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Comprehensive molecular, probiotic, and quorum-sensing characterization of anti-listerial lactic acid bacteria, and application as bioprotective in a food (milk) model

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ABSTRACT

Listeria monocytogenes is a major foodborne pathogen that adversely affects the food industry. In this study, 6 anti-listerial lactic acid bacteria (LAB) isolates were screened. These anti-listerial LAB isolates were identified via 16S rRNA gene sequencing and analyzed via repetitive extragenic palindromic-PCR. Probiotic assessment of these isolates, comprising an evaluation of the antibiotic susceptibility, tolerance to lysozyme, simulated gastric and intestinal juices, and gut conditions (low pH, bile salts, and 0.4% phenol), was carried out. Most of the isolates were resistant to streptomycin, vancomycin, gentamycin, kanamycin, and ciprofloxacin. All of the isolates were negative for virulence genes, including *agg*, *ccf*, *cylA*, *cylB*, *cylLL*, *cylLS*, *cylM*, *esp*, and *gelE*, and hemolytic activity. Furthermore, autoinducer-2 (a quorum-sensing molecule) was detected and quantified via HPLC with fluorescence detection after derivatization with 2,3-diaminonaphthalene. Metabolites profiles of the *Lactobacillus sakei* D.7 and *Lactobacillus plantarum* I.60 were observed and presented various organic acids linked with antibacterial activity. Moreover, freeze-dried cell-free supernatants from *Lb. sakei* (55 mg/mL) and *Lb. plantarum* (40 mg/mL) showed different minimum effective concentration (MEC) against *L. monocytogenes* in the food model (whole milk). In summary, these anti-listerial LAB isolates do not pose a risk to consumer health, are eco-friendly, and may be promising candidates for future use as bioprotective cultures and new probiotics to control contamination by *L. monocytogenes* in the food and dairy industries.

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INTRODUCTION

Listeria monocytogenes is the causative agent of deadly foodborne listeriosis outbreaks (David and Cossart, 2017). This pathogen often contaminates food items during their production, processing, packaging, and storage (Carpentier and Cerf, 2011). In light of the problems stemming from the transmission of foodborne pathogenic bacteria through the food chain, novel approaches are needed to control foodborne pathogens associated with food and food contact surfaces in the food industry (Gómez et al., 2016). Given the presence of lactic acid bacteria (**LAB**) probiotics in foods, in agricultural products, or in the gastrointestinal tract (**GIT**) of mammals, and their use as starter cultures in food production, these may be a promising way to prevent the growth of pathogens (Winkelströter and De Martinis, 2013).

Potential probiotics can be isolated from the food matrices in which those microorganisms are used (Das et al., 2016; Ayyash et al., 2018). Kimchi is a Korean traditional fermented food, and it is a rich source of LAB isolates with high probiotic potential characteristics (Wen et al., 2016). Lactic acid bacteria produce antimicrobial compounds and organic acids that prevent pathogenic bacterial growth, in addition to exhibiting health-promoting effects as a result of their probiotic functions in fermented foods (Oh and Jung, 2015). In 2002, an FAO and WHO joint panel defined probiotics as "live microorganisms which, when administered in adequate amounts, provide a beneficial effect on the host health" (FAO/WHO, 2002). Lactic acid bacteria are the largest and best-known group of probiotics to have been characterized (Rivera-Espinoza and Gallardo-Navarro,

2010). Consequently, several probiotic criteria are being considered to investigate the probiotic characteristics of new LAB isolates as potential probiotic candidates, including the ability to tolerate low-acid and bile conditions, lysozyme resistance, pathogen inhibition activity, nonhemolytic activity, sensitivity to antibiotics, and the ability to survive during ingestion and transit through the adverse environment of the human GIT (Hossain et al., 2017). Moreover, several studies have highlighted the danger associated with the potential for transfer of the antibiotic resistance gene profile of lactobacilli strains to other resident microbiota in the host gut and hence to pathogenic bacteria. Although most LAB probiotic bacteria have been awarded "generally regarded as safe" (GRAS) status (Leuschner et al., 2010), an examination of the antibiotic resistance characteristics from a safety perspective is nonetheless paramount when assessing probiotic bacteria (Lavilla-Lerma et al., 2013).

Cell-to-cell signaling, known as quorum sensing (**QS**), is one of several microbial communication mechanisms that may occur during food fermentation (Ivey et al., 2013). Autoinducer-2 (**AI-2**) is a LuxS-dependent, QS, universal signaling molecule, produced by the activity of LuxS enzyme (*luxS* gene-encoded). LuxS converts *S*-ribosyl-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione (**DPD**), which is the precursor of AI-2. Gram-negative and gram-positive bacteria recognize and produce AI-2, which mediates both intra- and interspecies communication. The role of the *luxS* gene in bacterial QS and biofilm formation has been well documented (Kaper and Sperandio, 2005; Toushik et al., 2020). LuxS homologs have been identified in the genomes of various lactobacilli (Lebeer et al., 2007). It is proposed that the activity of AI-2 could affect the balance of gut microbiota (Thompson et al., 2015). Although few lactobacilli produce the AI-2 QS molecule, the ability of *Lactobacillus acidophilus* to adhere and adapt to the intestinal environment is enhanced by the AI-2 QS system (Buck et al., 2009). Additionally, *Lactobacillus* spp. use this system not only to respond to environmental stress but also to regulate their growth and metabolism (Lebeer et al., 2007, 2008; Yeo et al., 2015). For example, AI-2 synthesis, biofilm formation, metabolic activity, and pleiotropic effects on growth are regulated by the *luxS* gene in *Lactobacillus rhamnosus* GG (Lebeer et al., 2007; Moslehi-Jenabian et al., 2009).

The use of LAB probiotics to control foodborne pathogens in the food industry is a novel field of research. Our objectives in this study were to (1) screen novel anti-listerial LAB strains collected from kimchi and evaluate their probiotic properties through a series of in vitro experiments; (2) determine their molecular characteristics; and (3) identify and determine the concentration of QS molecule (AI-2) in LAB isolates. A final aim of this study was to analyze the metabolites composition of 2 *Lactobacillus* spp. isolates and their application as bioprotective agents by assessing the activity of their lyophilized cell-free supernatants (**CFS**) against *L. monocytogenes* in vitro and in a food model (whole milk).

