levels of TNF-α were measured. The TNF-α levels of the blood from *L. sakei*- and *L. plantarum*-fed mice were significantly lower as compared with the LPS-only-injected control group and the other bacterial strain groups (Fig. 2A). Mice in the *L. plantarum*-fed group also maintained the best health conditions compared with the other bacterial strain groups and the control group during the study. This result suggests that *L. sakei* and *L. plantarum* can inhibit excessive inflammation caused by pathogen infection. To detect antigen-specific IgE, we fed mice for 14 days before OVA immunization. Seven days after immunization, blood was collected and the levels of anti-OVA IgE were measured. Blood from the *L. plantarum*-fed group showed slightly increased anti-OVA IgE levels as compared with the control group and the other bacterial strain groups (Fig. 2B), indicating that heat-killed *Lactobacillus* strains and *S. aureus* do not affect OVA-specific IgE expression.

Cytokine Balance Shifting by Bacterial Strains in LPS-Stimulated Splenocytes

To clarify the different immune responses *in vitro*, we obtained mouse splenocytes from OVA-immunized mice (two times in 14 days), which were orally administered with freeze-dried heat-killed bacteria for 2 weeks before the first OVA immunization. After extraction of the splenocytes, the cells were treated with OVA and LPS for 48 h and the culture supernatants were collected. OVA and LPS-mediated TNF-α and IL-12 production levels in the

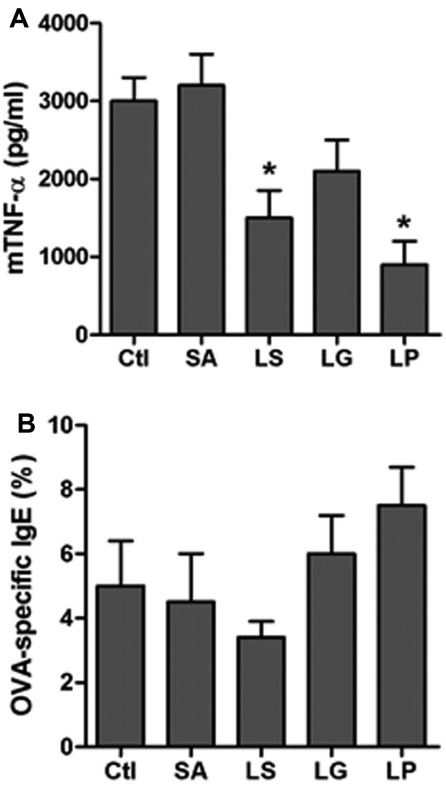

Fig. 2. Effect of *L. plantarum* an mouse TNF-α and IgE production.

(**A**) *L. plantarum* decreases TNF-α production in LPS-injected mice. Mice were orally administered 1×10^7 freeze-dried heat-killed bacteria in 1% skim milk (5 mg/day) for 2 weeks before LPS injection (10 mg/kg). Blood was collected 24 h after LPS injection and TNF-α levels were determined by ELISA. (**B**) *L. plantarum* increases OVA-specific IgE production in OVA immunized mice. Mice were orally administered with 1×10^7 freeze-dried heat-killed bacteria in 1% skim milk (5 mg/day) for 2 weeks before ovalbumin (OVA) (20 μg/mouse in alum) immunization. Blood was collected 7 days after OVA immunization and OVA-specific IgE levels were determined by ELISA. The control blood (CB) was obtained from mice that were immunized three times with OVA. CB levels were considered 100%. The data are represented as the mean ± SD from five mice per group. Statistical *p* values were determined using a two-tailed *t*-test: $*p < 0.05$ compared with the control. Ctl, control; SA, *Staphylococcus aureus*; LS, *Lactobacillus sakei*; LG, *Lactobacillus rhamnosus* GG; LP, *Lactobacillus plantarum*.

splenocytes of *L. plantarum*-fed mice were significantly decreased as compared with the other strains (Figs. 3A and 3B). On the other hand, IL-4 production from the splenocytes of *L. plantarum*-fed mice was significantly increased (Fig. 3C). These results suggest that orally administered *L. plantarum* can inhibit the endotoxin-mediated excessive Th1 response and it may drive the Th2 immune response.

