Differential Cytokine Regulatory Effect of Three Lactobacillus Strains Isolated from Fermented Foods

Yoon-Doo Lee†, Yi-Fan Hong1,2†, Boram Jeon1, Bong Jun Jung1, Dae Kyun Chung1,2,3*, and Hangeun Kim1,2*

1Graduate School of Biotechnology and Institute of Life Science and Resources, Kyung Hee University, Yongin 17104, Republic of Korea
2Skin Biotechnology Center, Kyung Hee University, Yongin 17104, Republic of Korea
3RNA Inc., College of Life Science, Kyung Hee University, Yongin 17104, Republic of Korea

Introduction

Lactic acid bacteria (LAB) have many beneficial effects in cancer, oxidative stress, maintenance of the normal microbiota of the digestive tract, gastroenteritis, and immune regulation [23]. Currently, it is known that LAB and their fermented products are effective at enhancing innate and adaptive immunity, including the prevention of gastric mucosal lesion development, alleviation of allergy development, and inhibition of intestinal pathogen infection [30]. Probiotics are microorganisms that are believed to provide health benefits when consumed. LAB, including members of the genus Lactobacillus, are members of the commensal microorganisms of the gastrointestinal tract of humans and mammals and are generally recognized as probiotics [5, 13]. Intestinal LAB promote the health of the host by modulating immune responses [2, 11]. They develop and maintain the homeostasis of the intestine-associated immune system via regular interactions with intestinal cells such as antigen-presenting cells and intestinal epithelial cells. In particular, bacteria belonging to the genus Lactobacillus are recognized by human cells and induce cytokine production [9, 30]. It is also reported that lactobacilli alleviate allergic reactions by maintaining the balance between Th helper type (Th) 1 and Th2 responses [33].

The effects of LAB on serum levels of cytokines and...
endotoxins in peritoneal dialysis patients have been examined previously. In patients receiving probiotics, serum levels of cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-5, and IL-6 and endotoxins were decreased after six months of administration, while the serum level of IL-10 was increased [32]. When LAB were administered to inflammatory bowel disease model mice, decreased levels of nitric oxide (NO) and interferon-gamma were observed, suggesting that LAB exerted anti-inflammatory effects and contributed to a rapid recovery of dextran sulfate sodium-induced acute colitis [27, 29]. Another study revealed that Lactobacillus treatment can manage organ damage associated with arthritis by down-regulating the levels of pro-inflammatory cytokines and up-regulating the levels of anti-inflammatory cytokines in serum [3]. It was also reported that L. plantarum and its cell wall components decrease pro-inflammatory cytokines such as TNF-α, IL-1β, IL-8, and NO, while they induce the anti-inflammatory cytokine IL-10 [17, 21, 28]. Furthermore, long-term administration of L. plantarum suppressed the tumor frequency, increased CD4+ T-cells in tumor tissue, and reduced the serum concentration of TNF-α [14].

Most studies have shown that LAB, including L. plantarum, have protective effects against pathogen-mediated diseases and various types of cancer. In this study, we focused on the immune characteristics of the L. plantarum K55-5 strain, which was isolated from kimchi (a Korean traditional fermented vegetable) and L. plantarum K55-5 was isolated from a dairy product. Strains were grown in MRS broth for 24 h at 37°C. After being centrifuged, cells were resuspended in sterilized water and concentrated to 10^6 cells/ml for this experiment. To make heat-killed bacteria, cells were incubated at 80°C for 30 min. Heat-treated bacteria (80°C, 30 min) were disrupted by microfluidizer (10,000–13,000 psi, 3–10°C) to make heat-killed lysates. After centrifugation of the cell lysates at 18,000 ×g for 30 min, the supernatants were collected.

### Materials and Methods

#### Materials

- Cyclophosphamide (CY, C7379), lipopolysaccharide (LPS, from Escherichia coli 055:B5), and concanavalin A (ConA, from Canavalia ensiformis) were purchased from Sigma-Aldrich (MO, USA). Mouse TNF-α High Sensitivity enzyme-linked immunosorbent assay (ELISA) (eBioscience, CA, USA), IL-10 and interferon (IFN)-γ ELISA kits (ENZO, NY, USA), Mouse IL-12 p70 IDELISA (ID Labs, NY, USA), IgA ELISA kit (Bethyl, TX, USA), and ACK lysing buffer (Lonza, MD, USA) were used in this study. Neutralization antibody for TLR2 was purchased from eBioscience, and signaling inhibitors for nuclear factor-kappa B (NF-κB), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 were purchased from InvivoGen (CA, USA).

