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# Evaluation of probiotic and anti-inflammatory properties of bacteriocinogenic *Pediococcus acidilactici* HW01 and *Leuconostoc citreum* HW02 from malted barley

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## Abstract

**Background** Bacteriocins are peptides with antibacterial properties that are released by lactic acid bacteria and affect genetically closely related bacteria. Settled bacteriocin-producing probiotics in the GI tract may contribute to bacteriocin formation, which can lead to pathogen inhibition and immune system regulation. The purpose of this study is to evaluate the probiotic capabilities of bacteriocin-producing LAB isolated from malted barley, *Pediococcus acidilactici* HW01, and *Leuconostoc citreum* HW02.

**Results** Both strains could survive in harsh GI conditions such as low pH, bile salt, pepsin, and lysozyme. Furthermore, exhibited non-haemolytic activity and tetracycline and erythromycin susceptibilities. In vitro adhesion of HW01 and HW02 strains to HT-29 cells was 76.28 and 75.99%, respectively. Auto-aggregation ability of HW01 and HW02 was about 14%, but their ability to aggregate with pathogens (*Escherichia coli*, *Salmonella enterica* ser. Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*) ranged from 32.0% to 43.5%. Regarding the anti-inflammatory effect, heat-killed bacteriocin-producing strains suppressed the mRNA expression levels of cytokines and chemokines in RAW 264.7 macrophages induced by LPS ( $P < 0.005$ ).

**Conclusions** Bacteriocin-producing *P. acidilactici* HW01 and *Leu. citreum* HW02 may be good probiotic candidates.

**Keywords** Bacteriocin-producing LAB, *Pediococcus acidilactici* HW01, *Leuconostoc citreum* HW02, Probiotic properties, Anti-inflammatory effect

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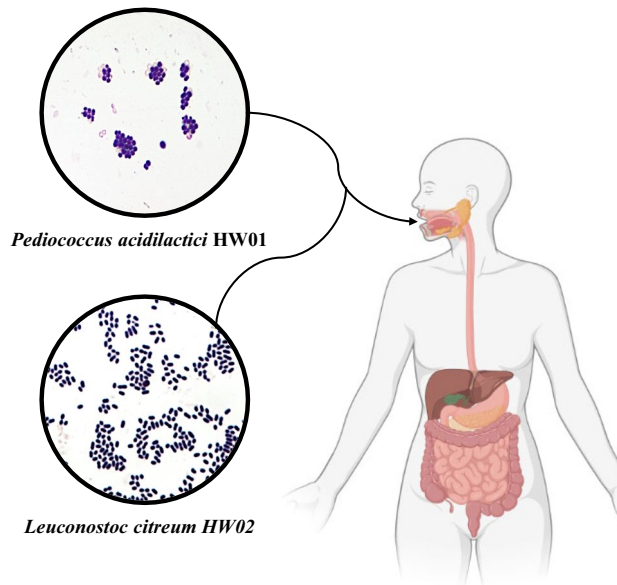
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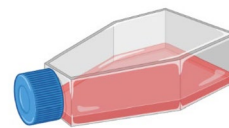
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## Graphical Abstract

### Bacteriocin-producing strains



- **Survival in GI conditions**
  - ✓ Low pH
  - ✓ Bile salt
  - ✓ Lysozyme
  - ✓ Stimulated gastric juice
  - ✓ Stimulated small intestinal fluid
- **Safety evaluation**
  - ✓ Haemolytic activity
  - ✓ Antibiotics susceptibility
- **Enzyme production**
- **Biochemical properties**
- **Hydrogen peroxide production**



HT-29, RAW 264.7 cells

- **Adhesion ability**
  - ✓ Auto-/Co-aggregation
  - ✓ Cell surface hydrophobicity
- **Anti-inflammatory effect**
  - ✓ Antioxidant
  - ✓ Real-time PCR
    - IL-1 $\beta$ , IL-6, MCP-1

## Introduction

Probiotics are defined by the Food and Agriculture Organization of the United Nations and the World Health Organization (WHO) as live microorganisms that originate from a food matrix and confer a health benefit to host cells when administered in adequate amounts in single or combination strains [1–3]. Probiotic strains that survive transit through the gastrointestinal (GI) tract and colonise in the intestinal tract provide health benefits to the host [4, 5]. The well-known health benefits of taking probiotics are the maintenance or restoration of the gut microbiome, immune regulation, intestinal barrier integrity maintenance, and protection against invasion by pathogenic bacteria [6]. To be approved as probiotics, the following aspects need to be proven: identify the microorganisms via 16S rRNA sequencing and fermentation ability of sugars, resistance to GI conditions (acid, bile salt), adhesion ability to mucus or human epithelial cells, antibacterial activity against pathogens, haemolytic activity, resistance to antibiotics [7–10].

Bacteriocins are antibacterial peptides secreted by bacteriocin-producing bacteria such as lactic acid bacteria, generally recognised as safe. They have bacteriocidal, bacteriostatic or bacteriolytic effects against genetically closely related bacteria with bacteriocin-producing bacteria [11–13]. In addition, bacteriocin has emerged

in interest because bacteriocins are harmless and safe due to being decomposed in the stomach by proteolytic enzymes [14]. However, the settlement of bacteriocinogenic probiotics in the GI tract may contribute to bacteriocin production and lead to pathogen invasion inhibition [13, 15, 16] and immune system regulation [13, 17]. For that reason, bacteriocinogenic probiotics may provide an alternative to antibiotics [18, 19].

The probiotic properties of bacteriocin-producing strains have attracted considerable attention. *Enterococcus faecalis* from the chicken GI tract and *Leuconostoc citreum* SJRP44 isolated from water-buffalo mozzarella cheese were identified as potential probiotics from assays of their inhibitory activities against pathogens, investigatory existence of virulence genes, auto-/co-aggregation abilities, resistance to antibiotics and survival rate in stimulated GI juice, among other properties [11, 20–22]. According to Qiao, Qiu [23], the bacteriocin-producing *Pediococcus acidilactici* strain exerted notable changes in the intestinal microflora and serum immune factors in mice.

