

## Anti-inflammatory Effect of Heat-Killed *Enterococcus faecalis*, EF-2001

Moon-Suk Choi<sup>1</sup>, Sang-Jin Chang<sup>1</sup>, Yuri Chae<sup>1</sup>, Myung-Hun Lee<sup>1</sup>, Wan-Joong Kim<sup>1</sup>, Masahiro Iwasa<sup>1,2</sup>, Kwon-II Han<sup>1,2</sup>, Wan-Jae Kim<sup>2</sup> and Tack-Joong Kim<sup>1,3\*</sup>

<sup>1</sup>Division of Biological Science and Technology, Yonsei University, Wonju 26493, Korea

<sup>2</sup>Research & Development Center, Korea BeRM Co. Ltd., Wonju 26361, Korea

<sup>3</sup>Research & Development Center, Doctor TJ Co. Ltd., Wonju 26493, Korea

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Inflammation is the most common condition in the human body. Tissue damage triggers inflammation, together with vasodilation and increased blood flow at the inflamed site, resulting in edema. Inflammatory responses are also triggered by lipopolysaccharide (LPS), a Toll-like receptor *Enterococcus faecalis*, a gram-positive organism, has been reported to possess immunomodulatory and preventive activities; however, its use may present risks of sepsis and other systemic infections. Heat-killed *Enterococcus faecalis* (EF-2001) has been reported to induce antitumor activity, but its effects on inflammation are not known. In the present study, we investigated the effect of EF-2001 on LPS-induced macrophage inflammatory responses. EF-2001 treatment reduced nitric oxide (NO) production, indicating suppression of inflammatory reactions. EF-2001 showed no cytotoxicity in macrophages. Further investigation of the anti-inflammatory mechanism of EF-2001 indicated that EF-2001 reduced the LPS-induced expression of inducible nitric oxide synthase and cyclooxygenase-2. EF-2001 also reduced the LPS induction of several inflammatory molecules involved in the nuclear factor- $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein kinase pathways, including ERK, JNK, and p38 phosphorylation, in a concentration-dependent manner. Additionally, EF-2001 inhibited Akt phosphorylation and increased the expression of the inhibitory  $\kappa$ B (I $\kappa$ B) protein, an inhibitor of NF- $\kappa$ B. EF-2001 also inhibited the nuclear translocation of p65. These results suggest that EF-2001 has anti-inflammatory properties and may be useful for treating inflammatory diseases.

**Key words** : Cyclooxygenase-2, EF-2001, *Enterococcus faecalis*, inducible nitric oxide synthase, inflammation

### Introduction

Inflammation is a series of processes involving various cells and cytokines and functions as a biological defense response to external stimuli [16]. During inflammation, the production of nitric oxide (NO) and prostaglandins (PGs) is increased. Increased NO and PGs are associated with hay fever, atherosclerosis, rheumatoid arthritis, Alzheimer's disease, and cancer, among other conditions [14]. These reactions can lead to infection in defective immune systems due to an insufficient immune response; if the inflammatory response to immunity is excessive, tissue degeneration disorders and tissue proliferation can result in dysfunction [2].

Toll-like receptors (TLRs) induce innate immunity and

subsequently acquired immunity. When agonists are attached to TLRs, they activate nuclear factor (NF)- $\kappa$ B through signaling pathways involving myeloid differential factor 88 or Toll/interleukin-1R domain-containing adapter inducing interferon- $\beta$  [29]. NF- $\kappa$ B plays an important role in immune responses in humans [13]. Activated NF- $\kappa$ B regulates the expression of several target genes and induces inflammation, leading to various diseases. NF- $\kappa$ B binds to the inhibitor of kappa B alpha (I $\kappa$ B $\alpha$ ) in the cytoplasm. Signals activated by various pathogens phosphorylate I $\kappa$ B $\alpha$ . Phosphorylated I $\kappa$ B $\alpha$  is degraded by the 26S proteasome to liberate NF- $\kappa$ B. NF- $\kappa$ B in the cytoplasm is translocated to the nucleus and binds DNA to stimulate target gene expression, leading to cancer or disease [5]. Therefore, if NF- $\kappa$ B can be inhibited, inflammation-related genes such as cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) can be reduced, thus preventing cancer and various other diseases. The MAPK signaling pathway, which is involved in the inflammatory response, includes major signaling pathways such as extracellular signal-regulated protein kinase (ERK), cjun terminal kinase (JNK), and p38 [15]. Activation of ERK,

#### \*Corresponding author

Tel : +82-33-760-2242, Fax : +82-33-760-2183

E-mail : ktj@yonsei.ac.kr

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JNK, and p38 by phosphorylation affects cell differentiation and division, leading to cell survival [26]. It is also known to play a role in regulating the inflammatory response.

