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ARTICLE

Functional Properties of Heat-Killed Lactic Acid Bacteria Isolated from Vietnamese Feces

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Abstract This study evaluated the functional properties of lactic acid bacteria (LAB) isolated from Vietnamese feces under various heat-treatment conditions by assessing their antioxidant activity, lipoteichoic acid (LTA) content, and immunomodulatory effects. Among the six LAB strains isolated, four (*Ligilactobacillus salivarius* V4, *Limosilactobacillus fermentum* V8, *Lactiplantibacillus plantarum* V10, and V11) were selected based on their acid and bile resistance, proteolytic activity, and β-galactosidase activity. Heat treatment (65℃ for 60 min or 95℃ for 10 min) completely inactivated LAB, reducing protein and phosphate content as treatment intensity increased. Antioxidant assays [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), 2,2-diphenyl-1-picrylhydrazyl, and ferric reducing antioxidant power] revealed comparable or superior activity in heat-treated (HT) cells compared to live cells, with *L. plantarum* V10 showing the highest activity. LTA content analysis of HT *L. plantarum* V10 showed the highest LTA content (1,244 ppm) at 65℃ for 60 min, surpassing that of the commercial dead cell *Enterococcus faecalis* EF-2001. Anti-inflammatory activity tests indicated no cytotoxicity at concentrations below 10^7 CFU/mL, and HT cells treated at 65°C for 60 min showed the highest inhibition of nitric oxide and the lowest expression levels of inflammatory cytokines [tumor necrosis factor-α, interleukin (IL)-1β, IL-6]. Consequently, 65°C for 60 min was established as the optimal heat-treatment condition. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis following digestive enzyme treatment revealed that HT *L. plantarum* V10 was not degraded by amylase or pepsin, but was partially degraded by oxgall. These findings suggest that HT *L. plantarum* V10 is a promising functional ingredient with antioxidant and immunoregulatory properties and is especially suitable for the Southeast Asian market.

Keywords lactic acid bacteria, heat-killed, lipoteichoic acid, antioxidant, anti-inflammatory

Introduction

The World Health Organization defines probiotics as "living microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Aljohani et al., 2024). Probiotics promote human health by maintaining the intestinal microbiota balance, enhancing immune function, and preventing conditions such as diarrhea, inflammatory bowel disease, and hypercholesterolemia (Hill et al., 2014; Sanders et al., 2019). These benefits have driven the global popularity of probiotics, making them essential health supplements and functional foods (Kechagia et al., 2013).

Recently, the Southeast Asian probiotic market has grown owing to increasing awareness of immunity enhancement and gut health benefits (China Research & Intelligence, 2023). However, the applications of heat-killed lactic acid bacteria (HK-LAB) are limited. HK-LAB inactivated via heat treatment retain health-promoting properties and offer advantages, such as stability and safety, for functional food applications (Piqué et al., 2019; Siciliano et al., 2021). Localized LAB strains, which reflect the unique gut microbiota of Southeast Asia, can improve product efficacy and market acceptance (Lee et al., 2017b).

Unlike live probiotics, HK-LAB do not proliferate in the gut, thus minimizing microbiota disruption (Sugahara et al., 2017). Heat treatment enhances functional properties by releasing intracellular antioxidants that reduce oxidative stress (Liu and Pan, 2010). HK-LAB also suppress pro-inflammatory cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6] and increase anti-inflammatory cytokines (IL-10), supporting immune modulation (Choi et al., 2018). Lipoteichoic acid (LTA), a major cell wall component of Gram-positive bacteria, interacts with Toll-like receptor-2 (TLR-2) to modulate inflammatory responses and promote immune balance (Kang et al., 2016).

Despite these benefits, research on HK-LAB, particularly on LTA content under varying heat treatment conditions, remains limited. In this study, we isolated LAB strains from Vietnamese feces to produce HK-LAB, and compared their functional properties with those of live cells. Through LTA analysis and cellular assays, we evaluated their potential as immunomodulatory ingredients for functional foods targeted in Southeast Asian markets.

Materials and Methods

Screening and identification of lactic acid bacteria

The selected strains were screened based on colony shape, Gram staining, and microscopic morphology. The selected strains were confirmed by 16S-rRNA gene sequencing based on sequence homology against 16S-rRNA gene sequences in the GenBank database (Macrogen, Seoul, Korea).

Tolerance of lactic acid bacteria to artificial gastric juice and bile

Tolerance to artificial gastric juice and bile was measured using the method described by Vinderola and Reinheimer (2003). To investigate the survival of LAB strains under acidic condition, each strain of LAB was harvested by centrifugation at 6,000×g for 10 min, washed with PBS (pH 7.4), and inoculated (1%) into MRS broth acidified to pH adjusted 3.0, 2.0 and 1.5 (using HCl) containing 1,000 units of pepsin (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37℃ for 2 h. Strains inoculated in non-acidified MRS broth were used as controls. Artificial bile acid tolerance was determined by cultivating the LABs in artificial bile. LAB cultivated in artificial gastric juice were harvested by centrifugation at 6,000×g for 10 min and washed with PBS (pH 7.4). The LABs were incubated at 37℃ for 24 h in MRS broth containing artificial bile (0.3% Oxgall; Becton Dickinson, Franklin Lakes, NJ, USA). The number of viable cells was determined by plating 1 mL aliquots of 10-fold serial dilutions in 0.85% saline on BCP agar. Plates were incubated at 37℃ for 48 h, and CFUs were estimated.

Proteolytic activity

Proteolytic activity was measured using a previously described method (Riffel and Brandelli, 2006) with some modifications. Selected LAB strains were screened for proteolytic activity using an agar well diffusion test on skim milk containing 2.0%

(w/v) agar (Becton Dickinson). After centrifugation at 5,000×g for 10 min, a 100 μL aliquot of the supernatant was loaded into 9 mm-diameter wells on skim milk agar plates. Proteolysis resulted in the formation of clear zones around the wells. Protease activity was determined by measuring the diameter of the clear zone area every 24 h over 72 h of incubation at 37℃.

β-Galactosidase activity

The *β*-galactosidase activity was measured as previously described (Jalili et al., 2009), with slight modifications. Cultures were grown in MRS broth at 37℃ for 18 h, and OD_{600} was recorded. A 1 mL culture aliquot was centrifuged at 12,000×g for 10 min at 4℃, washed twice with PBS (pH 7.4), and resuspended in 300 μL of 0.25 M Tris buffer (pH 8.0). Cell lysis was performed via freeze-thaw cycles (–80℃ for 30 min, room temperature for 30 min). After centrifugation (12,000×g, 3 min, 4℃), 30 μL of distilled water, 70 μL of 4 mg/mL o-nitrophenyl-*β*-D-galactopyranoside (Sigma-Aldrich), and 200 μL of Z buffer with *β*-mercaptoethanol were added to 100 μL of supernatant. The reaction was incubated at 37℃ for 30 min in the dark and stopped with 500 μL of 1 M sodium carbonate. Absorbance was measured at OD_{420} and OD_{550}, and *β*-galactosidase activity was calculated using the formula:

$$\text{Activity of } \beta\text{-galactosidase (unit/mL)} = (OD_{420} - 1.75 \times OD_{550}) / (t \times v \times OD_{600}) \quad (1)$$

Where t, v, OD_{420}, OD_{550}, and OD_{600} denote reaction time (min), sample volume (mL), and absorbance (420, 550, and 600 nm), respectively.

Heat shock treatment

LAB was subcultured three times at 16–18 h intervals in MRS broth, and the grown LAB was centrifuged at 10,000×g for 10 min at 4℃. After removing the supernatant, PBS (pH 7.4) was added to the cells. This process was repeated twice to harvest at 10^9 CFU/mL. For the heat shock treatment, cells were treated at different temperatures and times, and the viable cell count was measured.

Protein quantification

The heat-treated dead cells were centrifuged at 18,510×g for 10 min at 4℃; then only the supernatant was taken and used as a sample. Protein was quantified using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and colorimetric methods (Klotz et al., 2017). Ten microliter of samples were added to a 96-well cell culture plate, and then 190 μL of reagent was added to each well. After incubation at room temperature for 30 min, absorbance was measured at 562 nm using a microplate reader.

Phosphate assay

The heat-treated dead cells were centrifuged at 18,510×g for 10 min at 4℃; then only the supernatant was taken and used as a sample. Phosphate analysis was performed using a phosphate assay kit (Sigma-Aldrich) and colorimetric methods. Fifty microliter of samples were added to a 96-well cell culture plate, and then 100 μL of reagent was added to each well. After incubation at room temperature for 30 min, the absorbance of the solution was measured at 620 nm using a microplate reader.

