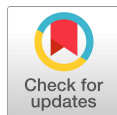# ARTICLE

Antioxidant and Anti-Inflammatory Activities of Heat-Treated Lactic Acid Bacteria and Quantitative Analysis of Lipoteichoic Acid under Different Heat Conditions

Yu-Bin Jeon, Sang-Jae Lee, Seo-Eun Song, In-Bae Kim, Cheol-Hyun Kim*

Department of Animal Resource & Science, Dankook University, Cheonan, Korea

*Corresponding author :
Cheol-Hyun Kim
Department of Animal Resource & Science, Dankook University, Cheonan, Korea
Tel : +82-41-529-6264
Fax : +82-41-529-6264
E-mail : hichkim@dankook.ac.kr

ORCID
Yu-Bin Jeon
https://orcid.org/0000-0002-5562-0958
Sang-Jae Lee
https://orcid.org/0009-0001-6877-5983
Seo-Eun Song
https://orcid.org/0009-0006-8584-1686
In-Bae Kim
https://orcid.org/0009-0006-3989-1543
Cheol-Hyun Kim
https://orcid.org/0000-0002-1905-9948

Abstract

In this study, we investigated the functional properties of kimchi-derived heat-treated lactic acid bacteria (LAB), focusing on their antioxidant and anti-inflammatory properties. We selected five LAB strains, i.e., *Lactiplantibacillus plantarum* DK211, *Lacticaseibacillus casei* DK301, *L. casei* DK302, *Lacticaseibacillus paracasei* DK310, and *L. paracasei* DK311based on their physiological activities. Heat treatment at 65℃ for 60 min resulted in the highest antioxidant activity, with DK211 and DK310 strains outperforming the commercial control strain. *In vitro* assays confirmed higher than 80% cell viability, significant nitric oxide (NO) inhibition, and reduced pro-inflammatory cytokine levels. High-performance liquid chromatography revealed that DK211 and DK310 contained higher lipoteichoic acid levels than the commercial strain. These results suggest that heat-treated LAB retained their bioactive properties, supporting their potential use as functional food ingredients with antioxidant and anti-inflammatory properties.

Keywords

Introduction

Probiotics are live microbes that are beneficial to human health and require safety, gastrointestinal endurance, adhesion to intestinal cells, and other biological functions [1]. Its known effects include lowering cholesterol levels, improving gastrointestinal diseases, anticancer activity, blood pressure regulation, immune modulation, constipation relief, and allergy improvement [2].

Recent studies on dead cells have emerged and enhanced probiotic efficiency. Dead cells lose their bacterial viability through chemical or physical methods that disrupt their DNA and cell membranes [3]. Recent studies have highlighted the immunomodulatory functions of probiotics, dead cells, and their metabolites [4], with dead cells proving to be more stable and easier to use than probiotics [5].

Heat is commonly used method for non-viable preparations. Even after heat treatment, probiotic bacteria and their extracts maintain their probiotic properties in the intestine, ensuring safer preparation [6,7]. Heat inactivation conditions vary widely (5–60 min at 60℃–121℃), depending on the strain and expected benefits [3]. *Lactobacillus* and *Bifidobacterium* species, which are commonly used as heat-treated dead cells, exhibit diverse physiological activities and retain their functionality [8].

Dead cells possess antimicrobial, antioxidant, and immunomodulatory properties with physiological, immunological, neuro-hormonal, and metabolic effects [6]. Heat treatment decomposes the cell wall, facilitating antioxidant release [9], whereas sugars and proteins generate Maillard reaction products with antioxidant activity [10]. Heat-treated *Lactobacillus* strains suppress NO and cytokine production in lipopolysaccharide (LPS)-induced RAW 264.7 cells, confirming their immunomodulatory potential [11].

Lipoteichoic acid (LTA), a cell wall component of gram-positive bacteria, induces cytokine production (TNF-α, IL-1β, and IL-8) and affects gut microbiota, anti-aging, sepsis, and cholesterol levels [12]. Unlike LPS from gram-negative bacteria, LTAs from *Lactobacillus* strains exhibit diverse immunoregulatory effects. They promote immune responses by activating macrophages and spleen cells. LTA regulates cell wall autolysis and binds to immune receptors, enhancing immunity by, acting as an anti-inflammatory agent [13,14].

Despite the increasing interest in dead cells, research on LTA from gram-positive lactic acid bacteria (LAB) remains limited compared to that on pathogenic gram-positive bacteria [15]. Studies on the physiological effects of heat-treated dead cells are limited, particularly regarding LTA, which is a key component of dead cells. Furthermore, LTA content analysis during LAB heat treatment has yet to be reported. Therefore, in this study, LAB were heat-treated to prepare dead cells, their functional properties were compared with those of live cells, and their immunological properties were analyzed through cell experiments and LTA content measurements.

Materials and Methods

1. Isolation and identification of lactic acid bacteria from kimchi

LAB were isolated from homemade kimchi collected from various regions of Korea. First, a 10 mL of kimchi sample was mixed with 40 mL of phosphate buffered saline (PBS; pH 7.4), homogenized and serially diluted with 0.85% Saline. Dillutions (100 μL) were plated onto De Man-Rogosa-Sharpe (MRS; BD Difco, USA) agar with 0.02% sodium azide and incubated at 37℃ for 48 h. The LAB colonies were cultured in MRS broth and preserved at ‑70℃ in MRS with 20% glycerol.

2. Screening and identification of lactic acid bacteria

The selected strains were evaluated based on their colony shape, Gram staining results, and microscopic morphology. The identified strains were further analyzed using 16S rRNA gene sequencing, and their sequence homology was compared with 16S rRNA gene sequences available in GenBank (Macrogen, Korea).

3. Tolerance to artificial gastric juice and bile

Gastric juice and bile tolerance were evaluated as described by [16]. Selected strains were subjected to different stress conditions to assess their resistance. For the acid tolerance assay, cultures were introduced into MRS broth containing 0.3% pepsin, which

had been adjusted to pH 2.0 using 1 N HCl, and subsequently incubated at 37℃ for 2 h. After incubation at 37℃ for 48 h, single colonies on MRS agar plates were examined. To determine bile tolerance, cultures were inoculated into MRS broth containing 0.3% oxgall and incubated at 37℃ for 24 h. The survival rate was assessed using a previously described method.

4. Heat treatment

LAB were subcultured three times at 16–18 h intervals in MRS broth, then harvested by centrifugation at 8,000×g for 10 min at 4℃. The supernatant was discarded and replaced with an equivalent volume of PBS (pH 7.4) and this washing process was repeated twice. The final bacterial concentration was adjusted to 10^{11} colony forming units (CFU)/mL. For the heat treatment, the cells were exposed to 65℃ and 95℃ for 5–60 min, and the viable cell count was determined. The survival rate was calculated using the following formula:

(log index value of experimental group / log index value of control) × 100.

5. Measurement of lipoteichoic acid (ELISA)

The heat-treated dead cells were centrifuged at 18,510×g for 10 min at 4℃, and the supernatant was collected for analysis. LTA analysis was performed using a general Lipoteichoic Acid ELISA kit (Mybiosource, USA) according to the manufacturer's instructions, and absorbance was measured at 450±2 nm using a microplate reader (Thermo Fisher Scientific, USA).

6. Protein quantification of live and dead cells

The heat-treated dead cells were centrifuged at 18,510×g for 10 min at 4℃, and the supernatant was collected for analysis. Protein quantification was conducted using the Total Protein Assay kit (Abnova, Taiwan) according to manufacturer's instruction, and absorbance was measured at 734 nm using a microplate reader.

7. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid radical savenging activity

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) diammonium salt) radical scavenging activity of live and dead cells was assessed following the method described by [17] with slight modifications. ABTS (Sigma-Aldrich, USA) was dissolved in distilled water (D.W.) to prepare a ABTS solution (7 mM). A potassium persulfate (Sigma-Aldrich) solution (2.45 mM) was prepared seperately. The ABTS solution and potassium persulfate were then mixed at a 1:1 (v/v) ratio and stored in the dark at room temperature for 12–16 h to allow radical formation. Following the incubation period, the ABTS reagent was diluted in PBS (pH 7.4) and adjusted until the absorbance was measured as 0.70±0.02 at 734 nm using a microplate reader. Subsequently, the ABTS reagent and live/dead cell samples were mixed at a 9:1 (v/v) ratio and incubated in the dark at room temperature for 6 min. After the reaction, the absorbance was measured

at 734 nm, and the results were expressed in terms of the Trolox (Sigma-Aldrich) equivalent antioxidant activity (reported in mM).