*Enterococcus faecalis*, a gram-positive organism, has been reported to possess immunomodulatory and preventive activities [27]. However, its use may present risks such as sepsis and other systemic infections [9]. Heat-killed *Enterococcus faecalis* has been reported to induce antitumor activity [31], and anti-atopic activity [6], but its effects on inflammation are not known.

In this study, we investigated the effects of heat-killed *E. faecalis* (EF-2001) on NO production and the underlying anti-inflammatory mechanisms.

## Materials and Methods

### Cell culture

Raw 264.7 cells used to examine the inflammatory responses were obtained from American Type Culture Collection (Manassas, VA, USA). These cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Welgene, Daegu, South Korea) containing 10% fetal bovine serum and 1% penicillin and streptomycin at 37°C and 5% CO<sub>2</sub>.

### Heat-killed *Enterococcus faecalis* (EF-2001)

EF-2001 originally isolated from healthy human feces is a commercially available probiotic from Nihon Berumu Co., Ltd. (Tokyo, Japan) and is supplied as a heat-killed, dried powder. One gram of dried EF-2001 contains  $7.5 \times 10^{12}$  units prior to being heat-killed [5, 6].

### Nitric oxide measurement

Raw 264.7 cells were cultured in a 24-well plate at a density of  $1 \times 10^5$  cells/ml for 24 hr. Each well contained different concentrations of EF-2001 (0, 50, 100, 250 µg/ml) and the plate was incubated for 30 min in an incubator. Lipopolysaccharide (LPS) was added to each well at a concentration of 100 ng/ml, and the plate was incubated in an incubator for 18 hr. Next, 500 µl of solution from each well was transferred to a microcentrifuge tube and centrifuged (3,000 rpm, 3 min). The supernatant (100 µl) was dispensed into a 96-well plate and 100 µl of Griess reagent (1% sulfanilamide, 0.1% N-(1-Naphthyl) ethylenediamine dihydro-chloride, 2.5% phosphoric acid) was added to each well; the reaction was conducted at room temperature for 10 min. Absorbance was measured at 595 nm using a microplate reader (BioTek

Instruments, Inc., Winooski, VT, USA). The concentration was measured using NaNO<sub>2</sub> as a standard.

### Cell viability

Raw 264.7 cells were seeded into 96-well plates at a density of  $1 \times 10^5$  cells/ml and cultured for 24 hr. EF-2001 was added to each well at various concentrations (0, 50, 100, 250 µg/ml) and cultured in an incubator for 24 hr. Ez-cytox (10 µl) was added to each well followed by further incubation for 1 hr. Cytotoxicity was measured at 595 nm using a microplate reader.

### Western blotting

Raw 264.7 cells were plated into 6-well plates at a density of  $1 \times 10^5$  cells/ml and cultured for 24 hr in an incubator. EF-2001 was added to the culture at 0, 50, 100, and 250 µg/ml for 30 min. LPS was then added to each well at a concentration of 100 ng/ml and incubated for the appropriate time depending on the protein to be investigated. For the detection of COX-2, LPS treatment was for 12 hr, that for i-NOS was 6 hr, for IκB, ERK, and p38, it was 30 min, and for Akt and JNK were 45 min. After washing twice with cold phosphate-buffered saline (PBS; pH 7.2), 200 µl of pro-prep (iNtRON Biotechnology, Inc., Seoul, Korea) was added to each well and reacted in the freezer for 1 hr. After sonication 2-3 times, the protein concentration was quantified using the Bradford (Bio-Rad, Hercules, CA, USA) assay with bovine serum albumin (BSA) as a standard. The SDS-polyacrylamide gel percentage was adjusted according to the protein size to be analyzed and electrophoresis was performed at 100 V for approximately 2 hr. Antibody treatment was performed with primary antibodies at a 1:2,500 ratio in an overnight reaction. After washing three times for 10 min with Tris-buffered saline containing Tween 20, the secondary antibody was added at a ratio of 1:5,000. Enhanced chemiluminescence was measured using an LAS 4000 system (GE Healthcare, Little Chalfont, UK).

