

Original Article

Characteristics and immuno-modulatory effects of *Enterococcus faecium* JS1-8 isolated from Kimchi

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Characteristics and immuno-modulatory effects of *Enterococcus (E.) faecium* JS1-8 isolated from Kimchi were investigated for potential probiotic use. We measured their acid, bile, and heat tolerances, adhesion properties in Caco-2 cells, antimicrobial activity against pathogenic bacteria, and bacteriocin-like substance-producing activity. Immuno-modulatory effects of *E. faecium* JS1-8 were measured by determination of nitric oxide (NO), nuclear factor- κ B (NF- κ B), interleukin-1 β (IL-1 β), and tumor necrosis factor- α (TNF- α) production in RAW 264.7 cells or RAW BLUE cells. JS1-8 survived at pH 2.0 for 2 hr and showed tolerance to 0.3% oxgall bile salt, and it survived after exposure for 5 min at 80°C. JS1-8 showed high antimicrobial inhibition zones to *Staphylococcus aureus* (460 mm), *Listeria monocytogenes* (310 mm), *Salmonella enteritidis* (280 mm), and *E. coli* (150 mm). Bacteriocin-like substance produced by JS1-8 showed a broad spectrum of activity against *Listeria monocytogenes* KCCM 40307 and *Lactobacillus sake* KCCM 40264. Low concentration (1×10^7 CFU/mL) of heat-killed *E. faecium* JS1-8 induced statistically higher production of NO than *Lactobacillus rhamnosus* GG (LGG), which is a well-known immuno-modulatory lactic acid bacteria. Low and high (5×10^7 CFU/mL) concentrations of JS1-8 induced statistically higher production of NF- κ B than that produced by LGG. We also found that JS1-8 increased production of pro-inflammatory cytokines such as IL-1 β and TNF- α in a concentration-dependent manner. As a result, *E. faecium* JS1-8 could be used as a useful probiotic for controlling pathogens and enhancing host immune responses.

Key words: *Enterococcus faecium*, bacteriocin, probiotics, immuno-modulatory effect, Kimchi

Introduction

Probiotics have been defined as “live microbial feed supplement beneficial to the host (man or animal) by improving the microbial balance within its body” by Fuller [1]. In order to be used as beneficial probiotics it should have no pathogenicity and exhibition of specific properties such as gastric acid and bile tolerance, adhesion to intestinal epithelial cell surfaces, production of antimicrobial substances and immunomodulation effects [2].

Kimchi is well known as a traditional Korean fermented food. Kimchi fermentation is initiated by various endogenous microorganisms in the raw materials [3]. Many lactic acid bacteria (LAB) are involved in the fermentation of Kimchi such as *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp. and *Enterococcus* spp. [3]. *Enterococcus faecium* JS1-8 strain was isolated from the Kimchi. Kimchi are known to possess several beneficial effects to health such as anti-diabetic effect, anti-oxidant action, anti-obesity effect, anti-cancer activity and immunomodulatory effects [4-8]. Although probiotic characteristics of *Enterococcus faecium* have been studied previously [9-11], there is little information on the immunomodulatory effects of *Enterococcus faecium* isolated from Kimchi.

Macrophages are phagocytes that play a central role in all phases of host defense including immune responses to infection. Macrophages can be activated by bacteria, lipopolysaccharides (LPS), and cytokines and activated macrophages regulate host immunity. Nitric oxide (NO) and pro-inflammatory cytokines such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) are produced by macrophages [12]. NO is a short lived free radical that either kills or prevents the growth of bacteria and

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tumor. IL-1 β is a member of the interleukin 1 family of cytokines. This cytokine is an important mediator of the proinflammatory response and is involved in a variety of cellular responses, including cell proliferation, differentiation, and apoptosis. TNF- α is mainly secreted by macrophages and cellular immunity activity and mediates resistance to infectious disease by controlling inflammation and pathogenic bacteria [13]. Nuclear factor κ B (NF- κ B) is a protein complex that controls transcription of DNA. It is involved in activation of pro-inflammatory genes. NF- κ B plays a key role in regulating the immune response to infection by cytokine production [14].