8. 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity of live and dead cell was evaluated according to the method described by [18] with some modifications DPPH radical solution (0.2 mM) was prepared for use in the experiment by mixing DPPH (Thermo Fisher Scientific) and ethanol (Samchun Pure Chemical, Korea). Next, the DPPH solution and live/dead cell samples were mixed in a 1:1 ratio (v/v) and incubated at room temperature in the dark for 30 min. The absorbance of the solution was measured at 517 nm, and the results were expressed in units of ascorbic acid (Sigma-Aldrich) equivalent capacity in millimolar (mM).

9. Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) of live and dead cells was determined using the method described by [19] with slight modifications. An acetate buffer solution (300 mM) was prepared by dissolving sodium acetate trihydrate (Sigma-Aldrich) in acetic acid (Samchun Pure Chemical). A HCl solution (40 mM) was prepared by mixing HCl (Samchun Pure Chemical) in D.W., whereas a 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) solution (10 mM) was prepared by dissolving TPTZ (Sigma-Aldrich) in D.W. Additionally, a 20 mM iron (III) chloride hexahydrate solution was prepared by dissolving 97% iron (III) chloride hexahydrate (A.C.S reagent; Sigma-Aldrich) in ethanol (Samchun Pure Chemical). To prepare the TPTZ reagent, 40 mM HCl and 10 mM TPTZ were mixed at a 1:1 (v/v) ratio. Then, the acetate buffer solution, TPTZ reagent, and iron (III) chloride hexahydrate solution were then combined in a 10:1:1 (v/v) ratio to create the FRAP working solution, which was pre-warmed at 37℃ before use. For the assay, the FRAP solution and live/dead cell samples were mixed at a 19:1 (v/v) ratio and allowed to react in the dark at room temperature for 30 min. After incubation, absorbance was recorded at 593 nm, and the results were expressed in terms of ascorbic acid (Sigma-Aldrich) equivalent capacity (mM).

10. Cell culture

RAW 264.7 cells (KCTC, Korea) were maintained in Dulbecco's Modified Eagle's Medium (Welgene, Korea) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich). Cultures were incubated at 37℃ in a 5% CO_2 environment to ensure optimal growth conditions.

11. Cell viability (MTT assay)

RAW 264.7 cells were seeded into a 96-well cell culture plate at a density of 1×10^5 cells/well and incubated for 16–18 h for adhesion. After removing the medium, live/dead cell sample (100 μL) was added and incubated at 37℃ with 5% CO_2 for 20–22 h. After incubation, the medium was removed, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-

tetrazolium bromide (MTT) (Sigma-Aldrich) solution (100 μL) was added to each well to a final concentration of 5 mg/mL. The plate was further incubated at 37℃ in a 5% CO_2 incubator for 2–3 h to allow formazan crystal formation. Once the reaction was complete, the medium was discarded, and 100 μL of dimethyl sulfoxide (Biosesang, Korea) was added to each well to fully dissolve the purple formazan crystals formed by the reduction reaction. The absorbance was then measured at 540 nm using following the method described by [20].

12. Measurements of nitric oxide (NO assay)

NO levels were determined by measuring nitrite in the cell culture supernatant using the griess reagent assay. RAW 264.7 cells were seeded into a 96-well cell culture plate at a density of 1×10^5 cells/well and incubated for 16–18 h. After removing the medium, live/dead cell samples at various concentrations were added. Thereafter each sample (100 μL) was dispensed into the wells and incubated at 37℃ with 5% CO_2 for 2–3 h. Subsequently, 100 μL of LPS (Sigma-Aldrich) at a final concentration of 100 ng/mL was introduced into each well, and the plate was further incubated for 20–22 h. After incubation, cell-free supernatant (100 μL) was transferred to a new plate, mixed with an equal volume of Griess reagent, and incubated in the dark for 15 min absorbance was measured at 540 nm using a microplate reader.

13. Cytokine measurements (ELISA)

The cytokine content in the supernatant of RAW 264.7 cell cultures was measured using a mouse TNF-α, IL-1α, and IL-6 ELISA kit (Komabiotech, Korea) following the manufacturer's instructions, and absorbance was measured at 450 nm using a microplate reader.

14. Lipoteichoic acid extraction and high-performance liquid chromatography (HPLC) analysis

The LTA extraction and high-performance liquid chromatography (HPLC) analysis were conducted following the method described by [21] with slight modifications. Heat-treated dead cells (200 mL) were centrifuged at 18,510×g for 20 min at 4℃ and resuspended twice in 20 mL of 0.1 M sodium citrate (pH 4.7; Sigma-Aldrich) and sonicated at 400 W for 5 min. After centrifugation, the pellet was extracted using 10 mL of 0.1 M sodium citrate and 10 mL of 1-butanol, shaken at room temperature for 30 min and centrifuged. The lower aqueous phase was collected and evaporated using a rotary evaporator, resuspended in 10 mL of D.W. and lyophilized. For HPLC analysis, 10 mg of lyophilized LTA sample was dissolved in 0.5 mL of 45% 1-propanol in 0.1 M ammonium acetate (pH 4.7; Sigma-Aldrich), centrifuged at 17,000×g for 60 min, and analyzed by reverse-phase (RP)-HPLC (Waters, USA) on a C8 column (Symmetry® C8, 3.9 mm×150 mm, particle size 5 μm, Waters) with a mobile phase of 45% 1-propanol in 0.1 M ammonium acetate (pH 4.7) (Table 1).

Table 1. HPLC condition for lipoteichoic acid (LTA) analysis

Instrument	Symmetry® C$_8$ column
Column	Symmetry® C$_8$ column
	(3.9 mm×150 mm, particle size 5 μm)
Mobile phase	0.1 M ammonium acetate (pH 4.7): 1-propanol
	(5.5 : 4.5, v/v)
Detector	UV/visible detector (215 nm)
Flow rate	1 mL/min
Injection volume	15.00 µL

Results

1. Isolation of lactic acid bacteria from kimchi

After 48 h of incubation, colonies of LAB were observed on the MRS agar plates. Five strains were isolated from kimchi samples and preserved at −70℃ in a mixture of 20% glycerol and MRS broth for long-term storage.

2. Screening and identification of lactic acid bacteria

For the Gram staining assay, the morphological characteristics of the isolated strains were examined under a microscope, which revealed that all strains were gram-positive. Additionally, 16s rRNA gene sequencing was performed to identify five strains, belonging to various *Lactobacillus* species. The 16s rRNA gene sequences of these strains are listed in Table 2.

3. Tolerance of lactic acid bacteria to artificial gastric juice and ile salts

Tolerance of the isolated strains to artificial gastric juice and bile salts was evaluated under simulated gastrointestinal conditions. Since the human stomach typically processes food within 20–120 min [22], the viability of LAB was tested after 2 h exposure to environments with pH 1.5, 2.0, and 3.0 at 37℃, followed by plate counting using BCP agar. The results demonstrated that LAB exhibited strong resistance to simulated gastric juice conditions (Table 3), consistent with findings by [23], which indicated that probiotic strain tolerance is strain dependent. Additionally, bacterial resistance to intestinal bile salts was assessed, because bile tolerance is a crucial factor in probiotic selection. Previous studies [24,25] have reported that human intestinal bile oxgall concentrations typically range from 0.3%–0.5%. To mimic these conditions, an artificial

Table 2. Identification of lactic acid bacteria isolated kimchi by 16s-rRNA sequence

Strain	16s-r-RNA sequence	
	Species	% ID
LA-5	*Lactobacillus acidophilus* 16S ribosomal RNA gene, complete sequence	99
DK109	*Lactiplantibacillus plantarum* strain VITDM15 16S ribosomal RNA gene, partial sequence	99
DK211	*Lacticaseibacillus casei* strain ML7 16S ribosomal RNA gene, partial sequence	99
DK212	*Lacticaseibacillus casei* strain cw3 16S ribosomal RNA gene, partial sequence	99
DK215	*Lacticaseibacillus paracasei* strain RS2 16S ribosomal RNA gene, partial sequence	99
DK310	*Lacticaseibacillus paracasei* strain CAU5144 16S ribosomal RNA gene, partial sequence	100

Table 3. Acid tolerance and bile tolerance of strains isolated from kimchi when incubated for 2 h at pH 1.5, 2.0 and 3.0