### Confocal microscopy

A cover glass was pre-sterilized and placed on a 6-well plate. Raw 264.7 cells were seeded at a density of  $1 \times 10^5$  cells/ml and cultured for 4 hr. The cells were incubated for 30 min in an incubator with EF-2001 (0, 50, 100, 250 µg/ml) in each well of a 6-well plate. LPS was then added to each well at a concentration of 100 ng/ml. Thereafter, the cells were incubated in an incubator for 18 hr. Each well was

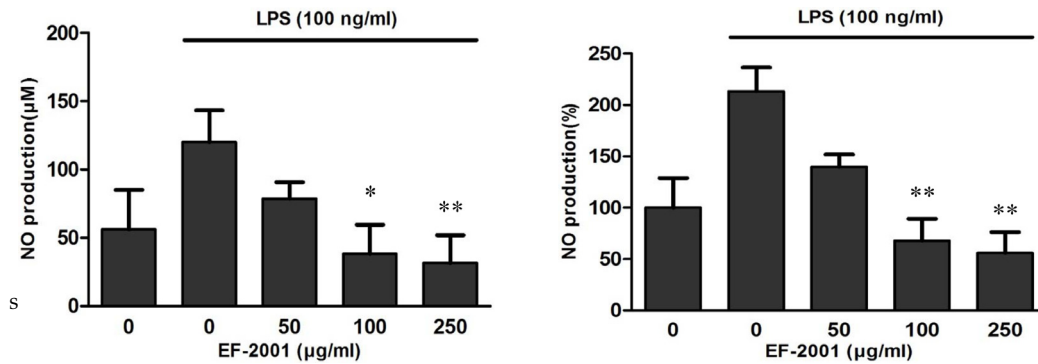


Fig. 1. Effects of EF-2001 on nitric oxide in LPS activated RAW 264.7 cells. LPS-stimulated RAW 264.7 cells were pre-treated with various concentrations (50, 100, and 250 μg/ml) of EF-2001 for 30 min before incubation with or without 100 ng/ml LPS for 18 hr. NO production was measured using Griess reagent and by ELISA. Each value indicates the mean ± SD (n = 4). \**p*<0.05, \*\**p*<0.01.

washed twice with 1× PBS and fixed with 4% paraformaldehyde for 10 min. After washing twice with 1× PBS, 0.2% Triton X-100 was added for 20 min to increase cell permeability. After washing twice with 1× PBS and blocking with 3% BSA for 30 min, p65 anti-rabbit monoclonal antibody (1:100) diluted in 3% BSA solution was added and the cells were incubated overnight. After washing with 1× PBS, Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (1:300) diluted in 3% BSA solution was added and incubated for 1 hr. After staining the cells, images were captured on a Carl Zeiss LSM710 confocal microscope (Carl Zeiss, Oberkochen, Germany).

**Statistical analysis**

Experimental results are expressed as the means ± standard deviation. Statistical analysis of all data was performed by one-way analysis of variance (one-way ANOVA) and multiple comparisons were followed by a Tukey - Kramer *post-hoc* analysis; *p*<0.05 was considered statistically significant.

**Results**

**Inhibitory effect of EF-2001 on NO production**

LPS is an endotoxin present in the extracellular membrane of gram-negative bacteria that stimulates macrophages such as RAW 264.7 and increases cytokine production to secrete inflammatory mediators such as NO. In this study, RAW 264.7 cells induced by LPS showed rapidly increased NO concentrations compared to cells without LPS treatment. However, EF-2001 decreased LPS-induced NO production in a concentration-dependent manner (Fig. 1).

**Cytotoxicity of EF-2001**

To analyze the cytotoxicity of EF-2001, Raw 264.7 cells were treated with EF-2001 (0, 50, 100, and 250 μg/ml) for 23 hr. No toxicity was observed with any of the tested concentrations of EF-2001 (Fig. 2).