Antioxidant activity

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) powder (7 mM, Sigma-Aldrich) in ethanol was prepared to investigate ABTS radical scavenging activity. Then, ABTS and 2.45 mM potassium persulfate (Sigma-Aldrich) were mixed in a 1:1 (v/v) ratio to prepare an ABTS solution. The resulting ABTS solution was stored at room temperature for 16 h. The ABTS•+ stock solution was diluted with ethanol to an absorbance of 0.7±0.02 at 734 nm. Nine hundred microliter of the diluted ABTS•+ solution was added to 100 μL of live and dead cell samples and reacted at 37℃ for 10 min. Absorbance was measured at 734 nm after centrifugation at 12,000×g for 10 min and was calculated as follows (Hussen and Endalew, 2023).

$$\text{ABTS radical scavenging activity (\%)} = (1 - A_{sample} / A_{control}) \times 100 \quad (2)$$

2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity was measured using a previously reported method (Han et al., 2020) with slight modifications. First, 0.4 mM DPPH was prepared in ethanol. Subsequently, 1.25 mL of DPPH solution was mixed with 500 μL of live and dead cell samples and reacted in the dark at room temperature for 30 min. Absorbance was measured at 517 nm after centrifugation at 12,000×g for 10 min, and was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = (1 - A_{sample} / A_{control}) \times 100 \quad (3)$$

Ferric reducing antioxidant power

Ferric-reducing antioxidant power (FRAP) was measured using a previously reported method (Aregbe et al., 2024). To prepare the FRAP solution, an acetate buffer (300 mM, pH 3.6) was prepared by mixing sodium acetate trihydrate (Sigma-Aldrich) and acetic acid (J.T. Baker, Phillipsburg, NJ, USA). Then, a 10 mM TPTZ was prepared by dissolving 2,4,6-tri (2-pyridyl)-1,3,5-triazine (Sigma-Aldrich) in 40 mM HCl (DAEJUNG, Siheung, Korea). Finally, a 20 mM Iron(III) chloride hexahydrate (Sigma-Aldrich) solution was prepared by dissolving Iron(III) chloride hexahydrate (97% A.C.S reagent; Sigma-Aldrich) solution was prepared in ethanol (Samchun Chemical, Seoul, Korea). The three solutions were mixed in a ratio of 10:1:1 (v/v). For the assay, 1 mL of the FRAP solution was mixed with 30 μL of live and dead cell samples or standard solution. The mixture was vortexed and reacted at 37℃ for 10 min in the dark. The absorbance was measured at 593 nm after centrifugation at 12,000×g for 10 min. The antioxidant capacity was expressed as Trolox (Sigma-Aldrich) equivalents (mM).

Lipoteichoic acid analysis of heat-killed lactic acid bacteria with high-performance liquid chromatography

LTA extraction and high-performance liquid chromatography (HPLC) analyses were performed as previously described (Lu et al., 2022; Tadmor et al., 2014). Five liter of dead cells were centrifuged at 10,000×g for 10 min at 4℃ and suspended with 50 mL of 0.1 M sodium citrate buffer (pH 4.7; Sigma-Aldrich). The samples were sonicated at 400 W for 30 min in an ice-cold bath. The suspension was then mixed with 50 mL of n-butyl alcohol and shaken at room temperature for 30 min. After centrifugation, the supernatant from the lower aqueous phase was collected and separated into two phases. The samples were then lyophilized. The lyophilized LTA sample was mixed with 5 mL of 10% 1-propanol in 0.1 M ammonium acetate (pH 4.7; Sigma-Aldrich) and centrifuged at 17,000×g for 60 min. The supernatant was subjected to reverse high-performance liquid chromatography (RP-HPLC; Waters, Milford, CT, USA) on a C_{18} analytical column (YMC Hydrosphere C_{18} column, 4.6 mm×250 mm, particle size 5 um; YMC, Kyoto, Japan) using 10% 1-propanol in 0.1 M ammonium acetate (pH 4.7; Table 1).

Anti-inflammatory effects

Cell culture

RAW 264.7 cell (KCTC, Seoul, Korea) are mouse macrophage cells. RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with heat-inactivated 10% FBS and 1% penicillin at 37℃, in a 5% CO_2 atmosphere. The cells were subcultured and plated at 80%–90% of confluency.

Cell viability [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay]

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Khanna et al., 2020), with minor modifications. RAW 264.7 cells were seeded at 5×10^4 cells/well in 96-well cell culture plates for 24 h. After removing the medium, RAW 264.7 cells were treated with various concentrations of live and dead cells in serum-free medium for 24 h. After that, 10 μL of MTT (Sigma-Aldrich) solution was added to each well. Incubation was continued for 2 h of incubation (37℃, 5% CO_2). After removing the supernatant, 150 μL of dimethyl sulfoxide was added to each well. The cell culture plate was shaken for 30 min to dissolve the formazan crystals formed by the reaction between the MTT reagent and the metabolites of viable cells. Absorbance was measured at a wavelength of 540 nm using a microplate reader, and cell viability was expressed as a percentage of the untreated control cells.

Measurements of nitric oxide assay

Nitric oxide (NO) production was measured as nitrite using the Griess reaction (Sun et al., 2003). RAW 264.7 cells were seeded at 1×10^6 cells/well in 24-well cell culture plates and incubated for 24 h. After removing the medium, RAW 264.7 cells were treated with various concentrations of live and dead cells in serum-free medium for 2 h. After that, lipopolysaccharide (LPS; 1 μg/mL) was added to each well and incubated for 24 h. Following the incubation, 100 μL of the reaction mixture was extracted and added to a 96-well microplate followed by the addition of Griess reagent. The mixture was shaken in the dark for 20–30 min at room temperature and the absorbance was measured at 540 nm. A fresh culture medium was used as a blank control.

Measurement of cytokines production (enzyme-linked immunosorbent assay)

RAW 264.7 cells were seeded at 1×10^6 cells/well in 24-well cell culture plates and incubated for 24 h. After removing the

Table 1. Lipoteichoic acid (LTA) analysis conditions (HPLC)

Instrument	Condition
Column	YMC Hydrosphere C_{18} column (YMC, Kyoto, Japan)
Mobile phase	0.1M ammonium acetate (pH 4.7): 1-propanol=9:1 (v/v)
Detector (detection)	Photodiode Array Detector W996 (257 nm; Waters, Milford, CT, USA)
Flow rate	0.8 mL/min
Injection volume	15 μL

HPLC, high-performance liquid chromatography.

medium, RAW 264.7 cells were treated with various concentrations of live and dead cells in a serum-free medium for 2 h. Subsequently, LPS was added at a final concentration of 1 μg/mL, and the cells were incubated for 24 h. Then, the supernatant was collected and cytokine levels were measured using Mouse TNF-α, IL-1β, and Mouse IL-6 enzyme-linked immunosorbent assay (ELISA) kits (Thermo Fisher Scientific) according to the manufacturer's instructions. The production levels of TNF-α, IL-1β, and IL-6 were determined using ELISA (Wang et al., 2020).

Degradation of heat-killed lactic acid bacteria by digestive enzyme

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the Laemmli method (Laemmli, 1970). Dead cell degradation was assessed by SDS-PAGE, as previously described method (Eslami et al., 2013) with minor modifications, using digestive enzymes such as amylase, pepsin, and oxgall. The molecular weights of dead cells were estimated using 12% acrylamide gels and a mini-protein tetra electrophoresis system (Bio-Rad, Hercules, CA, USA). The protein bands were stained with Coomassie Brilliant Blue R-250 (Bio-Rad) using Precision Plus Protein Dual Xtra Standards (Bio-Rad) as molar mass standards. Ten percentage of α-Amylase (Novozymes, Bagsvaerd, Denmark) was added to dead cell sample and reacted at 37℃ for 5, 15, and 30 min. After centrifugation at 10,000×g for 10 min and 4℃, the decomposition efficiency was measured through SDS-PAGE using supernatant. The remaining pellet was added with the same amount of PBS buffer as the supernatant, adjusted to pH 3.0 using HCl, and then added pepsin (1,000 unit/mL; Sigma-Aldrich) and reacted at 37℃ for 30, 60, 120 min. Centrifugation was performed as described above and SDS-PAGE was performed. After mixing the same amount of PBS buffer as the supernatant in the remaining pellet, 0.3% oxgall (BD Difco) was added and reacted at 37℃ for 6, 12, 24 h, followed by centrifugation as described above, and SDS-PAGE was measured with the supernatant.

Results

Screening and identification of lactic acid bacteria

Gram staining was performed to determine the morphological characteristics of each strain, and all isolates were confirmed to be Gram-positive. Six strains were identified using 16S rRNA sequencing. Based on the 16S rRNA sequence analysis, the isolates were classified as one strain of *Ligilactobacillus salivarius*, four strains of *Lactiplantibacillus plantarum*, and one strain of *Limosilactobacillus fermentum*. The strains were named *L. salivarius* V4, *L. plantarum* V7, V10, V11, and V12, and *L. fermentum* V8 (Table 2).

Table 2. Identification of lactic acid bacteria isolated from Vietnamese feces with the 16S-rRNA sequence

Strain	16S-rRNA sequence	
	Species	% ID
V4	*Ligilactobacillus salivarius* KLB242 16S ribosomal RNA gene, partial sequence	99
V7	*Lactiplantibacillus plantarum* strain Gt2 16S ribosomal RNA gene, partial sequence	99
V8	*Limosilactobacillus fermentum* strain BB102 16S ribosomal RNA gene, partial sequence	98
V10	*L. plantarum* HAU-6 16S ribosomal RNA gene, partial sequence	99
V11	*L. plantarum* EyLan2 16S ribosomal RNA gene, complete sequence	97
V12	*L. plantarum* AFPSH2 16S ribosomal RNA gene, partial sequence	98

Tolerance of lactic acid bacteria to artificial gastric juice and bile

Survival of LAB strains isolated from Vietnamese feces was evaluated using artificial gastric fluid and bile salts (Table 3). The results demonstrated that the LAB strains exhibited excellent resistance to artificial gastric juice, with varying survival rates among the strains. Among these strains, *L. plantarum* V10 showed the highest survival rate. These findings are consistent with those reported by Mechai et al. (2014), who observed that the resistance of probiotic strains is strain-dependent.