Strains	Counting (CFU/mL)			
	0 h	2 h		
		pH 1.5	pH 2.0	pH 3.0
LA-5	$9.01×10^{11}$	$2.21×10^7$	$4.12×10^8$	$1.68×10^9$
DK109	$4.43×10^{11}$	$1.12×10^7$	$8.51×10^7$	$1.26×10^9$
DK211	$8.15×10^{11}$	$1.04×10^7$	$1.42×10^8$	$5.44×10^9$
DK212	$6.52×10^{11}$	$1.42×10^7$	$6.98×10^7$	$9.54×10^8$
DK215	$3.02×10^{11}$	$2.66×10^7$	$1.19×10^8$	$1.82×10^9$
DK310	$4.59×10^{11}$	$3.97×10^7$	$1.92×10^8$	$2.88×10^9$

LA-5, *Lactobacillus acidophilus* LA-5 (Chr. Hansen, Denmark); DK109, *Lactiplantibacillus plantarum*. DK211, 212, *Lacticaseibacillus casei*; DK215, 310, *Lacticaseibacillus paracasei*; CFU, colony forming units.

digestive environment was created, with oxgall concentrations of 0.3%, and the number of viable bacteria was determined. These strains demonstrated good resistance to artificial bile juice (Table 4), supporting findings of previous studies [25,26] that highlighted strain-specific variations in LAB survival rates under artificial bile concentrations.

4. Heat treatment

Five strains of lactic acid bacteria (LAB) were heat-treated at 65℃ for 5, 15, 30, and 60 min and at 95℃ for 5 min and 10 min to induce bacterial inactivation. Thereafter the number of viable bacteria was determined (Figs. 1 and 2). The results confirmed that complete inactivation occurred at 65℃ for 30 min or longer and at 95℃ for 5 min or longer.

5. Measurement of lipoteichoic acid (ELISA)

The experimental results showed that LTA expression was highest at 65℃ for 60 min. Additionally, DK211, DK215, and DK310 exhibited higher LTA expression rates under

Table 4. Acid tolerance and bile tolerance of strains isolated from kimchi when incubated for 24 h at pH 1.5, 2.0 and 3.0

Strains	Counting (CFU/mL)		
	24 h (oxgall 0.3%)		
	pH 1.5	pH 2.0	pH 3.0
LA-5	$3.32×10^4$	$2.91×10^5$	$3.71×10^6$
DK109	$4.04×10^4$	$1.21×10^5$	$1.18×10^6$
DK211	$4.51×10^4$	$3.11×10^5$	$5.91×10^7$
DK212	$8.59×10^3$	$8.76×10^4$	$3.64×10^6$
DK215	$7.24×10^3$	$8.23×10^4$	$1.91×10^7$
DK310	$3.82×10^4$	$1.08×10^5$	$2.82×10^6$

LA-5, *Lactobacillus acidophilus* LA-5 (Chr. Hansen, Denmark); DK109, *Lactiplantibacillus plantarum*. DK211, 212, *Lacticaseibacillus casei*; DK215, 310, *Lacticaseibacillus paracasei*; CFU, colony forming units.
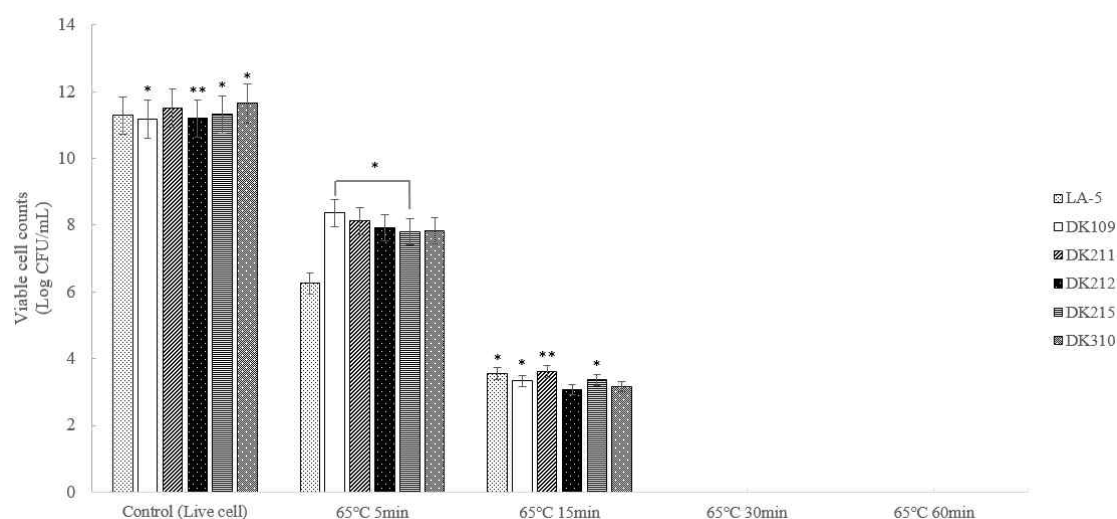
Fig. 1. Total viable counts of heat treated at 65℃, 5, 15, 30, 60 min. Statistical difference [*] p<0.05; [**] p<0.01; [***] p<0.001. LA-5, *Lactobacillus acidophilus* LA-5 (Chr. Hansen, Denmark); DK109, *Lactiplantibacillus plantarum*; DK211, 212, *Lacticaseibacillus casei*; DK215, 310, *Lacticaseibacillus paracasei*.
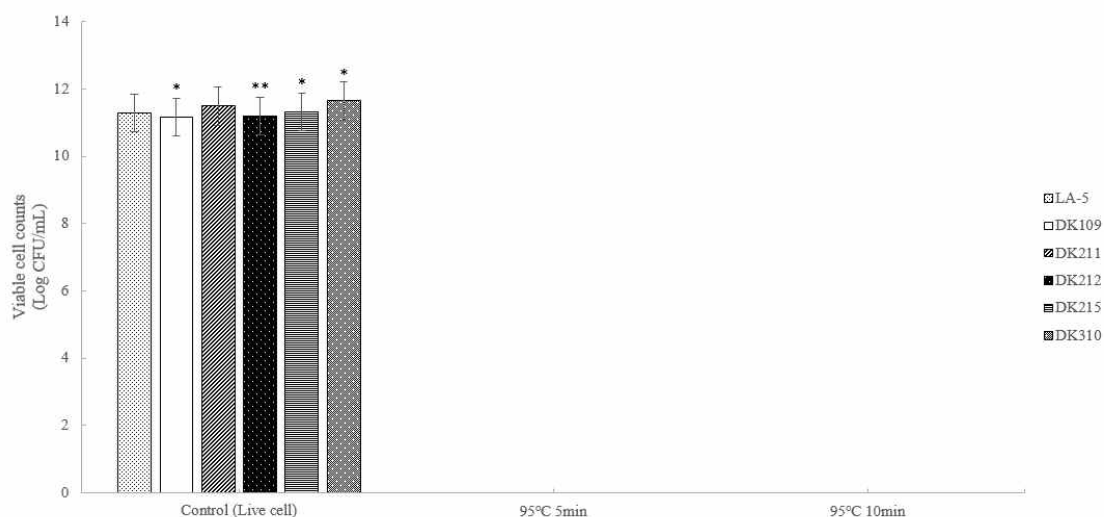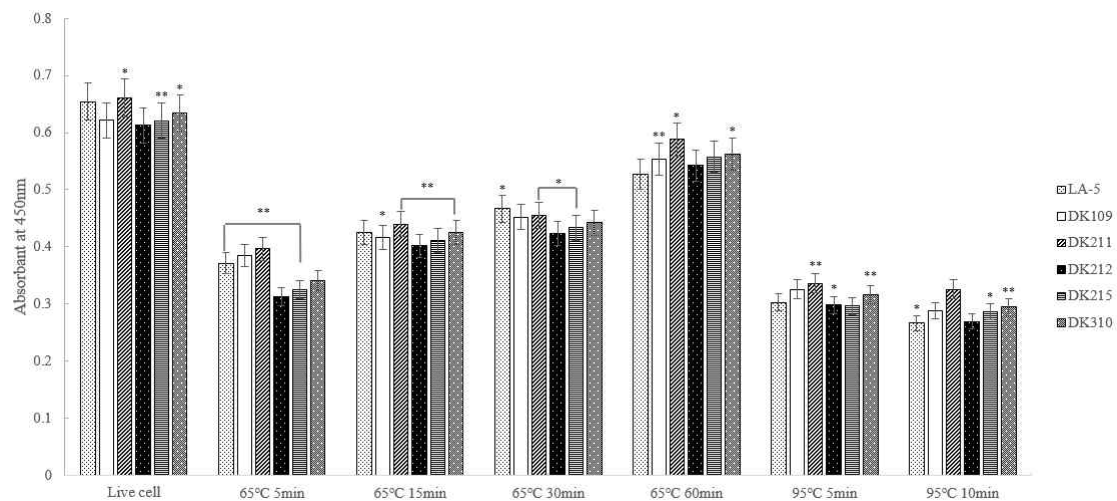

Fig. 2. Total viable counts of heat shock at 95℃, 5, 10 min. Statistical difference [*] p<0.05; [**] p<0.01; [***] p<0.001. LA-5, *Lactobacillus acidophilus* LA-5 (Chr. Hansen, Denmark); DK109, *Lactiplantibacillus plantarum*; DK211, 212, *Lacticaseibacillus casei*; DK215, 310, *Lacticaseibacillus paracasei*.

treatment at 65℃ for 60 min and 95℃ for 10 min compared to the commercial strain LA-5 (Fig. 3).