**Effect of EF-2001 on iNOS and COX-2 protein expression in Raw 264.7 cells induced by LPS**

iNOS directly and rapidly increases NO levels in activated macrophages and plays an important role in inflammatory diseases [12, 17]. Furthermore, prostaglandins such as COX-2 play a physiologically important role in inflammation, edema, pain, and fever [7, 10, 11]. Because EF-2001 inhibited NO production, the effect of EF-2001 on iNOS and COX-2 protein expression was examined. LPS-induced iNOS protein expression was decreased by EF-2001 in a concen-

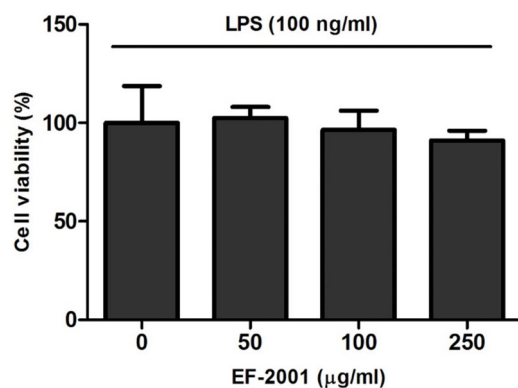


Fig. 2 Effects of EF-2001 on cell viability in RAW 264.7 cells. Raw 264.7 cells were treated with various concentrations (50, 100, and 250 μg/ml) of EF-2001 for 23 hr. Cell viability was measured by ELISA using EZ-cytox kits. Each value indicates the mean ± SD (n=4).

tration-dependent manner (Fig. 3A). In addition, COX-2 expression was inhibited by EF-2001 (Fig. 3B). Thus, we suggest that EF-2001 inhibited NO production by decreasing iNOS and COX-2 protein levels.

**Effect of EF-2001 on MAPK activation**

As an effect of EF-2001, the degree of phosphorylation of ERK, JNK, and p38, which are downstream molecules of the MAP kinase pathway, was measured. Raw 264.7 cells were treated with EF-2001 at various concentrations and then stimulated with LPS. The results showed that ERK, JNK, and p38 phosphorylation levels were gradually decreased upon EF-2001 treatment compared to those in the group treated with LPS alone (Fig. 4). These results suggest that EF-2001 decreases the degree of phosphorylation of MAP kinase, which may suppress the inflammatory response.

**Effect of EF-2001 on Akt-NF-κB activation**

We investigated whether another pathway, the Akt-NF-κB pathway, was affected by EF-2001. First, we investigated the degree of phosphorylation of Akt, an upstream molecule in the NF-κB pathway [28]. When Akt phosphorylation was compared to that in the group treated with LPS alone after treatment with EF-2001, Akt phosphorylation was found to be decreased by EF-2001 (Fig. 5). Thus, EF-2001 inhibited

the phosphorylation of Akt, which may affect the NF-κB pathway.

NF-κB is inhibited when complexed with IκB in the cytoplasm, but becomes activated when IκB is eliminated through phosphorylation [3, 8]. In general, NF-κB is activated by LPS-induced phosphorylation and the p65 subunit acts as a transcription factor to induce the expression of specific genes [22].

As shown in Fig. 6, in the group treated with LPS alone, the protein expression level of IκB was dramatically decreased compared to that in the group without any treatment (Fig. 6). The expression level of IκB was increased compared to that in the control group treated with LPS alone after treatment with EF-2001. We then investigated whether EF-2001 affects the activation of NF-κB by confocal microscopy. Activated NF-κB translocates into the nucleus following elimination of IκB. As shown in Fig. 7, p65 labeled with Alexa 488 (green) was only observed in the cytoplasm in the untreated group. However, in the group treated with LPS, p65 migrated into the nucleus. When EF-2001 concentrations of 50, 100, and 250 μg/ml were used to treat the cells, p65 was found in the cytoplasm. This suggests that the nuclear migration of p65 is inhibited by EF-2001 treatment. Thus, EF-2001 blocked both the Akt-NF-κB pathway and the MAPK pathway.