MATERIALS AND METHODS

Screening of Anti-Listerial LAB Isolates

Thirty-four LAB isolates were procured from the Food Microbiology Laboratory, Chung-Ang University, South Korea (Hossain et al., 2020). The strains were previously isolated from commercial kimchi, available in the local market in Anseong, South Korea; cultured in de Man, Rogosa, and Sharpe (**MRS**) agar medium; and incubated aerobically at 30°C for 48 h. The isolates were stored in MRS broth comprising 15% glycerol at −80°C for further use. A spot-on-lawn procedure was performed to investigate the anti-listerial effect of the LAB against *L. monocytogenes* ATCC 19113, ATCC 19117, and ATCC 15313, as a pathogenic indicator. Briefly, the *L. monocytogenes* strains were cultured overnight in tryptic soy broth (**TSB**) at 30°C, and 100 μL of the culture (10^8 cfu/mL) was spread on brain heart infusion (BHI) agar plates. Tested LAB cultures were poured (10 μL) on the agar plates and incubated aerobically overnight at 30°C to assess the degree of inhibition. The zones of inhibition were then examined as per the protocol outlined by Hossain et al. (2020).

Strains

The probiotic strains *Lb. acidophilus* KACC 12419 and *Lactobacillus paracasei* KACC 12427 were collected from the Korean Agricultural Culture Collection (**KACC**), South Korea. These 2 probiotic strains were used in this study as reference strains for comparison of the probiotic, as well as other characteristics. The pathogenic strains *Enterococcus faecalis* ATCC 51299 and ATCC 29212 were used as positive controls to identify virulence genes.

Molecular Characterization

Genus-Specific PCR. The LAB isolate genotypes were identified using a *Lactobacillus* genus-specific primer pair (forward: LbLMA1, 5′-CTCAAAACTAAACAAAGTTTC-3′; reverse: R16-1, 5′-CTTGTACACACCGCCCGTCA-3′), as previously reported by Dubernet et al. (2002). Following Mizan et al. (2016), total DNA of the isolate was purified using the DNeasy

Blood and Tissue Kit (Qiagen). The PCR reactions (25 μL) carried a mixture of PCR mix (12 μL; Genetic Technologies), each of the primers (2 μL, 10 μ*M*), DNA template (2 μL), and remaining double-distilled water. DNA amplification was performed as follows: initial denaturation (95°C for 5 min), followed by 30 cycles consisting of denaturation (95°C for 30 s), annealing (55°C for 30 s), extension (72°C for 30 s), and a final extension step (72°C for 7 min) and then cooling to 4°C (Dubernet et al., 2002).

LAB Identification at Species Level. Genotypic identification at the species level was identified using the 16S rRNA gene sequencing method. The fragment of the gene was amplified by PCR using a universal primer pair: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), 1492R (5′-GGTTACCTTGTTACGACTT-3′, Bioneer Corp.; Wilson, Blitchington, and Greene, 1990; Angmo et al., 2016). DNA sequencing was performed by a commercial company (SolGent, Daejon, South Korea). The sequences were analogized with the existing reference sequences in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

Genetic Fingerprinting by Repetitive Extragenic Palindromic-PCR. Repetitive extragenic palindromic (**REP**)-PCR fingerprinting was used to compare chromosomal DNA in the LAB isolates using only a single primer $(GTG)_5$, (5′-GTGGTGGTGGTGGTG-3′), as reported previously (Al Kassaa et al., 2014; Ouali et al., 2014). The REP-PCR program involved an initial step of denaturation at 95°C for 4 min, followed by 29 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 8 min. The program was ended by an extra extension step at 72°C for 16 min. According to Mizan et al. (2017), the obtained REP-PCR products were analyzed by gel electrophoresis (1.5% agarose gel) with 1× Tris-acetate-EDTA buffer solution at 100 V for 1 h. The amplified DNA was stained with 1% ethidium bromide (Bio-Rad Laboratories) and scanned using a charge-coupling device camera (Gel Doc XR system, Bio-Rad Laboratories). The obtained DNA fingerprints were assayed employing the FPQuest software (Bio-Rad Laboratories). Similarities between the digital profiles were assessed by calculating the Pearson correlation coefficient, and an average linkage dendrogram was acquired by using an unweighted pair group method with arithmetic mean.

Tests for LAB Safety Considerations

Screening for Virulence Factors. Virulence factors in the LAB isolates were detected by PCR with previously described primer pairs and conditions (Table 1). All primers used in this study were synthesized by a commercial company, Bioneer Corp. (South Korea). Total DNA purification and PCR reactions followed Mizan et al. (2016), as mentioned previously. Amplification reactions were as follows: an initial cycle of 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at a suitable temperature depending on the melting temperature of the primers for 1 min, elongation at 72°C for 2 min, a final extension step of 72°C for 7 min, and then cooling to 4°C (Eaton and Gasson, 2001; Semedo et al., 2003; Vankerckhoven et al., 2004; Gómez et al., 2016). All experiments were performed in triplicate with 3 or more biological replicates. Amplified products were detected by gel electrophoresis (1.5% agarose gel) at 100 V for 30 min. A marker of 100 bp (Biofact Co. Ltd.) was used as the standard to determine the molecular weight (Mizan et al., 2016). The positive controls were *Enterococcus faecalis* ATCC 51299 and ATCC 29212 (Nami et al., 2015).

Antimicrobial Susceptibility. Antibiotic susceptibility was evaluated by a disk diffusion procedure considering the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012). The antibiotics used in our experiment are listed in Table 2. Antibiotics were chosen based on the recommendations of the European Food Safety Authority (EFSA, 2012). Mueller-Hinton agar medium was overlaid with 100 μL of overnight LAB cultures, and the antibiotic disks were then placed on the Mueller-Hinton agar plates. After incubation at 30°C for 24 h, inhibition zone diameters (mm) were calculated to assess susceptibility based on the CLSI criteria (CLSI, 2012). Isolates were categorized as susceptible (≥21 mm), moderately susceptible (16–20 mm), or resistant (≤15 mm; Reuben et al., 2020). When a bacterial strain is inhibited at a specific antimicrobial concentration equal to or lower than the established cutoff value, it is defined as susceptible, and when it is not inhibited at a concentration higher than the established cutoff value, it is defined as resistant (Yépez et al., 2017).