This study aims to assess the probiotics properties of bacteriocin-producing LAB *P. acidilactici* HW01 and *Leu. citreum* HW02 isolated from malted barley. For this purpose, the survival rate in acidic, bile salt and artificial GI conditions; safety evaluation, such as haemolytic

activity and resistance to antibiotics; enzyme production; carbohydrate fermentation ability; cell surface properties, including hydrophobicity and auto-/co-aggregation; adhesion ability to human epithelial cells; antioxidant capacity and anti-inflammatory effect in the RAW 264.7 macrophage cell line were analysed.

## Materials and methods

### Bacteria and cell line and culture conditions

Two bacteriocin-producing LAB, *P. acidilactici* HW01 (HW01) and *Leu. citreum* HW02 (HW02), were isolated and characterised in our previous research [11, 18]. *Lactobacillus rhamnosus* GG (KCTC 5033) (LGG; Korean Collection for Type Culture [KCTC], Jeongseup, Korea), a well-known probiotic strain, was used as a reference strain. All LAB were grown in de Man – Rogosa – Sharpe (MRS) medium (Difco, Spark, MD, USA). *Escherichia coli* KCTC 1039 and *Salmonella enterica* ser. Typhimurium KCTC 1925 were cultured in Luria – Bertani medium, and *Staphylococcus aureus* ATCC 29213 and *Listeria monocytogenes* KCTC 3569 were cultured in brain heart infusion medium, respectively. All bacteria were cultured at 37 °C for 24 h.

The human epithelial colorectal cell line HT-29 (HTB-38) and the *Mus musculus* macrophage cell line RAW 264.7 (TIB-17) were obtained from the American Type Culture Collection (ATCC). Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, HyClone, Logan, UT, USA) with 10% foetal bovine serum (FBS, Gibco, Burlington, ON, Canada), 100 U/mL of penicillin and 100 µg/mL of streptomycin (HyClone) and incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere condition.

### Survival in acidic, bile salt and artificial GI conditions

To determine the survival rate of LAB in acidic and bile salt conditions, overnight cultured HW01, HW02 and LGG were inoculated at a final concentration of 1 × 10<sup>7</sup> CFU/mL in MRS broth with pH adjustment to 2.5 using 5N HCl or inoculated in MRS broth containing 0.3% or 1% bile salt, respectively, and then incubated at 37 °C for 3 h. For the determination of the survival rate of LAB in artificial GI conditions, the probiotic candidates and the reference strain were prepared as described above. All bacteria were inoculated in an electrolyte solution at a concentration of 1 × 10<sup>7</sup> CFU/mL. For simulated gastric juice, the electrolyte solution (sodium chloride, 6.2 g/L; potassium chloride, 2.2 g/L; calcium chloride, 0.22 g/L; sodium bicarbonate, 1.2 g/L; pH 2.5) was mixed with 0.3% pepsin, and for simulated small intestinal fluid, 0.1% pancreatin and 0.3% bile salt were mixed with an electrolyte solution (sodium chloride, 5 g/L; potassium chloride, 0.6 g/L; calcium chloride 0.25 g/L; pH 7.0). Consequently, the electrolyte solution was incubated at 37 °C

for 3 h. The viable cell was measured every hour during the incubation time, and after 3 h of incubation, the survival rate was calculated using the following equation:

$$\frac{N_t}{N_i} \times 100,$$

where  $N_i$  and  $N_t$  are the viable cell numbers before and after treatment, respectively.

### Resistance to lysozyme

The evaluation of the lysozyme resistance method followed as the bacteriocin-producing LAB was inoculated into a sterilized electrolyte solution (sodium chloride, 5 g/L; potassium chloride, 0.6 g/L; calcium chloride 0.25 g/L; pH 7.0) with lysozyme (100 mg/L). The control was prepared using the sterilized electrolyte solution without lysozyme. The survival rate of bacteriocin-producing LAB under lysozyme was measured using viable cell count after 2 h incubation at 37 °C.

### Safety evaluation

Haemolytic activity was investigated using Columbia 5% sheep blood agar (bioMérieux, Marcy-l'Étoile, France). The cultivated HW01, HW02 and LGG were streaked on Columbia 5% sheep blood agar and incubated at 37 °C for 24 h. The presence of a green zone around the colony suggested α-haemolysis, no effect on the agar plate was considered γ-haemolysis and displaying a clear zone around the colony was classified as β-haemolysis. The antibiotic susceptibility of the LAB was determined by the disc diffusion assay [24] with some modifications. Susceptibility to the following six antibiotics was evaluated: 10 µg/mL of ampicillin, gentamicin and streptomycin, 15 µg/mL of erythromycin, and 30 µg/mL of kanamycin, tetracycline and chloramphenicol. All antibiotics were purchased from Sigma-Aldrich, except gentamycin and erythromycin, which were obtained from Enzo Life Sciences (Farmingdale, NY, USA). Briefly, sterilised paper discs (8 mm in diameter) were placed on MRS agar seeded with each strain (10<sup>7</sup> CFU/mL), and 100 µL of each antibiotic was placed on the paper disc, followed by incubation at 37 °C for 24 h. The clear zone around each paper disc was measured after incubation and classified as sensitive (≥ 20 mm), intermediate (15 – 19 mm) or resistant (≤ 14 mm).

### Evaluation of enzyme production and biochemical properties

The API ZYM kit was used to evaluate the enzyme production of each LAB strain, and the API 50 CHL kit was used to assess the biochemical properties of each LAB strain. All API kits (bioMérieux) were used following the manufacturer's instructions.