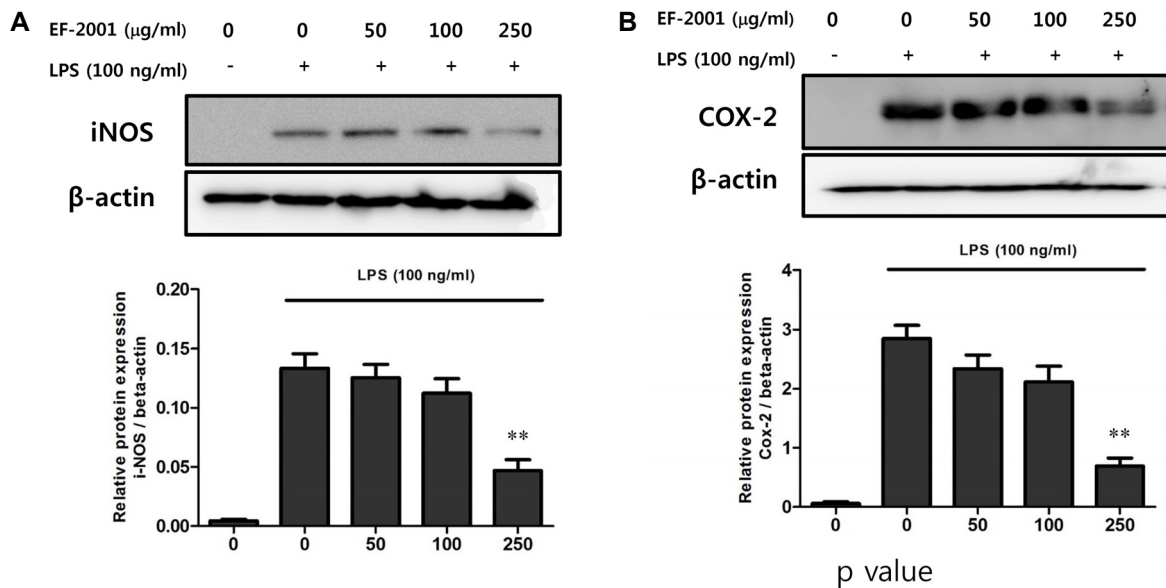


Fig. 3. Inhibition of LPS-induced iNOS and COX-2 expression by EF-2001. (A), (B) RAW 264.7 cells were pre-treated with various concentrations (50, 100, and 250 μg/ml) of EF-2001 for 30 min before incubation with or without 100 ng/ml LPS for 18 hr. Protein expression of iNOS and COX-2 was analyzed by Western blotting, and each image was analyzed using ImageQuant LAS 4000. Each value indicates the mean ± SD (n=4). \*\*p<0.01.