#### Cell Culture and Stimulation

- RAW 264.7, a mouse macrophage cell line, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and antibiotics such as penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were cultured at 37°C with 5% CO₂ in a humidified incubator.

#### Preparation of Lactic Acid Bacteria

- L. sakei K101 and L. plantarum K8 strains were isolated from kimchi (a Korean traditional fermented vegetable) and L. plantarum K55-5 was isolated from a dairy product. Strains were grown in MRS broth for 24 h at 37°C. After being centrifuged, cells were resuspended in sterilized water and concentrated to 10^6 cells/ml for this experiment. To make heat-killed bacteria, cells were incubated at 80°C for 30 min. Heat-treated bacteria (80°C, 30 min) were disrupted by microfluidizer (10,000–13,000 psi, 3–10°C) to make heat-killed lysates. After centrifugation of the cell lysates at 18,000 ×g for 30 min, the supernatants were collected.

### Isolation and Culture of Splenocytes

- Spleens were isolated from normal female BALB/c mice. Each spleen was suspended and filtered through a sieve mesh. Single-cell spleen suspensions were pooled in serum-free RPMI 1640 medium. Red blood cells were lysed with ACK lysing buffer. Total numbers of splenocytes, dyed with crystal violet, were counted under light microscopy. The cells were cultured in 24-well plates (2 × 10^5 cells/well) at 37°C in RPMI 1640 supplemented with 10% FBS and penicillin/streptomycin. To determine the immunomodulation effect of splenocytes, cells were incubated with L. sakei K101, L. plantarum K8, and L. plantarum K55-5.

### In Vivo Mouse Study

- Seven-week-old female BALB/c mice were grouped into four groups (16 mice per group). One group was a non-treated group and the others were immunosuppressed and treated with LAB. Immunosuppressed mice were generated by intraperitoneal injection with cyclophosphamide (200 mg/kg) and were orally administered L. sakei K101 (1 × 10^8 CFU/day), L. plantarum K55-5 (1 × 10^8 CFU/day), or saline for 7 days. After the treatment, fecal samples and mouse serum were collected and the spleens were isolated to determine cytokine production. The mice were cared for and used in accordance with the guidelines of the animal ethics committee at Global Campus of Kyung Hee University (KHU-14-021).

### Natural Killer Cell Cytotoxic Activity

- Effector cells (isolated spleen cells) and targets (YAC-1) cells were co-cultured at an effector:target ratio of 50:1 for 24 h. Cytotoxicity was assessed using an LDH-cytotoxicity assay kit (bioWorld, MN, USA), according to the manufacturer’s instructions. Briefly, cells were harvested and washed with cold PBS, and then homogenized in 2 volumes of cold assay buffer. After centrifugation at 10,000 ×g
Differential Cytokine Production by \textit{Lactobacillus} Strains

### Determination of Cytokines

To measure the levels of IL-10, IL-12, IFN-\(\gamma\), and TNF-\(\alpha\) by ELISA (R&D Systems, MN, USA), splenocytes were stimulated with \textit{L. sakei} K101, \textit{L. plantarum} K8, and \textit{L. plantarum} K55-5. Cytokine levels were determined from splenocyte culture supernatants or mouse serum using commercial ELISA kits.

### Preparation of LTA

Highly pure and structurally intact LTA was isolated from \textit{L. plantarum} K8 (KCTC10887BP), \textit{L. sakei} K101 (KCCM11175P), \textit{L. rhamnosus} GG, and \textit{L. plantarum} K55-5 by \(n\)-butanol extraction, as described previously [16]. The purity of the purified LTA was determined by measuring the protein contamination and endotoxin content through conventional silver staining after SDS-PAGE and the \textit{Limulus} amebocyte lysate assay (BioWhittaker, MD, USA), respectively.

### Statistics

In vitro experiments were performed at least three times. The data shown are representative results of triplicate experiments and are presented as the mean \pm SD. Differences were judged to be statistically significant when the \(p\) value was < 0.05. In vivo data were analyzed by one-way analysis of variance, followed by the Kruskal-Wallis multiple comparison test. Results were presented as the mean \pm standard error.