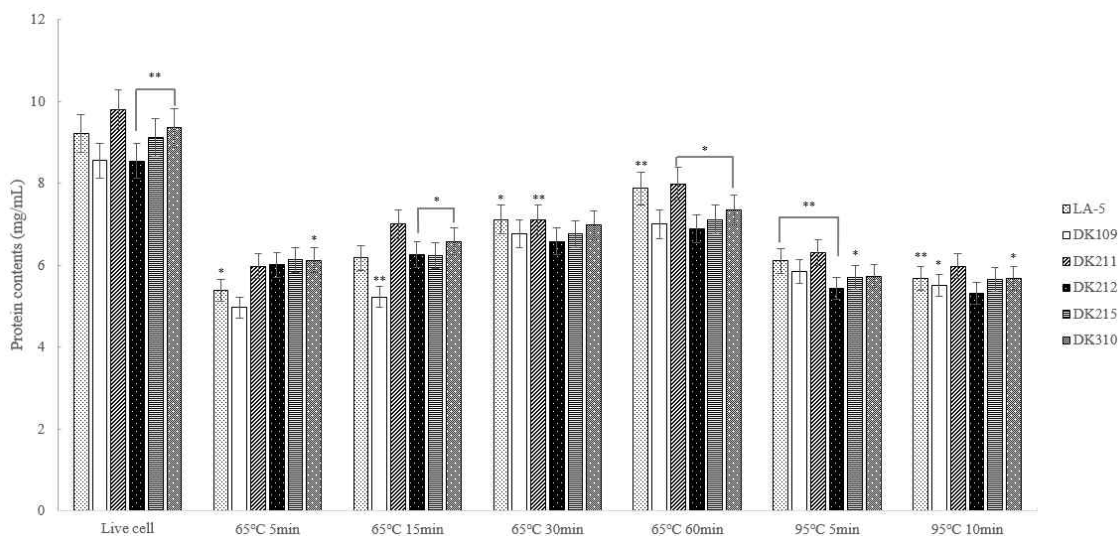## 6. Protein quantification

The quantitative protein analysis revealed that protein content in dead cells heat-treated at 95℃ was lower than in those heat-treated at 65℃. This suggests that heat treatment at 95℃ led to protein degradation, resulting in reduction in protein content (Fig. 4).
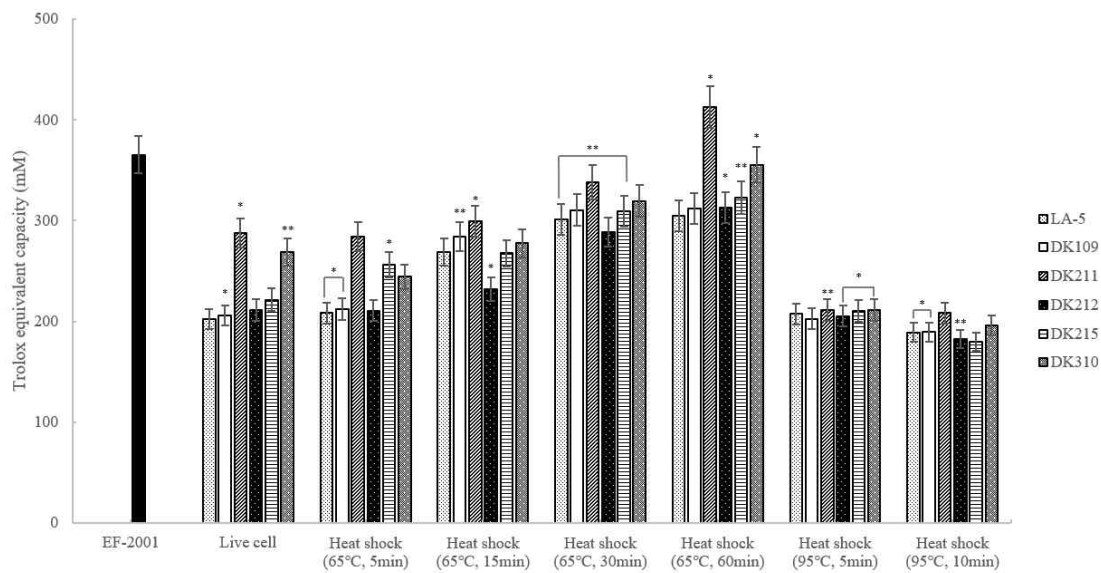
Fig. 3. Lipoteichoic acid analysis of live and dead (heat shock) cell. Statistical difference * $p<0.05$; ** $p<0.01$; *** $p<0.001$. LA-5, *Lactobacillus acidophilus* LA-5 (Chr. Hansen, Denmark); DK109, *Lactiplantibacillus plantarum*; DK211, 212, *Lacticaseibacillus casei*; DK215, 310, *Lacticaseibacillus paracasei*.

Fig. 4. Protein quantification of live and dead cell. Statistical difference * $p<0.05$; ** $p<0.01$; *** $p<0.001$. LA-5, *Lactobacillus acidophilus* LA-5 (Chr. Hansen, Denmark); DK109, *Lactiplantibacillus plantarum*; DK211, 212, *Lacticaseibacillus casei*; DK215, 310, *Lacticaseibacillus paracasei*.

7. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity

ABTS radical scavenging activity was measured and heat-treated DK211 and DK310 exhibited higher antioxidant activity than both LA-5 and live cells. Among the tested strains, DK211 and DK310 demonstrated greater ABTS radical scavenging activity under the same heat-treatment conditions. In particular, DK211 heat-treated at 65℃ for 60 min demonstrated higher ABTS radical scavenging activity than *Enterococcus faecalis* (EF-2001), a commercially available heat-killed probiotc strain (Fig. 5).

Fig. 5. ABTS radical scavenging activities for live and dead cell. Statistical difference * p<0.05; ** p<0.01; *** p<0.001. EF-2001: *Enterococcus faecalis* EF-2001 (Nihon Berumu, Japan); LA-5, *Lactobacillus acidophilus* LA-5 (Chr. Hansen, Denmark); DK109, *Lactiplantibacillus plantarum*; DK211, 212, *Lacticaseibacillus casei*; DK215, 310, *Lacticaseibacillus paracasei*. ABTS, 2,2'-azino- bis(3-ethylbenzothiazoline-6-sulfonic acid).