### Hydrogen peroxide production

The hydrogen peroxide production of bacteriocin-producing LAB was measured. Briefly, the supernatants were obtained by centrifuging an overnight culture of LAB. The produced hydrogen peroxide by the LAB in the supernatants was measured using an OxiTec Hydrogen Peroxide/Peroxidase Assay kit (Biomax, Gyeonggi, Korea) and followed the manufacturer's instructions.

### Cell surface hydrophobicity

The surface hydrophobicity of LAB was evaluated using xylene and chloroform. The LAB was cultured in MRS overnight and washed twice under centrifugation. The collected cell was re-suspended in phosphate-buffered saline (PBS) to an optical density at 600 nm ( $OD_{600}$ ) of  $0.6 \pm 0.05$ . The cell suspension was mixed with an equal volume of xylene or chloroform and then vortexed for 2 min. After 30 min incubation at room temperature, the  $OD_{600}$  of the aqueous phase was measured. The hydrophobicity was calculated using the following equation:

$$\frac{A_0 - A_{30}}{A_0} \times 100,$$

where  $A_0$  and  $A_{30}$  are  $OD_{600}$  at 0 and 30 min, respectively.

### Adhesion ability to HT-29 cells

To evaluate the adhesion ability of the strains to the HT-29 cell line, the HT-29 cells were seeded at a concentration of  $5 \times 10^5$  cells/mL in a 24-well plate and cultured until fully confluent. The LAB suspension was prepared at  $1 \times 10^7$  CFU/mL in DMEM without penicillin and streptomycin. The LAB suspension was inoculated into HT-29 cells and incubated at 37 °C for 1 h. After incubation, the supernatant was discarded, and the pellet was washed twice using PBS to remove non-adherent bacteria cells. Then, 0.5% Triton X-100 (Sigma–Aldrich) was treated to detach the adherent bacterial cells from the HT-29 cells. The number of adherent bacterial cells was measured by the viable cell count using MRS agar. The adhesion ability was determined using the Equation [7].

### Auto-aggregation and co-aggregation

Auto-aggregation and co-aggregation assays were conducted to determine the aggregation ability of bacteriocin-producing LAB. Bacteriocin-producing LAB and pathogenic bacteria (*E. coli*, *S. Typhimurium*, *L. monocytogenes* and *S. aureus*) were grown for 24 h at 37 °C. The cells were harvested by centrifugation (as described above), washed twice, and re-suspended in PBS to give an  $OD_{600}$  of  $1 \pm 0.05$ . For auto-aggregation ability, each LAB suspension (4 mL) was homogenised by vortex for 10 s. For co-aggregation ability determination, an equal volume (2 mL) of LAB suspension was

mixed with pathogenic bacterial suspension by vortex for 10 s. Each control tube containing 4 mL of each bacterial suspension (the LAB and the pathogens) was prepared and incubated in parallel. After 4 h of incubation, the  $OD_{600}$  of the mixture was measured. The percentage of auto-aggregation was calculated as follows:

$$\text{Auto-aggregation\%} = \left(1 - \frac{A_t}{A_0}\right) \times 100,$$

where  $A_0$  represents the absorbance at 0 h, and  $A_t$  represents the absorbance at 4 h. The percentage of co-aggregation was calculated as follows:

$$\text{Co-aggregation\%} = \frac{\left(\frac{Ax+Ay}{2}\right) - A(x+y)}{Ax + \frac{Ay}{2}} \times 100,$$

where  $x$  and  $y$  represent each of the two isolates in the control tube, and  $(x+y)$  the mixture.

### Antioxidant activity

Each LAB of a cell-free supernatant (CFS) was examined by the 1,1-diphenyl 2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays to determine its antioxidant activity. The CFS of LAB was collected by centrifugation of the overnight cultured LAB. The DPPH assay was based on the method of Yoshida, Mori [25] with a slight modification. Briefly, 200  $\mu$ M of DPPH (Sigma–Aldrich) in ethanol was mixed with an equal volume of the CFS and incubated for 30 min at room temperature in the dark. After incubation, the  $OD_{520}$  was measured. For the ABTS assay, 7 mM of ABTS (diammonium salt; Sigma–Aldrich) was mixed with 2.45 mM potassium persulphate in distilled water. The CFS of each LAB was added to the ABTS solution at a ratio of 1:200 and then incubated at room temperature for 1 h in the dark. The DPPH and ABTS free radical scavenging activities were calculated by the following equation:

$$\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

### Preparation of heat-killed bacteria

The pellet of each LAB was collected, washed and re-suspended in PBSR. Heat-killed bacteria were prepared by autoclaving (121 °C, 15 min), then collected in the pellet and re-suspended at a concentration of  $1 \times 10^8$  CFU/mL in DMEM containing FBS. The bacterial suspension was maintained at –80 °C for further study.



### Determination of the anti-inflammatory effect in RAW 264.7 cells

To determine the anti-inflammatory effect, the RAW 264.7 cells were seeded at a density of  $5 \times 10^5$  cells/mL in a 6-well plate and cultivated until 80% confluence. The cells were treated with heat-killed bacteria (final concentration at  $1 \times 10^7$  CFU/mL) in the presence of 1  $\mu$ g/mL of lipopolysaccharide (LPS) for 3 h. Subsequently, total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as described in the manufacturer's instructions. Complementary DNA (cDNA) was synthesised using the total RNA (2  $\mu$ g), reverse transcriptase (Promega, Madison, WI, USA) and random hexamers. The cDNA was stored at  $-20^\circ\text{C}$  until used for real-time PCR. Real-time PCR was performed with SYBR Green Realtime PCR Master Mix Plus (Toyobo Co., Ltd., Osaka, Japan) with primer pairs specific for mouse  $\beta$ -actin (forward primer: 5'-TAC AGCTTCACCACCACAGC-3', reverse primer: 5'-GGA AAAGAGCCTCAGGGCAT-3'), IL-1 $\beta$  (forward: 5'-CTC ACAAGCAGAGCACAAGC-3', reverse: 5'-TCTTGG CCGAGGACTAAGGA-3'), IL-6 (forward: 5'-TCCTAC CCCAATTTCCAATGCT-3', reverse: 5'-TCTGACCAC AGTGAGGAATGTC-3') and MCP-1 (forward: 5'-AGC CAACTCTCACTGAAGCC-3', reverse: 5'-TCTCCAGCC TACTCATTGGGA-3') using a QuantStudio 3 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Relative expression levels were normalised to  $\beta$ -actin by the  $2^{-\Delta\Delta C_t}$  method.