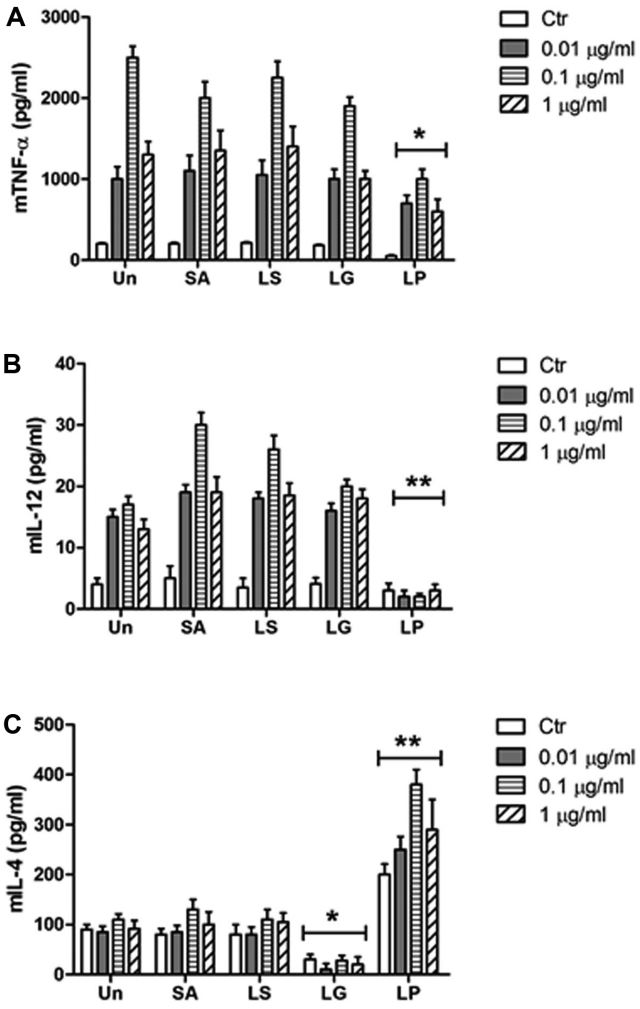

Fig. 3. Splenocytes from *L. plantarum*-administered mice decreased TNF-α and IL-12 production and increased IL-4 production.

Mice were orally administered freeze-dried heat-killed bacteria in 1% skim milk (5 mg/day) for 2 weeks before ovalbumin (OVA) (20 μg/mouse in alum, two times in 14 days) immunization. Mouse splenocytes were obtained from OVA-immunized mice and immunized with OVA (5.0 μg/ml) and LPS. Culture supernatants were collected 48 h after LPS treatment and the TNF-α (**A**), IL-12 (**B**), and IL-4 (**C**) levels were determined *via* ELISA. The data are represented as the mean ± SD from three groups. Statistical *p* values were determined using a two-tailed *t*-test: $*p < 0.05$; $**p < 0.01$ compared with untreated control cells. Ctl, control; SA, *Staphylococcus aureus*; LS, *Lactobacillus sakei*; LG, *Lactobacillus rhamnosus* GG; LP, *Lactobacillus plantarum*.

Effect of LTAs on Cytokine Production

To identify the immune regulatory effects of bacteria cell wall components, we purified lipoteichoic acid (LTA) from *L. sakei* (sLTA) and *L. plantarum* (pLTA). LTA isolated from *S. aureus* was used as a control (aLTA). Protein and endotoxin

contamination of the purified LTA were examined by silver staining (Fig. 4A) and LAL-testing (Table 1), respectively. The priming treatment of sLTA, pLTA, and aLTA followed by LPS retreatment in splenocytes isolated from OVA-immunized mice significantly decreased mouse TNF-α (Fig. 4B) and IL-4 (Fig. 4C) production. Interestingly, unlike heat-killed bacteria, the *Lactobacillus* LTAs inhibited IL-4 production. The discrepancy of these two experiments may be caused by the dosage of material used. Approximately, 100 μg/ml LTA corresponds to 6×10^{10} bacteria. However, IL-12 production was not affected by pLTA and sLTA. High aLTA doses significantly increased IL-12 production (Fig. 4D). Together, LTA isolated from *Lactobacillus* strains had an inhibitory effect on endotoxin-mediated inflammation, which may suggest that LTA downregulates both the Th1 and Th2 responses.