The objective of this study was to examine the probiotic characteristics of *Enterococcus faecium* JS1-8 isolated from Kimchi and investigate the immuno-modulatory effects such as NO, NF- κ B, IL-1 β and TNF- α production in RAW 264.7 cells or RAW BLUE cells.

Materials and Methods

Bacterial strains

E. faecium JS1-8 isolated from Kimchi was used in this study and *Lactobacillus rhamnosus* GG (LGG, ATCC 53103), a well known commercial probiotic strain, was used as reference strains. As the indicator strain for antimicrobial activities and bacteriocin like substance producing activity, five types of microorganisms (*Escherichia coli* KCTC 1682, *Salmonella enteritidis* KCCM 12021, *Staphylococcus aureus* KCTC 1621, *Listeria monocytogenes* KCTC 3569, *Lactobacillus sake* KCCM 40264) were employed. JS1-8, LGG and *L. sake* were grown for 18 hr at 37°C in MRS broth (Difco, USA). The other pathogenic strains were cultured in BHI (Brain Heart Infusion) broth (Difco, USA) for 18 hr at 37°C before each experiment.

Scanning electron microscopic observation

The monolayers of *E. faecium* JS1-8 were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 hr at room temperature. After washing with phosphate buffer, cells were fixed for 30 min with 2% OsO₄ in the same buffer and washed five times. After dehydrated in a graded series of ethanol, cells were dried in a critical-point dryer (JEOL JCPD-5, Japan) and coated with gold. Specimens were observed by HITACHI S-4500 scanning electron microscope (Hitachi, Japan) [15].

Bacteriocin like substance producing activity

In order to investigate the bacteriocin like substance producing activity, *E. faecium* JS1-8 strains were grown at 37°C in MRS broth for 30 hr. The bacterial cells were removed by centrifugation at 12,000 rpm for 15 min at 4°C and the supernatant was passed through a filter mem-

brane (0.45 μ m pore size) and then boiled for 10 min to inactivate the proteases. Ammonium sulfate was added to achieve 50% saturation. After precipitation for 24 hr at 4°C, the saturated solution was centrifuged 12,000 rpm for 20 min at 4°C. The pellet was resuspended in distilled water and dialyzed with a membrane with a 1 kDa cutoff (Spectrum Medical Inc., LA, USA) for 24 hr at 4°C. The antimicrobial activity of JS1-8 crude bacteriocin against several pathogenic bacteria and lactic acid bacteria (*E. coli*, KCTC 1682, *S. enteritidis* KCCM 12021, *S. aureus* KCTC 1621, *L. monocytogenes* KCTC 3569, *L. sake* KCCM 40264) were tested using spot-on-lawn method. Bacteriocin activity was expressed in terms of arbitrary units per ml (AU/mL) [16].

Acid, bile salt and heat tolerance

Acid and bile salt tolerance tests were conducted by a modified method of Kimoto *et al* [17]. To test tolerance at low pH value, JS1-8 strain suspended in MRS broth were adjusted with 0.05 M sodium phosphate buffer from pH 2.0 to pH 4.0 and incubated at 37°C for 2 hr. Bile salt tolerance was determined by cultivating on MRS plate containing 0.3% (w/v) oxgall (Difco Laboratories, USA). To assay the heat resistance, suspension of JS1-8 strain was aseptically inoculated into MRS broth and subjected to water bath at 70°C and 80°C for 5 min, respectively. After incubation for 48 hr at 37°C on MRS plate, viable cells were determined as acid, bile salt and heat tolerance [18].