Resistance to intestinal bile salts is a critical factor for probiotic selection. As the concentration of human bile typically ranges from 0.3% to 0.5%, an artificial bile acid environment was simulated using 0.3% OxGall (Hu et al., 2018). Resistance to artificial bile acids was higher in the LAB strains than in the commercial strain *L. acidophilus* LA-5. Notably, *L. plantarum* V10 exhibited superior resistance to bile acids (Table 4).

Table 3. **Survival of lactic acid bacteria in artificial gastric acid**

Strains	Viable cell counts (CFU/mL)			
	pH 7.0 (control)	pH 3.0	pH 2.0	pH 1.5
LA-5	$3.01\times10^{9}\pm0.1\times10^{9}$	$1.68\times10^{8}\pm0.21\times10^{8}$	$4.12\times10^{7}\pm0.08\times10^{7}$	$9.61\times10^{4}\pm0.06\times10^{4}$
V4	$2.70\times10^{9}\pm0.02\times10^{9}$	$6.56\times10^{8}\pm0.08\times10^{8}$	$8.53\times10^{6}\pm0.06\times10^{6}$	$6.52\times10^{5}\pm0.26\times10^{5}$
V7	$2.30\times10^{9}\pm0.3\times10^{9}$	$2.56\times10^{7}\pm0.12\times10^{7}$	$1.23\times10^{6}\pm0.13\times10^{6}$	$7.78\times10^{4}\pm0.14\times10^{4}$
V8	$3.90\times10^{9}\pm0.06\times10^{9}$	$2.72\times10^{8}\pm0.24\times10^{8}$	$1.23\times10^{7}\pm0.32\times10^{7}$	$7.78\times10^{5}\pm0.04\times10^{5}$
V10	$6.67\times10^{10}\pm0.04\times10^{10}$	$2.64\times10^{9}\pm0.06\times10^{9}$	$3.89\times10^{7}\pm0.04\times10^{7}$	$1.22\times10^{5}\pm0.08\times10^{5}$
V11	$2.24\times10^{9}\pm0.02\times10^{9}$	$8.51\times10^{8}\pm0.4\times10^{8}$	$2.53\times10^{6}\pm0.08\times10^{6}$	$2.41\times10^{5}\pm0.4\times10^{5}$
V12	$4.21\times10^{8}\pm0.12\times10^{8}$	$5.02\times10^{6}\pm0.31\times10^{6}$	$8.26\times10^{6}\pm0.23\times10^{6}$	$2.56\times10^{5}\pm0.31\times10^{5}$

All value were mean of triplicates.
All samples incubated at 37°C for 2 h in broth containing 0.3% pepsin.
LA-5: *Lactobacillus acidophilus* (commercial strain); V4: *Ligilactobacillus salivarius*; V7, 10, 11, 12: *Lactiplantibacillus plantarum*; V8: *Limosilactobacillus fermentum.*

Table 4. **Bile tolerance of lactic acid bacteria for 24 hours**

Strains	Viable cell counts (CFU/mL)			
	pH 7.0 (control)	pH 3.0	pH 2.0	pH 1.5
LA-5	$8.68\times10^{9}\pm0.4\times10^{9}$	$1.38\times10^{7}\pm0.08\times10^{7}$	$7.53\times10^{6}\pm0.12\times10^{6}$	$7.32\times10^{5}\pm0.04\times10^{5}$
V4	$7.61\times10^{9}\pm0.22\times10^{9}$	$5.21\times10^{7}\pm0.04\times10^{7}$	$8.51\times10^{6}\pm0.1\times10^{6}$	$1.53\times10^{5}\pm0.06\times10^{5}$
V7	$5.01\times10^{8}\pm0.08\times10^{8}$	$2.56\times10^{6}\pm0.1\times10^{6}$	$7.31\times10^{5}\pm0.04\times10^{5}$	$6.59\times10^{4}\pm0.04\times10^{4}$
V8	$2.01\times10^{8}\pm0.04\times10^{8}$	$8.26\times10^{7}\pm0.02\times10^{7}$	$3.78\times10^{5}\pm0.02\times10^{5}$	$1.08\times10^{4}\pm0.08\times10^{4}$
V10	$6.61\times10^{9}\pm0.06\times10^{9}$	$3.52\times10^{8}\pm0.08\times10^{8}$	$5.36\times10^{6}\pm0.02\times10^{6}$	$2.41\times10^{5}\pm0.01\times10^{5}$
V11	$1.35\times10^{8}\pm0.07\times10^{8}$	$8.51\times10^{7}\pm0.2\times10^{7}$	$2.53\times10^{6}\pm0.08\times10^{6}$	$8.07\times10^{3}\pm0.12\times10^{3}$
V12	$5.46\times10^{8}\pm0.1\times10^{8}$	$2.39\times10^{6}\pm0.12\times10^{6}$	$3.66\times10^{6}\pm0.2\times10^{6}$	$7.59\times10^{5}\pm0.08\times10^{5}$

All value were mean of triplicates.
All samples incubated at 37°C for 24 h in broth containing 0.3% oxgall.
LA-5: *Lactobacillus acidophilus* (commercial strain); V4: *Ligilactobacillus salivarius*; V7, 10, 11, 12: *Lactiplantibacillus plantarum*; V8: *Limosilactobacillus fermentum.*

Proteolytic activity

Bacterial proteolytic activity was assessed based on the ability to produce clear zones on skimmed milk agar. A clear zone was observed around the wells for these six strains, indicating proteolytic activity. Among the six strains tested, *L. salivarius* V4 and *L. plantarum* V10 exhibited the highest proteolytic activities on skim milk agar (Table 5).

β-Galactosidase activity

β-Galactosidase plays an important role in the hydrolysis of lactose (Brás et al., 2010). This enzyme is widely used to improve lactose digestion and to convert lactose into short-chain fatty acids that are beneficial to the host (Naidu et al., 1999). *L. salivarius* V4, *L. fermentum* V8, and *L. plantarum* V10 showed higher *β*-galactosidase activity than the commercial strain *L. acidophilus* LA-5 (Fig. 1).

Table 5. Measurement of proteolytic activity of lactic acid bacteria

Sample	24 h	48 h	72 h
LA-5	8.00±0.12	10.00±0.11	15.00±0.21
V4	11.00±0.11	14.00±0.18	21.00±0.09
V7	8.00±0.08	10.00±0.22	13.00±0.09
V8	9.00±0.11	11.00±0.13	17.00±0.11
V10	12.00±0.11	16.00±0.18	20.00±0.08
V11	10.00±0.13	15.00±0.16	17.00±0.02
V12	8.00±0.12	10.00±0.18	12.00±0.22

All value were mean±SD of triplicates.
Diameter of clear zone: mm.
All samples incubated at 37℃.
LA-5: *Lactobacillus acidophilus* (commercial strain); V4: *Ligilactobacillus salivarius*; V7, 10, 11, 12: *Lactiplantibacillus plantarum*; V8: *Limosilactobacillus fermentum*.

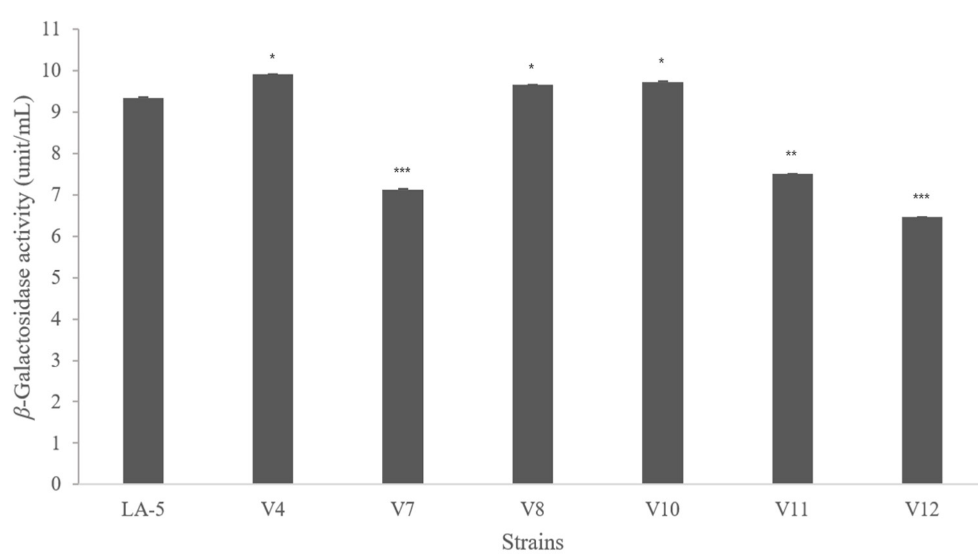

Fig. 1. β-galactosidase activity of lactic acid bacteria isolated from Vietnamese feces. LA-5: *Lactobacillus acidophilus* (commercial strain); V4: *Ligilactobacillus salivarius*; V7, 10, 11, 12: *Lactiplantibacillus plantarum*; V8: *Limosilactobacillus fermentum*. Statistical difference: * p<0.05, ** p<0.01, *** p<0.001.

Selection of heat-killed lactic acid bacteria

Heat shock treatment

To prepare dead cell, four types of lactic acid bacteria isolated from Vietnamese feces were subjected to heat treatment at 65℃ for 5, 15, 30, and 60 min and at 95℃ for 5 and 10 min, after which viable cell counts were performed. As a result, all bacterial cells were completely killed after 60 min at 65℃ and 10 min at 95℃ (Fig. 2). Therefore, in subsequent experiments, dead cells were prepared by heat treatment at 65℃ for 60 and 90 min and at 95℃ for 10 and 20 min, ensuring complete inactivation and the experiments were conducted.