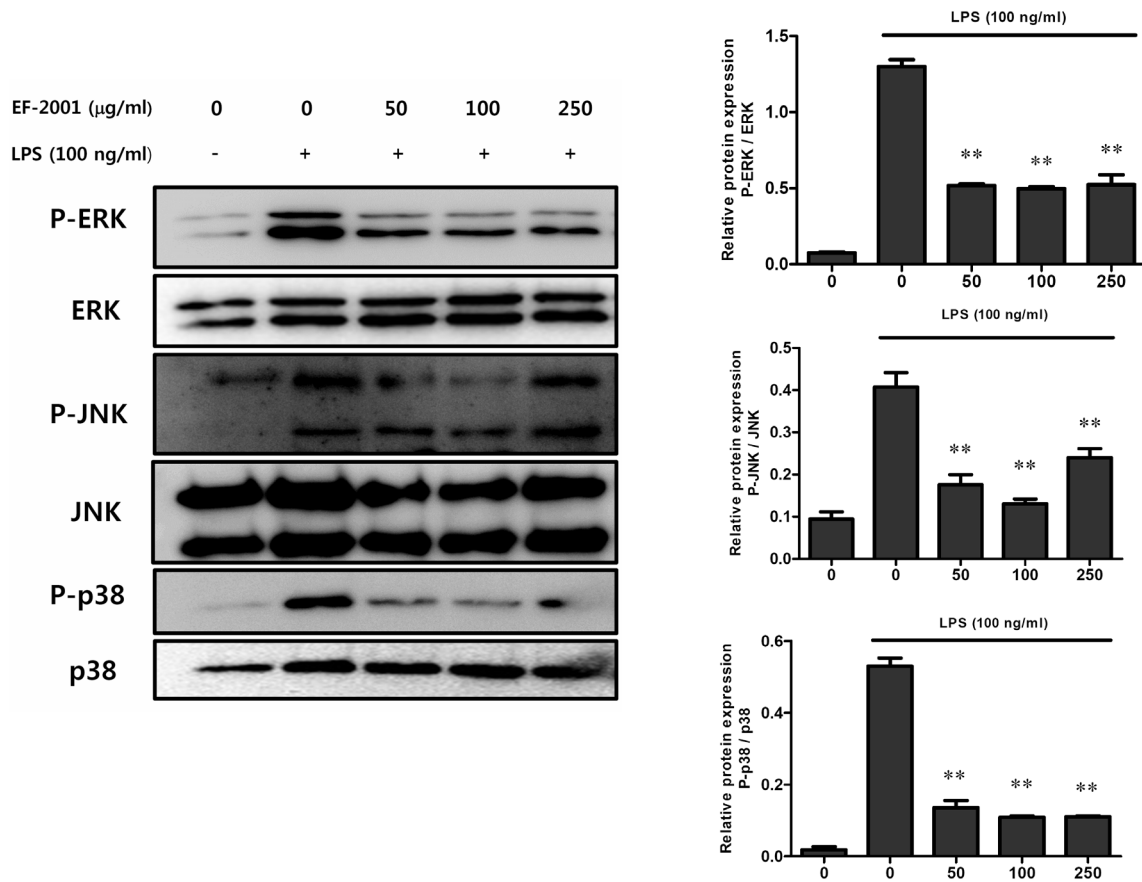


Fig. 4. Effect of EF-2001 on the LPS-induced MAP kinase pathway in Raw 264.7 cells. Raw 264.7 cells were pre-treated with various concentrations (50, 100, and 250 μg/ml) of EF-2001 for 30 min, followed by stimulation with LPS (100 ng/ml) for ERK (30 min), JNK (45 min), and p38 (30 min). Phosphorylation of ERK, JNK, and p38 was analyzed by Western blotting, and each image was analyzed using ImageQuant LAS 4000. Each value indicates the mean ± SD (n=4). \*\*p<0.01.

### Discussion

Inflammation is a defense mechanism that occurs when chemical substances and noxious stimuli are introduced into tissues or living organisms. If inflammation continues to be excessive, the condition becomes chronic [4].

Although EF-2001 was reported to have an effect on atopy [5, 6], its effects on inflammation were unclear. Therefore, in this study, we investigated whether activation of macrophages was inhibited by EF-2001. Although NO is an important substance involved in vasodilation and neurotransmission, excessive NO formation is related to the pathogenesis of various diseases including inflammation and cancer [1, 30]. By measuring the NO produced during inflammation, we confirmed that EF-2001 decreased NO production in a concentration-dependent manner (Fig. 1). Next, after the decrease of NO concentration was confirmed, iNOS and COX-2 experiments were carried out. iNOS produces

a much higher concentration of NO than neuronal NOS and endothelial NOS [25]. iNOS and COX-2 protein expressions were suppressed following treatment of the cells with EF-2001 (Fig. 3). These results suggest that EF-2001 inhibits all inflammatory mediators and anti-inflammatory processes.

We then examined the MAPK pathway and Akt-NF-κB pathway that are associated with inflammatory signaling [20]. Proteins in both pathways were evaluated for the degree of phosphorylation. Firstly, phosphorylation of three molecules, ERK, JNK, and p38 was confirmed in the MAPK pathway. The degree of phosphorylation of these molecules induced by LPS in EF-2001-treated cells was decreased (Fig. 4). These results suggest that activation of active protein-1, the main transcription factor in the MAPK pathway, is reduced [18, 19]. In a subsequent study on the Akt-NF-κB pathway, NF-κB translocation from the cytoplasm to the nucleus and its function as a transcription factor was examined; NF-κB cannot act as a transcription factor if its translocation