Antimicrobial activity

The antagonistic activity of *E. faecium* JS1-8 strain was investigated by the modified spot on lawn method [19]. On top of each agar containing JS1-8, 7 mL of soft agar (0.7%, w/v) mixed with the indicator strain (10⁷ CFU/mL, *E. coli*, KCTC 1682, *S. enteritidis* KCCM 12021, *S. aureus* KCTC 1621, *L. monocytogenes* KCTC 3569) was poured and followed by spotting 20 μ L of concentrated culture supernatant upon each paper disc. The extent of the antimicrobial activity was measured by the diameter (mm) of the inhibition zone.

Adhesion property to Caco-2 cells

Human enterocyte-like Caco-2 cells were purchased from Korean Cell Line Bank (KCLB, Korea). Caco-2 cells were grown in DMEM (Gibco, USA), supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, USA) and 1% (v/v) streptomycin and penicillin (Gibco, USA). The number of adherent *E. faecium* JS1-8 per Caco-2 cell was determined by the average number of bacteria attached to the 10 Caco-2 cells. Adhesion property to Caco-2 cells was conducted according to Kimoto's methods [17].

Activation of macrophages cells

RAW 264.7 macrophages (5×10^5 cells/well) cells (KCLB) and RAW BLUE (5×10^5 cells/well) cells (InvivoGen) were cultured in DMEM supplemented with 10% FBS, 1% 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco). JS1-8 and LGG strain were killed by heating at 110°C for 15 min. All heat-killed probiotics were diluted to 5×10^8 CFU/mL for high concentration dosage or 1×10^8 CFU/mL for low concentration dosage. In contrast to probiotics, PBS was added as a negative control and LPS (100 or 1000 ng/mL; Sigma, USA) was added as a positive control in PBS containing wells. After 48 hr, the culture supernatants were collected for use in determining the productions of NO, NF- κ B and cytokines (IL-1 β and TNF- α) [12].

Measurement of NO production

Nitric oxide productions were analyzed using Griess reagent (Promega, USA). Briefly, 50 μ L of macrophage culture supernatant was mixed with 100 μ L of Griess reagent and incubated at room temperature for 10 min in the dark. Samples were measured in triplicate. The absorbance was measured at 540 nm using a microplate reader (Tecan, Austria). NO levels in the supernatant were calculated using the generated standard curve of nitrite (0~100 μ M sodium nitrite) [12].

Cytokine measurements by ELISA (IL-1 β and TNF- α)

Productions of cytokines (IL-1 β and TNF- α) were mea-

sured by mouse ELISA kits (eBioscience, USA) according to the manufacturer's instructions. Each assay was performed in triplicate with 450 nm in a microplate reader. Cytokine levels were then calculated with the standard curves of each cytokine (0~1,000 pg/mL for IL-1 β and TNF- α) [12].

NF- κ B Assay

RAW BLUE cells were plated 5×10^5 cell/mL in each well of 12-well. Probiotics (100 μ L volume containing 1×10^7 CFU/mL or 5×10^7 CFU/mL JS1-8 and LGG) were then added to the wells and incubated for 48 hr. As a positive control, 100 ng/mL lipopolysaccharide (LPS; Sigma, USA) was added. Medium samples (20 μ L) were mixed with QUANTI-Blue (InvivoGen, San Diego, USA) medium (200 μ L) in 96-well plates at 37°C for 15 min. The results of the secreted embryonic alkaline phosphatase (SEAP) activity were measured by reading absorbance at 620 nm using microplate reader (Tecan, Austria) [18].

Statistical analysis

Statistical analysis of the results was performed by SPSS for Windows version 12.0 (SPSS Inc., USA). Significant differences between groups were tested by ANOVA and compared using Duncan's tests ($P < 0.05$).

