Anti-inflammatory Effect of Lactobacillus Plantarum IDCC 3501 and Its Safety Evaluation

Soo-Yeon Yang  
Ildong Bioscience

Seung A Chae  
Ildong Bioscience

Won Yeong Bang  
Ildong Bioscience

O-Hyun Ban  
Ildong Bioscience

Soo-Jung Kim  
Chonnam National University

Young Hoon Jung  
Kyungpook National University  https://orcid.org/0000-0002-0781-3608

Jungwoo Yang  
Ildong Bioscience

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Abstract

This study investigated the anti-inflammatory activity of *L. plantarum* IDCC 3501 isolated from kimchi (Korean fermented food) and its safety. When lipopolysaccharide (LPS)-induced RAW 264.7 macrophages were treated with cell-free supernatant from *L. plantarum* IDCC 3501, the mRNA expression level of inflammatory markers (i.e., TNF-α, IL-1β, and IL-6) was significantly reduced. The decreased cell viability by LPS was recovered and NO production in LPS-induced cell was also decreased. The genes responsible for antibiotic resistance and virulence were not detected from the genome analysis of this strain. Consistent with this, minimal inhibitory concentrations against various antibiotics, biogenic amines and D-lactate production, as well as enzymatic and hemolysis activities indicated that *L. plantarum* IDCC 3501 did not produce any harmful compounds during fermentation. Also, no acute toxicity and mortality were observed in a murine mouse model when feeding with *L. plantarum* IDCC 3501. Based on our findings, *L. plantarum* IDCC 3501 is safe and beneficial for human consumption.

Introduction

*Lactobacillus plantarum* is a major widespread species among the genus, *Lactobacillus*, due to its ecological niches, existing in dairy products, fermented foods, and host's mouth and intestinal tract [1]. As a facultatively heterofermentative species, *L. plantarum* has various beneficial health effects, such as antimicrobial activity [2], antiobesity [3], immune-boosting [4], and anti-inflammatory effects [5]. For this reason, *L. plantarum* has been used in various industrial food fermenters as a starter. As a probiotic, this species typically possess acid/bile tolerance and intestinal adhesion activity [6–7], and alleviating acute and chronic inflammation.

Inflammation is an immunity-mediated response to viral or pathogenic infection, toxic compounds, or irradiation [8]. This response is typically regulated by proinflammatory mediators (e.g., iNOS and COX-2) and cytokines (e.g., TNF-α, IL-1β, and IL-6) [9], resulting in the recruitment of immune cells (e.g., macrophages) and systemic responses. Mainly, gut inflammation often causes acute symptoms, such as diarrhea, gastric bleeding, and abdominal pain and chronic symptoms, such as inflammatory bowel disease and colorectal cancer [10]. Therefore, the anti-inflammatory effect of probiotics has been intensively studied in the last decades to improve gut health. For example, *L. plantarum* has significant positive effects on Crohn's disease and ulcerative colitis by modulating the intestinal microbiota, suppressing pathogens, and boosting the immune system [11–12].

Previously, *L. plantarum* IDCC 3501 was reported to have the following characteristics as probiotics: coaggregation with pathogens, 25.0–66.1%; hydrophobicity, 39.2%; acid tolerance, and 84.9%; antibacterial effects against nine pathogens including *E. coli* O157:H7 and *Salmonella Typhimurium* [13]. However, the safety of *L. plantarum* IDCC 3501 needs to be carefully examined for human consumption on the strain basis.

In this study, the anti-inflammatory effects of *L. plantarum* IDCC 3501 and its safety were investigated. Here, cell viability, nitric oxide (NO) production, and the expression of inflammatory markers were investigated in LPS-stimulated RAW 264.7 macrophages. *In vitro* and *in vivo* studies, including hemolysis, minimal inhibitory concentration (MICs) tests, whole-genome analysis, and single-dose acute oral toxicity test in rats, were performed.