Table 1. LAL test to confirm endotoxin contamination in LTAs.

LTAs	*S. aureus*	*L. sakei*	*L. rhamnosus* GG	*L. plantarum*
OD 405 nm	0.031	0.021	0.025	0.020
EU/ml	0.045	0.028	0.037	0.026

LAL, *Limulus* amebocyte lysate; LTAs were isolated from *S. aureus*, *L. sakei*, *L. rhamnosus* GG, and *L. plantarum*.

Discussion

Numerous reports have suggested that LAB influence the immune system and have the potential to promote beneficial effects in some immune disorders [14]. Our study focused on the immune characteristics of two *Lactobacillus* strains that were isolated from *kimchi*. In the present study, *L. sakei* showed greater immune-inducing potential than the other bacterial strains under *in vitro* conditions. The immune-inducing potential of *L. plantarum*, however, was very low. Thus, our results suggest that *L. sakei* could be a powerful candidate for an immune-stimulating LAB probiotic. It is known that LPS is a powerful immune-stimulating material [25]. It is supposed that the LTA of *L. sakei* is an immune booster like LPS, since sLTA dramatically increased pro-inflammatory cytokine production (data not shown). More investigation is needed

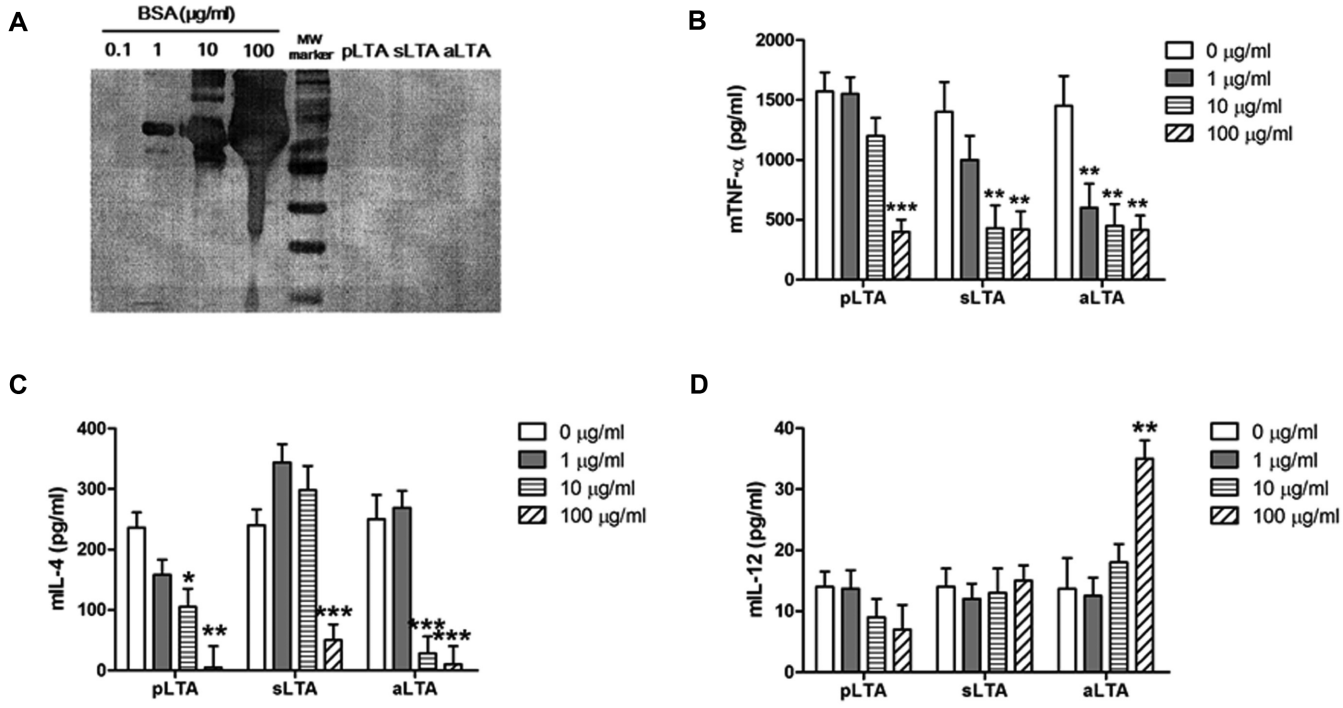

Fig. 4. The different cytokine production potential by LTA priming treatment in mouse splenocytes.
(**A**) LTAs were prepared as described in the Materials and Methods. The contamination of purified LTAs was determined by silver staining. The splenocytes were obtained from ovalbumin (20 μg/mouse in alum)-immunized mice (two times in 14 days). LTAs were pretreated at the indicated concentrations in mouse splenocytes (1×10^6 cells/ml) for 24 h, and then 0.1 μg/ml LPS was retreated for 24 h. Culture supernatants were collected and the TNF-α (**B**), IL-14 (**C**), and IL-12 (**D**) levels were determined by ELISA. Statistical p values were determined using a two-tailed t-test: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with LTA-untreated control cells.

characteristics of *L. sakei* and *L. sakei* LTA were studied only in *in vitro* conditions; some reports state that comprehensive *in vivo* results are needed to clarify the present findings. We also focused on the characteristics of *L. plantarum* that are involved in the immune stimulating potential that showed no effects compared with the other LAB.