Results

Characteristics of *Enterococcus faecium* JS1-8 for probiotic use

E. faecium JS1-8 strain was tested for their ability to survive under acidic conditions (pH 2.0) and for their bile tolerance (Table 1). JS1-8 could survive at pH 2.0 after 2 hr of exposure and also showed tolerance to 0.3% of oxgall bile salt. In the heat tolerance test, the JS1-8 was able to survive at 70°C and 80°C for 5 min, respectively. The average number of JS1-8 and LGG attached to the 10 Caco-2 cells were 81 and 100, respectively. JS1-8 strain showed adherence to the Caco-2 cells, but *L. rhamnosus* GG, a well-known commercial strain, had better adhesion capacity. Table 2 showed high antimicrobial inhibition zone of *E. faecium* JS1-8 strain to *Staphylococcus*

Table 1. General characteristics of *Enterococcus faecium* JS1-8 for probiotic use

Characteristics	Results
Cell Morphology	Coccus
Gram stain	+
Acid tolerance: pH 2	+
Bile tolerance to 0.3% of bile	+
Heat tolerance at 70°C, 5min	+
Heat tolerance at 80°C, 5min	+
Adhesion to Caco-2 cell: JS1-8*	81
LGG	100
Antimicrobial activities to:	
<i>Escherichia coli</i> KCTC 1682	150 mm
<i>Listeria monocytogenes</i> KCTC 3569	310 mm
<i>Staphylococcus aureus</i> KCTC 1621	460 mm
<i>Salmonella enteritidis</i> KCCM 12021	280 mm

*The adhesion capacity expressed as the average number of bacteria attached to the 10 Caco-2 cells.

Table 2. Antimicrobial spectrum of the Bacteriocin-like substance produced by *Enterococcus faecium* JS1-8

Indicator strains	Spot-on-lawn method (AU/mL)
<i>Escherichia coli</i> KCTC 1682	–
<i>Listeria monocytogenes</i> KCTC 3569	3,200
<i>Staphylococcus aureus</i> KCTC 1621	–
<i>Salmonella enteritidis</i> KCCM 12021	–
<i>Lactobacillus sake</i> KCCM 40264	400

aureus (460 mm), *Listeria monocytogenes* (310 mm), *Salmonella enteritidis* (280 mm), and *E. coli* (150 mm).

Bacteriocin- like substance produced by *E. faecium* JS1-8

The antimicrobial activity of bacteriocin- like substance produced by *E. faecium* JS1-8 against several Gram-positive and Gram-negative bacteria was tested using modified spot-on-lawn method. The *E. faecium* JS1-8 showed a spectrum of antimicrobial activity against *L. monocytogenes* and *L. sake*. The greatest activity (3,200 AU/mL) was obtained from *L. monocytogenes* KCTC 3569. This bacteriocin-like substance did not show activity against Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium* (Table 2).

NO, NF- κ B and cytokine production in RAW 264.7 or RAW BLUE cells

We measured NO, NF- κ B and cytokines (IL-1 β and TNF- α) induced by heat-killed *E. faecium* JS1-8 in activated RAW 264.7 or RAW BLUE cells to examine the immunomodulatory properties of JS1-8. Low concentration (1×10^7 CFU/mL) of JS1-8 induced statistically higher production of NO than LGG. High concentration (5×10^7 CFU/mL) of JS1-8 produced statistically higher production of NO than LGG, but these results were not statistically different from the 100 ng/mL LPS as a treated positive control (Fig. 2A). NF- κ B production by *E. faecium* JS1-8 and LGG was increased in a dose-dependent manner. Low and high concentration of JS1-8 induced statistically higher production of NF- κ B than that produced by LGG, respectively (Fig. 2B). Low concentration of LGG induced 82.17 ± 8.58 pg/mL of IL-1 β production, which was significantly lower than that induced

by *E. faecium* JS1-8 (138.17 ± 6.33 pg/mL). High concentration of JS1-8 (5×10^7 CFU/mL) induced 354.83 ± 10.87 pg/mL of IL-1 β which is about 2-fold higher than that of LGG (162.75 ± 6.25 pg/mL) (Fig. 2C). Low concentration of JS1-8 induced significantly higher levels of TNF- α (51650 ± 3879.7 pg/mL) than the low concentration of LGG (8975 ± 375.0 pg/mL). High concentration of JS1-8 also produced statistically higher production of TNF- α than high concentration of LGG (47600 ± 1917.6 pg/mL) and 1000 ng/mL LPS (18800 ± 2560.7 pg/mL) (Fig. 2D).

