Antioxidant and Anti-Inflammatory Effect of Probiotic Lactobacillus plantarum KU15149 Derived from Korean Homemade Diced-Radish Kimchi

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Lactobacillus plantarum KU15149 was demonstrated to have probiotic behavior and functions, including antioxidant and anti-inflammatory activity. L. plantarum KU15149 obtained from homemade diced-radish kimchi has a high survival rate under artificial gastric acid (pH 2.5, 0.3% pepsin) and bile salt (0.3% oxgall) conditions. However, L. plantarum KU15149 did not produce β-glucuronidase, which is known to be a carcinogenic enzyme with resistance to several antibiotics, such as gentamycin, kanamycin, streptomycin, tetracycline, and ciprofloxacin. L. plantarum KU15149 strongly adhered to HT-29 cells and had high antioxidant activity in terms of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging and β-carotene bleaching assays.

L. plantarum KU15149 also exhibited a pronounced inhibition of nitric oxide (NO) production, along with expression of nitric oxide synthase (iNOS) and cyclooxygenase -2 (COX-2) as well as pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, when RAW 264.7 cells were stimulated with LPS. Therefore, L. plantarum KU15149 exhibited pharmaceutical functionality as a potential probiotic.

Keywords: Probiotics, kimchi, Lactobacillus plantarum, antioxidant, anti-inflammatory

Introduction

Oxidative stress is caused by a pro-oxidant and antioxidant imbalance that leads to the generation of toxic reactive oxygen species (ROS), such as hydrogen peroxide, organic hydroperoxides, superoxide, hydroxyl radicals, and nitric oxide. Persistent oxidative damage of tissue and cellular components can cause several diseases and accelerate the aging process in humans [1].

Inflammatory reactions and ROS perform indispensable physiological functions in immune defense and cell signaling [2]. However, undue or continued ROS production and inflammation may result in a number of health problems, such as type 2 diabetes, cardiovascular disease, osteoporosis, insulin resistance, inflammatory bowel disease, arthritis, and asthma [3]. Therefore, ROS levels and inflammatory responses are important for reducing the risk of related chronic diseases.

The inflammatory response in the body is a defense against risk stimuli, including microbial infections, endotoxins, and tissue damage, and it is necessary to restore the normal structure and function of tissues. Normal inflammatory responses have a regulatory process during which the production of pro-inflammatory mediators decreases over time, while anti-inflammatory mediators rise in number, thereby limiting the inflammatory response itself [4]. Macrophages, one of the cell types involved in the body’s inflammatory response, play an important role in this inflammatory response. Macrophages are activated by pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and lipopolysaccharide (LPS), and exposure to LPS stimulation, which is a bacterial cell membrane component. In addition, macrophages produce various inflammatory mediators, such as nitric oxide (NO) through expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase -2 (COX-2) [5].

Probiotics are defined as live microorganisms that when administered in appropriate amounts, confer a beneficial effect upon the host [6] and contribute to the regulation of immune responses [7]. Lactic acid bacteria (LAB) have been used as probiotics and can survive under strong acid and bile salt conditions while adhering to the cells of the intestinal tract [8]. LAB strains are also generally known to be non-pathogenic and sensitive to antibiotics [9]. In addition, several studies have reported that probiotics have antimicrobial, antioxidant, anticancer, antiallergy, and immune-enhancing activities [10,11]. Probiotics have also been reported to have several positive and beneficial effects on human health [12].
Kimchi is a Korean traditional fermented vegetable product that is gaining popularity as a functional food as it contains various LAB, such as *Lactobacillus plantarum* and *Leuconostoc mesenteroides* [13]. In particular, LAB strains isolated from kimchi can survive in the intestines due to their strong resistance to acid and bile salt conditions [14]. Kimchi LAB are probiotic strains that exert various beneficial effects, such as antioxidant, antimicrobial, antimutagenic, anticancer, immune-regulation, anti-inflammatory, anti-allergic, anti-obesity, cholesterol-lowering, and lipid-lowering activities [15].