Protein quantification

As a result of protein quantification analysis revealed that the protein content of dead cells heat-treated at 95℃ was lower than that of cells killed heat-treated at 65℃. Furthermore, as the duration of the heat treatment increased, the protein content decreased. This result, shows that when heat treatment at 95℃, the protein was decomposed and the protein content was reduced (Fig. 3).

Phosphate assay

Conducting quantitative phosphate analysis to measure the content of LTA indirectly revealed that the content was lower at 95℃ compared to the lactic acid bacteria heat-treated at 65℃, and similarly, the longer the heat treatment time, the lower the content. However, at 95℃, it was confirmed that there was no difference over time (Fig. 4).

Antioxidant activity

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity

The Trolox equivalent antioxidant capacity assay measures the ability of a compound to eliminate or scavenge radicals

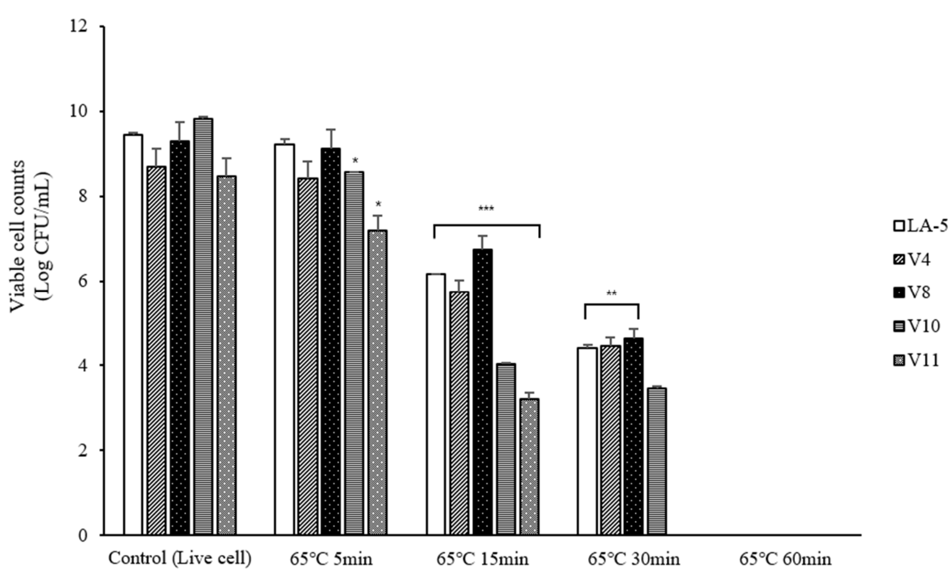

Fig. 2. Total viable counts of heat shock at 65℃ 5, 15, 30, 60 min, 95℃ 5, 10 min. LA-5: *Lactobacillus acidophilus* (commercial strain); V4: *Ligilactobacillus salivarius*; V8: *Limosilactobacillus fermentum*; V10, 11: *Lactiplantibacillus plantarum.* Statistical difference: * p<0.05, ** p<0.01, *** p<0.001.

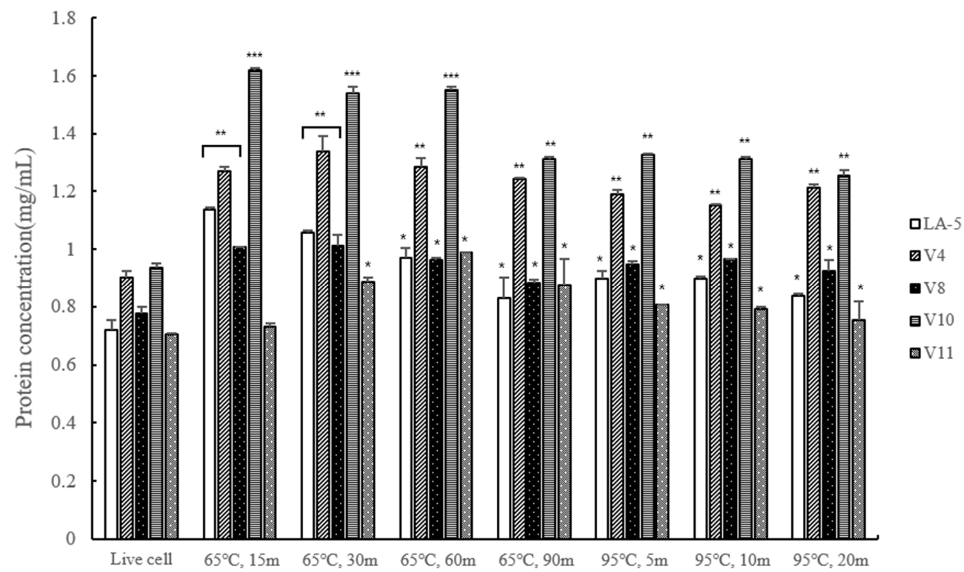

Fig. 3. Protein quantification contents of live and dead cells. LA-5: *Lactobacillus acidophilus* (commercial strain); V4: *Ligilactobacillus salivarius*; V8: *Limosilactobacillus fermentum*; V10, 11: *Lactiplantibacillus plantarum.* Statistical difference: * p<0.05, ** p<0.01, *** p<0.001.

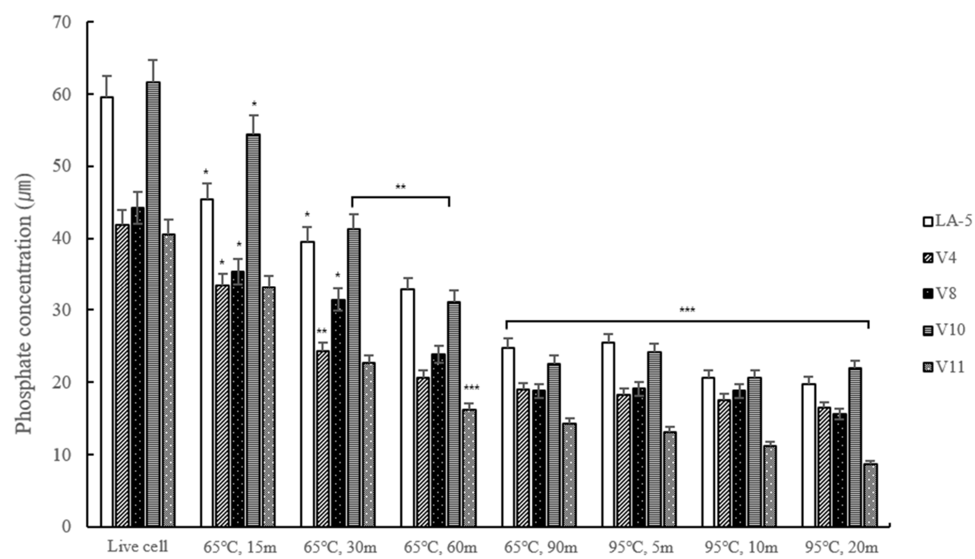

Fig. 4. Phosphate contents of live and dead cells. LA-5: *Lactobacillus acidophilus* (commercial strain); V4: *Ligilactobacillus salivarius*; V8: *Limosilactobacillus fermentum*; V10, 11: *Lactiplantibacillus plantarum.* Statistical difference: * p<0.05, ** p<0.01, *** p<0.001.

compared to Trolox as an antioxidant reference (Leung et al., 2009). The results of the ABTS assay are shown in Fig. 5A. Heat-treated dead cells showed higher antioxidant capacity than live cells. In particular, ABTS radical scavenging activity was higher in V4 and V10 heat-treated at 65℃ for 60 min than *Enterococcus faecalis* EF-2001, a commercial strain of dead cells.

2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity

DPPH is a stable free radical that has been widely accepted as a tool for estimating the free radical-scavenging activity of antioxidants (Lin and Chang, 2000). Based on the Trolox standard curve, DPPH radical scavenging activity values were statistically analyzed. According to the results (Fig. 5B), heat-treated dead cells at 65℃ for 60 min showed higher antioxidant