Hemolytic Activity. Hemolytic capability was assessed by following the methodology of Pieniz et al. (2014), with slight modification. Overnight plate cultures of the LAB colonies were streaked on the surface of blood agar plates containing 7% defibrinized sheep blood and incubated at 30°C for 24 to 48 h. Following incubation, the hemolysis zone surrounding the colonies was investigated. When greenish zones around the colonies (α-hemolysis) or when no effect on the blood agar plates (γ-hemolysis) was observed, the strains were deemed to be nonhemolytic. By contrast, strains producing blood lysis zones around the LAB colonies were considered to be hemolytic (β-hemolysis; Oh and Jung, 2015; Ku et al., 2020).

Table 1. Primers used for screening of virulence factors genes

Virulence gene	Gene function	Primer[1]	Sequence 5′–3′	Amplicon size (bp)	Reference
agg	Aggregation substance	agg-F	AAGAAAAAGAAGTAGACCAAC	1,553	Eaton and Gasson (2001)
		agg-R	AAACGGCAAGACAAGTAAATA		
gelE	Gelatinase	gelE-F	ACCCCGTATCATTGGTTT	419	Eaton and Gasson (2001)
		gelE-R	ACGCATTGCTTTTCCATC		
esp	Cell wall–associated protein	esp-F	TTGCTAATGCTACTCCACGACC	933	Eaton and Gasson (2001)
		esp-R	GCGTCAACACTTGCATTGCCGAA		
ccf	Facilitation of conjugation	ccf-F	GGGAATTGAGTAGTGAAGAAG	543	Eaton and Gasson (2001)
		ccf-R	AGCCGCTAAAATCGGTAAAAT		
cylA	Activation of cytolysin	cylA-F	ACTCGGGGATTGATAGGC	688	Vankerckhoven et al. (2004)
		cylA-R	GCTGCTAAAGCTGCGCTT		
cylB	Transport of cytolysin	cylB-F	AAGTACACTAGTACAACTAAGGGA	2,020	Semedo et al. (2003)
		cylB-R	ACAGTGAACGATATAACTCGCTATT		
cylM	Post-translational modification of cytolysin	cylM-F	AAAAGGAGTGCTTACATGGAAGAT	2,940	Semedo et al. (2003)
		cylM-R	CATAACCCACACCACTGATTCC		
cylLL	Cytolysin precursor	cylLL-F	GATGGAGGGTAAGAATTATGG	253	Semedo et al. (2003)
		cylLL-R	GCTTCACCTCACTAAGTTTTATAG		
cylLS	Cytolysin precursor	cylLS-F	GAAGCACAGTGCTAAATAAGG	240	Semedo et al. (2003)
		cylLS-R	GTATAAGAGGGCTAGTTTCAC		

[1]F = forward; R = reverse.

Table 2. Antibiotic susceptibility assay for lactic acid bacteria (LAB) isolates[1]

Antibiotic	Disc content	LAB isolate (diameter of inhibition zone, in mm)							
		B.67	D.7	I.60	J.27	M.2	M.21	KACC 12419	KACC 12427
Penicillin G	10 U	S (25)	R (10)	S (25)	MS (18)	R (12)	MS (17)	S (25)	S (28)
Ampicillin	10 µg	S (27)	S (30)	S (29)	S (31)	S (30)	S (29)	S (31)	S (32)
Gentamycin	10 µg	R (0)	R (9)	R (8)	R (8)	R (8)	R (9)	R (10)	R (12)
Ciprofloxacin	5 µg	MS (17)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	MS (17)
Vancomycin	30 µg	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)
Kanamycin	30 µg	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)
Streptomycin	10 µg	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)	R (0)
Chloramphenicol	30 µg	S (23)	MS (16)	MS (17)	MS (17)	MS (16)	MS (16)	MS (18)	MS (19)
Erythromycin	15 µg	S (24)	S (25)	S (27)	S (27)	S (26)	S (25)	S (29)	S (33)
Tetracycline	30 µg	S (27)	S (27)	S (22)	S (23)	S (24)	S (22)	S (28)	S (31)
Clindamycin	2 µg	S (25)	S (24)	S (27)	S (25)	S (25)	S (26)	S (32)	S (29)

[1]S = susceptible (≥21 mm); MS = moderately susceptible (16–20 mm); R = resistant (≤15 mm).

Resistance of LAB to Simulated Gastrointestinal Conditions

Acid Tolerance. The viability of the LAB isolates at pH 3.0 and 6.5 (control) was examined in accordance with the method previously described (Oh and Jung, 2015; Yadav et al., 2016), with slight modification. Overnight LAB cultures were centrifuged (10,000 × *g*, 4°C, 10 min) and washed 2 times with PBS (pH 7.0), and the pellets were resuspended in MRS broth fixed to pH 3.0 using a 1 *M* HCl solution. A control sample of LAB culture was adjusted to pH 6.5 in MRS broth. The cultures were incubated at 30°C for 3 h. Following incubation, cultures were withdrawn and serially diluted in 0.1% peptone water (**PW**; Oxoid) and spread on MRS agar plates, which were subsequently incubated at 30°C for 48 h. Acid resistance was investigated by colony counts on MRS agar plates, and the results were expressed as log colony-forming units per milliliter (cfu/mL).

Bile Salt Resistance. This assay was executed following the method of García-Ruiz et al. (2014) and Oh and Jung (2015), with some modifications. The cell pellet (obtained as described in the previous section) was resuspended in MRS broth (adjusted to 10^8 cfu/mL) supplemented with 0.5 and 1.0% bile salt (wt/vol; Oxoid). The cultures were then incubated at 30°C for 4 h, in keeping with the time that food spends in the small intestine. Bile salt resistance was examined based on the number of colonies on MRS agar plates, which were incubated at 30°C for 48 h.

Phenol Resistance. The method of Yadav et al. (2016) was implemented with slight modification to determine LAB viability in the presence of 0.4% phenol. Overnight LAB cultures (1% vol/vol) were inoculated in MRS broth with 0.4% phenol (Sigma-Aldrich). After incubation at 30°C for 0 and 24 h, LAB were enumerated by serial dilutions in 0.1% PW (Oxoid) and spreading the cultures on MRS agar plates, followed by incubation at 30°C for 48 h.