*L. plantarum* is the most important and prominent microorganism involved in the middle and latter steps of kimchi fermentation [16]. *L. plantarum* is a generally recognized as safe (GRAS) microorganism and has been used as a probiotic strain and fermentation starter in various foods, including meat, dairy products, plant-based products, and coffee [17]. Furthermore, various studies have reported that *L. plantarum* has immune stimulation, inflammatory reduction, and antioxidant activities [18].

Therefore, the aim of the present study was to determine the probiotic properties, safety, and functional effects of LAB strains isolated from homemade diced-radish kimchi. Further, the antioxidant and anti-inflammatory activities of the isolated strains were investigated.

**Material and Methods**

**Chemicals and Reagents**

MRS and oxgall were purchased from Becton Dickinson Biosciences (USA). Pepsin, ascorbic acid, β-carotene, linoleic acid, chloroform, Tween 80, Triton X-100, 2,2-diphenyl-1-picrylhydrazil (DPPH), lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and Griess reagent were purchased from Sigma Chemical Co., Ltd. (USA). Dulbecco’s Modified Eagle’s Medium (DMEM), RPMI1640, water, antibiotics, fetal bovine serum (FBS), phosphate-buffered saline (PBS), and 1% streptomycin/penicillin solution were purchased from HyClone Laboratories, Inc. (USA). The API ZYM kit was purchased from bioMérieux (France). Specific primers used for performing the real-time polymerase chain reaction (RT-PCR) were purchased from Bionics (Korea).

**Bacterial Strains and Sample Preparations**

*Lactobacillus plantarum* KU15149 was isolated from homemade diced-radish kimchi. Kimchi samples (1 g) were diluted and inoculated on MRS agar at 37°C for 24 h. The selected strain was inoculated and incubated in MRS broth at 37°C for 24 h and identified as *L. plantarum* by 16S rRNA gene sequencing. The commercial probiotic strain, *L. rhamnosus* GG (LGG), was obtained from the Korean Collection for Type Cultures (Korea) and used as a reference probiotic strain. To harvest the LAB cells, bacterial cell cultures were centrifuged at 12,000 ×g for 10 min and washed three times with PBS. The LAB cells were suspended in PBS.

**Cell Cultures**

HT-29 (human colon adenocarcinoma) and RAW 264.7 (murine macrophage) cell lines were obtained from the Korean Cell Line Bank (Korea) and respectively cultured in RPMI and DMEM supplemented with 10% FBS and 1% streptomycin/penicillin solution at 37°C in a humidified atmosphere containing 5% CO₂.

**Tolerance to Artificial Gastric Conditions of LAB Strains**

The tolerance of LAB strains to artificial gastric conditions was evaluated as described by Lee et al. [19]. The LAB strains were incubated in an overnight culture and resuspended in the MRS medium (pH 2.5) containing 0.3% (w/v) pepsin and oxgall, and then incubated at 37°C for 3 h and 24 h. The number of surviving bacteria was determined by counting viable cells on the MRS plates.

**Enzyme Production of LAB Strains**

Enzyme production was assessed using the API ZYM kit. The LAB strains were centrifuged at 12,000 ×g for 10 min, and the cell pellet was resuspended in PBS at 10⁹ CFU/ml and added to the cultures. After inoculation, the mixtures were incubated at 37°C for 4 h and reagents zym A and zym B were each added. The level of enzyme activity was determined as 0 (no activity) to 5 (≥ 40 nM) based on the color change.

**Adhesion of LAB Strains to HT-29 Cells**

The adhesion ability of LAB strains to HT-29 cells was described as the percentage of viable bacteria remaining as compared to the initial bacterial counts added. Adherence to HT-29 cells of LAB strains was assessed by the methodology of Son et al. [11]. For the adhesion assays, 1 × 10⁵ cells/ml of HT-29 cells were seeded onto a 24-well cell culture plate and incubated at 37°C for 24 h. The LAB strains were centrifuged at 12,000 ×g for 10 min and washed twice with PBS before being inoculated on the wells at approximately 10⁶ CFU/ml and incubated for 2 h at 37°C. Non-adhesive bacteria were removed by washing three times with PBS. Next, 1 ml of 1% (v/v) Triton X-100 was added into each well and incubated for 10 min at 37°C. Following incubation, the cells were separated from the wells. The number of adherent bacterial cells was indicated by counting viable cells on the MRS plates.