8. 2,2−diphenyl−1−picrylhydrazyl free radical scavenging activity

The DPPH assay was used to evaluate the antioxidant activity of heat-treated LAB strains. As shown in Fig. 6, dead cells heat-treated at 65℃ for 30 and 60 min exhibited antioxidant activity comparable to or higher than that of live cells. Notably, DK211 and DK310 heat-treated at 65℃ for 60 min demonstrated greater DPPH radical scavenging activity than EF-2001, a commercially available heat-killed probiotic strain.
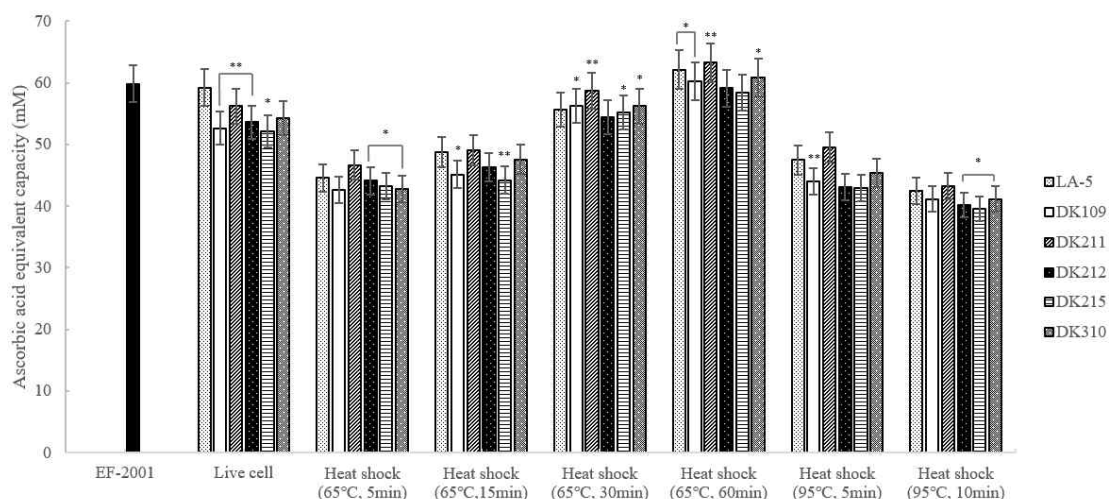
Fig. 6. DPPH free radical scavenging activities for live and dead cell. Statistical difference * p<0.05; ** p<0.01; *** p<0.001. EF-2001: *Enterococcus faecalis* EF-2001 (Nihon Berumu, Japan); LA-5, *Lactobacillus acidophilus* LA-5 (Chr. Hansen, Denmark); DK109, *Lactiplantibacillus plantarum*; DK211, 212, *Lacticaseibacillus casei*; DK215, 310, *Lacticaseibacillus paracasei*. DPPH, 2,2-diphenyl-1-picrylhydrazyl.

## 9. Ferric reducing antioxidant power (FRAP assay)

The FRAP assay was used to assess the antioxidant activity of heat-treated LAB strains by measuring their electron donation ability. As shown in Fig. 7, all DK strains exhibited higher iron-reducing capacity than LA-5 under the same heat-treatment conditions. Notably, DK211, DK212, and DK310 heat-treated at 65℃ for 60 min demonstrated significantly greater FRAP activity than EF-2001, a commercially available heat-killed probiotic strain. Based on these findings, 65℃ for 60 min was selected as the optimal heat treatment condition for further evaluation of anti-inflammatory effects.

10. Cell viability (MTT assay)

The MTT assay was used to assess the viability of RAW 264.7 macrophages after treatment with live and heat-treated LAB cells. As shown in Fig. 8, live cells exhibited no cytotoxicity or growth-promoting effects at any concentration except 10^{11} CFU/mL, while dead cells showed no cytotoxic effects across all tested concentrations. Notably, both live and dead cells maintained over 80% viability, confirming that they do not induce cytotoxic effects in macrophages.

11. Nitric oxide (NO) assay

NO assay was performed to evaluate the anti-inflammatory effects of heat-treated LAB strains in LPS-stimulated RAW 264.7 macrophages. As shown in Fig. 9, LPS treatment significantly increased NO production, whereas treatment with both live and dead LAB cells effectively suppressed NO production in a concentration-dependent manner. These findings suggest that heat-treated LAB strains exhibit anti-inflammatory potential by reducing excessive NO production associated with inflammation.
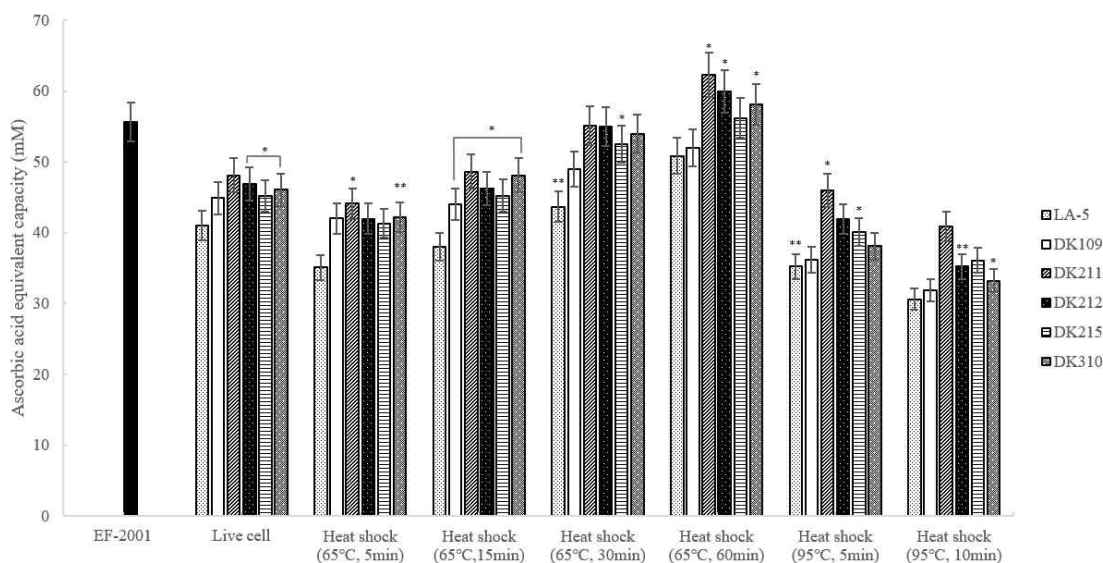
Fig. 7. FRAP activities for live and dead cell. Statistical difference * $p<0.05$; ** $p<0.01$; *** $p<0.001$. EF-2001: *Enterococcus faecalis* EF-2001 (Nihon Berumu, Japan); LA-5, *Lactobacillus acidophilus* LA-5 (Chr. Hansen, Denmark); DK109, *Lactiplantibacillus plantarum*; DK211, 212, *Lacticaseibacillus casei*; DK215, 310, *Lacticaseibacillus paracasei*. FRAP, ferric reducing antioxidant power.

(A)

(B)
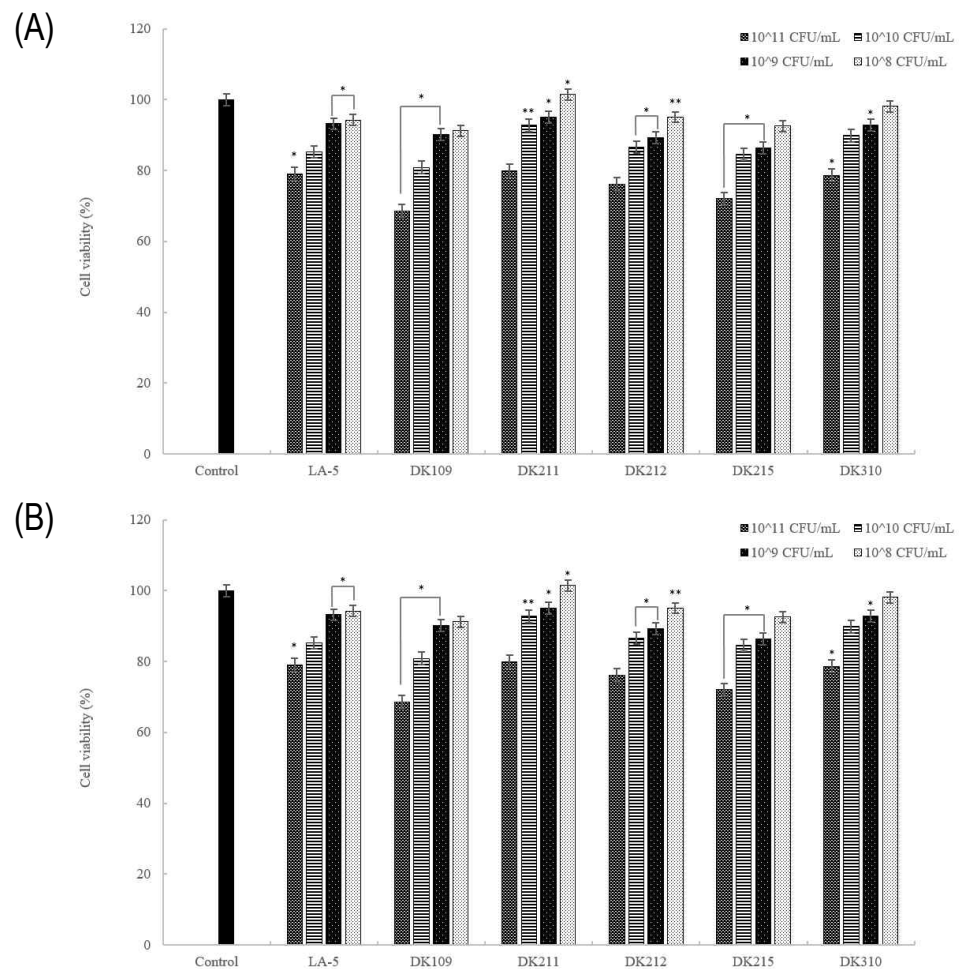
Fig. 8. Viability of RAW 264.7 cells treated with live and dead cell. Statistical difference * $p<0.05$; ** $p<0.01$; *** $p<0.001$. A: Viability of RAW 264.7 cells treated with live cell; B: Viability of RAW 264.7 cells treated with dead cell; LA-5, *Lactobacillus acidophilus* LA-5 (Chr. Hansen, Denmark); EF-2001: *Enterococcus faecalis* EF-2001 (Nihon Berumu, Japan); DK109, *Lactiplantibacillus plantarum*; DK211, 212, *Lacticaseibacillus casei*; DK215, 310, *Lacticaseibacillus paracasei*.