### Statistical analysis

The results are expressed as mean  $\pm$  standard deviation from independent triplicate experiments. Statistical significance was analysed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test ( $P < 0.05$ ; IBM SPSS ver. 25.0; SPSS, Inc., Chicago, IL, USA) or the unpaired  $t$ -test ( $P < 0.005$ ; GraphPad Prism 5, GraphPad Prism Software, Inc., La Jolla, CA, USA).

## Results and discussion

### Survival in GI conditions

To be used as probiotics, isolated strains must have a survival ability under acidic conditions high bile salt concentrations [26]. Therefore, the tolerances to acidic or bile salt or artificial gastric juice of strains were tested (Table 1). When the bacteriocin-producing strains (HW01 and HW02) were exposed to acidic conditions (pH 2.5), both strains displayed better resistance than the reference strain, LGG, the most studied probiotic bacteria, with survival rates of over 99.80% for HW01 and HW02 and 97.17% for LGG. The strains HW01 and HW02 were also highly tolerant to both concentrations of bile salt (0.3% and 1%), with a

survival rate of over 100%. In several previous studies [26, 27], *Leuconostoc* spp. exhibited a better survival ability at less than a concentration of 0.4%, and *P. acidilactici* showed an above 90% survival rate under pH 3 and 0.3% bile salt concentration which is similar to our results. On the other hand, *P. acidilactici* B14 showed a much lower survival rate of 45.9% and 72% at pH 2.5 and 0.3% bile salt concentration, respectively [28]. The change in the survival rate of all strains was tested under simulated GI conditions. All strains were able to withstand both simulated stomach and small intestine conditions. Based on the experiments, bacteriocin-producing strains (HW01 and HW02), which maintained viability in acidic, bile salt and proteolytic enzyme conditions, satisfied the requirements of probiotics. After ingestion, probiotics must remain viable under the harsh conditions of the GI tract, such as the acidic environment of the stomach or exposure to bile salt, to colonise the large intestine [29]. During passage through acidic or bile salt conditions, the cytoplasmic pH in the probiotic is reduced by highly acidic conditions, and lipids and fatty acids in the cell membrane may be damaged by disruption due to bile salts [30–32]. In the results on the survival in simulated GI tract conditions, the bacteriocin-producing LAB strains HW01 and HW02 were shown to remain viable under those harsh conditions, an important criterion for the selection of potential probiotics.

### Lysozyme tolerance

Lysozyme, in saliva, is the first barrier confronted with probiotics in oral and has an antimicrobial effect on gram-positive bacteria by disruption of the cell wall and subsequent cell lysis [33]. Probiotics should be tolerant to lysozyme since generally, probiotics are administered orally [34, 35]. In our study, bacteriocin-producing HW01 and HW02 were exposed to lysozyme (100 mg/L) for 2 h, the survival rate was  $99.66 \pm 1.62\%$  and  $99.20 \pm 1.07\%$ , respectively (Table 1). Additionally, *Lb. rhamnosus* GG, as control, showed a  $101.81 \pm 1.90\%$  survival rate against lysozyme. According to Sirichokchatchawan, Pupa [36], *P. acidilactici* isolated from pig faeces exhibited high resistance (average 78% after lysozyme treatment at 120 min) to lysozyme. *Leu. citreum* isolated from bovine and turkey meat sausages showed good resistance toward lysozyme with an above 88% survival rate [37]. The concentration of lysozyme is known up to 180  $\mu$ g/mL in saliva [33]. In the present study, consideration of testing in the concentration of lysozyme at 100 mg/mL, two bacteriocin-producing LAB exhibited lysozyme tolerance levels that are high enough to allow them to live in salivary conditions.