Discussion

Potential probiotic strains should have tolerance for low pH/gastric juice and high pH/bile juice in order to successfully pass through the stomach and small intestine [20]. In the present study, *E. faecium* JS1-8 survived at pH 2 for 2 hr and showed resistance against 0.3 % of bile salt condition. This is in good agreement with the results of the study by Qianglei Tan *et al* [21], who found that *E. faecium* YF5 was tolerant to gastrointestinal transit *in vitro*.

Heat tolerance of probiotic bacteria is a desirable characteristic for stability during heat-processing. The heat tolerance of JS1-8 was tested in 70°C and 80°C for 5 minutes, respectively and survived well for an exposure of 5 min at 80°C. But Hosseini *et al* showed that all of the *Enterococcus* strains tested were killed after 5 min at 70°C and 80°C except only at 65°C [9]. As a result, although *Enterococcus* strains are a mesophilic bacteria, it seems to be flexible to heat treatment depending on the stains.

Adherent strains of probiotic bacteria are likely to

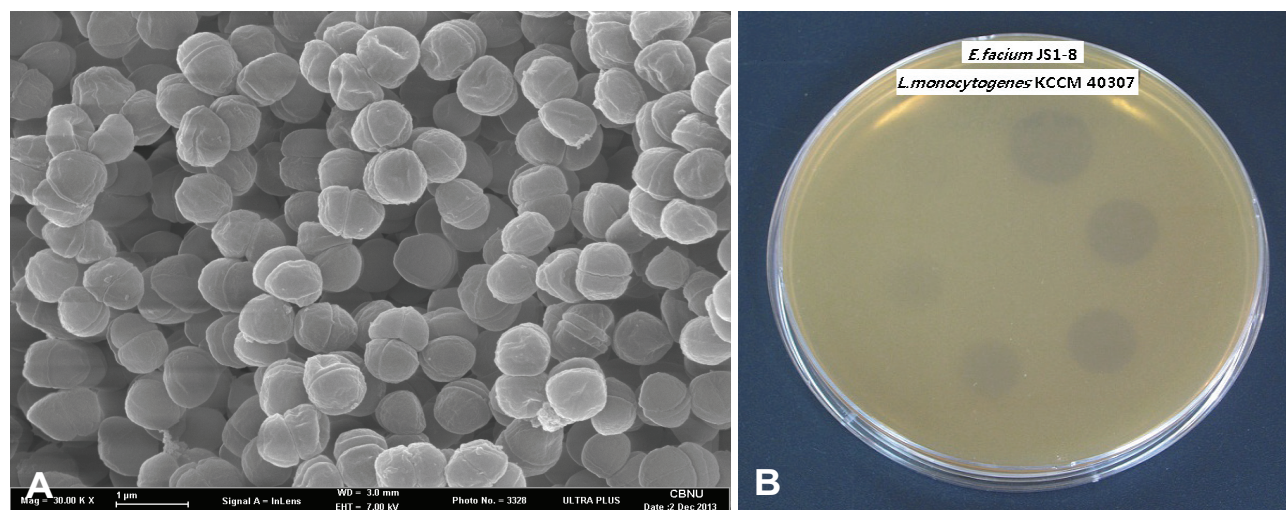


Fig. 1. Scanning electron micrograph of *Enterococcus faecium* JS1-8 strain (A) and growth inhibition of *Listeria monocytogenes* KCTC 3569 by the bacteriocin-like substance produced by *Enterococcus faecium* JS1-8 (B).

persist longer in the intestinal tract and thus may have better chance to act through metabolism, antagonism against pathogens and modulation of the immune system than non-adherent strains [10]. *E. faecium* JS1-8 strain showed similar adhesion property compared with LGG, a well-known probiotic commercial strain.