Material And Methods

**Bacterial strain, culture conditions, and preparation of cell-free supernatants**

*Lactobacillus plantarum* IDCC 3501 (ATCC BAA-2838), isolated from kimchi (Korean fermented food), has been manufactured in Ildong Bioscience (Pyeongtaek, Korea) since 2015. *L. plantarum* IDCC 3501 and *Lactobacillus rhamnosus* GG were anaerobically cultured in De Man, Rogosa, and Sharpe (MRS; BD Difco, Franklin Lakes, NJ, USA) medium at 37°C in
a static incubator. *Staphylococcus aureus* ATCC 25923 was used as a positive strain for hemolysis assay, and it was cultured in brain heart infusion (BHI; BD Difco) medium at 37°C with shaking at 200 rpm. Supernatants of 16 h-cultured *L. plantarum* IDCC 3501 and *L. rhamnosus* GG (positive control) were prepared by centrifugation at 8000 rpm and filtered using a 0.22 µm syringe filters (Merck Millipore, Burlington, MA, USA).

**Cell culture**

RAW 264.7 cells were purchased from Korean Cell Line Bank (Seoul, Korea) and were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, MA, USA), and supplemented with 10% (v/v) fetal bovine serum and 1% (w/v) penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

**Macrophage viability assay**

The viability of RAW 264.7 cells was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, the cells (1 × 10⁵ cells·well⁻¹) were seeded in a 96-well plate for 24 h and treated with *L. plantarum* IDCC 3501 cell-free supernatants for 2 h. Then, MTT was added to each well and incubated for 4 h at 37°C. After removal of media and MTT, dimethyl sulfoxide (DMSO) was added to the well to dissolve the formazan. Finally, the developed color was measured at 540 nm using a microplate reader (BioTek, Winooski, VT, USA).

**Measurement of nitric oxide production**

Nitric oxide contents were determined by the Griess reaction (Promega, Madison, WI, USA). Briefly, 50 µL of cell supernatant was collected and mixed with 50 µL of Griess A (1% sulfanilamide) and 50 µL of Griess B (0.1% N-1-naphthylethylenediamine dihydrochloride). The mixture was then incubated at room temperature for 10 min, and nitric oxide contents were determined at 540 nm according to the calibration curve.

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

Total RNA from RAW 264.7 cells treated with LPS and supernatant of either *L. plantarum* IDCC 3501 or *L. rhamnosus* GG, was isolated using TRIzol reagent (ThermoFisher Scientific, Waltham, MA, USA). Reverse transcription (Superscript IV First-Strand Synthesis System; ThermoFisher Scientific, MA, USA) was then performed to synthesize cDNA with 2 µg of total RNA and random hexamers. Finally, PCR was performed using the primers listed in Table S1, and the bands were analyzed and quantified using Image Lab (Bio-Rad, Hercules, CA, USA).

**Gene search for antibiotic resistance and virulence**

The complete genome sequence of *L. plantarum* IDCC 3501 was previously reported, consisting of a circular 3,242,587 bp chromosome with a GC content of 44.52% [14] (GenBank accession no. CP031702). The assembled sequence was compared with the reference sequences in the ResFinder database, using ResFinder v.3.2 (https://cge.cbs.dtu.dk/services/ResFinder). The search parameters were sequence identity greater than 80% and a 60% coverage. Virulence genes were searched using the BLASTn algorithm with VFDB database [15]. The identification thresholds were identity greater than 70%, coverage greater than 70%, and E-value less than 1E⁻⁵.

**Determination of MIC**

The susceptibility of *L. plantarum* IDCC 3501 to ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol was evaluated. A single colony was inoculated into MRS broth and cultured for 16–18 h. The cultured cells and antibiotic solution were mixed in a 96-well plate to obtain an initial cell density of 5 × 10⁵ CFU mL⁻¹ and antibiotic concentration of 0.125–1,024 µg mL⁻¹. The plate was then anaerobically incubated at 37°C for 20 h. Finally, optical density was measured using a microplate reader (BioTek, Winooski, VT, USA), and MICs for each antibiotic were determined as the lowest concentration that completely inhibited cell growth.