**Table 1** Tolerance of LAB strains to acid, bile salt and simulated gastrointestinal conditions

	Strain		
	<i>Lb. rhamnosus</i> GG	<i>P. acidilactici</i> HW01	<i>Leu. citreum</i> HW02
Acid (pH 2.5)			
Viable cell number (Log <sub>10</sub> CFU/mL)			
0 h	6.59 ± 0.05	6.14 ± 0.09	6.15 ± 0.03
1 h	6.59 ± 0.02	6.20 ± 0.01	6.26 ± 0.03
2 h	6.47 ± 0.01	6.14 ± 0.02	6.14 ± 0.03
3 h	6.41 ± 0.02	6.13 ± 0.06	6.14 ± 0.05
Survival rate (%)	97.17 ± 1.06 <sup>a</sup>	99.80 ± 0.61 <sup>b</sup>	99.85 ± 0.62 <sup>b</sup>
Bile salt (0.3%)			
Viable cell number (Log <sub>10</sub> CFU/mL)			
0 h	7.05 ± 0.06	7.01 ± 0.06	7.15 ± 0.01
1 h	7.09 ± 0.06	7.13 ± 0.04	7.19 ± 0.01
2 h	7.13 ± 0.06	7.50 ± 0.11	7.42 ± 0.05
3 h	7.27 ± 0.08	7.54 ± 0.07	7.88 ± 0.06
Survival rate (%)	104.50 ± 1.74 <sup>a</sup>	106.39 ± 0.74 <sup>a</sup>	110.86 ± 1.50 <sup>b</sup>
Bile salt (1%)			
Viable cell number (Log <sub>10</sub> CFU/mL)			
0 h	7.10 ± 0.05	7.01 ± 0.06	7.10 ± 0.04
1 h	7.12 ± 0.08	6.84 ± 0.12	7.30 ± 0.07
2 h	7.13 ± 0.13	7.50 ± 0.11	7.43 ± 0.10
3 h	7.24 ± 0.06	7.54 ± 0.07	7.64 ± 0.07
Survival rate (%)	102.03 ± 1.35 <sup>a</sup>	106.40 ± 1.65 <sup>b</sup>	107.63 ± 0.89 <sup>b</sup>
Lysozyme (100 mg/L)			
Viable cell number (Log <sub>10</sub> CFU/mL)			
C*	8.93 ± 0.11	9.12 ± 0.13	9.04 ± 0.04
L*	9.09 ± 0.07	9.08 ± 0.17	8.97 ± 0.07
Survival rate (%)	101.81 ± 1.90 <sup>b</sup>	99.66 ± 1.62 <sup>a</sup>	99.20 ± 1.07 <sup>a</sup>
Simulated gastrointestinal condition			
Simulated stomach fluid (pH 2.5)			
Viable cell number (Log <sub>10</sub> CFU/mL)			
0 h	7.30 ± 0.07	7.50 ± 0.08	7.50 ± 0.05
1 h	7.26 ± 0.01	7.42 ± 0.07	7.44 ± 0.08
2 h	7.26 ± 0.14	7.38 ± 0.05	7.44 ± 0.14
3 h	7.38 ± 0.02	7.50 ± 0.03	7.55 ± 0.02
Survival rate (%)	101.04 ± 1.12 <sup>a</sup>	100.05 ± 0.91 <sup>a</sup>	100.60 ± 0.92 <sup>a</sup>
Simulated small intestinal fluid			
Viable cell number (Log <sub>10</sub> CFU/mL)			
0 h	6.58 ± 0.17	6.29 ± 0.10	6.06 ± 0.08
1 h	6.48 ± 0.20	6.13 ± 0.12	6.16 ± 0.13
2 h	6.22 ± 0.17	6.14 ± 0.12	6.16 ± 0.12
3 h	6.21 ± 0.15	6.16 ± 0.11	6.26 ± 0.13
Survival rate (%)	94.40 ± 2.40 <sup>a</sup>	97.43 ± 2.98 <sup>a</sup>	103.69 ± 2.26 <sup>b</sup>

All values are shown as mean ± S.D. (Standard deviation) (n = 3)

Lowercase letters (series "a–c") indicate significant (Duncan's range test,  $p < 0.05$ ) differences in the same row

C\* and L\* indicate control (without lysozyme) and lysozyme treatment (100 mg/L) samples, respectively

**Table 2** Antibiotics susceptibility and hemolysis of strains

Antibiotics susceptibility									Hemolysis
Strain	AMP (10 µg/mL)	S (10 µg/mL)	K (30 µg/mL)	T (30 µg/mL)	C (30 µg/mL)	E (15 µg/mL)	G (10 µg/mL)		
<i>Lb. rhamnosus</i> GG	R	R	R	S	I	S	R	Y	
<i>P. acidilactici</i> HW01	R	R	R	S	R	S	R	Y	
<i>Leu. Citreum</i> HW02	R	R	R	S	R	S	R	Y	

CTR control, AMP ampicillin, S streptomycin, K kanamycin, T tetracycline, C chloramphenicol, E erythromycin, G gentamycin

S Sensitivity, I Intermediate, R Resistant

### Evaluation of haemolytic activity and antibiotic susceptibility of LAB

Evaluation of the safety of the strain is integral for the development of potential probiotics. Therefore, we demonstrated the safety of the strains through antibiotic resistance and haemolytic activity (Table 2). To this end, the in vivo haemolytic activity of HW01 and HW02 was tested using Colombia sheep blood agar. Both strains exhibited  $\gamma$ -haemolytic activity, which is safe to be acknowledged in probiotic strains [38].

All strains were tested for susceptibility towards ampicillin, streptomycin, kanamycin, tetracycline, chloramphenicol, erythromycin and gentamycin. Both bacteriocin-producing strains showed susceptibility to tetracycline and erythromycin, which are protein synthesis inhibitors [39, 40]. The use of antibiotic-resistant probiotics is controversial due to the potential for the transfer of antibiotic resistance caused by endogenous or horizontal gene transfer acquired by chromosomal mutations [41, 42]. However, other studies have reported that antibiotic resistance is beneficial for controlling intestinal infections caused by pathogenic bacteria and restoring microbial homeostasis in the GI tract. This means that antibiotic resistance can have advantages for both medically prescribed antibiotic treatment and prophylaxis [43, 44].

### Assessment of enzyme production and biochemical properties

The profile of enzymes produced by bacteriocin-producing LAB was evaluated using the ZYM kit (Table 3). HW01 and HW02 did not produce alkaline phosphatase, trypsin,  $\alpha$ -galactosidase,  $\beta$ -glucuronidase,  $\beta$ -glucosidase,  $\alpha$ -glucosidase, *N*-acetyl- $\beta$ -glucosaminidase,  $\alpha$ -mannosidase and  $\alpha$ -fucosidase. Among them,  $\beta$ -glucuronidase is well-known as a carcinogenic enzyme, which can cause or increase the possibility of carcinogenesis in the liver or colon due to the hydrolysis of glucuronide to reactive metabolites [45, 46]. The carbohydrate metabolic pattern of the probiotic candidates was evaluated using the API 50 CHL kit (Table 4). Both HW01 and HW02 were capable of metabolising L-arabinose, ribose, D-xylose, galactose, glucose, fructose, mannose, *N*-acetyl-glucosamine, esculin, cellobiose, sucrose, trehalose and tagatose. It is important to understand the carbohydrate metabolic abilities of LAB because it plays a key role in colonisation and multiplication in various ecological systems, including the intestine [47, 48].