12. Cytokine measurements (ELISA)

Maintaining the immune balance requires direct and indirect interactions between immune cells, with cytokines playing crucial roles in the proliferation, differentiation, and functional regulation of immune responses. Many diseases are associated with inflammation, where inflammatory cells secrete inflammatory cytokines in response to harmful environmental factors, viruses, and drugs [27]. In this study, the expression on levels of TNF-α, IL-1α, and IL-6 were measured using ELISA, and results showed that their production was significantly lower in the treated live and dead cells than in the LPS (+) control group (Fig. 10). Additionally, dead cells exhibited lower TNF-α levels than live cells, suggesting that their effect on macrophages may help prevent the activation of pro-inflammatory cytokines induced by TNF-α expression. These findings indicate that both live and dead cells have the potential to exert anti-inflammatory effects.
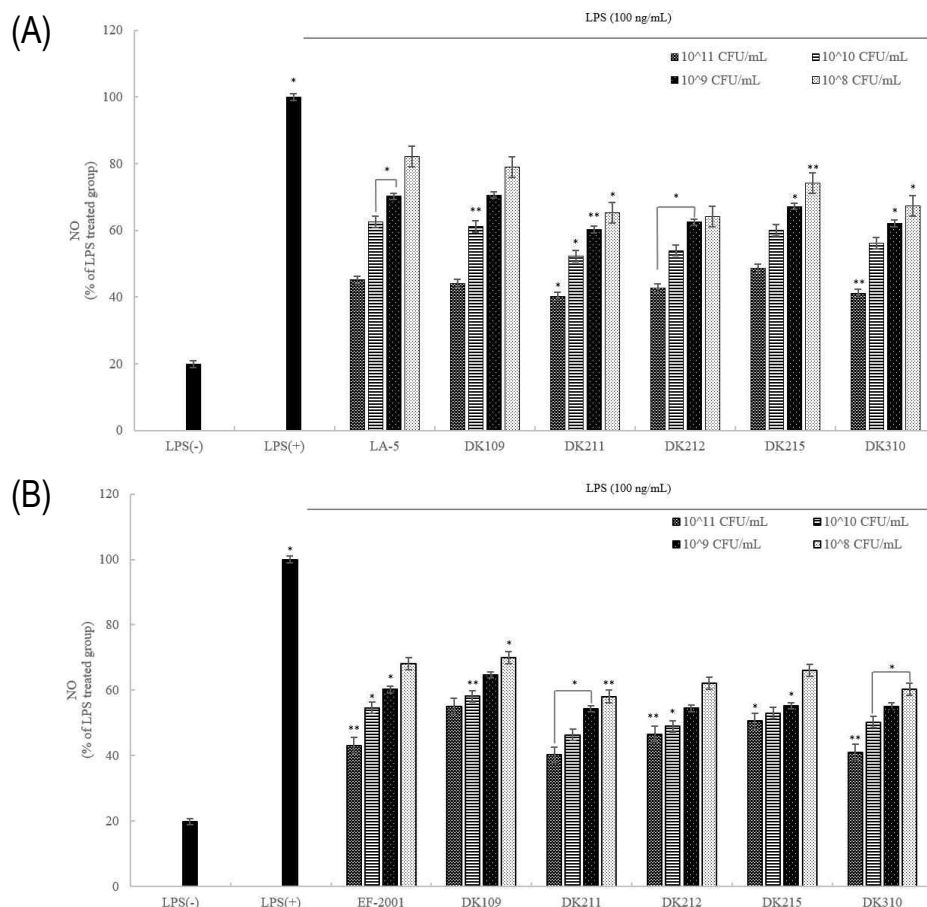
Fig. 9. Inhibitory effects of live and dead cells on LPS-induced nitric oxide production in RAW 264.7 cells. Statistical difference [*] $p<0.05$; [**] $p<0.01$; [***] $p<0.001$. A: Result of nitric oxide production of RAW 264.7 cells treated with live cell; B: result of nitric oxide production of RAW 264.7 cells treated with dead cell; LA-5, *Lactobacillus acidophilus* LA-5 (Chr. Hansen, Denmark); EF-2001: *Enterococcus faecalis* EF-2001 (Nihon Berumu, Japan); DK109, *Lactiplantibacillus plantarum*; DK211, 212, *Lacticaseibacillus casei*; DK215, 310, *Lacticaseibacillus paracasei*. LPS, lipopolysaccharide.

13. Lipoteichoic acid analysis of dead cells (HPLC)

LTA is a cell wall component of gram-positive bacteria, although numerous studies have been conducted, most studies have focused on pathogenic gram-positive bacteria, with limited investigations into LTA in LAB. In the present study, LTA was extracted using butanol and analyzed using a Symmetry C8 column in a RP-HPLC system. The LTA content in dead cells was quantified, and it was revealed that the commercial strain EF-2001 contained 2.67 mg/mL, whereas DK211 and DK310 contained 3.74 mg/mL and 3.72 mg/mL, respectively (Table 5). These findings confirmed that DK211 and DK310 had higher LTA content than the commercial strain EF-2001.

Discussion

This study examined the functional and immune effects of live and heat-treated dead

(A)

(B)
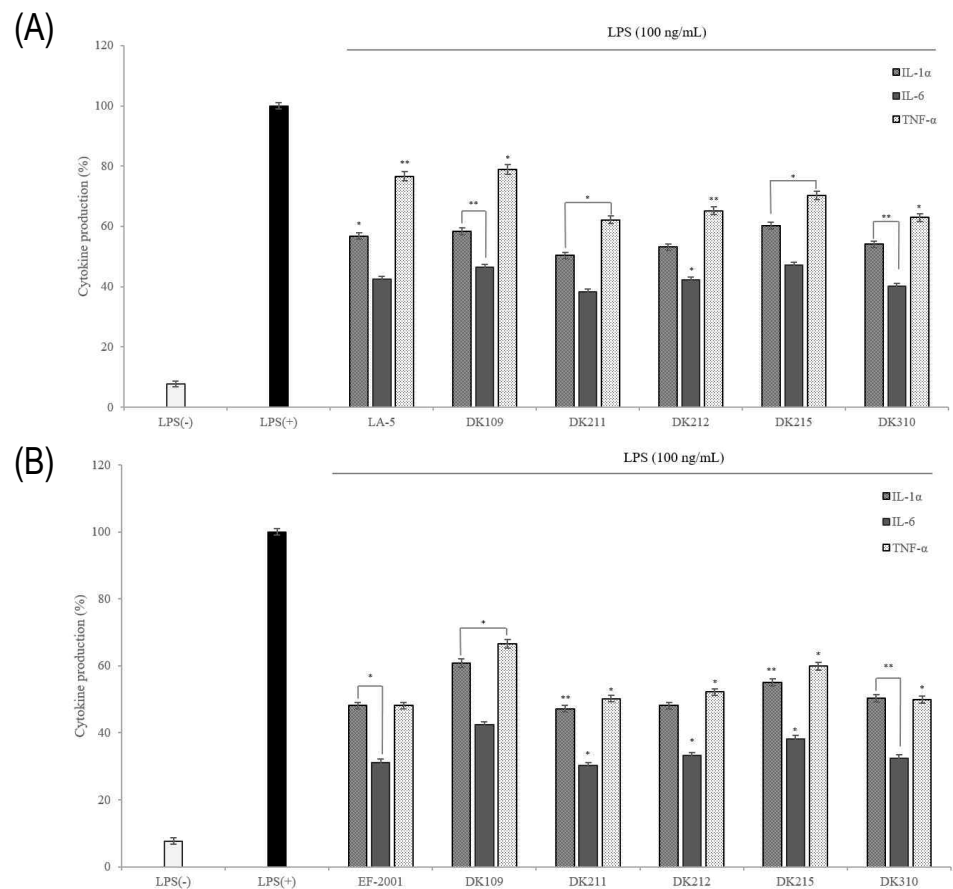
Fig. 10. Comparison of IL-1α, IL-6, and TNF-α production in LPS-stimulated RAW 264.7 cell treated with live and dead cells. Statistical difference * p<0.05; ** p<0.01; *** p<0.001. LA-5, *Lactobacillus acidophilus* LA-5 (Chr. Hansen, Denmark); EF-2001: *Enterococcus faecalis* EF-2001 (Nihon Berumu, Japan); DK109, *Lactiplantibacillus plantarum*; DK211, 212, *Lacticaseibacillus casei*; DK215, 310, *Lacticaseibacillus paracasei*. LPS, lipopolysaccharide.