**Biogenic amine and lactate concentration determination**
The supernatants from *L. plantarum* IDCC 3501 were collected by centrifugation at 6,000 rpm for 5 min at 4°C and were filtered using a 0.2-µm pore-size membrane. Then, 1 mL of supernatant was mixed with 200 µL of saturated NaHCO₃, 20 µL of 2 M NaOH, and 0.5 mL of dansyl chloride (10 mg mL⁻¹ acetone), and the mixture was incubated at 70°C for 10 min for derivatization. The derivatized sample was added with 200 µL of proline (100 mg mL⁻¹ H₂O) and incubated in a dark room for 15 min. The sample was made up to 5 mL with acetonitrile and filtered with a 0.45 µm membrane. Finally, biogenic amines were analyzed using high-performance liquid chromatography (HPLC; LC-NETI/ADC, Jasco, Macclesfield, UK) equipped with an Athena C18 column (4.6 mm × 250 mm, ANPEL Laboratory analysis, Shanghai, China). Aqueous acetonitrile solution (67:33 of H₂O) was used as the mobile phase at 0.8 mL min⁻¹. Peaks were detected at 254 nm using a UV detector (UV-2075 Plus, Jasco, Macclesfield, UK) and quantified according to the calibration curves of each biogenic amine.

The quantities of L- and D-lactate in *L. plantarum* IDCC 3501 supernatant were measured using an assay kit (Megazyme, Bray, Ireland) according to the manufacturer's protocol.

**Hemolytic and extracellular enzyme activities**

*L. plantarum* IDCC 3501 and *Staphylococcus aureus* ATCC 25923 were streaked on sheep blood agar plates (BBL Microbiology Systems, Cockeysville, MD) and incubated at 37°C overnight. Then, β-hemolytic activity was determined by observing clear zones around colonies.

Enzyme activities of *L. plantarum* IDCC 3501 were assessed using the API-ZYM kit (BIOMÉRIUX, Marcy-l’Étoile, France), which can evaluate 19 hydrolytic enzyme activities according to the manufacturer's protocol.

**Acute oral toxicity test**

Acute oral toxicity test was performed by Korea Testing and Research Institute (Hwasun-gun, Jeollanam-do, Korea) according to the OECD guidelines (Test No. 425) [16]. Briefly, 12 Crl::CD(SD) female rats aged 9–10 weeks were divided into 4 groups of 3 rats each. Each group's rats were orally administered at 300 mg kg⁻¹ or 2000 mg kg⁻¹, corresponding to 3.4 × 10¹¹–3.6 × 10¹¹ CFU and 2.3 × 10¹²–3.4 × 10¹² CFU, respectively. Then, toxicity, morality, and body changes were observed for 14 d. Finally, 100-mL isoflurane was treated to euthanize the rats, and an autopsy for the examination of organs was performed.

**Results And Discussion**

**Effects of L. plantarum IDCC 3501 on cell viability**

The cytotoxicity of the supernatants of *L. plantarum* IDCC 3501 on RAW 264.7 cells, which inflammation was induced by 1 µg per mL of lipopolysaccharide (LPS), was determined using MTT assay (Fig. 1A). The supernatants of *L. rhamnosus GG* were also applied as a control. After treatment using the supernatants of *L. plantarum* IDCC 3501, the decreased cell viability by LPS was recovered, showing no negative effect on cell viability. Like cell viability results, LPS addition showed a remarkable increase in NO production, which shows early stimulation exerted on activated macrophages (Fig. 1B). However, the supernatants of *L. plantarum* IDCC 3501 considerably inhibited NO production in a level of more than 60%. Thus, both cell viability and NO production results suggested that *L. plantarum* IDCC 3501 supernatants positively affected the relief of oxidative stress in cells.