**Antibiotic Sensitivity of LAB Strains**

Sensitivity of the LAB strains was measured according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [20]. The disc diffusion method was applied to determine sensitivity to clinical antibiotics, such as ciprofloxacin (5 mg), gentamicin (10 mg), ampicillin (10 mg), streptomycin (10 mg), tetracycline (30 mg), kanamycin (30 mg), doxycycline (30 mg), and chloramphenicol (30 mg). Each LAB strain, at a concentration of...
$10^7$ CFU/ml, was spread on MRS agar, and paper discs containing the antibiotics were placed on plates after a few minutes. Subsequent to incubation for 24 h at 37°C, the diameters of the inhibition zones were measured.

**Preparation of Bacterial Cells**

The LAB strains were grown in MRS broth overnight, centrifuged at 12,000 ×g for 10 min, and washed twice with PBS. The washed bacterial cells were resuspended in PBS to a final concentration of $10^8$ CFU/ml.

**Free Radical-Scavenging Activity toward DPPH of the LAB Strains**

Antioxidant activity was determined by DPPH free radical-scavenging activity according to Yang et al.’s method [21]. A volume of 0.4 mM of DPPH solution was prepared in methanol, and 2 ml of bacterial cells or distilled water (control) were mixed with the same volume of this solution. The mixtures were incubated for 30 min at room temperature in the dark. The absorbance of the supernatant was measured at 517 nm after centrifugation at 12,000 ×g for 10 min, and calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

**β-Carotene Bleaching Assay**

A β-carotene bleaching assay was conducted as described by Kachouri et al. [22] with some modifications. β-carotene solution was prepared by mixing 3 mg of β-carotene, linoleic acid (66 μl), and Tween 80 (300 μl) with chloroform (10 ml). Chloroform was then removed in a rotary evaporator at 40°C under vacuum and the remaining solution was diluted with 100 ml of distilled water. For the assay, 200 μl of samples or distilled water (control) was mixed with 4 ml of this solution and incubated in a water bath at 50°C for 2 h. Next, absorbance was measured at 470 nm for 0 and 2 h, and inhibition activity was calculated as follows:

$$\text{Inhibition activity of β-carotene oxidation (\%)} = \left(1 - \frac{A_{\text{sample}, 2h} - A_{\text{control}, 2h}}{A_{\text{control}, 0h} - A_{\text{control}, 2h}}\right) \times 100$$

**Cell Viability of RAW 264.7 Cells by LAB Strains**

The effect of LAB strains on the viability of RAW 264.7 cells was evaluated using the MTT assay according to the method described by Han et al. [23] with some modifications. RAW 264.7 cells (1 × 10^6 cells/ml) were plated on 96-well cell plates for 24 h. Next, LAB strains were treated with 10^7 CFU/ml and incubated for 44 h. After aspiration of supernatant for determination, cells were treated with MTT solution (2.5 mg/ml in PBS) and incubated for 4 h. Upon discarding the supernatant, DMSO was added to each well and the generated formazan deposits were dissolved. Absorbance of each well was measured at 570 nm using a microplate reader. Cell viability was calculated as a percentage of the absorbance.

**NO Production in RAW 264.7 Cells**

The production of NO in LPS-induced RAW 264.7 cells was assessed according to the methods of Lee et al. [19]. RAW 264.7 cells (2 × 10^5 cells/well) were plated in 96-well plates and treated with LAB strains (10^7 CFU/ml) and LPS (1 μg/ml) for 24 h. The supernatant for determination, cells were treated with MTT solution (2.5 mg/ml in PBS) and incubated for 4 h. Upon discarding the supernatant, DMSO was added to each well and the generated formazan deposits were dissolved. Absorbance of each well was measured at 570 nm using a microplate reader. NO production was calculated through comparison with the standard curve constructed with sodium nitrate as a standard.