In the present study, antibacterial activity was measured by the diameter of transparent inhibition zone against various food-borne pathogens such as *Escherichia coli* KCTC 1682, *Salmonella enteritidis* KCCM 12021, *Staphylococcus aureus* KCTC 1621 and *Listeria monocytogenes* KCTC 3569. These results revealed that the culture medium of *E. faecium* JS1-8 can effectively inhibit the tested pathogens. Previous study has demonstrated that probiotic strains usually have antibacterial effects because these strains produce several metabolic

compounds such as organic acids, fatty acids, H₂O₂, or bacteriocins [22]. Du Toit M *et al* reported that *E. faecium* inhibited a wider range of indicator bacteria, including *Listeria monocytogenes*, *Listeria innocua*, *Clostridium sporogenes*, *Clostridium tyrobutyricum* and *Propionibacterium* spp [11].

In this study, only Gram-positive *L. monocytogenes* and *L. sake* were inhibited by the bacteriocin-like substances of *E. faecium* JS1-8. Generally, Gram-negative bacteria are not sensitive to bacteriocins produced by Gram-positive bacteria. Although the cytoplasmic membrane should be susceptible, the outer membrane protects the cell from bacteriocins. The outer membrane of Gram-negative bacteria acts as a permeability barrier for the cell [23]. But bacteriocin-like substances of *E. faecium* JS1-8 might be used to inhibit some pathogenic bacteria