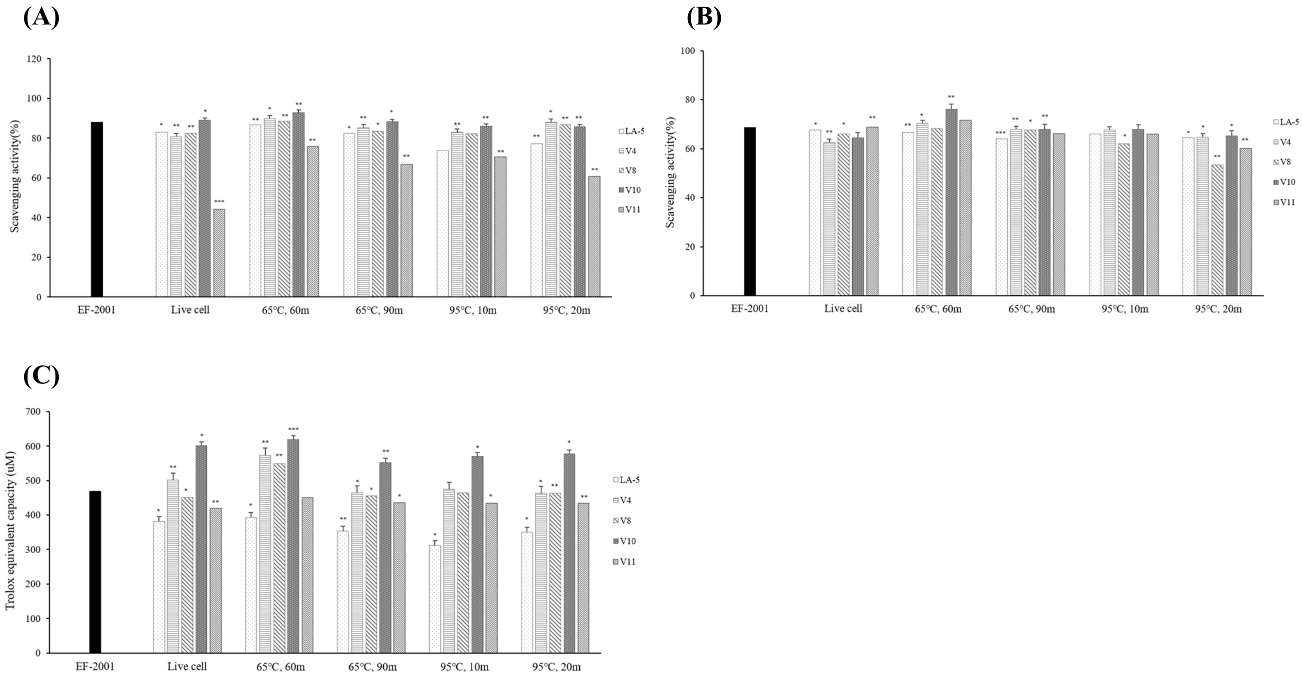

Fig. 5. Antioxidant activities of live and dead cells. Antioxidant activities of live and dead cells: (A) ABTS radical scavenging activity; (B) DPPH radical scavenging activity; (C) Ferric reducing antioxidant power. LA-5: *Lactobacillus acidophilus* (commercial strain; live cell); EF-2001: *Enterococcus faecalis* (commercial strain; dead cell); V4: *Ligilactobacillus salivarius*; V8: *Limosilactobacillus fermentum*; V10, 11: *Lactiplantibacillus plantarum*. Statistical difference: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl.

activity than live cells. Except for heat-treated dead cells at 65℃ for 60 min, the other heat-treated dead cells showed antioxidant activity similar to live cells. In addition, it was confirmed that V4, V10, and V11 heat-treated at 65℃ for 60 min showed higher antioxidant activity than *E. faecalis* EF-2001, a commercial strain of dead cells.

Ferric reducing antioxidant power

FRAP analysis is a method of verifying oxidative activity through electron donation ability; when ferric tripyridyltriazine (Fe^{3+}-TPTZ), which acts as an oxidizing agent, and antioxidants react, the reduction to blue ferrous tripyridyltriazine (Fe^{2+}-TPTZ) is quantified through absorbance measurement (Spiegel et al., 2020). When compared to LA-5 heat treated under the same conditions as the V strain, all strains showed higher iron-reducing abilities than LA-5. It was confirmed that the V4, V8, and V10 strains heat-treated at 65℃ for 60 min showed higher iron-reducing ability than *E. faecalis* EF-2001, the commercial strain of dead cells (Fig. 5C).

In the three antioxidant assays (ABTS, DPPH, and FRAP), V10 showed the highest antioxidant activity. Thus, *L. plantarum* V10 was selected as the final strain.

Lipoteichoic acid analysis of heat-killed lactic acid bacteria with high-performance liquid chromatography

LTA is a key component of the cell wall in Gram-positive bacteria, and it has been the focus of numerous recent studies. While considerable research has been conducted on the LTA of pathogenic Gram-positive bacteria, studies on the LTA of LAB remain limited.

The LTA of dead cells was extracted and linearity was obtained through HPLC analysis with R^2=0.9953. Based on the standard curve of LTA from *Staphylococcus aureus* (Sigma-Aldrich), the LTA content was statistically analyzed. As a result of analyzing the LTA content of heat-treated dead cells, it was confirmed that the commercial strain EF-2001 was 552 ppm, V10 heat-treated at 65℃ for 60 min was 1,244 ppm, V10 heat-treated at 65℃ for 90 min was 617 ppm, V10 heat-treated at 95℃ for 10 min was 582 ppm and V10 heat-treated at 95℃ for 20 min was 414 ppm (Table 6). It was confirmed that the LTA content of heat-treated at 65℃ for 60 min was the highest. Except for heat-treated dead cells at 95℃ for 20 min, the other heat-treated dead cells were higher than that of commercial strains EF-2001. As the LTA content decreased, the peak resolution also decreased (Fig. 6).

Anti-inflammatory effects

Cell viability [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay]

MTT is an indicator of mitochondrial activity and is primarily used as an index of cell survival. Active mitochondrial dehydrogenase in viable cells cleaves MTT to produce formazan, which is directly correlated with the number of metabolically

Table 6. Lipoteichoic acid (LTA) content of dead cells

Sample	Retention time (min)	LTA content (ppm)	Area	Height
EF-2001	3.925	552	583,674	47,376
V10 65℃, 60 min	3.826	1,244	1,351,848	112,684
V10 65℃, 90 min	3.907	617	656,332	49,783
V10 95℃, 10 min	3.973	582	616,983	542,557
V10 95℃, 20 min	3.97	414	430,692	38,837

EF-2001: *Enterococcus faecalis* (commercial strain; dead cell); V10: *Lactiplantibacillus plantarum*.

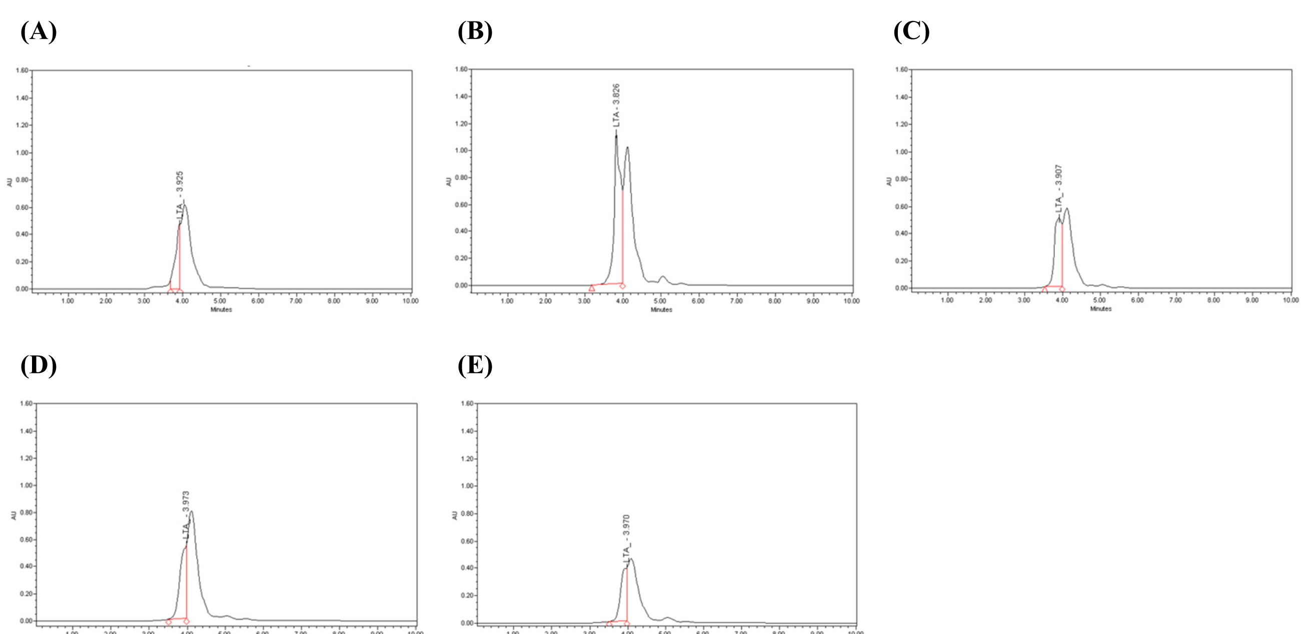

Fig. 6. Chromatogram of lipoteichoic acid from dead cell was analyzed with HPLC equipped with YMC Hydrosphere C_{18} column and PDA Detector (YMC, Kyoto, Japan). (A) EF-2001; *Enterococcus faecalis* EF-2001 (Nihon Berumu, Tokyo, Japan). (B) V10 treated 65℃ for 60 min; *Lactiplantibacillus plantarum*. (C) V10 treated 65℃ for 90 min; *L. plantarum*. (D) V10 treated 95℃ for 10 min; *L. plantarum*. (E) V10 treated 95℃ for 20 min; *L. plantarum*. HPLC, high-performance liquid chromatography.

active cells (Carreño et al., 2021). According to the experimental results, live cells showed no cytotoxic or growth-promoting activity at any concentration. In addition, the heat-treated dead cells were not able to confirm cytotoxicity or growth-promoting activity at the remaining concentration except for the concentration of 10^7 CFU/mL of heat-treated dead cell at 95℃ for 20 min (Fig. 7). Live cells and heat-treated dead cells showed 80% or more cell viability; therefore, it was confirmed that live and dead cells were not cytotoxic to macrophages.

Measurement of nitric oxide assay

NO is a signaling molecule produced by various cells, including macrophages, that plays a crucial role in immune responses. NO production contributes to the elimination of pathogens and modulation of inflammatory processes (Lee et al., 2017a).

To evaluate their anti-inflammatory potential, the effects of live and heat-treated dead cells on NO production were examined in LPS-induced RAW 264.7 cells. Compared to the LPS-negative group, treatment with LPS significantly increased NO production. However, treatment with live cells and heat-treated dead cells inhibited NO production in a dose-dependent manner, except for 10^7 CFU/mL live cells (Fig. 8).