Lysozyme Resistance. This assay was executed following the method of Zago et al. (2011) and Yadav et al. (2016), with slight modification. Overnight LAB cultures were centrifuged (10,000 × *g*, 4°C, 10 min) and washed 2 times with PBS (pH 7.0), and the pellets were resuspended in 2 mL of Ringer solution (Sigma-Aldrich). A 1% bacterial suspension was then inoculated into a sterile electrolyte solution ($CaCl_2$ 0.22 g/L, NaCl 6.2 g/L, KCl 2.2 g/L, $NaHCO_3$ 1.2 g/L) supplemented with 100 mg/L lysozyme (Sigma-Aldrich). A LAB culture in sterile electrolyte solution without lysozyme was used as a control. Viable bacterial cell counts were calculated after 2 h of incubation at 30°C by the plate-count method on MRS agar plates, which were incubated at 30°C for 48 h.

Resistance to Pepsin and Pancreatin. The survival of LAB isolates in stress conditions with pepsin and pancreatin was assessed, as described by Osmanagaoglu et al. (2010) and Ben Taheur et al. (2016), with some modifications. Overnight LAB cultures were centrifuged (10,000 × *g*, 4°C, 10 min); after washing the pellets with PBS (pH 7.0), they were resuspended in PBS (pH 3.0) with pepsin (3 mg/mL; Sigma-Aldrich) and PBS (pH 8.0) with pancreatin (1 mg/mL; Sigma-Aldrich), respectively. The samples were then incubated at 30°C with pepsin (3 h) and pancreatin (4 h) to reflect the amount of time that food spends in the stomach and the small intestine, respectively. Viable bacterial cell counts were investigated after the desired incubation period, using colony counts on MRS agar plates, which were incubated at 30°C for 48 h.

Detection and Determination of the Concentration of AI-2 QS Molecules

AI-2 Determination by Bioluminescence Assay. For autoinducer bioassays, LAB isolates were cultivated in modified MRS medium (glucose replaced by galactose in the growth medium; DeKeersmaecker and Vanderleyden, 2003). Following centrifugation, the CFS was collected and sterilized using syringe filters (pore size 0.2 μm), the pH was fixed to 7.0 using a 5 *N* NaOH solution, and the samples were stored at −20°C. The AI-2 activity was assessed using a modified bioluminescence assay (Mizan et al., 2016). *Vibrio harveyi* BB170, which produces bioluminescence strictly in response to AI-2, was used as a reporter strain (Taga and Xavier, 2011). *Vibrio harveyi* BB120 was used as a positive control due to the production of AI-1 and AI-2 (Mizan et al., 2016). Both strains were grown overnight in Luria-Bertani broth (2% NaCl) at 30°C with aeration (200 rpm). *Vibrio harveyi* BB120 was centrifuged (10,000 × *g*, 4°C, 10 min), and the resultant CFS was then filtered (0.2 μm) and kept at −20°C. After overnight growth, *V. harveyi* BB170 was diluted (1:5,000) with fresh autoinducer bioassay medium to achieve 10^5 cfu/mL. The CFS (500 μL) of either LAB or *V. harveyi* BB120 was mixed well with diluted *V. harveyi* BB170 (4.5 mL) in a 50-mL Falcon tube and incubated at 30°C with aeration (200 rpm) for 15 h. The CFS of BB120 was used as the positive control for AI-2 detection, and sterile autoinducer bioassay medium was used as the negative control. Afterward, 100-μL aliquots were shifted to a white 96-well microtiter plate, and the luminescence was calculated every 1 h using a luminometer device (GloMax 96 Microplate Luminometer, Promega). The

readings were depicted as relative light units (Mizan et al., 2016).

AI-2 Detection by HPLC With Fluorescence Detection. Detection of AI-2 was performed as previously reported by Song et al. (2014) and Mizan et al. (2017), with some modifications. A DPD solution (3 mg/mL, prepared by dissolving in ultrapure water; Omm Scientific Inc.), 2,3-diaminonaphthalene (**DAN**; J&K Scientific Ltd.), HPLC-grade formic acid and acetonitrile (both from Sigma-Aldrich), and HPLC-grade water (Daejung Chemicals and Metals Co. Ltd.) were sourced. The working solution of 500 ng/mL DPD was generated by dilution of the DPD stock solution (0.3 mg/mL). The DAN solution was prepared by dissolving 10 mg of DAN in 50 mL of 0.1 *M* HCl. A total of 400 μL of standard solution (500 ng/mL DPD) or post-treatment supernatant was transferred to 2-mL autosampler screw-cap vials (Agilent Technologies) containing an equal volume of DAN solution. After thorough mixing for 2 min, the samples were incubated in a water bath at 90°C for 40 min, cooled to room temperature, and analyzed directly via HPLC with fluorescence detection (**HPLC-FLD**). The Varian HPLC system was equipped with dual pumps, a fluorescence detector, and a Zorbax SB-C18 reverse-phase column (250 mm × 4.6 mm, 5 μm; Agilent Technologies) set at 30°C. The sample injection volume was 20 μL. The chromatographic separation condition was set up as previously reported by Mizan et al. (2017). The mobile phase contained solvents (A) 0.1% formic acid and (B) acetonitrile, with a flow rate of 0.8 mL/min. The following gradient elution profile was used: 70% A, 30% B at 0 min; 70% A, 30% B at 4 min; 35% A, 65% B at 12 min; 35% A, 65% B at 20 min; 70% A, 30% B at 24 min; and 70% A, 30% B at 27 min. The excitation and emission wavelengths of the fluorescence detector were set at 271 and 503 nm, respectively.

Analysis of Metabolite Composition in LAB Supernatant. Metabolite profiles of the LAB supernatant were prepared following the procedure and conditions of Park et al. (2010), with slight modification. Ribitol, glycolic acid, acetic acid, lactic acid, glucose, fructose, and chloroform were purchased from Sigma-Aldrich. Methanol and HPLC-grade acetonitrile were purchased from J. T. Baker. Ultrapure water was obtained from a water purification system (Milli-Q Direct 8 system, Merck Millipore). Briefly, a mixture solution was prepared using 1 mL CSF of LAB isolates with methanol (14 mL), ribitol (80 μg), and glycolic acid (80 μg). Ribitol and glycolic acid were added to the mixture as internal standards. The mixture was heated for 25 min at 70°C in a water bath and cooled at room temperature for 30 min. Then the mixture was concentrated to a final volume of 0.1 mL via rotary evaporator (N-1110, Eyela). The concentrates were desiccated in a vacuum oven at 40°C for 4 h. The desiccated extracts were mixed with 100 μL of *N,O*-bis (trimethylsilyl) trifluoroacetamide comprising 1% trimethylchlorosilane (Supelco) and 100 μL of acetonitrile. The extracts were heated (70°C for 20 min) in a water bath and cooled (10 min) in order to proceed to GC-MS analysis.