Table 5. Lipoteichoic acid concentration in each sample

Sample	LTA content (mg/mL)
EF-2001	2.67
Dead cell DK211	3.74
Dead cell DK310	3.72

EF-2001, *Enterococcus faecalis* EF-2001 (Nihon Berumu, Japan); DK211, *Lacticaseibacillus casei*; DK310, *Lacticaseibacillus paracasei*; LTA, lipoteichoic acid.

LAB cells by analyzing their properties under different heat treatment conditions. Five kimchi-derived LAB strains were identified as one *Lactiplantibacillus plantarum* strain, two *Lacticaseibacillus casei* strains, and two *Lacticaseibacillus paracasei* strains, selected based on their physiological activity. All strains demonstrated strong resistance to human digestive enzymes, maintaining stability at pH 1.5 and tolerating artificial gastric juice and bile salts.

The bacteria were completely inactivated at 65℃ for 30 min and 95℃ for more than

5 min. LTA expression was analyzed in heat-treated LAB lysates, revealing that LTA expression increased with longer heat treatment durations, with DK211 and DK310 exhibiting the highest levels. Protein content analysis showed that LTA expression at 95℃ was lower than at 65℃ after 60 min, likely due to protein degradation at higher temperatures. Similarly, protein content was highest at 65℃ for 60 min, confirming that excessive heat leads to protein breakdown and reduced LTA expression.

Antioxidant activity varied by assay but consistently showed that heat-treated cells at 65℃ for 60 min had stronger activity than live cells. DK211, DK212, and DK310 exhibited greater antioxidant activity compared with EF-2001 in ABTS, DPPH, and FRAP assays. Heat-treated cells contain bioactive components such as intracellular proteins, polysaccharides, peptides, and polyphenols, which have been shown to contribute to the scavenging of reactive oxygen species (ROS) and thereby exert antioxidant effects [28]. In addition, heat treatment facilitates the release of various bioactive molecules, including exopolysaccharides, peptidoglycans, and LTA, which are presumed to play an important role in enhancing antioxidant capacity [7,29-31].

Among these components, LTA has received considerable attention as a key determinant of both antioxidant and immunomodulatory activity. LTA from pathogenic bacteria, such as *Staphylococcus aureus*, is known to activate Toll-like receptor 2 (TLR2)-mediated NF-κB signaling, thereby upregulating pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, and simultaneously promoting ROS generation, inflammation, and cellular damage [32,33]. Furthermore, LTA stimulation in macrophages and microglial cells has been reported to increase NO production and inducible nitric oxide synthase expression, while reducing the activity of antioxidant enzymes [34].

Conversely, LTA derived from probiotic *Lactobacillus* strains exhibits potent anti-inflammatory and antioxidant activities. For example, in a mouse model, administration of LTA purified from *L. paracasei* significantly decreased oxidative stress markers such as myeloperoxidase, NO, and malondialdehyde, while simultaneously increasing the levels of major antioxidant factors, including total superoxide dismutase and reduced glutathione, thereby enhancing overall antioxidant capacity [35]. Similarly, β-carotene bleaching assays confirmed antioxidant potential, as Heat treated *L. plantarum* exhibited strong activities, underscoring their potential as functional paraprobiotics [36].

In line with these reports, our results also demonstrated that heat-treated LAB cells downregulated cytokine production in LPS-induced macrophages. Expression of TNF-α, IL-1β, and IL-6 was significantly decreased compared with the control strain, consistent with observations that heat treatment modulates cytokine production depending on the amount of LTA released [37]. Furthermore, LTAs isolated from three different *Lactobacillus* strains exhibited distinct regulatory effects in RAW 264.7 cells, increasing anti-inflammatory IL-10 secretion while suppressing TNF-α and IL-6 expression, consistent with previous reports [38].

Taken together, these findings indicate that the immunomodulatory effects of LTA derived from *Lactobacillus* strains is not uniform but strain-specific, reflecting structural heterogeneity. Multi-spectrometric analyses of *L. plantarum* LTAs confirmed clear

strain-dependent variability in chemical composition and architecture, providing a mechanistic explanation for divergent immunological functions [39]. Importantly, this supports the hypothesis that LTA structure influences the interaction between microbe-associated molecular patterns and host pattern recognition receptors, thereby modulating downstream cytokine expression via NF-κB and MAPK signaling cascades.

The commercial strain *E. faecalis* EF-2001 has previously been recognized for its immunomodulatory properties, including anti-inflammatory and anti-allergic effects [40]. In our study, DK211 and DK310 not only showed comparable functional effects but also contained higher levels of LTA compared to EF-2001, suggesting their potential as candidate paraprobiotics. Since LTA bioactivity varies widely depending on bacterial species [41,42], further studies should aim to establish standardized purification protocols and reliable quantitative methods, along with in vivo validation models for their functional activities.

Collectively, our study highlights the potential of heat-treated LAB, particularly DK211 and DK310, as promising paraprobiotics with enhanced antioxidant and immunomodulatory properties. Notably, analysis of LTA extracted from heat-treated *Lactobacillus* strains (DK211, DK310) and *Enterococcus faecalis* EF-2001 via RP-HPLC revealed higher LTA levels in DK211 and DK310 compared to EF-2001. These findings, together with previous reports on the antioxidant and anti-inflammatory properties of LTA, indirectly suggest that dead cells may exhibit greater functional activity than live cells. However, further *in vivo* investigations are needed to confirm their efficacy and to elucidate the precise molecular mechanisms of LAB-derived LTA in immune regulation.

Conflict of Interest

The authors declare no potential conflict of interest.

Acknowledgements

References

1. Hwang CH, Kim KT, Lee NK, Paik HD. Immune-enhancing effect of heat-treated Levilactobacillus brevis KU15159 in RAW 264.7 cells. Probiotics Antimicrob Proteins. 2023;15:175-184.

2. Manning TS, Gibson GR. Microbial-gut interactions in health and disease. Probiotics. Best Pract Res Clin Gastroenterol. 2004;18:287-298.

3. de Almada CN, Martinez RC, Sant'Ana AS. Paraprobiotics: evidences on their ability to modify biological responses, inactivation methods and perspectives on their

application in foods. Trends Food Sci Technol. 2016;58:96-114.

4. Li N, Russell WM, Douglas-Escobar M, Hauser N, Lopez M, Neu J. Live and heat-killed Lactobacillus rhamnosus GG: effects on proinflammatory and anti-inflammatory cytokines/chemokines in gastrostomy-fed infant rats. Pediatr Res. 2009;66:203-207.

5. Choi MS, Chang SJ, Chae Y, Lee MH, Kim WJ, Iwasa M, et al. Anti-inflammatory effect of heat-killed Enterococcus faecalis, EF-2001. J Life Sci. 2018;28:1361-1368.

6. Aggarwal S, Sabharwal V, Kaushik P, Joshi A, Aayushi A, Suri M. Postbiotics: from emerging concept to application. Front Sustain Food Syst. 2022;6:408.

7. Piqué N, Berlanga M, Miñana-Galbis D. Health benefits of heat-killed (Tyndallized) probiotics: an overview. Int J Mol Sci. 2019;20:2534.