Measurement of cytokines production (enzyme-linked immunosorbent assay)

To maintain the immune balance, there must be direct and indirect interactions between immune cells. Cytokines can induce the proliferation, differentiation, and changes in the function and activity of various immune cells. Diseases are mostly associated with inflammation and inflammatory cells secrete substances that induce inflammation. The substance secreted at this time is called an inflammatory cytokine, which increases when environmentally harmful factors, viruses, and drugs enter the body (Barland et al., 2004). In this study, the expression levels of TNF-*α*, IL-1*β*, and IL-6 were measured.

According to the results of cytokine measurement using ELISA, three cytokines (TNF-*α*, IL-1*β*, and IL-6) production was significantly lower than LPS (+) group in live and dead cells (Fig. 9). Lower levels of TNF-*α* cytokines were confirmed in dead cells than in live cells. The low expression level of TNF-*α* from macrophage effect by strains prevents the activation of

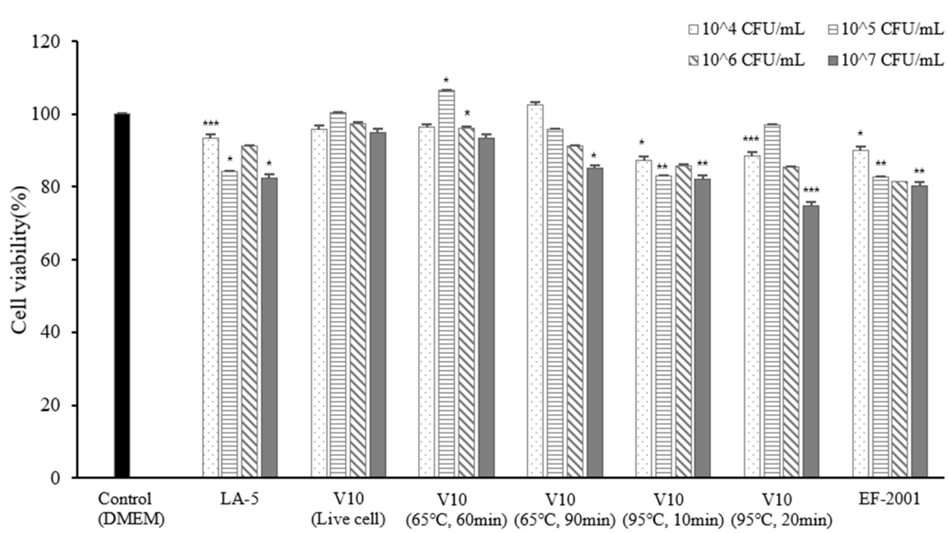

Fig. 7. Cell viability of RAW 264.7 cell treated with live and dead cells. Control: DMEM. LA-5: *Lactobacillus acidophilus*; Live cell (Chr.Hansen, Hørshol, Denmark). EF-2001: *Enterococcus faecalis*; Dead cell (Nihon Berumu, Tokyo, Japan). V10: *Lactiplantibacillus plantarum*. Statistical difference: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. DMEM, Dulbecco's modified Eagle's medium.

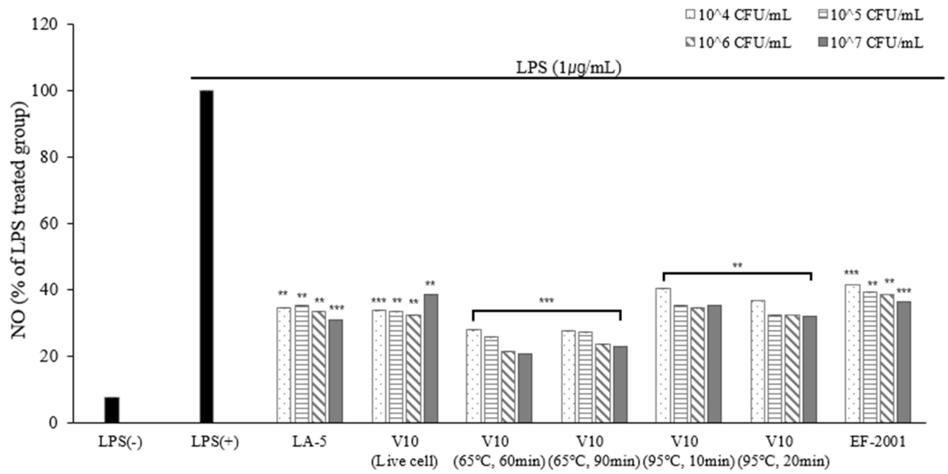

Fig. 8. Effects of live and dead cells on production of nitric oxide in LPS-induced RAW 264.7 cell. LA-5: *Lactobacillus acidophilus*; Live cell (Chr.Hansen, Hørshol, Denmark). EF-2001: *Enterococcus faecalis;* Dead cell (Nihon Berumu, Tokyo, Japan). V10: *Lactiplantibacillus plantarum*. Statistical difference: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. NO, nitric oxide; LPS, lipopolysaccharide.

pro-inflammatory cytokines which are produced by the expression of TNF-*α*. Based on these results, anti-inflammatory activity can be found in both live and dead cells. In addition, it was confirmed that the cytokine levels of the three cytokines (TNF-*α*, IL-1*β*, and IL-6) were the lowest in heat-treated dead cells at 65℃ for 60 min.

Therefore, the final heat treatment condition for dead cells was set to 65℃ for 60 min to indirectly evaluate the degradation rate of the dead cells via digestive enzyme treatment.

Degradation of heat-killed lactic acid bacteria by digestive enzyme

SDS-PAGE analysis was performed using commercial enzymes (amylase, pepsin, and oxgall) to indirectly evaluate whether heat-treated dead cells underwent decomposition in the human digestive system following ingestion.

Following treatment with 10% amylase, the degradation of dead cells was evaluated under various conditions (5, 15, and 30 min) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. No evidence of cell decomposition was observed under any of these conditions. Considering that the average duration of mastication during human digestion is approximately 5 min, the amylase treatment time was standardized to 5 min.

Subsequent treatment with 0.3% pepsin (pH 3.0) was performed, and the degradation of dead cells was assessed using SDS-PAGE at different time intervals (30, 60, and 120 min). No degradation was observed under any of these conditions. Because the average gastric transit time in humans is approximately 30 min, the pepsin treatment time was set to 30 min.

SDS-PAGE analysis was conducted to assess degradation during oxgall treatment (0.3%) over extended periods (6, 12, and 24 h). The results indicated the partial decomposition of dead cells, with some molecules degraded into smaller fragments (Fig. 10).

In conclusion, dead heat-treated cells did not undergo degradation upon treatment with 10% amylase or 0.3% pepsin (pH 3.0), confirming their ability to reach the intestine. However, partial decomposition into smaller molecular fragments was observed after treatment with 0.3% oxgall.

Discussion

Heat-treated dead cells with antioxidant and immune-enhancing properties are known to have beneficial physiological and

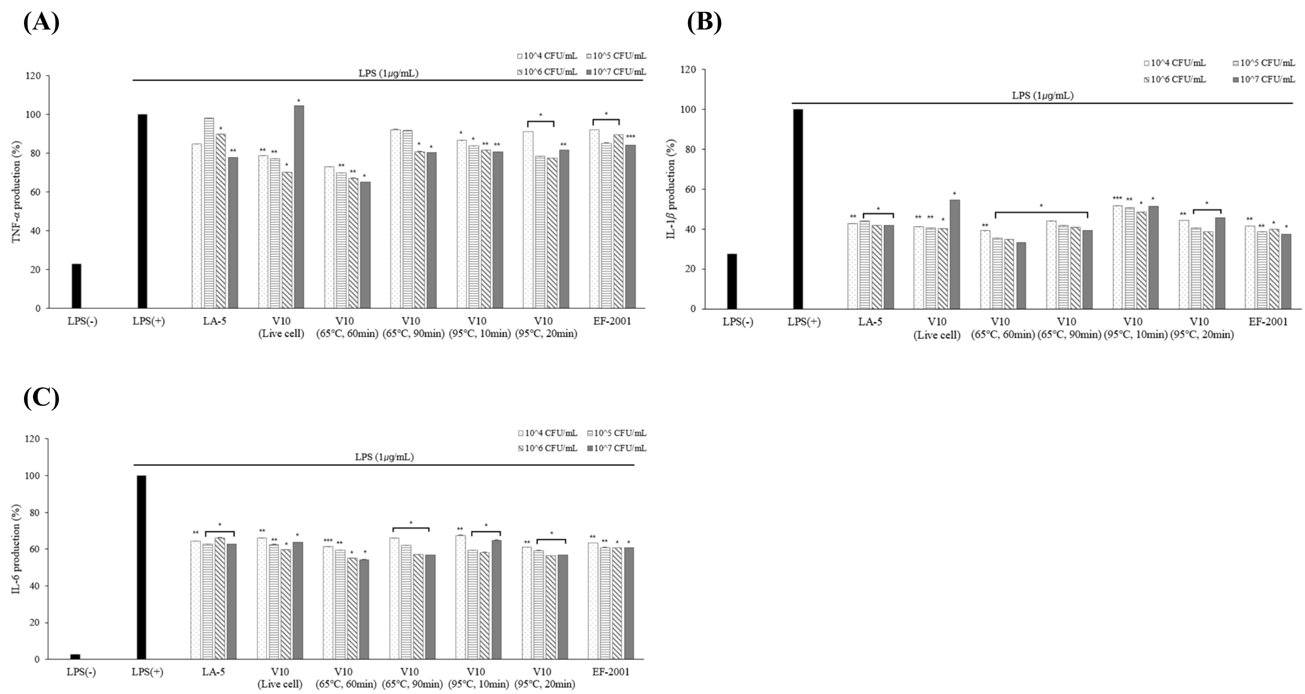

Fig. 9. TNF-*α* (A), IL-1*β* (B) and IL-6 (C) production in LPS-induced RAW 264.7 cell treated with live and dead cells. LA-5: *Lactobacillus acidophilus*; Live cell (Chr.Hansen, Hørshol, Denmark). EF-2001: *Enterococcus faecalis*; Dead cell (Nihon Berumu, Tokyo, Japan). V10: *Lactiplantibacillus plantarum.* Statistical difference: * p<0.05; ** p<0.01; *** p<0.001. TNF, tumor necrosis factor; LPS, lipopolysaccharide; IL, interleukin.

immunological effects. In particular, the functional properties of dead heat-treated cells vary with temperature and time conditions (de Almada et al., 2016). In this study, we confirmed the functional and immunological effects of HK-LAB isolated from Vietnamese feces by varying the heat-treatment temperature and time. In addition, tolerance to decomposition in the human gastrointestinal tract has been indirectly confirmed using commercial enzymes.