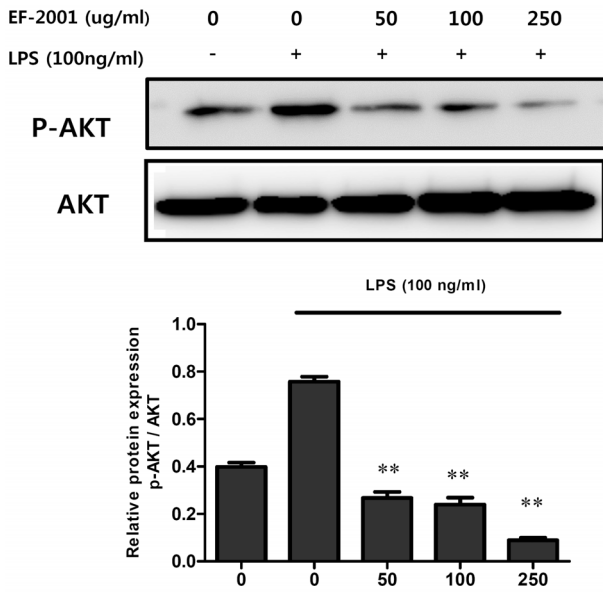


Fig. 5. Effect of EF-2001 on LPS-induced phosphorylation of Akt in Raw 264.7 cells. Raw 264.7 cells were pre-treated with various concentrations (50, 100, and 250  $\mu\text{g/ml}$ ) of EF-2001 for 30 min followed by stimulation with LPS (100 ng/ml) for 45 min. Phosphorylation of Akt was analyzed by Western blotting, each image was analyzed using ImageQuant LAS 4000. Each value indicates the mean  $\pm$  SD (n=4). \*\* $p < 0.01$ .

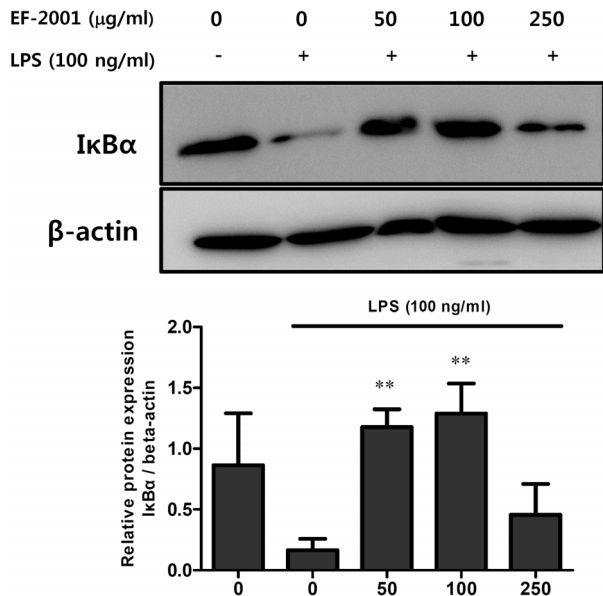


Fig. 6. Effect of EF-2001 on LPS-induced phosphorylation of IκB in Raw 264.7 cells. Raw 264.7 cells were pre-treated with various concentrations (50, 100, and 250  $\mu\text{g/ml}$ ) of EF-2001 for 30 min followed by stimulation with LPS (100 ng/ml) for 30 min. Phosphorylation of IκB was analyzed by Western blotting, each image was analyzed using ImageQuant LAS 4000. Each value indicates the mean  $\pm$  SD (n=4). \*\* $p < 0.01$ .

is inhibited [22]. Akt, the NF- $\kappa\text{B}$  upstream signal, directly regulates IκB kinase, which activates IκB, an inhibitor of NF- $\kappa\text{B}$  [24]. EF-2001 inhibited LPS-induced Akt phosphorylation (Fig. 5) and increased IκB expression (Fig. 6). Furthermore, EF-2001 inhibited p65 nuclear translocation (Fig. 7). Thus, EF-2001 blocked both the Akt-NF- $\kappa\text{B}$  pathway and the MAPK pathway.

The heat-killed *Enterococcus faecalis* used in this study was isolated from the feces of a healthy person and sold as a commercially available probiotic. A probiotic is a micro-organism that provides health benefits. Safety concerns in treatment with probiotics have not been clarified. However, some studies have reported complications when patients are treated with certain bacteria, and thus, further studies are needed to confirm that specific probiotics are safe [23]. Heat-treated lactic acid bacteria can be easily stored and have a long shelf life. Recently, heat-killed lactic acid bacteria were reported to exhibit beneficial immune-modulating effects [21]. Taken together, this study suggests that heat-killed *Enterococcus faecalis* may be used as a therapeutic agent for various inflammatory diseases.