### Production of hydrogen peroxide

In this study, the amount of hydrogen peroxide was measured using an overnight culture broth of LAB (Table 5). *Lb. rhamnosus* GG as control produced hydrogen peroxide with 109.49  $\mu$ M while *P. acidilactici* HW01 and *Leu.*

*citreum* HW02 produced 155.10  $\mu$ M and 129.90  $\mu$ M, respectively. In another study,  $2.53 \pm 1.5$   $\mu$ g/mL of H<sub>2</sub>O<sub>2</sub> was produced by *P. acidilactici* isolated from kefir [49]. The hydrogen peroxide generates hydroxyl radical that may occur the growth inhibition of pathogens and leads to gene damage by reacting with the nucleic acid. Since probiotics exert antimicrobial activity toward pathogenic bacteria including GI tract pathogens such as *S. aureus* and *Listeria* spp. by secretion of hydrogen peroxide as well as other substances such as lactic acid, bacteriocin, etc., verification of produced hydrogen peroxide is quite important in probiotics properties [4, 50, 51].

### Cell surface hydrophobicity and auto-/co-aggregation abilities

The results of the physicochemical properties of the bacterial cell surface are displayed in Table 5. With the consideration that the attachment and interaction abilities of the bacteria to the host cell depend on the hydrophobicity of the bacterial cell membrane [50], the cell surface hydrophobicity towards xylene and chloroform was evaluated. Both bacteriocin-producing strains (HW01 and HW02) exhibited lower affinities than the LGG strain to chloroform, which has a characteristic as an electron-acceptor, showing 14.03%, 14.01% and 79.12%, respectively. However, compared to chloroform, all three strains showed a higher adhesion to xylene of approximately 90%. Having good affinities towards hydrocarbons, such as xylene, indicates that the bacterial cell surface is hydrophobic. A higher hydrophobicity facilitates the hydrophobic interaction of probiotic bacteria with the intestinal epithelial cells, resulting in better adhesion abilities to the host cells [52–55].

Along with hydrophobicity, auto-aggregation ability is related to the adherence of bacteria to the colon epithelial cells, and biofilm formation, whereas co-aggregation ability is associated with the prevention of pathogenic bacteria adherence [31, 56, 57]. In our study, the auto-/co-aggregation abilities of all three strains were tested (Table 5). The bacteriocin-producing bacteria (HW01 and HW02) showed roughly 20% auto-aggregation abilities, while the LGG strain showed better auto-aggregation ability by over 40%. Conversely, the co-aggregation competence against *E. coli*, *S. Typhimurium*, *L. monocytogenes* and *S. aureus* was 38.87%, 32.04%, 36.92% and 43.02% for HW01 and 39.14%, 35.23%, 38.65% and 43.50% for HW02, respectively. Compared to the co-aggregation ability of the LGG strain, HW01 and HW02 showed comparable or better performance. According to previous studies, the auto- and co-aggregation abilities of probiotic candidates increase with time [27, 58–60]. Thus, the tested bacterial strains also may expect to show an improvement in aggregation ability over time. In the



**Table 3** Enzyme production profiles by API ZYM kit of bacteriocin-producing LAB strains

	Enzyme	Results <sup>1</sup>		
		<i>Lb. rhamnosus</i> GG	<i>P. acidilactici</i> HW01	<i>Leu. citreum</i> HW02
1	Control	0 <sup>1</sup>	0	0
2	Alkaline phosphatase	1	0	0
3	Esterase (C4)	3	1	1
4	Esterase Lipase (C8)	2	1	1
5	Lipase (C14)	0	1	1
6	Leucine arylamidase	5	5	5
7	Valine arylamidase	4	3	4
8	Crystine arylamidase	3	2	2
9	Trypsin	0	0	0
10	$\alpha$ -chymotrypsin	1	0	1
11	Acid phosphatase	3	2	3
12	Naphthol-AS-BI-phosphohydrolase	1	2	3
13	$\alpha$ -galactosidase	1	0	0
14	$\beta$ -glucuronidase	0	0	0
15	$\beta$ -glucosidase	0	0	0
16	$\alpha$ -glucosidase	1	0	0
17	$\beta$ -glucosidase	5	4	5
18	N-acetyl- $\beta$ -glucosaminidase	0	0	0
19	$\alpha$ -mannosidase	0	0	0
20	$\alpha$ -fucosidase	2	0	0

<sup>1</sup> 0, 0 nmol; 1, 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol; 5, = 40 nmol

previous studies, bacteriocin-producing strains HW01 and HW02 exhibited antimicrobial effects against *E. coli*, *L. monocytogenes*, *S. Typhimurium*, and *S. aureus*. These antimicrobial effects were presumed as owing to bacteriocin and other antimicrobial substances such as H<sub>2</sub>O<sub>2</sub>, organic acid, etc. [11, 18, 61]. In addition, strains HW01 and HW02 are inferred to produce class II and class IV bacteriocin, respectively [11, 18]. Based on the results of the co-aggregation assay and antimicrobial abilities, the *P. acidilactici* HW01 and *Leu. citreum* HW02 might be estimated to be capable of not only inhibiting the pathogen adhesion but be able to inhibit pre-colonized pathogenic bacteria in the GI tract.

#### In vitro adhesion ability to HT-29 cells

The ability to adhere to the intestinal epithelial cells, such as HT-29 or Caco-2 cells, is one of the criteria for evaluating new probiotic strains [62]. In the present study, the adhesion ability to the HT-29 cell line was evaluated (Table 5). The results showed that the adhesion ability of bacteriocin-producing HW01, HW02 and the indicator strain LGG ranged from 75 to 81%. These results concur with the findings of Oh and Jung that *P. acidilactici* SW05 adhered well to HT-29 cells. According to Silva

et al., *Leu. citreum* CIATEJ BI-49.1 isolated from artisanal *tejuino* showed a 25.4% adhesion ability to HT-29 cells, whereas our results show that *Leu. citreum* HW02 exhibits a much higher 75.99%. The adhesion properties of probiotics have relevance to the hydrophobicity of cell surfaces and the profiles of extracellular compounds or proteins. Furthermore, the in vitro adhesion to the human epithelial cells is perceived to be correlated with actual colonisation or persistence in humans. However, some factors can affect the adhesion ability during the assay, such as the period of bacterial growth, the density of the bacterial suspension, buffer, pH, and washing intensity of unattached bacterial cells [63, 64]. Therefore, exhibiting good adhesion ability in vitro does not always lead to good adhesion ability in vivo [65], and further study is needed to assess the adhesion ability in vivo.