The GC-MS analysis was accomplished using a 7890B GC coupled with a 5977B mass selective detector and a DB-5MS fused silica column (30-m × 0.25-mm internal diameter × 0.25-μm film thickness). Metabolite levels in the LAB CFS were calculated based on a previous study (Lee et al., 2014).

Within sugar profiles, contents of glucose and sucrose were quantified using a 1260 Infinity HPLC system coupled with a refractive index detector and a YMC-Pack polyamine II column (4.6 mm × 250 mm, 5 μm; YMC Co. Ltd.) set at 35°C. For experimental analysis, filtered CFS extract (0.22 μm) and a mixture of water and acetonitrile (25:75 vol/vol) solution as mobile phase (1.0 mL/min flow rate) were prepared. Moreover, the contents of acetic acid and lactic acid in the LAB CFS were determined via a 1260 Infinity HPLC system coupled with a photodiode array detector (G1115A, Agilent Technologies) and a Grace Prevail organic acid column (4.6 mm × 150 mm, 5 μm; Hichrom Ltd.) set at 40°C. A solution of 25 m*M* K_2HPO_4 (pH 2.8) was used as mobile phase in the column, with 0.8 mL/min flow rate (Park et al., 2010).

Challenge Study in Whole Milk Food Model

Preparation of CFS and Freeze-Drying. *Lactobacillus sakei* D.7 and *Lactobacillus plantarum* I.60 isolates were cultured in MRS broth on a shaking incubator (220 rpm) at 30°C, respectively; then the bacterial suspensions were centrifuged (10,000 × *g*, 4°C, 10 min). The CFS was harvested and lyophilized on a pilot-scale freeze-dryer (ilShin Biobase Co. Ltd.) at freezing temperature of −45°C, pump pressure of 40 mTorr, and shelf temperature of 14.7°C; resultant CFS (approximately 25 g/L freeze-dried sample from culture supernatant) was used in the next assay, as described by Moradi et al. (2019), with some modifications.

Challenge of Freeze-Dried CFS Against Listeria monocytogenes in Whole Milk (Food) Model. *Listeria monocytogenes* strain ATCC 19113 was used in this study. It was kept at −80°C in TSB containing 15% (vol/vol) glycerol and activated by subculturing 2 times in TSB under aerobic conditions at 30°C for 24 h. Subsequently, *L. monocytogenes* culture was centrifuged (10,000 × *g*, 4°C, 10 min). The resultant pellets were washed 3 times with sterile PBS and resuspended in 0.1% PW (Oxoid), and the *L. monocytogenes* concentration

was confirmed by plating the inoculum onto PALCAM agar (Oxoid; Sadekuzzaman et al., 2017). Minimal effective concentrations (**MEC**) of *Lb. sakei* D.7 and *Lb. plantarum* I.60 were measured, respectively, according to Hartmann et al. (2011) and Moradi et al. (2019), with slight modification. *Listeria monocytogenes* was mixed with 10 mL of pasteurized whole milk in a sterile 50-mL Falcon tube to deliver an ultimate concentration of ~4 log cfu/mL. Then, the freeze-dried CFS from 2 *Lactobacillus* spp. was mixed in the concentrations range of 10 to 100 mg/mL. The experimental samples were thoroughly homogeneous and kept at 4°C for 6 d. For enumeration of *L. monocytogenes* cells, bacterial cell suspension was serially diluted in 0.1% PW and spread onto PALCAM agar plates; then plates were kept at 30°C for 48 h (Hossain et al., 2020). According to Moradi et al. (2019), MEC was defined as the CFS concentration that reduces the initial count of *L. monocytogenes* to under the detection limit of 10 cfu/mL bacteria of whole milk in the storage condition at 4°C for 3 d. A similar study was also conducted with TSB broth instead of whole milk.

Transmission Electron Microscopy Analysis. The effect of freeze-dried CFS on the intracellular organization of *L. monocytogenes* cells was evaluated under transmission electron microscopy (**TEM**) based on the method of Cui et al. (2018) and Ashrafudoulla et al. (2020), with slight modifications. *Lactobacillus* CFS at MEC concentration was mixed with *L. monocytogenes* suspension for 3 h; *L. monocytogenes* upon exposure to MRS broth (LAB growth medium) without CFS was accounted as the control. After 3 h, the *L. monocytogenes* cells were collected by centrifugation (10,000 × *g*, 4°C, 10 min) and washed thrice with PBS. Then the bacterial suspension was put on filter paper and contacted with copper mesh for 1 min. The copper mesh was stained (1 min) for microscopic examination (JEM-2100, Jeol Ltd.).

Statistical Analysis

Statistical analysis was evaluated by performing an ANOVA and Duncan's new multiple tests at $P < 0.05$, using SAS version 9.2 software (SAS Institute Inc.). GraphPad Prism 5.03 for Windows software (GraphPad Software Inc.) was also used.

RESULTS AND DISCUSSION

Comprehensive Molecular Characterization of Anti-Listerial LAB

Of the 34 LAB isolates, 6 exhibited anti-listerial activity and were typed using 16S rRNA sequencing. According to the 16S rRNA gene analysis, LAB isolates were 99% homologous to the partial 16S rRNA gene of *Lactobacillus curvatus*, isolate B.67 (accession no. MH304289); *Lb. sakei*, isolate D.7 (accession no. MH304290); *Lb. plantarum*, isolate I.60 (accession no. MH304291); *Leuconostoc mesenteroides*, isolate J.27 (accession no. MH304292); *Lb. plantarum*, isolate M.2 (accession no. MH304293); and *Lb. plantarum*, isolate M.21 (accession no. MH304294; Hossain et al., 2020).