### Results

\textit{L. sakei} K101 and \textit{L. plantarum} K55-5 Increased Cytokine Production in the Splenocytes Isolated from Mouse

Splenocytes isolated from BALB/c mice were stimulated with the indicated dose of live \textit{L. sakei} K101, \textit{L. plantarum} K55-5, or \textit{L. plantarum} K8. One \(\mu g/ml\) LPS and 5 \(\mu g/ml\) ConA were used as positive controls. \textit{L. sakei} K101 increased IL-10, IL-12, IFN-\(\gamma\), and TNF-\(\alpha\) levels in a dose-dependent manner, although their production levels were lower than those produced by \textit{L. plantarum} K55-5. \textit{L. plantarum} K55-5

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Cytokine expression in the splenocytes isolated from mice. Splenocytes were isolated from BALB/c mouse and treated with the indicated dose of \textit{L. sakei} K101, \textit{L. plantarum} K55-5, \textit{L. plantarum} K8, 1 \(\mu g/ml\) LPS, and 5 \(\mu g/ml\) ConA for 24 h. IL-10 (A), IL-12 (B), IFN-\(\gamma\) (C), and TNF-\(\alpha\) (D) expression levels from the culture supernatants were examined by the ELISA method. *\(p < 0.05\); **\(p < 0.01\); ***\(p < 0.001\) compared with the control (Ctr).}
\end{figure}
caused dose-dependent increases in IL-10, IFN-γ, and TNF-α. Interestingly, *L. plantarum* K55-5-mediated IL-12 production was highest at $10^7$ CFU and decreased at $10^8$ CFU (Figs. 1A to 1D). On the other hand, cytokine production by *L. plantarum* K8 was no altered. These data suggest that both *L. sakei* K101 and *L. plantarum* K55-5 have the ability to regulate cytokine production in mouse splenocytes. Similar results were shown in peritoneal macrophages isolated from mice (data not shown).

**Cytokine Expression Was Increased in the *L. sakei* K101- and *L. plantarum* K55-5-Fed Immunosuppressed Mice**

To suppress the natural immunity, BALB/c mice were intraperitoneally injected with cyclophosphamide (CY) and then mice were orally administered with LAB for 7 days. Cytokine expression was examined by ELISA using splenocytes culture supernatants. The expression levels of IL-10 were not significantly altered (Fig. 2A). Cytokine expression, such as IL-12, IFN-γ, and TNF-α, was significantly increased in *L. sakei* K101- and *L. plantarum* K55-5-treated mice as compared with the Saline control (Figs. 2B to 2D), whereas they were not significantly increased by *L. plantarum* K8 except TNF-α (Fig. 2D).

Next, the level of cytokine in the blood samples that were collected from immunosuppressed mice fed with LAB was examined. The serum IL-10 level was slightly increased by *L. sakei* K101 and *L. plantarum* K55-5 as compared with mice in the immunosuppressed control (CY+Saline) group (Fig. 3A), whereas the level of other cytokines was significantly altered by *L. sakei* K101 and *L. plantarum* K55-5 (Figs. 3B to 3D). The serum TNF-α level in *L. plantarum* K8-fed mice was also increased as compared with the Saline control (Fig. 3D). These results suggest that *L. sakei* K101 and *L. plantarum* K55-5 can increase immune activity in immunosuppressed mice.

**NK Cell Cytotoxic Activity Was Increased in the *L. sakei* K101- and *L. plantarum* K55-5-Fed Immunosuppressed Mice**

NK cells are a type of cytotoxic lymphocyte of the innate immune system that control tumors and microbial infections [31]. To examine whether NK cell activity can be affected by orally administered LAB, mouse splenocytes were isolated from LAB-fed immunosuppressed mice, and

![Fig. 2. Cytokine expression in the splenocytes isolated from LAB-fed mice.](image-url)

The level of IL-10 (A), IL-12 (B), IFN-γ (C), and TNF-α (D) from the splenocytes was estimated by ELISA. *p < 0.05; **p < 0.01; compared with the Saline control (CY+Saline).
incubated with YAC-1 cells at a ratio of 50:1 for 24 h. The body and spleen weights were not different between control and experimental groups (Figs. 4A and 4B). The NK cell cytotoxicity was examined by a modified lactate dehydrogenase (LDH) release assay. NK cell cytotoxicity was decreased by Cy+Saline treatment and it was increased in LAB-fed mice, but \textit{L. plantarum} K8 did not affect NK cell activity (Fig. 4C). \textit{L. sakei} K101 and \textit{L. plantarum} K55-5 increased NK cell cytotoxicity by 3.8- and 4.3-fold, respectively.