#### Antioxidant activities of bacteriocin-producing LAB

According to published research, probiotics effectively reduce the duration and frequency of diarrhoea, stimulate the immunocytes, prevent cancer and decrease adverse metabolites, such as carcinogenic enzymes and ammonium. In addition to these favourable effects, probiotics have been shown to display antioxidant activities,

**Table 4** Biochemical properties of bacteriocin-producing LAB using API 50 CHL kit

Carbohydrates	Results		Carbohydrates	Results	
	<i>P. acidilactici</i> HW01	<i>Leuc. citreum</i> HW02		<i>P. acidilactici</i> HW01	<i>Leuc. citreum</i> HW02
Control	—	—	Esculin	+	+
Glycerol	—	—	Salicin	—	+
Erythritol	—	—	Cellobiose	+	+
D-Arabinose	—	—	Maltose	—	+
L-Arabinose	+	+	Lactose	—	—
Ribose	+	+	Melibiose	—	—
D-Xylose	+	+	Sucrose	+	+
L-Xylose	—	—	Trehalose	+	+
Adonitol	—	—	Inulin	—	—
β-methyl-D-Xylopyranose	—	—	Melezitose	—	—
Galactose	+	+	Raffinose	—	—
Glucose	+	+	Starch	—	—
Fructose	+	+	Glycogen	—	—
Mannose	+	+	Xylitol	—	—
Sorbose	—	—	Gentiobiose	—	+
Rhamnose	—	—	Turanose	—	+
Dulcitol	—	—	Lyxose	—	—
Inositol	—	—	Tagatose	+	+
Mannitol	—	+	D-Fucose	—	—
Sorbitol	—	—	L-Fucose	—	—
α-Methyl-D-Mannoside	—	—	D-Arabitol	—	—
α-Methyl-D-Glucoside	—	+	L-Arabitol	—	—
N-Acetyl-Glucosamine	+	+	Gluconate	—	+
Amygdalin	—	+	2-Ketone-Gluconate	—	+
Arbutin	—	+	5-Keto-Gluconate	—	+

such as scavenging hydroxyl radicals, peroxide radicals and the superoxide anion [66].

Thus, in this study, the antioxidant properties of LAB were measured (Table 5) using the CFS of each LAB strain based on the DPPH and ABTS assays. The LGG strain, the most well-known probiotic, exhibited the lowest DPPH free radical scavenging activity (15.59%), whereas its ABTS cation radical eliminating activity was 77.33%. In comparison, both bacteriocin-producing strains (HW01 and HW02) showed higher scavenging capacities towards DPPH radicals (25.79% and 37.36%) but similar values towards ABTS cation radicals (77.61 and 85.74%).

The antioxidative effect of probiotics results from producing numerous metabolites, including folate, butyrate and glutathione, as well as their metal ion chelating ability and antioxidant enzymes, such as superoxide dismutase [66, 67]. Thus, further studies are needed to support our results on the antioxidative capacities of LAB, for example, to determine the DPPH and ABTS assays using intact

cells, the glutathione assay and the ferrous ion chelating activity.

#### Anti-inflammatory effect of bacteriocin-producing LAB

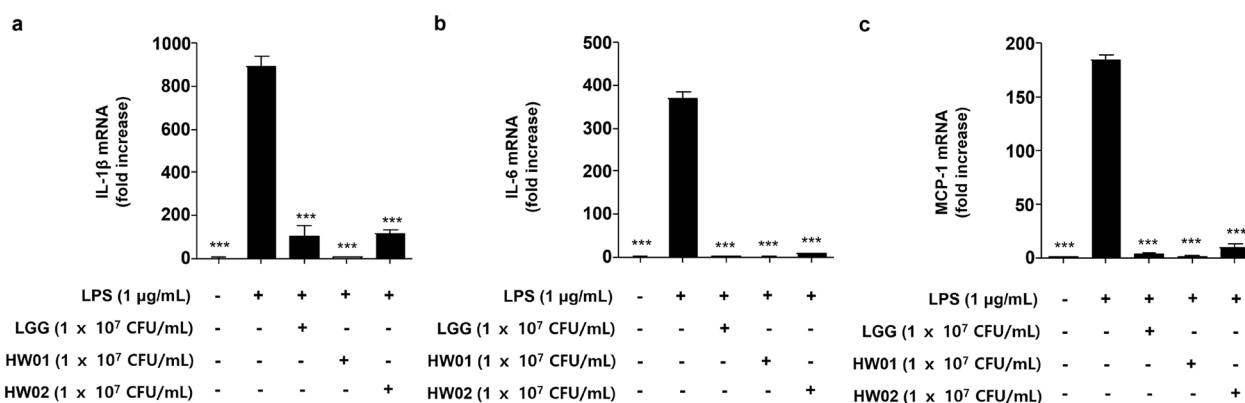
Probiotics are live bacteria giving beneficial effects on the host. In the case of treatment to a host with a feeble immune system, probiotics might be causative of inflammatory responses such as diarrhoea, immoderate immune stimulation and etc. Probiotics with antibiotic resistance genes might transfer the genes to pathogenic bacteria in the GI tract [68–70]. Dead or heat-killed microorganisms are also well-known to give health-promoting to humans [3]. For this reason, in the present study, heat-killed bacteriocin-producing strains were used to measure the anti-inflammatory abilities (Fig. 1). RAW 264.7 murine macrophage cells have been extensively used because of their function as antigen-presenting cells, which engage in the first step of innate immunity by inducing the secretion of intercellular signalling cytokines, such as IL-8, IL-6, NO and COX-2 [45].