Dubernet et al. (2002) proposed a method that allows amplification of 250-bp DNA fragments originating from LAB isolates, which confirmed that the isolates belonged to *Lactobacillus* genus. Moreover, Ouali et al. (2014) and Yadav et al. (2016) implemented this means of confirming the presence of *Lactobacillus*. In the current study, when DNA from the LAB isolates was used as a template, a ~250-bp PCR product was acquired for all the tested *Lactobacillus* genera, whereas the genus *Leuconostoc* did not generate any product. Our tests on the 6 LAB isolates typed and detected using 16S rRNA sequencing revealed that 5 isolates belonged to the genus *Lactobacillus*, and one isolate belonged to the genus *Leuconostoc*.

We applied REP-PCR to study the genetic relatedness of LAB isolates. Amplification of the LAB genomic DNA by REP-PCR yielded fragments ranging from 1,000 to 3,000 bp (Figure 1). Analysis of the $(GTG)_5$-PCR fingerprint band patterns is shown in Figure 1. Based on this and numerical analysis, 2 major groups were identified. Group 1 contained 6 LAB isolates (B.67, D.7, I.60, J.27, M.2, and M.21), including the KACC 12419 and KACC 12427 strains and was composed of numerous subgroups that exhibited 54.9% similarity to the other group, which contained the *Ent. faecalis* reference strains ATCC 29212 and ATCC 51299. A previous REP-PCR study depicted both intraspecific differences in the same species of LAB strains and interspecific differences among other LAB species (Ouali et al., 2014). The current study made a similar observation, showing intraspecific and interspecific differences among the LAB strains by genotypic REP-PCR fingerprinting. Moreover, genotypic intraspecific and interspecific differences in LAB strains have been reported by Al Kassaa et al. (2014), and Al Atya et al. (2015) also established genetic relatedness among LAB strains via REP-PCR fingerprinting.

Safety Consideration Tests for LAB

In terms of the safety of applying LAB to food, the inspection of virulence genes present in LAB strains is of paramount importance. The evaluation of virulence genes in the LAB isolates by PCR is presented in Table 3. All of the studied isolates were negative

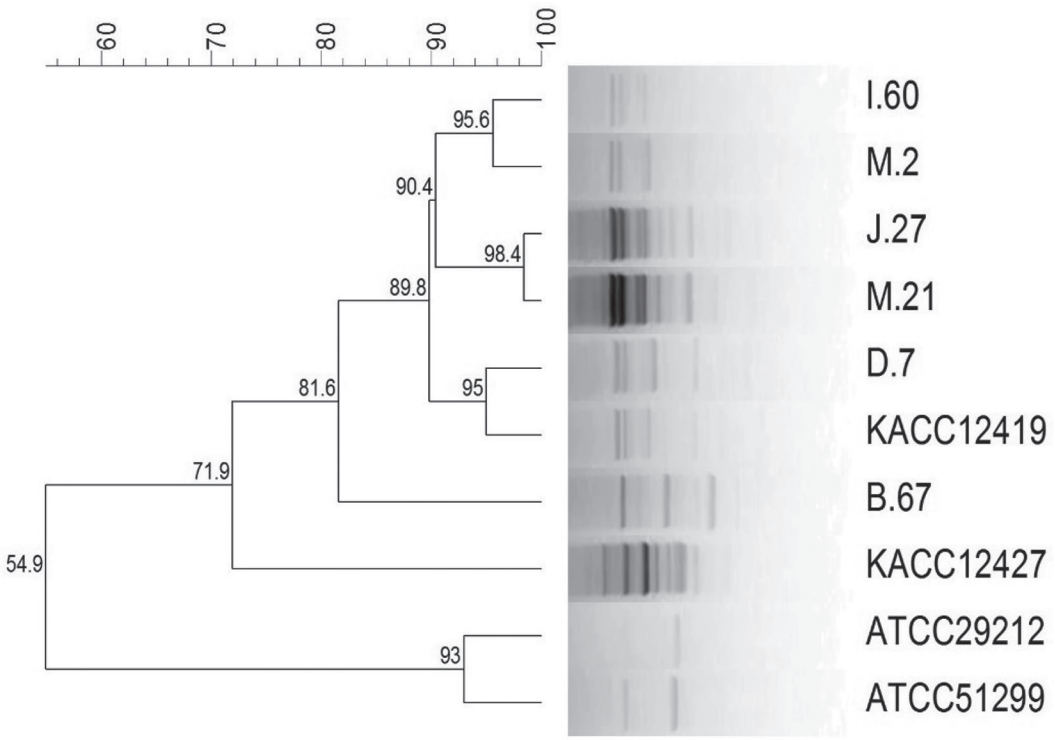

Figure 1. Repetitive extragenic palindromic PCR results for the lactic acid bacteria isolates and reference strains *Lactobacillus acidophilus* KACC 12419, *Lactobacillus paracasei* KACC 12427, and *Enterococcus faecalis* ATCC 51299 and ATCC 29212.

for *agg*, *cylA*, *cylB*, *ccf*, *cylLL*, *cylLS*, *cylM*, *esp*, and *gelE*, except for *Lb. paracasei* KACC 12427, which was positive for *esp*. The *esp* gene encodes a cell wall–associated protein that is associated with the secretion of microbial substances involved in colonization and adherence to biotic and nonbiotic surfaces contributing to biofilm formation (Valenzuela et al., 2009). Moreover, Popović et al. (2018) stated that the presence of the *esp* gene in dairy enterococci isolates correlated more with biofilm formation ability and adhesion properties than virulence. Cebrián et al. (2012) and Popović et al. (2018) recommended that several virulence factors, such as *agg* and *esp*, may play a beneficial role for probiotic bacteria. However, the presence of the *esp* gene in some *Lactobacillus* strains does not seems to deserve consideration as a risk indicator, because this gene is also found in starter *Lactobacillus* strains with a long record of safe use, including several *Lactococcus lactis* and *Lactobacillus casei* strains (Gómez et al., 2016). However, these authors also determined that *agg*, *gelE*, *ccf*, *cylLL*, *cylLS*, *cylM*, *cylA*, and *cylB* were not present in the LAB strains that they tested, which is similar to what we found. Negative PCR results do not necessarily denote the absence of genes, and detecting genes by PCR can also give false negative results, especially when the sequences are not known and the alleles are likely divergent among strains; therefore, the results need to be verified by southern blot or whole-genome sequencing (Mizan et al., 2016).