Six strains used in this study were derived from Vietnamese feces. One strain of *L. salivarius*, four strains of *L. plantarum*, and one strain of *L. fermentum* were selected through basic physiological activity verification. Of the six strains tested, four with potential for use as commercial lactic acid bacteria were selected through verification of their basic physiological activity.

All bacteria did not survive under heat treatment conditions of 65℃ for 60 min or 95℃ for 10 min or longer. In protein quantification, the protein content was higher at 65℃ compared to 95℃, and the longer the heat treatment time, the lower the protein content. It means that the protein content was reduced due to degradation of the protein at 95℃. In addition, to indirectly measure the LTA content, we conducted quantitative analysis of phosphate, a component of LTA. The results indicated that the phosphate content was lower in heat-treated dead cells at 95℃ compared to those treated at 65℃, indirectly suggesting a higher LTA content at 65℃.

The antioxidant activity of the dead cells was confirmed for each heat treatment condition and compared with that of live bacteria. *E. faecalis* (EF-2001) is a Gram-positive bacterium with immunomodulatory and preventive properties. According to the research results of Choi et al. (2018), heat-killed *E. faecalis* (EF-2001) has various beneficial effects on human health, including anti-allergic, anti-inflammatory, and antitumor activities. Through the results of antioxidant activities (ABTS, DPPH, FRAP), heat-treated dead cells at 65℃ for 60 min showed higher antioxidant activities than the live cells and EF-2001. V10 strain showed the highest antioxidant activity. These results are similar to those of other studies, showing that dead

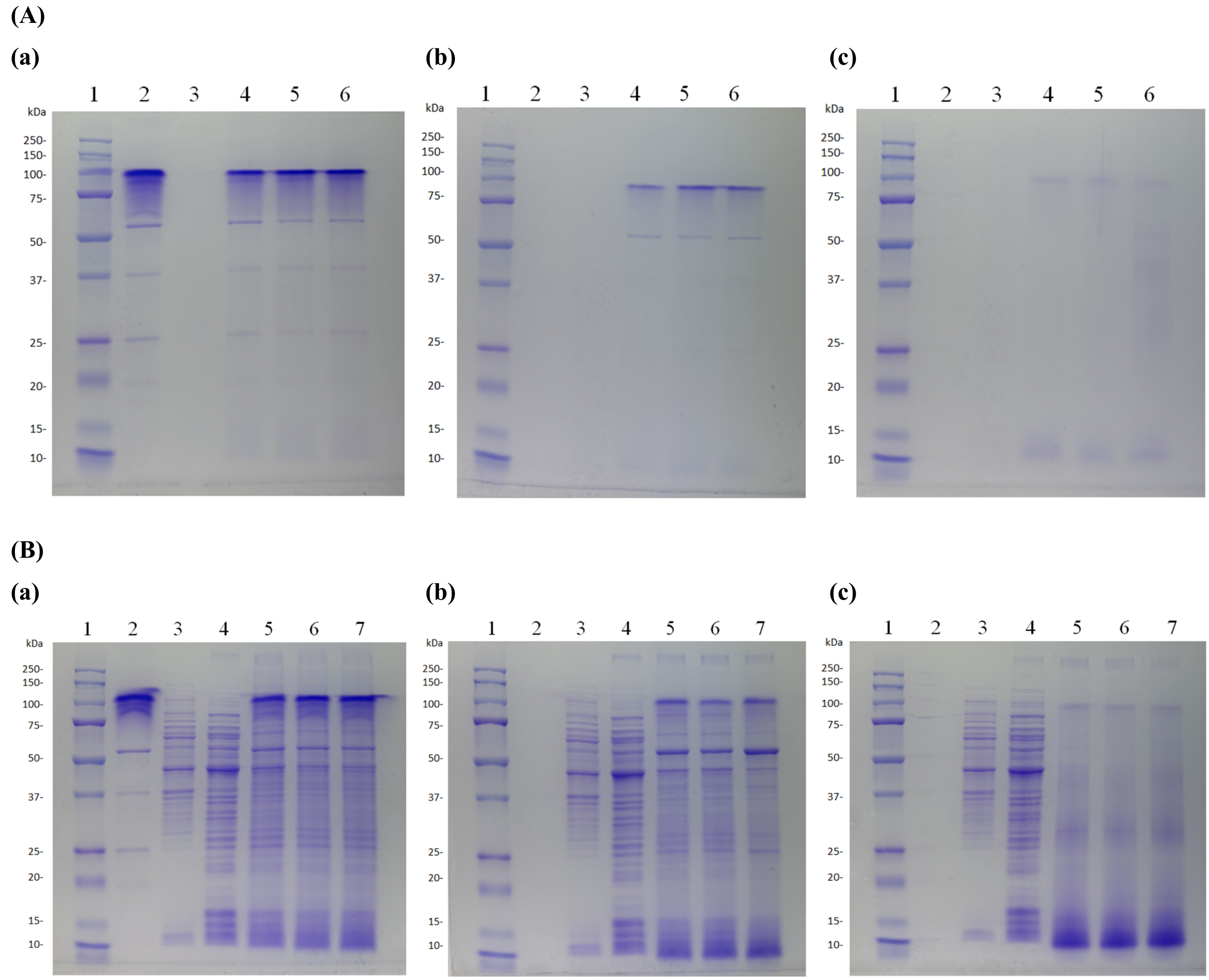

Fig. 10. SDS-PAGE patterns of EF-2001 (A) and V10 (B) by digestive enzyme at different time points. (A) EF-2001: *Enterococcus faecalis*; Dead cell commercial strain (Nihon Berumu, Tokyo, Japan). (B) V10: *Lactiplantibacillus plantarum*. (a) Treatment by 10% α-amylase. (b) Treatment by 0.3% pepsin (pH 3.0). (c) Treatment by 0.3% oxgall. Lane 1: Molecular weight marker (Precision Plus Protein Dual Xtra Standards, Bio-Rad, Hercules, CA, USA). Lane 2: Standard [(a) 10% α-amylase; (b) 0.3% pepsin; (c) 0.3% oxgall]. Lane 3: (A) *Enterococcus faecalis* EF-2001, (B) *Lactobacillus plantarum* V10; Live cell. Lane 4: (A) Enzyme-treated EF-2001 at different time points [(a) 10% α-amylase–5 min; (b) 0.3% pepsin–30 min; (c) 0.3% oxgall–6 h]; (B) *Lactobacillus plantarum* V10; Dead cell. Lane 5: (A) Enzyme-treated EF-2001 at different time points [(a) 10% α-amylase–15 min; (b) 0.3% pepsin–60 min; (c) 0.3% oxgall–12 h]; (B) Enzyme-treated V10 at different time points [(a) 10% α-amylase–5 min; (b) 0.3% pepsin–30 min; (c) 0.3% oxgall–6 h]. Lane 6: (A) Enzyme-treated EF-2001 at different time points [(a) 10% α-amylase–30 min; (b) 0.3% pepsin–120 min; (c) 0.3% oxgall–24 h]; (B) Enzyme-treated V10 at different time points [(a) 10% α-amylase–15 min; (b) 0.3% pepsin–60 min; (c) 0.3% oxgall–12 h]. Lane 7: (B) Enzyme-treated V10 at different time points [(a) 10% α-amylase–30 min; (b) 0.3% pepsin–120 min; (c) 0.3% oxgall–24 h]. SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

cells have a better antioxidant effect than live cells (Jang et al., 2018).

A previous study showed that extracted and purified LTA, a cell wall component of dead cells, inhibited cytokine production and analyzed the structure of LTA according to strain in Gram-positive bacteria (Lu et al., 2022; Morath et al., 2001). However, few studies have confirmed LTA content of LTA, HPLC analysis was performed to confirm the LTA content of dead cells following heat treatment. As a result of the analysis, except for the dead cells treated at 95°C for 20 min, the LTA content of heat-treated dead cells was higher than that of the commercial strain EF-2001, and the dead cells treated at 65℃ for 60 min showed the highest LTA content.

Subsequently, the immunomodulatory effect of dead cells was confirmed. As a result of NO analysis, NO production was inhibited as the concentration of the dead cells increased, and the dead cells treated at 65℃ for 60 min showed the lowest oxidation inhibition rate. In addition, the cytokines (TNF-α, IL-1β, and IL-6) of the dead cells were significantly lower than those of the LPS (+) group in a concentration-dependent manner, and, similar to the NO results, were the lowest in the dead cells heat-treated at 65℃ for 60 min. This is consistent with previous studies showing that dead cells have similar or higher immune effects than live cells (Li et al., 2009). Based on these findings, the LTA content is expected to be closely associated with the immune response, highlighting the need for further research in this area.