**Table 5** The measurement of adhesion, hydrophobicity, auto/co-aggregation, antioxidant activity and hydrogen peroxide production of probiotic bacterial strains

Subject	LAB strain		
	<i>Lb. rhamnosus</i> GG	<i>P. acidilactici</i> HW01	<i>Leu. citreum</i> HW02
Adhesion ability (%)	81.09 ± 2.58 <sup>a</sup>	76.28 ± 0.25 <sup>b</sup>	75.99 ± 0.28 <sup>b</sup>
Cell surface hydrophobicity			
Chloroform	79.12 ± 1.90 <sup>b</sup>	14.03 ± 0.28 <sup>a</sup>	14.10 ± 2.07 <sup>a</sup>
Xylene	90.51 ± 0.07 <sup>a</sup>	90.80 ± 0.07 <sup>a</sup>	89.60 ± 1.30 <sup>a</sup>
Auto-aggregation (%)	48.82 ± 0.32 <sup>c</sup>	21.07 ± 0.26 <sup>a</sup>	22.77 ± 0.48 <sup>b</sup>
Co-aggregation (%)			
<i>E. coli</i>	36.85 ± 0.80 <sup>a</sup>	38.87 ± 0.29 <sup>b</sup>	39.14 ± 0.39 <sup>b</sup>
<i>S. Typhimurium</i>	30.54 ± 1.17 <sup>a</sup>	32.04 ± 0.83 <sup>a</sup>	35.23 ± 0.30 <sup>b</sup>
<i>S. aureus</i>	38.28 ± 1.04 <sup>ab</sup>	36.92 ± 0.72 <sup>a</sup>	38.65 ± 0.39 <sup>b</sup>
<i>L. monocytogenes</i>	40.45 ± 1.12 <sup>a</sup>	43.02 ± 0.64 <sup>b</sup>	43.50 ± 0.60 <sup>b</sup>
Antioxidant activity (%)			
DPPH	15.59 ± 2.87 <sup>a</sup>	25.79 ± 0.10 <sup>b</sup>	37.36 ± 5.60 <sup>c</sup>
ABTS	77.33 ± 1.56 <sup>a</sup>	77.61 ± 0.72 <sup>a</sup>	85.74 ± 1.94 <sup>b</sup>
Hydrogen peroxide production (μM)	109.49 ± 2.95 <sup>a</sup>	155.10 ± 2.77 <sup>b</sup>	129.90 ± 1.64 <sup>c</sup>

All values are shown as mean ± S.D. (Standard deviation) (n = 3)

Lowercase letters (series “a–c”) indicate significant (Duncan’s range test,  $p < 0.05$ ) differences in the same row



**Fig. 1** Anti-inflammatory cytokines and chemokine expression in RAW 264.7 cells treated with lipopolysaccharide (LPS) and putative probiotics by real-time PCR. **a** IL-1β, **b** IL-6, **c** MCP-1. \*\*\* $P < 0.005$ , compared with the LPS-treated group

When the cytotoxicity of the LAB to RAW 264.7 cells was tested, no cytotoxicity was observed (data not shown). RAW 264.7 cells were simultaneously treated with the bacteriocin-producing strains, LGG strain and LPS, and the cytokine expressions were ascertained. IL-1β and IL-6 represented the anti-inflammatory cytokines in this study, and MCP-1 was the anti-inflammatory cytokine. All strains showed a decrease in the expression of IL-1β and MCP-1. Upon LPS treatment of RAW 264.7 cells, the mRNA expression level of cytokines and MCP-1 increased, while heat-killed LAB suppressed the expression of cytokines and MCP-1 induced by LPS (\*\* $P < 0.005$ ). The HW02 strain exhibited a similar ability to LGG regarding IL-1β

expression and less inhibition ability of MCP-1 and IL-6 expression than other strains. Meanwhile, the HW01 strain suppressed the chemokine MCP-1 and the cytokine IL-6 expressions as much as the control. Recently, the anti-inflammatory effect of heat-killed bacteria has been reported. Choi, Chang [71] reported that heat-killed *E. faecalis* EF-2001 dose-dependently suppressed iNOS and COX-2 expression in LPS-induced RAW 264.7 cells. According to Jhong, Tsai [72], heat-killed *Lactobacillus paracasei* GMNL-653 reduced the levels of NO and IL-6 in LPS-stimulated RAW 264.7 cells. We investigated the effectiveness of the heat-killed bacteriocin-producing strains on the suppression of cytokine expression at the

mRNA level. However, further study on the quantification of secreted cytokines at the protein level or an anti-inflammatory signalling pathway, not only suppression at the mRNA expression level, would be required to understand the anti-inflammatory characteristics of the strains.

## Conclusions

This study evaluated the probiotic properties of two bacteriocin-producing LAB, *P. acidilactici* HW01 and *Leu. citreum* HW02, isolated from malted barley. Both strains were able to endure harsh conditions, such as exposure to acid, bile salt and proteolytic enzymes, which increases the possibility of their viability and settlement in the large intestine. Through the evaluation of their safety, cell surface properties, adhesion ability to human epithelial cells, antioxidant activity and anti-inflammatory effects in vitro in macrophage cells, both strains were suggested as possible probiotic candidates. However, further study, such as an in vivo trial in an animal model, will be needed to prove their usefulness as probiotics.

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## Author contributions

HA: Formal analysis, Investigation, Methodology; GL: Formal analysis; WL: Methodology; MK: Methodology; K-GL: Supervision; Validation; Investigation; Project administration.

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## Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Declarations

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no conflicts of interest.

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