Safety and nonpathogenicity are important criteria for potential probiotics. Moreover, lack of hemolytic activity is a key demand (Oh and Jung, 2015). Our 5 tested LAB isolates displayed no hemolysis (γ-hemolysis) when cultivated on sheep blood plate (Table 3). In addition, only B.67 showed α-hemolysis activity, which might be attributed to the secretion of hemolytic enzymes by this isolate and is considered negative for hemolytic activity according to Semedo et al. (2003). We also confirmed that the LAB isolates were negative for gelatinase activity. Thus, the tested isolates were found to exert neither of these activities, indicating that the tested strains are not pathogenic and are safe for consumption. These are crucial factors when the strain is intended for use as a probiotic for human health. Bermudez-Humarán and Langella (2012) demonstrated that hemolytic activity is regarded as a disadvantage in probiotic isolates. The absence of hemolytic activity in selected LAB has been reported previously (Ben Taheur et al., 2016). Moreover, LAB

Table 3. Virulence genes and hemolytic activity of the lactic acid bacteria (LAB) isolates

LAB isolates and strains	Virulence gene[1]									Hemolytic activity
	agg	*gelE*	*esp*	*ccf*	*cylLL*	*cylLS*	*cylM*	*cylB*	*cylA*	
B.67	−	−	−	−	−	−	−	−	−	α
D.7	−	−	−	−	−	−	−	−	−	γ
I.60	−	−	−	−	−	−	−	−	−	γ
J.27	−	−	−	−	−	−	−	−	−	γ
M.2	−	−	−	−	−	−	−	−	−	γ
M.21	−	−	−	−	−	−	−	−	−	γ
Lactobacillus acidophilus KACC 12419	−	−	−	−	−	−	−	−	−	γ
Lactobacillus paracasei KACC 12427	−	−	+	−	−	−	−	−	−	γ
Enterococcus faecalis ATCC 29212 (positive control)	−	+	−	−	+	−	−	−	+	γ
Ent. faecalis ATCC 51299 (positive control)	+	+	−	+	+	−	−	−	−	β

[1]+ = positive for virulence gene; − = negative for virulence gene.

strains have also been shown to be negative for gelatinase activity (Gómez et al., 2016).

To be considered safe for human consumption, probiotic strains also need to be assessed in terms of antibiotic susceptibility (FAO/WHO, 2002; EFSA, 2012). Table 2 shows the antibiotic susceptibility of the LAB isolates. All of the LAB isolates were resistant to kanamycin, gentamycin, streptomycin, and vancomycin. In addition, only 2 isolates, B.67 and KACC 12427, were moderately sensitive to ciprofloxacin, whereas the other isolates appeared to be resistant. However, all of the LAB isolates exhibited maximum susceptibility to ampicillin, tetracycline, erythromycin, and clindamycin, and all of them were moderately susceptible to chloramphenicol, except B.67, which was susceptible. In terms of penicillin G, 2 of the isolates were resistant (D.7, M.2), and 2 were moderately susceptible (J.27, M.21); all of the other isolates were susceptible. Resistance profiles generally varied among the LAB isolates, as antibiotic activity is species- and strain-dependent. Moreover, it has been noted that antibiotic resistance in *Lactobacillus* is species- and strain-dependent (Danielsen and Wind, 2003). The source and geographical location of LAB also contribute to the antibiotic susceptibility patterns of potential probiotic strains (Al Kassaa et al., 2014). Several studies have found that *Lactobacillus* spp. are resistant to tetracycline, chloramphenicol, and erythromycin (Gotcheva et al., 2002), whereas other studies have demonstrated sensitivity to these antibiotics with the same species (Arici et al., 2004). The resistance of lactobacilli to ciprofloxacin and vancomycin was investigated by (Vay et al., 2007), and it was noted that most LAB strains appeared to be resistant to vancomycin, which is considered to be an inherent property (Gotcheva et al., 2002). The absence of antibiotic-targeting sites in LAB might be responsible for resistance to particular agents (DeLisle and Perl, 2003). The natural resistance to various classes of antibiotics is possibly related to cell wall structure and membrane permeability (Ammor et al., 2007). However, this attribute might represent a competitive advantage. The intrinsic antibiotic resistance of LAB probiotics suggests their application for both therapeutic and preventive purposes in the treatment and control of intestinal infections, especially when they are administered together with antibiotics, because intestinal microflora recovery becomes enhanced (Jose et al., 2015). Therefore, for the strains that expressed antibiotic resistance, future genetic studies are required to confirm whether this resistance is because of obtained antimicrobial determinants. The nature of the resistance demands further experiments before any of the resistant strains can be regarded as safe for human use.

Resistance of LAB to Simulated Gastrointestinal Conditions

Adequate survival in response to stress due to exposure to acid and bile salts is of paramount importance for the application of probiotic bacteria in the food industry (Chalas et al., 2016). The low pH (2.5–3.5) in the stomach is a definitive chemical boundary that impedes the passage of bacteria into the GIT. Generally, the passage of food via the stomach takes 2 to 4 h. We observed a sharp decline in LAB viability after a 3-h exposure at low pH (3.0) compared with the control condition (pH 6.5; Table 4). Montville and Matthews (2013) reported that acid- and bile salt-resistant mechanisms depended on the strain and species. Angmo et al. (2016) demonstrated that several LAB isolates survive equally well in vitro at pH 3.0 and 7.0.

The human GIT carries bile salt, which ranges in levels from 0.3 to 0.5%. Luo et al. (2012) have reported that resistance to bile salts helps strains to colonize the host GIT. Bile salts exert a detrimental effect on living cells by causing damage to their cell membranes. Accordingly, tolerance to bile salts is essential for probiotic bacteria to colonize the small intestine and exert their beneficial effects on the host (Argyri et al., 2013). The LAB isolates exhibited good tolerance to bile salts at 0.5 and 1% (Table 4). However, a sharp decline in cell viability was observed as the bile salt concentration increased. These study results are similar with those noted by García-Ruiz et al. (2014). Interestingly, few previous studies have discovered a relationship between a tolerance of high bile salt concentration and the strength of *Lactobacillus* to hydrolyze bile salts (Argyri et al., 2013).