**The effect of L. plantarum IDCC 3501 on pro-inflammatory cytokines**

Activated macrophages which were regulated by LPS-induced pro-inflammatory cytokine mediators could release various cytokines to enhance immune defense mechanisms, such as TNF-α, IL-1β, and IL-6 in RAW 264.7 cells. To demonstrate that *L. plantarum* IDCC 3501 inhibits LPS-induced nitrite and PEG₂ production, iNOS and COX-2 expression were investigated using semi-RT-PCR (Fig. 2A and B). The expression levels of both iNOS and COX-2 were significantly increased in LPS-
induced cells. *L. plantarum* IDCC 3501 decreased iNOS and COX-2 expression by 26.5% and 55.5% than the values of LPS-induced cells, respectively. Although, TNF-α, IL-1β, and IL-6 were inhibited by 38.2%, 37.9%, and 73.8%, respectively (Fig. 2C–E), IL-6 was the mostly affected among these cytokines.

There are many reports about whole cells (e.g., alive or heat-killed cells) or cell wall components of lactic acid bacteria. Recently, much attention has been paid to cell-free supernatants containing biologically active metabolites which are secreted by live bacteria [17]. Previous studies have shown anti-inflammatory response on intestinal epithelial cells and macrophages by reducing proinflammatory mediators or cytokines [18]. Among them, the deregulation of IL-6 has been implicated in the pathogenesis of many diseases, especially Crohn’s disease and colon cancer [19]. According to our findings, the supernatants of *L. plantarum* IDCC 3501 exert anti-inflammatory effects because it leads to iNOS, COX-2, TNF-α, IL-1β, and IL-6 inhibition under viable conditions (Fig. 2). Thus, cell-free bacterial supernatants are promising anti-inflammatory agents for treating gut inflammation. The administration of our strains to infected mice can clarify the effects for further study.

### Whole-genome analysis and antibiotic susceptibility of *L. plantarum* IDCC 3501

In lactic acid bacteria, antibiotic resistance and virulence factors are easily acquired from an exogenous source. Gene transfer commonly involves bacterial conjugative plasmids, transposable elements, and integron systems [20–21]. Thus, whole-genome analysis is becoming an essential procedure for safety assessment in the probiotic industry. Notably, *L. plantarum* IDCC 3501 has no antibiotic resistance genes and virulence factors [15]. Meanwhile, *L. plantarum* IDCC 3501 showed susceptibility to all antibiotics, except kanamycin (Table 1). *Lactobacillus* strains are susceptible to antibiotics related to protein synthesis (e.g., chloramphenicol, erythromycin, clindamycin, and tetracycline) but are aminoglycosides-resistant (neomycin, kanamycin, streptomycin, and gentamicin) [22]. For example, approximately 79% of isolated probiotic strains showed kanamycin resistance [23]. Thus, *L. Plantarum* IDCC 3501 resistance to kanamycin might be considered aminoglycoside intrinsic because of membrane impermeability by potential efflux mechanisms [24].

| Antibiotic   | AMP | VAN | GEN | KAN | STR | ERY | CLI | TET | CHL |
|--------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Breakpoint   | 2   | n.r. | 16  | 64  | n.r. | 1   | 4   | 32  | 8   |
| (µg mL⁻¹)ᵃ   | 0.25–0.5 | > 512 | 8–16 | 256 | 32 | < 0.125 | 0.5 | 4 | 2–8 |
| MIC          | no  | no  | no  | no  | no  | no  | no  | no  | no  |
| Antibiotic resistance gene | no | no | no | no | no | no | no | no | no |

ᵃEFSA (2018)

n.r.: not required.

**Abbreviations:** AMP, ampicillin; VAN, vancomycin; GEN, gentamycin; KAN, kanamycin; STR, streptomycin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; CHL, chloramphenicol; MIC, minimal inhibitory concentration.