Finally, SDS-PAGE was performed to indirectly evaluate whether heat-treated dead cells decompose in a simulated gastrointestinal environment. The results confirmed denaturation when artificial digestive enzymes were used in the presence of oxgall. Dead cells can be partially degraded by digestive enzymes while retaining their immune-stimulatory effects, thereby improving the intestinal environment (Taverniti and Guglielmetti, 2011). This finding indirectly suggests that heat-treated dead cells can maintain their immunomodulatory properties even when partially decomposed during digestion.

Conclusion

In this study, we confirmed the immunological effects of heat-treated dead cells isolated from Vietnamese feces, and demonstrated that heat-treated dead cells with immunomodulatory functions in the Southeast Asian market can be utilized as functional food ingredients.

Recently, active research has been conducted on the immunological effects of heat treatment on dead cells. However, studies that specifically analyze the content of LTA, a cell wall component of dead cells, remain insufficient. Further research in this area is required. In addition, because the method of inducing immune responses varies depending on the structural characteristics of LTA, additional research is needed to analyze not only the content, but also the structural characteristics of LTA. This study provides basic data for the development of functional food ingredients that utilize dead cells in the Southeast Asian market.

Conflicts of Interest

The authors declare no potential conflicts of interest.

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Author Contributions

Conceptualization: Lee JW. Data curation: Lee JW, Kim CH. Formal analysis: Lee JW, Song SE. Methodology: Lee JW, Oh DG, Kim DJ. Software: Song SE, Kim IB. Validation: Lee JW, Song SE, Kim IB. Investigation: Lee JW, Song SE. Writing - original draft: Lee JW, Song SE, Kim IB. Writing - review & editing: Lee JW, Song SE, Kim IB, Oh DG, Kim DJ, Kim CH.

Ethics Approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

References

Aljohani A, Rashwan N, Vasani S, Alkhawashki A, Wu TT, Lu X, Castillo DA, Xiao J. 2024. The health benefits of probiotic *Lactiplantibacillus plantarum*: A systematic review and meta-analysis. Probiotics Antimicrob Proteins (in press). doi: 10.1007/s12602-024-10287-3

Aregbe AY, Boasiako TA, Xiong YQ, Rahman MH, Ma Y. 2024. Probiotic *Lactobacillus plantarum* LP28 and *Saccharomyces cerevisiae* improve the bioactive content and quality of fruit-based rice beverage. Food Sci Nutr 12:9340-9352.

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

Brás NF, Fernandes PA, Ramos MJ. 2010. QM/MM studies on the β-galactosidase catalytic mechanism: Hydrolysis and transglycosylation reactions. J Chem Theory Comput 6:421-433.

Carreño EA, Alberto AVP, de Souza CAM, de Mello HL, Henriques-Pons A, Alves LA. 2021. Considerations and technical pitfalls in the employment of the MTT assay to evaluate photosensitizers for photodynamic therapy. Appl Sci 11:2603.

China Research & Intelligence. 2023. Research report on Southeast Asia probiotics industry 2023-2032. China Research & Intelligence, Shanghai, China.

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

de Almada CN, Almada CN, Martinez RCR, Sant'Ana AS. 2016. Paraprobiotics: Evidences on their ability to modify biological responses, inactivation methods and perspectives on their application in foods. Trends Food Sci Technol 58:96-114.

Eslami N, Kermanshahi RK, Erfan M. 2013. Studying the stability of S-layer protein of *Lactobacillus acidophilus* ATCC 4356 in simulated gastrointestinal fluids using SDS-PAGE and circular dichroism. Iran J Pharm Res 12:47-56.

Han KJ, Lee JE, Lee NK, Paik HD. 2020. Antioxidant and anti-inflammatory effect of probiotic *Lactobacillus plantarum* KU15149 derived from Korean homemade diced-radish kimchi. J Microbiol Biotechnol 30:591-598.

Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME. 2014. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 11:506-514.

Hu PL, Yuan YH, Yue TL, Guo CF. 2018. A new method for the *in vitro* determination of the bile tolerance of potentially probiotic lactobacilli. Appl Microbiol Biotechnol 102:1903-1910.

Hussen EM, Endalew SA. 2023. *In vitro* antioxidant and free-radical scavenging activities of polar leaf extracts of *Vernonia amygdalina*. BMC Complement Med Ther 23:146.

Jalili H, Razavi SH, Safari M, Malcata FX. 2009. Enhancement of growth rate and β-galactosidase activity, and variation in organic acid profile of *Bifidobacterium animalis* subsp. *lactis* Bb 12. Enzyme Microb Technol 45:469-476.

Jang HJ, Song MW, Lee NK, Paik HD. 2018. Antioxidant effects of live and heat-killed probiotic *Lactobacillus plantarum* Ln1 isolated from kimchi. J Food Sci Technol 55:3174-3180.

Kang SS, Sim JR, Yun CH, Han SH. 2016. Lipoteichoic acids as a major virulence factor causing inflammatory responses via

Toll-like receptor 2. Arch Pharm Res 39:1519-1529.

Kechagia M, Basoulis D, Konstantopoulou S, Dimitriadi D, Gyftopoulou K, Skarmoutsou N, Fakiri EM. 2013. Health benefits of probiotics: A review. Int Sch Res Notices 2013;481651.

Khanna S, Bishnoi M, Kondepudi KK, Shukla G. 2020. Isolation, characterization and anti-inflammatory mechanism of probiotics in lipopolysaccharide-stimulated RAW 264.7 macrophages. World J Microbiol Biotechnol 36:74.

Klotz C, O'Flaherty S, Goh YJ, Barrangou R. 2017. Investigating the effect of growth phase on the surface-layer associated proteome of *Lactobacillus acidophilus* using quantitative proteomics. Front Microbiol 8:2174.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lee M, Rey K, Besler K, Wang C, Choy J. 2017a. Immunobiology of nitric oxide and regulation of inducible nitric oxide synthase. In Macrophages: Origin, functions and biointervention. Kloc M (ed). Springer, Cham, Switzerland. pp 181-207.

Lee YK, Conway P, Pettersson S, Balakrish Nair G, Surono I, Egayanti Y, Sofia Amarra M. 2017b. ILSI Southeast Asia region conference proceedings: The gut, its microbes and health: Relevance for Asia. Asia Pacific J Clin Nutr 26:957-971.

Leung PH, Zhao S, Ho KP, Wu JY. 2009. Chemical properties and antioxidant activity of exopolysaccharides from mycelial culture of *Cordyceps sinensis* fungus Cs-HK1. Food Chem 114:1251-1256.

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

Lin MY, Chang FJ. 2000. Antioxidative effect of intestinal bacteria *Bifidobacterium longum* ATCC 15708 and *Lactobacillus acidophilus* ATCC 4356. Dig Dis Sci 45:1617-1622.

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

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

Mechai A, Debabza M, Kirane D. 2014. Screening of technological and probiotic properties of lactic acid bacteria isolated from Algerian traditional fermented milk products. Int Food Res J 21:2497-2504.

Morath S, Geyer A, Hartung T. 2001. Structure–function relationship of cytokine induction by lipoteichoic acid from *Staphylococcus aureus*. J Exp Med 193:393-398.

Naidu AS, Bidlack WR, Clemens RA. 1999. Probiotic spectra of lactic acid bacteria (LAB). Crit Rev Food Sci Nutr 39:13-126.

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

Riffel A, Brandelli A. 2006. Keratinolytic bacteria isolated from feather waste. Braz J Microbiol 37:395-399.

Sanders ME, Merenstein DJ, Reid G, Gibson GR, Rastall RA. 2019. Probiotics and prebiotics in intestinal health and disease: From biology to the clinic. Nat Rev Gastroenterol Hepatol 16:605-616.

Siciliano RA, Reale A, Mazzeo MF, Morandi S. 2021. Paraprobiotics: A new perspective for functional foods and nutraceuticals. Nutrients 13:1225.

Spiegel M, Kapusta K, Kołodziejczyk W, Saloni J, Żbikowska B, Hill GA, Sroka Z. 2020. Antioxidant activity of selected phenolic acids–ferric reducing antioxidant power assay and QSAR analysis of the structural features. Molecules 25:3088.

Sugahara H, Yao R, Odamaki T, Xiao JZ. 2017. Differences between live and heat-killed bifidobacteria in the regulation of immune function and the intestinal environment. Benef Microbes 8:463-472.

Sun J, Zhang X, Broderick M, Fein H. 2003. Measurement of nitric oxide production in biological systems by using Griess reaction assay. Sensors 3:276-284.

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

Taverniti V, Guglielmetti S. 2011. The immunomodulatory properties of probiotic microorganisms beyond their viability (ghost probiotics: Proposal of paraprobiotic concept). Genes Nutr 6:261-274.

Vinderola CG, Reinheimer JA. 2003. Lactic acid starter and probiotic bacteria: A comparative "*in vitro*" study of probiotic characteristics and biological barrier resistance. Food Res Int 36:895-904.

Wang J, Fang X, Wu T, Fang L, Liu C, Min W. 2020. *In vitro* immunomodulatory effects of acidic exopolysaccharide produced by *Lactobacillus plantarum* JLAU103 on RAW264.7 macrophages. Int J Biol Macromol 156:1308-1315.