**Anti-Inflammatory Effect of LAB Strains**

The anti-inflammatory effect of LAB strains was measured as described by Lee et al. [19] and Son et al. [24]. RAW 264.7 cells were seeded on a 6-well plate (1 × 10^6 cells/ml) and incubated for 24 h and then added into LPS-

### Table 1. Primer sequences related to anti-inflammatory effect used in real-time PCR.

| Primer | Primer sequence (5’–3’)
|--------|-------------------------|
| TNF-α  | Sense 5’TGG ACC TCG TCA GGG TCT AGT TG-3’ |
|        | Antisense 5’CCT GTA GCC CAC TGC GTA GC-3’ |
| iNOS   | Sense 5’CCC TTC CGA AGT TGC TGG CAG CAG C-3’ |
|        | Antisense 5’GGC TGT CAG AGC CTC GTG CCT GTG G-3’ |
| COX-2  | Sense 5’CAC TAC ATC CTG ACC CAC TT-3’ |
|        | Antisense 5’ATG CTC CTG CTT GAG TAT GT-3’ |
| IL-1β  | Sense 5’CAT GAT GAG GAC ATG AGC ACC C-3’ |
|        | Antisense 5’GTC TGC AGA CTC AAA CTC CAC C-3’ |
| IL-6   | Sense 5’GTA CTC CAG AAG ACC AGA GG-3’ |
|        | Antisense 5’TGC TGG TGG CAA CCA CGG CC-3’ |
| β-Actin| Sense 5’GGG GGA AGA AGA TGG GGC AGT-3’ |
|        | Antisense 5’GGG GGA AGA AGA TGG GGC AGT-3’ |

*TNF-α, tumor necrosis factor-α; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; IL-1β, interleukin-1β; IL-6, interleukin-6.*
stimulated (1 μg/ml) and LAB strains (10^7 CFU/ml). RNA was isolated from RAW 264.7 cells treated with samples using the RNeasy Mini Kit (QIAGEN) and cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The expression levels of iNOS and cytokines related to anti-inflammatory effects were measured using synthesized cDNA as a template and a PCR mixture containing SYBR Green PCR Master Mix using RT-PCR (PikoReal 96, Thermo Scientific Pierce). RT-PCR was performed as per the following conditions: 95°C for 2 min followed by 40 cycles of 95°C for 5 sec and 60°C for 15 sec. The results were analyzed after normalization with β-actin as a reference gene and calculated using the 2–ΔΔCt method. The melting curve analysis was performed to assess reaction specificity. The primer sequences are listed in Table 1.

Statistical Analysis
All experiments were repeated in triplicate and presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) and Duncan’s multiple range test were applied to determine the degree of significant differences. Values were considered significant at p < 0.05, and all analyses were conducted with the Statistical Package for the Social Sciences (SPSS), version 24 (IBM, USA) program.

Results and Discussions
Tolerance to Gastric Conditions of LAB Strains
Tolerance to artificial gastric conditions are characteristic of LAB strains required for probiotic properties in the intestine [25]. The tolerance of L. rhamnosus GG, L. plantarum KU15149 and L. brevis KU15176 to artificial gastric acids and bile salts is indicated in Table 2. All LAB strains exhibited high resistance at 0.2 log CFU to artificial gastric conditions, including pH 2.5 and 0.3% pepsin. L. rhamnosus GG exhibited the highest resistance to 0.3% oxgall. The viable cell counts of L. plantarum KU15149 and L. brevis KU15176 were slightly decreased by 1.53 and 0.92 log CFU with bile salts, respectively. L. plantarum Lb41 isolated from kimchi was reduced by 0.06 log CFU/ml and 1.36 log CFU/ml under strongly acidic and bile salt conditions, respectively [17]. Therefore, L. plantarum KU15149 and L. brevis KU15176 possessed probiotic properties due to resistance to artificial gastric conditions, and these strains could be expected to survive in the gastrointestinal tract.