According to hemolytic activity results identified as a virulence factor, *L. plantarum* IDCC 3501 showed no hemolysis zones on the sheep blood agar plates, unlike *Staphylococcus aureus* ATCC 25923 which was used as a positive control showing a distinct hemolytic zone (Fig. S1). In conclusion, *L. plantarum* IDCC 3501 was safe regarding antibiotic resistance and virulence factors.
In vitro safety evaluation of *L. plantarum* IDCC 3501

In this study, observations of biogenic amines (BAs), D-lactate, and potential toxin-producing enzymes in *L. plantarum* IDCC 3501 were evaluated. BAs are low molecular weight compounds derived by the decarboxylation of amino acids. BAs are present in various foods and beverages, such as meat, fish, cheese, and vegetables [25]. However, BAs are precursors for forming *N*-nitroso compounds known as cancer-causing agents [26]. Also, high BA consumption may cause symptoms, such as headache, heart palpitations, vomiting, diarrhea, and hypertensive crises in humans and animals [27]. In this study, BAs’ production including tyramine, histamine, putrescine, 2-phenethyamine, and cadaverine, was not observed during *L. plantarum* IDCC 3501 fermentation (data not shown).

Lactate, a key molecule produced during the fermentation of lactic acid bacteria, exists in two forms, D-lactate and L-lactate. In humans, more than 99% of lactate found in the blood is L-lactate. Even though D-lactate appears in human tissue, it possesses D-2-hydroxy acid dehydrogenase that converts D-lactate to pyruvate, resulting in a decrease in acidosis risk [28]. In this study, *L. plantarum* IDCC 3501 produced 20.1 g L⁻¹ of L-lactate (99.85%) and 0.03 g L⁻¹ of D-lactate (0.15%) (Table 2). D-lactate production in *L. plantarum* IDCC 3501 was much lower than in other lactic acid bacteria. Next, an essential criterion in safety assessment is the absence of harmful enzymatic activity. For example, α-chymotrypsin has been associated with virulence and local tissue injury during infective endocarditis [29]. β-glucuronidase can cleave glucuronic acid-conjugated carcinogens related to toxic compounds [30]. Also, β-glucosidase may have damaging effects on the colon [31]. In this study, *L. plantarum* IDCC 3501 had no α-chymotrypsin and β-glucuronidase (Table 3 and Fig. S2). Although a low level of β-glucosidase activity was shown, it was relatively low compared with other lactic acid bacteria. In contrast, β-galactosidase expressed in probiotics has contributed to the relief of lactose maldigestion symptoms [32]. Also, aminopeptidases, such as leucine arylamidase, valine arylamidase, and cystine arylamidase, are involved in cheese ripening by supplying free amino acids that further affect metabolism of cheese flavor [33]. Conclusively, *L. plantarum* IDCC 3501 retained these beneficial enzymes (Table 3 and Fig. S2).

| Strain               | D-lactate       | L-lactate       |
|----------------------|-----------------|-----------------|
| *L. plantarum* IDCC 3501 | 0.03 ± 0.00 g L⁻¹ | 20.15 ± 0.41 g L⁻¹ |
|                      | (0.15%)         | (99.85%)        |

Table 2

The ratio of D- to L-lactate
### Table 3
Enzymatic activities of *L. plantarum* IDCC 3501

| Enzyme                        | IDCC 3501 | KCC-10<sup>a</sup> | 0147<sup>b</sup> | 0612<sup>b</sup> | JCM 1057<sup>c</sup> |
|-------------------------------|-----------|---------------------|------------------|------------------|-----------------------|
| Alkaline phosphate            | -         | -                   | +                | -                | -                     |
| Esterase (C4)                 | -         | +                   | +                | -                | +                     |
| Esterase lipase (C8)          | -         | +                   | +                | -                | -                     |
| Lipase (C14)                  | -         | -                   | -                | -                | -                     |
| Leucine arylamidase           | +         | +                   | +                | +                | +                     |
| Valine arylamidase            | +         | +                   | +                | +                | +                     |
| Cystine arylamidase           | -         | +                   | +                | -                | -                     |
| Trypsin                       | -         | +                   | -                | -                | -                     |
| α-Chymotrypsin                | -         | -                   | -                | -                | -                     |
| Acid phosphatase              | +         | -                   | +                | +                | +                     |
| Naphthol-AS-BI-phosphohydrolase| +         | +                   | +                | +                | +                     |
| α-Galactosidase               | +         | +                   | -                | -                | -                     |
| β-Galactosidase               | +         | -                   | +                | +                | +                     |
| β-Glucuronidase               | -         | +                   | -                | -                | -                     |
| α-Glucosidase                 | +         | -                   | +                | -                | +                     |
| β-Glucosidase                 | +         | +                   | +                | +                | +                     |
| N-acetyl-β-glucosaminidase    | +         | +                   | +                | -                | +                     |
| α-Mannosidase                 | -         | -                   | -                | -                | -                     |
| α-Fucosidase                  | -         | -                   | -                | -                | -                     |