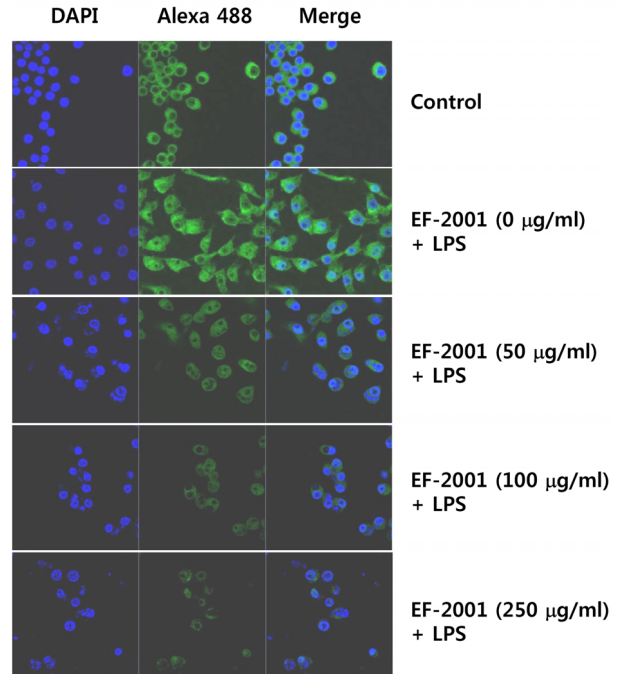


Fig. 7. Effect of EF-2001 on LPS-induced translocation of p65 in Raw 264.7 cells. Raw 264.7 cells stimulated with LPS (100 ng/ml) in the presence or absence of EF-2001 (50, 100, 250  $\mu\text{g/ml}$ ) for 18 hr. p65 localization was visualized by confocal microscopy after immunofluorescence staining with Alexa 488 (green). Cells were stained with DAPI for visualization of nuclei (blue).

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**초록 : 열처리 사균체 엔테로코커스 패칼리스 EF-2001의 항염증 효과**

최문석<sup>1</sup> · 장상진<sup>1</sup> · 채유리<sup>1</sup> · 이명현<sup>1</sup> · 김완중<sup>1</sup> · 이와사키 마사히로<sup>1,2</sup> · 한권일<sup>1,2</sup> · 김완재<sup>2</sup> · 김택중<sup>1,3\*</sup>  
 (<sup>1</sup>연세대학교 생명과학기술학부, <sup>2</sup>한국베름(주) 연구개발센터, <sup>3</sup>㈜닥터티제이 기술연구소)

염증은 인체에서 가장 흔히 나타나는 증상으로 조직이 손상되면 염증 반응이 발생하고 염증 부위에서 혈관 확장 과 혈류가 증가하여 부종이 생긴다. Lipopolysaccharide (LPS)는 Toll-like receptor 4에 의해 인식되고 염증 반응을 일으킨다. 열로 사멸시킨 *Enterococcus faecalis* 사균체(EF-2001)는 면역 조절 및 예방 활동을 하는 것으로 사전 보고되었고, 항 종양 효과가 있다고 보고되었지만 염증에 미치는 영향에 대해서는 지금까지 연구되지 않았다. 본 연구에서는 LPS에 의한 대식세포 염증 반응에 대한 EF-2001의 효과에 대해 연구하였다. 연구결과에서 EF-2001은 LPS에 의해 유도된 산화 질소의 생성을 감소시켰다. 우리는 EF-2001의 세포 독성이 있는지 확인했으며, 산화 질소의 감소는 세포독성에 의한 것이 아님을 확인하였다. 또한 이러한 EF-2001의 항염증 효과에 대한 분자기전을 연구 하였다. LPS에 의한 유도된 iNOS와 COX-2의 발현은 EF-2001에 의해 감소되었다. 더해진 분자기작 분석에서 EF-2001은 LPS로 유도된 ERK, JNK 및 p38 인산화를 농도 의존적으로 억제하였다. 더해진 실험에서 EF-2001은 Akt 인산화를 억제하고 NF-κB 억제제인 IκB 단백질 발현을 증가시켰다. 또한, EF-2001은 p65의 핵으로의 이동을 억제함을 알 수 있었다. 따라서, 이러한 결과는 EF-2001이 항염증 효과를 가지며 염증 질환 치료에 유용 할 수 활용 될 수 있음을 시사한다.