To investigate the differences in immune reactions under *in vivo* conditions between each strain that showed significantly different reactions under *in vitro* conditions, BALB/c mice were orally administered with freeze-dried heat-killed bacteria. Two weeks later, mice were injected with OVA and LPS to induce sepsis. The *L. sakei*- and *L. plantarum*-fed mice displayed reduced septic shock effects in the LPS-injected condition as compared with those of the other bacterial strains and the control. Further study to detect blood immunoglobulin levels in the OVA-challenged mice revealed that *L. plantarum* represses the Th1 immune response. The levels of total OVA-specific IgG and total IgE were not changed significantly (data not shown) in *L. plantarum*-fed mice, but the OVA-specific IgE level was slightly increased. This result may represent the increase in the Th2 immune response, which represses Th1 polarization. In previous studies, it was reported that treatment with *L. plantarum* increased IL-12 levels in splenocytes [10]. However, heat-killed *L. plantarum*-mediated mouse IL-12 blood levels were moderately increased in the current study. The differences between these two experiments could have been due to the direct treatment of bacteria to splenocytes, which may increase high levels of cytokine production, whereas indirect treatment *via* oral administration may not cause the same effect. In the present study, we also noticed that the influence of the effects of bacterial strains on the immune response was entirely consistent in both the *in vivo* and *in vitro* conditions.

Numerous reports have considered the influence of LAB on the Th1 and Th2 immune responses [3]. The current study demonstrates a repression of the Th1 immune response, and the Th1 immune response is considered to induce inflammatory reactions. It could, therefore, be assumed that *L. plantarum* shows potential for inducing anti-inflammatory effects. It is known that excessive inflammation induces various immune disorders [5]. *L. plantarum* is supposed to have beneficial influences on inflammatory disorders, and there is some possibility of using *L. plantarum* as a therapeutic bacterial strain. Previously, we reported that *L. plantarum* and its LTA have potential as a medical treatment material for septic shock conditions in an *in vitro* environment [18, 19]. There must, therefore, be more studies investigating the effects of *L. plantarum* on excessive inflammatory conditions.

In conclusion, we focused on two LAB strains, *L. sakei* and *L. plantarum*, which were isolated from *kimchi*. We characterized the immune stimulating effects of *L. sakei in vitro* and the Th1 immune response-repressing influence of *L. plantarum*. These two strains are predicted to have the potential to induce beneficial health effects. However, larger numbers of LAB strains remain to be examined in *kimchi*, and further studies are needed to discover their characteristics.

Acknowledgments

This work was supported by a grant from Kyung Hee University in 2011 (KHU-20110263).

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