<sup>a</sup> ref. 33; <sup>b</sup> ref. 34; <sup>c</sup> ref. 35

### In vivo safety evaluation of *L. plantarum* IDCC 3501 by oral administration

A 14-d oral acute toxicity study in female rats aged 9–10 weeks was performed to investigate the oral consumption safety of *L. plantarum* IDCC 3501 (Table 4). *L. plantarum* IDCC 3501 did not cause any toxic symptoms or mortality, and the rats lived up to 14 d after administering 300 mg or 2000 mg single dose per kg body weight. Also, there were no significant behavior changes, skin effects, impairment in feed intake, and body weight. Thus, these results show that *L. plantarum* IDCC 3501 is safe, both *in vitro* and *in vivo*. 
Table 4

Body weight evolution of the tested rats by \textit{L. plantarum} IDCC 3501 and its dosage

| Group           | Dose (g kg\(^{-1}\) BW\(^1\)) | Day after administration |
|-----------------|---------------------------------|---------------------------|
|                 | 0  | 1   | 3   | 7   | 14  |
| 9 week-aged     | 300| 214.6 ± 5.1 | 234.5 ± 4.0 | 245.6 ± 4.6 | 249.8 ± 3.3 | 258.3 ± 8.3 |
|                 | 2000| 223.4 ± 11.4 | 241.5 ± 15.5 | 249.1 ± 12.0 | 263.6 ± 15.0 | 267.9 ± 23.0 |
| 10 week-aged    | 300| 234.4 ± 11.4 | 257.6 ± 10.1 | 261.4 ± 10.2 | 269.0 ± 7.8 | 276.2 ± 8.9 |
|                 | 2000| 238.8 ± 7.1 | 254.8 ± 7.8 | 264.8 ± 5.1 | 267.5 ± 4.5 | 277.2 ± 5.1 |

\(^1\)BW, body weight

Based on the Student’s t-tests using STATISTICA 7.0, there was no significant difference among the rats

Conclusion

In this study, the anti-inflammatory effect was verified by deregulation of genes associated with proinflammatory mediators and cytokines in RAW 264.7 macrophage cells. Also, the safety of \textit{L. plantarum} IDCC 3501 was demonstrated with various \textit{in vitro} and \textit{in vivo} tests. Thus, \textit{L. plantarum} IDCC 3501 is considered safe and beneficial for consumption.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article and its additional information files.

Competing interests

The authors declare that they have no competing interests

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Authors’ contributions

S-Y Yang carried out the in vitro experiments and drafted the manuscript. SA Chae participated in the in vivo experiments. WY Bang carried out MIC assays. O-H Ban carried out genetic studies, S-J Kim participated in the design of the study, YH Jung conceived of the study and participated in its design and coordination, and J Yang managed this project and helped to finalize the manuscript. All authors read and approved the final manuscript.

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**Figures**
Figure 1

Effects of *L. plantarum* IDCC 3501 and *L. rhamnosus* GG on (A) cell viability and (B) NO production in LPS-induced RAW 264.7 cells. The results were expressed as mean ± standard deviation from three independent experiments. Different letters indicate significant differences at p-value < 0.05 by Duncan's studentized range test.
Figure 2

Effects of *L. plantarum* IDCC 3501 and *L. rhamnosus* GG on mRNA expression of (A) iNOS, (B) COX-2, (C) TNF-α, (D) IL-1β, and (E) IL-6 in LPS-induced RAW 264.7 cells. The results were expressed as mean ± standard deviation from three independent experiments. Different letters indicate significant differences at p-value < 0.05 by Duncan's studentized range test.

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