Enzymatic Activities of LAB Strains
Certain probiotic bacteria are of value as they express enzymes such as α-glucosidase, β-glucosidase, and β-
galactosidase. β-galactosidase hydrolyzes lactose into glucose and galactose in milk and alleviates the lactose intolerance problem experienced by some adults [26, 27]. In addition, probiotic bacteria should not produce enzymes such as β-glucuronidase, which has been associated with the induction of carcinogenesis, mutagens, and toxins [28]. The enzymatic activities of the LAB strains tested are shown in Table 3. Although L. rhamnosus GG, L. plantarum KU15149, and L. brevis KU15176 produced β-galactosidase, none of the LAB strains produced β-glucuronidase. A previous study indicated that probiotic L. plantarum FI10604 and L. brevis FI10700 also do not produce β-glucuronidase [11]. Lactococcus lactis KC24 produces various enzymes, such as acid phosphatase, β-galactosidase, and naphthol-AS-BI-phosphohydrolase, but does not produce β-glucuronidase [19].

Antibiotic Sensitivity of LAB Strains

The sensitivity of probiotic bacteria to antibiotics is a fundamental precondition because antibiotic-resistant strains may not be easily eliminated if required, and their antibiotic resistance may be transmitted to pathogenic or potentially pathogenic bacteria [29]. The antibiotic sensitivity of LAB strains is presented in Table 4. L. brevis KU15176 had the same sensitivity as L. rhamnosus GG, and in the case of L. plantarum KU15149, it had a similar sensitivity, except to tetracycline (30 mg) and chloramphenicol (30 mg). L. plantarum Ln4 has been shown to be sensitive to commercial antibiotics, such as ampicillin, chloramphenicol, doxycycline, and tetracycline [11]. Therefore, these results confirm that LAB strains are safe in accordance with the CLSI guidelines.

Adhesion of LAB Strains to HT-29 Cells

The adhesion of LAB strains to intestinal cells is the most important factor in their effective use [30]. LAB strain adhesion to HT-29 cells is shown in Fig. 1. L. plantarum KU15149 and L. brevis KU15176 demonstrated a similar ability to adhere to HT-29 cells. L. rhamnosus GG had the greatest adhesion ability (6.37%), followed by L. plantarum KU15149 (2.39%) and L. brevis KU15176 (2.61%). Song et al. [31] previously showed that L. brevis KCCM 12203P (6.84%) has a slightly higher adhesion ability to intestinal epithelial cells compared to that of L. rhamnosus GG (6.21%). Zhang et al. [32] showed that L. plantarum C32, C42, and C62 (< 2%) have diminished adhesion ability to intestinal epithelial cells compared to that of L. rhamnosus GG (4.30%). Therefore, although the adhesion ability of L. plantarum KU15149 (2.39%) and L. brevis KU15176 (2.61%) to intestinal cells was lower than that of the commercial strain, L. rhamnosus GG (6.21%), this was still considered sufficient for use as a functional probiotic.

Antioxidant Effect of LAB Strains

The critical role of LAB strains in antioxidant activity is protection from free radicals [33]. The antioxidant activity of LAB strains was assessed by DPPH free radical scavenging (Fig. 2A) and β-carotene bleaching assays (Fig. 2B).

Table 4. Antibiotic sensitivities of LAB strains.

| Antibiotics            | L. rhamnosus GG | L. plantarum KU15149 | L. brevis KU15176 |
|------------------------|-----------------|----------------------|-------------------|
| Ampicillin (10 mg)     | S               | S                    | S                 |
| Gentamycin (10 mg)     | R               | R                    | R                 |
| Kanamycin (30 mg)      | R               | R                    | R                 |
| Streptomycin (10 mg)   | R               | R                    | R                 |
| Tetracycline (30 mg)   | S               | R                    | S                 |
| Ciprofloxacin (30 mg)  | R               | R                    | R                 |
| Chloramphenicol (30 mg)| S               | I                    | S                 |
| Doxycycline (30 mg)    | S               | S                    | S                 |

Resistance was evaluated according to the CLSI breakpoints (CLSI, 2012). S, Susceptible; I, intermediate; R, resistant.