8. Deshpande G, Athalye-Jape G, Patole S. Para-probiotics for preterm neonates – the next frontier. Nutrients. 2018;10:871.

9. Liu CF, Pan TM. In vitro effects of lactic acid bacteria on cancer cell viability and antioxidant activity. J Food Drug Anal. 2010;18:77-86.

10. Liu CF, Tseng KC, Chiang SS, Lee HB, Hsu WH, Pan TM. Immunomodulatory and antioxidant potential of Lactobacillus exopolysaccharides. J Sci Food Agric. 2011;91:2284-2291.

11. Noh HJ, Park JM, Kwon YJ, Kim K, Park SY, Kim I, et al. Immunostimulatory effect of heat-killed probiotics on RAW264.7 macrophages. J Microbiol Biotechnol. 2022;32:638-644.

12. Hong YF, Lee YD, Park JY, Kim SJ, Lee YW, Jeon B, et al. Lipoteichoic acid isolated from Weissella cibaria increases cytokine production in human monocyte-like THP-1 cells and mouse splenocytes. J Microbiol Biotechnol. 2016;26:1198-1205.

13. Lee IC, Tomita S, Kleerebezem M, Bron PA. The quest for probiotic effector molecules—unraveling strain specificity at the molecular level. Pharmacol Res. 2013;69:61-74.

14. Kim YH, Lee B, Kang JH, Kang DJ. Development of tyndallized Lactobacillus rhamnosus IDCC 3201 with immunomodulation: optimization, validation, and in vitro evaluation. Korean Soc Biotechnol Bioeng J. 2017;32:271-278.

15. Stadelmaier A, Figueroa-Perez I, Deininger S, von Aulock S, Hartung T, Schmidt RR. A Staphylococcus aureus lipoteichoic acid (LTA) derived structural variant with two diacylglycerol residues. Bioorg Med Chem. 2006;14:6239-6254.

16. Yun H, Kim JD, Choi HC, Lee CW. Antibacterial activity of CNT-Ag and GO-Ag nanocomposites against Gram-negative and Gram-positive bacteria. Bull Korean Chem Soc. 2013;34:3261-3264.

17. Shan B, Cai YZ, Sun M, Corke H. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. J Agric Food Chem. 2005;53:7749-7759.

18. Tang W, Xing Z, Li C, Wang J, Wang Y. Molecular mechanisms and in vitro anti-oxidant effects of Lactobacillus plantarum MA2. Food Chem. 2017;221:1642-1649.

19. Chaiyasut C, Kumar T, Tipduangta P, Rungseevijitprapa W. Isoflavone content and antioxidant activity of Thai fermented soybean and its capsule formulation. Afr J

Biotechnol. 2010;9:4120-4126.

20. Sadeghi-Aliabadi H, Mohammadi F, Fazeli H, Mirlohi M. Effects of Lactobacillus plantarum A7 with probiotic potential on colon cancer and normal cells proliferation in comparison with a commercial strain. Iran J Basic Med Sci. 2014;17:815-819.

21. Tadmor K, Pozniak Y, Golani TB, Lobel L, Brenner M, Sigal N, et al. Listeria monocytogenes MDR transporters are involved in LTA synthesis and triggering of innate immunity during infection. Front Cell Infect Microbiol. 2014;4:16.

22. Renner HW, Münzner R. The possible role of probiotics as dietary antimutagens. Mutat Res Lett. 1991;262:239-245.

23. Mechai A, Debabza M, Kirane D. Screening of technological and probiotic properties of lactic acid bacteria isolated from Algerian traditional fermented milk products. Int Food Res J. 2014;21:2199-2208.

24. Minikiewicz P, Slangen CJ, Lagerwerf FM, Haverkamp J, Rollema HS, Visser S. Reversed-phase high-performance liquid chromatographic separation of bovine κ-casein macropeptide and characterization of isolated fractions. J Chromatogr A. 1996;743:123-135.

25. Romacho T, Glosse P, Richter I, Elsen M, Schoemaker MH, van Tol EA, et al. Nutritional ingredients modulate adipokine secretion and inflammation in human primary adipocytes. Nutrients. 2015;7:865-886.

26. Gu RX, Yang ZQ, Li ZH, Chen SL, Luo ZL. Probiotic properties of lactic acid bacteria isolated from stool samples of longevous people in regions of Hotan, Xinjiang and Bama, Guangxi, China. Anaerobe. 2008;14:313-317.

27. Barland CO, Zettersten E, Brown BS, Ye J, Elias PM, Ghadially R. Imiquimod-induced interleukin-1α stimulation improves barrier homeostasis in aged murine epidermis. J Invest Dermatol. 2004;122:330-336.

28. Hoffmann A, Kleniewska P, Pawliczak R. Antioxidative activity of probiotics. Arch Med Sci. 2019;17:792-804.

29. Akter S, Park JH, Jung HK. Potential health-promoting benefits of paraprobiotics, inactivated probiotic cells. J Microbiol Biotechnol. 2020;30:477-481.

30. Nataraj BH, Ali SA, Behare PV, Yadav H. Postbiotics-parabiotics: the new horizons in microbial biotherapy and functional foods. Microb Cell Fact. 2020;19:168.

31. Teame T, Wang A, Xie M, Zhang Z, Yang Y, Ding Q, et al. Paraprobiotics and postbiotics of probiotic Lactobacilli, their positive effects on the host and action mechanisms: a review. Front Nutr. 2020;7:570344.

32. Zhao G, Jiang K, Wu H, Qiu C, Deng G, Peng X. Polydatin reduces Staphylococcus aureus lipoteichoic acid-induced injury by attenuating reactive oxygen species generation and TLR2-NFκB signalling. J Cell Mol Med. 2017;21:2796-2808.

33. Jayakumar T, Yang CM, Yen TL, Hsu CY, Sheu JR, Hsia CW, et al. Anti-inflammatory mechanism of an alkaloid rutaecarpine in LTA-stimulated RAW 264.7 cells: pivotal role on NF-κB and ERK/p38 signaling molecules. Int J Mol Sci. 2022;23:5889.

34. Fernández-Rojas B, Vázquez-Cervantes GI, Pedraza-Chaverri J, Gutiérrez-Venegas G. Lipoteichoic acid reduces antioxidant enzymes in H9c2 cells. Toxicol Rep. 2020;7:101-108.

35. Zhang L, Liu J, Kong S, Chen N, Hung WL, Zhao W, et al. Lipoteichoic acid obtained from Lactobacillus paracasei via low-temperature pasteurization alleviates the macrophage inflammatory response by downregulating the NF-κB signaling pathway. J Funct Foods. 2023;107:105673.

36. You CB, Lee ES, Lee MK, Lee GY, Park H. Antioxidant and anti-inflammatory activities of heat-killed Lactiplantibacillus plantarum isolated from kimchi. Curr Top Lact Acid Bact Probiotics. 2022;8:66-78.

37. Lee JW, Song SE, Kim IB, Oh DG, Kim DJ, Kim CH. Functional properties of heat-killed lactic acid bacteria isolated from Vietnamese feces. Food Sci Anim Resour. 2025;45:890-909.

38. Lu Q, Guo Y, Yang G, Cui L, Wu Z, Zeng X, et al. Structure and anti-inflammation potential of lipoteichoic acids isolated from Lactobacillus strains. Foods. 2022;11:1610.

39. Jang KS, Baik JE, Han SH, Chung DK, Kim BG. Multi-spectrometric analyses of lipoteichoic acids isolated from Lactobacillus plantarum. Biochem Biophys Res Commun. 2011;407:823-830.

40. Chang SJ, Lee MH, Kim WJ, Chae Y, Iwasa M, Han KI, et al. Effect of heat-killed Enterococcus faecalis, EF-2001 on C2C12 myoblast damage induced by oxidative stress and muscle volume decreased by sciatic denervation in C57BL/6 mice. J Life Sci. 2019;29:215-222.

41. Jeong JH, Jang S, Jung BJ, Jang KS, Kim BG, Chung DK, et al. Differential immune-stimulatory effects of LTAs from different lactic acid bacteria via MAPK signaling pathway in RAW 264.7 cells. Immunobiology. 2015;220:460-466.

42. Leemans JC, Vervoordeldonk MJBM, Florquin S, van Kessel KP, van der Poll T. Differential role of interleukin-6 in lung inflammation induced by lipoteichoic acid and peptidoglycan from Staphylococcus aureus. Am J Respir Crit Care Med. 2002;165:1445-1450.