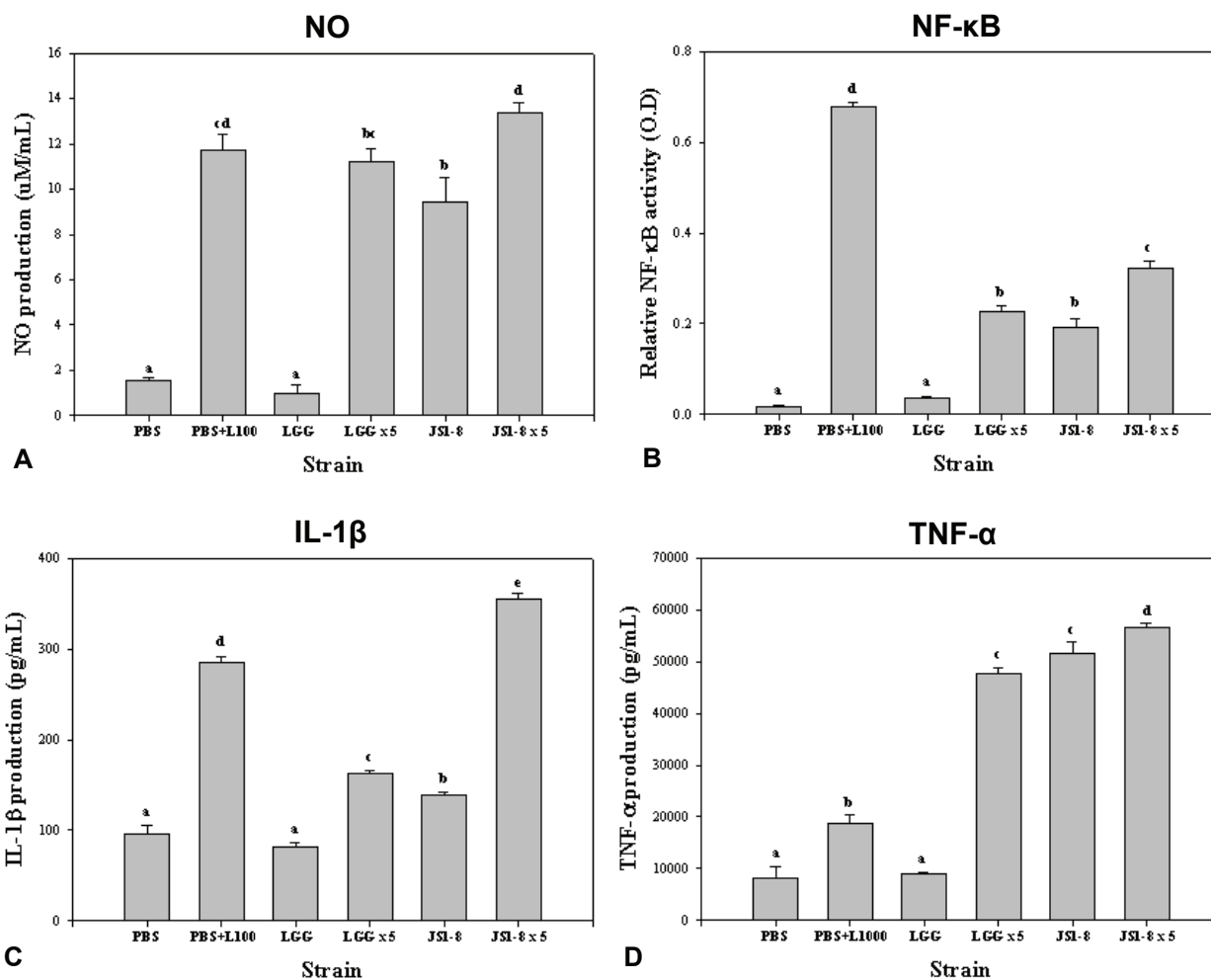


Fig. 2. Nitric oxide (NO) (A), NF-κB (B), IL-1β (C) and TNF-α (D) production induced by two concentrations of heat-killed *Enterococcus faecium* JS1-8 (JS1-8) and *Lactobacillus rhamnosus* GG (LGG) in RAW 264.7 or RAW BLUE cells. RAW 264.7 and RAW BLUE cells (5×10^5 cells/mL) were stimulated with probiotics or PBS for 48 hr. LGG and JW15 concentrations were 1×10^7 CFU/mL. LGG $\times 5$ and JW15 $\times 5$ concentrations were 5×10^7 CFU/mL. L100 or L1000 means LPS at 100 ng/mL or 1000 ng/mL, respectively. Data are mean \pm S.D. values of triplicates. Different superscript letters (a, b, c, d, e) indicate statistical differences as determined by ANOVA ($P < 0.05$).

for probiotic use in humans and animals.

We measured the immune-modulating effects of *E. faecium* JS1-8 by analyzing NO, NF- κ B and cytokines such as IL-1 β and TNF- α compared with the LGG strain which is one of the best studied probiotic strains with its immune responses. We used heat-killed JS1-8 and LGG to prevent the cell death caused by overgrowth of probiotic cells and low acid conditions during experiment. As a result, the heat-killed *E. faecium* JS1-8 increased the production of NO and NF- κ B. NO is a short lived free radical that either kills or prevents the growth of bacteria and it has been described to modulate intestinal barrier function, gut motility, iron transport, and be related to intestinal infection [24]. Activated NF- κ B causes regulation of the genes involved in inflammation, cell proliferation and apoptosis. Also we found that *E. faecium* JS1-8 increased the production of pro-inflammatory cytokine such as IL-1 β , and TNF- α in RAW 264.7 cells. These results show that *E. faecium* JS1-8 has stronger immunomodulating effects than LGG. Low (1×10^7 CFU/mL) and high (5×10^7 CFU/mL) concentration of JS1-8 induced statistically higher production of NF- κ B, IL-1 β and TNF- α than those produced by LGG, respectively.

In conclusion, *E. faecium* JS1-8 isolated from Kimchi showed excellent probiotic properties, wide spectrum of antimicrobial activity and enhancing host immune responses. *E. faecium* JS1-8 may potentially be used in feed and food industries as probiotic application to humans or animals and in natural biopreservatives. In addition, it could be a useful Kimchi starter and a health functional food for controlling food borne pathogens and modulating host immune responses.

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