Heat-Killed Bacterial Lysates Increased TNF-\(\alpha\) Production in the RAW 264.7 Cells

To identify which bacterial components affect cytokine production, TNF-\(\alpha\) expression was examined with live, heat-killed, bacterial lysates, and clear lysate supernatant in the RAW 264.7 cell line. As shown in Fig. 5A, heat-killed \textit{L. sakei} K101 and \textit{L. plantarum} K55-5 increased more TNF-\(\alpha\) production than live bacteria. When cells were treated with heat-killed bacterial lysates, more TNF-\(\alpha\) production was shown as compared with heat-killed bacteria, whereas clear supernatants from bacterial lysates of both LAB had no increased level of TNF-\(\alpha\) although they also increased its production (Fig. 5A). Heat-killed lysates of \textit{L. sakei} K101 and \textit{L. plantarum} K55-5 most significantly increased TNF-\(\alpha\) production when macrophages were treated with \(1 \times 10^8\) CFU/ml (Fig. 5B) for 6 h (Fig. 5C). On the other hand, heat-killed lysates of \textit{L. plantarum} K8 did not affect TNF-\(\alpha\) induction. These results indicate that bacterial cell wall components, but not others such as DNA, RNA, or protein, may affect TNF-\(\alpha\) production in RAW 264.7 cells.

LTA Increased TNF-\(\alpha\) Production through the TLR2-Mediated Signaling Pathway

To examine whether LTA, a component of gram-positive cell wall, contributes to cytokine induction, LTAs were isolated from \textit{L. plantarum} K8, \textit{L. rhamnosus} GG, \textit{L. sakei} K101, and \textit{L. plantarum} K55-5. Lipopolysaccharide (LPS) was used as a control. Both K101 and K55-5 LTA induced significant TNF-\(\alpha\) production, whereas \textit{L. plantarum} K8 and \textit{L. rhamnosus} GG did not affect TNF-\(\alpha\) production in RAW 264.7 cells (Fig. 6A). Next, signaling activation was examined.
in LTA-treated cells. As shown in Fig. 6B, *L. sakei* K101 and *L. plantarum* K55-5 increased the phosphorylation of NF-κB p65 and p38. When cells were treated with specific inhibitors, TNF-α production was significantly inhibited by NF-κB.
Differential Cytokine Production by Lactobacillus Strains

September 2016  Vol. 26  No. 9

Neutralization assay using anti-TLR2, -TLR4, and -CD14 antibodies revealed that the activation of NF-κB and p38 was mediated by the TLR2 signaling pathway (Fig. 6D). These results suggest that L. plantarum K55-5 LTA increases TNF-α production via NF-κB and p38 activated by the TLR2-mediated signaling pathway.

Discussion

LAB, especially probiotic resident bacteria of the large intestine, play an important role in promoting the health of the host, including modulation of immune responses. LAB are involved in the development and maintenance of homeostasis in the intestine-associated immune system. Cell wall components of LAB mediate the activation of immunocompetent cells in the intestinal tract [11]. Intestinal LAB interact with intestinal cells, which include antigen-presenting cells and intestinal epithelial cells [2, 12, 25]. They also moderate allergic reactions by maintaining the balance between Th1 and Th2 responses [4]. In the present study, L. sakei 101 and L. plantarum K55-5 showed an immune-inducing potential in the immunosuppressed mice model. The immune-inducing potential of L. plantarum K55-5 was higher than that of L. sakei 101, suggesting that L. plantarum K55-5 could be a powerful candidate for an immune-stimulating LAB probiotic. In particular, we observed increased levels of cytokines from blood and splenocytes and activation of NK cells from mice treated with L. plantarum K55-5. Lipoteichoic acid isolated from L. plantarum K55-5 induced a high level of TNF-α production. Thus, our data suggest that L. plantarum K55-5 may be used...
for the treatment of immune disorders.