Fig. 1. Adhesion of LAB strains to HT-29 cells. LGG, L. rhamnosus GG; KU15149, L. plantarum KU15149; KU15176, L. brevis KU15176. Error bars indicate standard deviation of three independent experiments. Letters indicate a significant difference between prospective and commercial probiotic strain. All values are expressed as the mean ± standard deviation. Values with different letters indicate significant differences for each characteristic (p < 0.05).
L. brevis KU15176 (47.09%) showed significantly higher DPPH free-radical scavenging activity than that of L. rhamnosus GG (36.60%), while the activity of L. plantarum KU15149 (37.73%) was similar to that of L. rhamnosus GG. In a prior study, L. brevis KU15153 (44.14%) has been shown to exhibit higher DPPH free-radical scavenging activity than L. rhamnosus GG (19.21%) [34]. The β-carotene bleaching inhibition activity of LAB strains is depicted in Fig. 2B. The β-carotene bleaching inhibition activity of L. plantarum KU15149 (39.57%) was higher than that of L. brevis KU15176 (16.65%) but not significantly different from that of L. rhamnosus GG (35.08%). Another study found that L. paraplantarum SC61 showed 35.64% inhibition of β-carotene bleaching activity [24].

Anti-Inflammatory Activity of LAB Strains
To confirm cytotoxicity in RAW 264.7 cells, the viability of LAB was determined using the MTT assay (data not shown). The LAB strains were shown to have higher viability at 10^7 CFU/ml than at 10^8 CFU/ml. Therefore, LAB strains were tested at a concentration of 10^7 CFU/ml for induction of NO synthesis in macrophages.

The NO-producing activity of L. plantarum KU15149 (2.25 μM) was the lowest in comparison to that of

Fig. 3. Production of (A) NO on LAB strains in lipopolysaccharide (LPS)-stimulated RAW264.7 cells and the relative expression of mRNA level of (B) TNF-α, (C) iNOS, (D) COX-2, (E) IL-1β, and (F) IL-6. LPS−, without LPS treatment; LPS+, treated with LPS (1 μg/ml); LGG, L. rhamnosus GG with LPS; KU15149, L. plantarum KU15149 with LPS. All values are expressed as the mean ± standard deviation and standardized against the β-actin housekeeping gene. Values with different letters indicate significant differences for each characteristic (p < 0.05).
L. rhamnosus GG (7.32 μM) and L. brevis KU15176 (25.57 μM) in stimulating LPS conditions (Fig. 3A). Lactococcus lactis NK34 has been reported to possess low levels of NO production [23] and L. plantarum 4B15 and 4M13 have been found to exert the greatest inhibitory effect on NO production [35].

LAB strains were analyzed for mRNA expression levels of TNF-α, iNOS, COX-2, IL-1β, and IL-6 from RAW 264.7 cells through RT-qPCR (Figs. 3B-3F). Both L. plantarum KU15149 and L. rhamnosus GG demonstrated significant decrease in all five mRNAs assessed when compared to the LPS+ control. L. plantarum KU15149 was associated with a significant decrease in relative expression of TNF-α and iNOS in comparison to L. rhamnosus GG, suggesting that L. plantarum KU15149 would be more effective in dampening an LPS-induced immune response; levels of COX-2, IL-1β, and IL-6 mRNA expression showed no significant difference between the two strains. Weissella cibaria JW15 has been shown to significantly inhibit the expression of pro-inflammatory cytokines compared to L. rhamnosus GG [36].

In conclusion, L. plantarum KU15149 isolated from homemade diced-radish kimchi was found to have probiotic properties including gastric acid and bile salt tolerance, enzyme activity, adhesion to intestinal cells, and antibiotic sensitivity. L. plantarum KU15149 had a high abundance of antioxidians, as measured by DPPH free-radical scavenging and β-carotene bleaching inhibition. In addition, L. plantarum KU15149 induced lower levels of NO and less expression of pro-inflammatory cytokine genes TNF-α and iNOS than did L. rhamnosus GG under LPS-induced conditions in macrophages. Therefore, L. plantarum KU15149 could be potentially used as a probiotic and anti-inflammatory ingredient.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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