Cytokines such as IL-10, IL-12, IFN-\(\gamma\), and TNF-\(\alpha\) play an important role in immune regulation. For example, IL-10 is known to repress pro-inflammatory responses and maintain normal tissue homeostasis [24]. IL-12 is composed of p35 and p40 subunits and is produced by activated monocytes, macrophages, and dendritic cells, resulting in the activation and differentiation of T cells. IL-12 is required for the induction of IFN-\(\gamma\) production and is critical for the induction of Th1 cells [6]. IFN-\(\gamma\) is produced mainly by T-cells and NK cells and serves as a key mediator of antiviral defenses and inflammation [1]. TNF-\(\alpha\) regulates immune cells and induces systemic inflammation. It is also involved in the induction of fever, cell death, tumorigenesis, viral replication, and sepsis. TNF-\(\alpha\) is primarily produced by activated macrophages, although many other cell types such as CD4\(^+\) lymphocytes, NK cells, neutrophils, mast cells, and eosinophils produce it [18]. Previously, we reported that L. sakei K101 has an immune regulatory effect [7, 10]. In that study, we compared heat-killed L. sakei 101 and L. plantarum K8, and found that L. sakei K101 and its LTA have a greater immune-stimulating potential in IL-12, IFN-\(\gamma\), and TNF-\(\alpha\) production, as compared with L. plantarum K8, in in vitro conditions. It is known that L. plantarum moderately induces pro-inflammatory cytokine production from macrophages. In fact, L. plantarum K8, isolated from kimchi, does not induce significant variation of cytokine production in macrophages [15, 16]. However, interestingly, L. plantarum K55-5, which was isolated from dairy food, showed a different cytokine regulatory effect as compared with L. plantarum K8. In the current study, we compared live L. sakei 101 and L. plantarum K8 together with L. plantarum K55-5 to examine their immune regulatory effects in the immunosuppressed mouse model and we found that L. plantarum K55-5 induced greater inflammatory cytokine production than did L. sakei K101. When we compared L. plantarum K55-5 with L. plantarum K8, L. plantarum K55-5 had a much better cytokine-inducing ability, suggesting that different strains of the same species of LAB have different immune regulatory effects [19].

The species-specific immune-stimulatory potency of different LAB could be due to structural differences in the LTA, a cell wall component of gram-positive bacteria [26]. For example, the contents of D-alanine in the repeating units of LTA were shown to be key determinants of the immunostimulating ability of the LTA [20]. The differences in lipid anchor structure in LTAs isolated from Staphylococcus aureus and L. plantarum result in different biological functions [8]. Removal of the acryl group of LTA from S. aureus diminishes immunostimulatory activity [22]. However, LTA isolated from L. plantarum without an acyl group did not inhibit LPS-induced TNF-\(\alpha\) production, whereas intact LTA showed an inhibition effect against LPS [16]. These studies suggest that different cell wall structures may contribute to different immune regulatory effects and that L. plantarum K8 and K55-5 strains may have different cell wall structures. In the unpublished studies, we found that LTA from L. plantarum K8 and K55-5 had different structures and TLR2 affinities. More studies, however, are needed to identify why the same L. plantarum genus have different cell wall structures and contribute different immune regulatory activities. Previously, we had shown that L. plantarum K8 and its LTA have some potential as a medical treatment for septic shock conditions in an in vitro environment [15, 16]. Through the studies, we observed that L. plantarum K8 had beneficial influences on inflammatory disorders, and that there was some possibility of using L. plantarum K8 as a therapeutic bacterial strain since L. plantarum K8 moderately induced the expression of inflammatory cytokines while it significantly inhibited pathogen-mediated excessive inflammation. At this moment, we only know that L. plantarum K55-5 has more immune-activating ability than L. plantarum K8. More properties of L. plantarum K55-5 should be characterized in further studies.

In conclusion, we have focused on three Lactobacillus strains, L. sakei K101 and L. plantarum K8, which were isolated from kimchi and L. plantarum K55-5, which was isolated from a dairy product. The immune-stimulating effects of the three strains were characterized in vivo using immunosuppressed mice and in vitro using RAW264.7 cell line studies, and we found that L. plantarum K55-5 had very strong immune-inducing activities as compared with L. plantarum K8 and L. sakei K101. Our data indicate that L. plantarum K55-5 has the potential to induce beneficial health effects in patients with some diseases related to immune suppression.

Acknowledgments

This research was supported by a grant from the regional innovation center program of the Ministry of Trade, Industry and Energy at the Skin Biotechnology Center of Kyung Hee University, Korea.

References

is associated with lack of protection from intravaginal simian immunodeficiency virus SIVmac239 challenge in simian-human immunodeficiency virus 89.6-immunized rhesus macaques. J. Virol. 78: 841-854.