The metabolism of aromatic amino acids by gut bacteria produces phenol as a byproduct, which can restrict LAB growth. Therefore, probiotic LAB must be able to withstand the phenolic bacteriostatic environment in the intestine. The LAB isolates were assessed for phenol (0.4%) resistance, and the results were variable (Table 5). The results show that B.67 was the most sensitive to phenol, whereas survival was comparable among the other strains. As mentioned previously, all of the LAB isolates were resistant to high concentrations (0.4%) of phenol, which is in accordance with the findings of Palaniswamy and Govindaswamy (2016).

All of the LAB isolates were resistant to lysozyme (100 mg/L) after a 2-h incubation (Table 5). Of the tested isolates, J.27, M.21, KACC 12419, and KACC 12427 exhibited the highest level of tolerance; with KACC 12419 and B.67 exhibiting the highest and lowest levels of tolerance, respectively. In keeping with this, the response of lactobacilli to lysozyme has been found to be species- and strain-specific (Dias et al., 2015). In

Table 4. Low pH and bile salt tolerance tests for lactic acid bacteria isolates[1]

Isolate	Low pH tolerance			Bile salt tolerance				
	Control (pH 6.5; log cfu/mL)	pH 3 (log cfu/mL)	Survival (%)	Control (log cfu/mL)	0.5% (log cfu/mL)	Survival (%)	1% (log cfu/mL)	Survival (%)
B.67	8.422 ± 0.09^{a}	7.455 ± 0.09^{a}	89	8.227 ± 0.07^{b}	7.741 ± 0.07^{b}	94	7.253 ± 0.10^{cd}	88
D.7	8.361 ± 0.07^{a}	7.710 ± 0.10^{a}	92	8.483 ± 0.06^{a}	8.011 ± 0.10^{a}	95	7.532 ± 0.05^{ab}	88
I.60	8.400 ± 0.11^{a}	7.580 ± 0.12^{a}	90	8.325 ± 0.07^{ab}	8.181 ± 0.08^{a}	98	7.707 ± 0.05^{a}	93
J.27	8.395 ± 0.10^{a}	7.651 ± 0.09^{a}	91	8.463 ± 0.04^{a}	8.124 ± 0.06^{a}	96	7.721 ± 0.06^{a}	91
M.2	8.455 ± 0.09^{a}	7.655 ± 0.12^{a}	91	8.481 ± 0.05^{a}	7.744 ± 0.04^{b}	91	7.110 ± 0.06^{d}	84
M.21	8.341 ± 0.09^{a}	7.515 ± 0.13^{a}	90	8.387 ± 0.09^{ab}	8.128 ± 0.05^{a}	97	7.562 ± 0.10^{ab}	90
Lactobacillus acidophilus KACC 12419	8.432 ± 0.11^{a}	7.550 ± 0.05^{a}	90	8.543 ± 0.06^{a}	8.187 ± 0.04^{a}	96	7.607 ± 0.07^{ab}	89
Lactobacillus paracasei KACC 12427	8.370 ± 0.10^{a}	7.495 ± 0.14^{a}	89	8.472 ± 0.07^{a}	8.097 ± 0.06^{a}	96	7.460 ± 0.07^{bc}	88

[a–d]For each column, different lowercase superscript letters indicate significant difference according to Duncan's multiple-range test ($P < 0.05$) between different strains.
[1]Values are presented as mean ± SEM (n = 3).

Table 5. Phenol (0.4%) tolerance and lysozyme (100 mg/L) resistance tests for lactic acid bacteria isolates[1]

Isolate	Phenol (0.4%) tolerance			Lysozyme resistant		
	0 h (log cfu/mL)	24 h (log cfu/mL)	Survival (%)	0 h (log cfu/mL)	2 h (log cfu/mL)	Survival (%)
B.67	7.455 ± 0.06^{c}	6.805 ± 0.05^{e}	92	8.360 ± 0.08^{a}	6.420 ± 0.12^{d}	77
D.7	7.658 ± 0.07^{ab}	7.563 ± 0.07^{d}	99	8.356 ± 0.07^{a}	6.452 ± 0.14^{d}	77
I.60	7.748 ± 0.09^{a}	7.846 ± 0.04^{abc}	101	8.440 ± 0.05^{a}	6.699 ± 0.06^{cd}	80
J.27	7.665 ± 0.05^{ab}	7.696 ± 0.09^{bcd}	101	8.391 ± 0.09^{a}	7.172 ± 0.14^{ab}	86
M.2	7.785 ± 0.03^{a}	7.882 ± 0.10^{ab}	101	8.356 ± 0.05^{a}	6.531 ± 0.13^{d}	78
M.21	7.531 ± 0.05^{bc}	7.664 ± 0.03^{cd}	102	8.473 ± 0.05^{a}	7.005 ± 0.07^{bc}	83
Lactobacillus acidophilus KACC 12419	7.783 ± 0.04^{a}	7.912 ± 0.06^{a}	102	8.455 ± 0.09^{a}	7.442 ± 0.12^{a}	88
Lactobacillus paracasei KACC 12427	7.792 ± 0.05^{a}	7.974 ± 0.04^{a}	103	8.476 ± 0.07^{a}	7.312 ± 0.09^{ab}	87

$^{a–e}$For each column, different lowercase superscript letters indicate significant difference according to Duncan's multiple-range test ($P < 0.05$) between different strains.

[1]Values are presented as mean ± SEM (n = 3).

other studies, *Lactobacillus* strains exhibited high resistance to lysozyme at 100 mg/L, which is comparable to the stressful environment to which microorganisms are exposed in the saliva (Zago et al., 2011). Furthermore, Angmo et al. (2016) examined the effect of lysozyme (100 μg/mL) on probiotic LAB, and they showed that the viability of all of the isolates was only slightly affected by lysozyme treatment, which is in accordance with our observations.

In our study, the examined LAB could resist exposure to pepsin and pancreatin (Table 6), and all of the isolates exhibited good survival after treatment. Similar to our findings, Chen et al. (2014) found that LAB exhibited good survival after pepsin and pancreatin treatment.

AI-2 Identification in LAB

We qualitatively measured the presence of AI-2 signaling molecules in LAB isolates by a modified bioluminescence assay (Figure 2). There are several indications that food (isolation source of microorganisms) and AI-2 expression levels are associated with AI-2 signaling in LAB strains. It is conjectured that AI-2 signaling activities may actively affect the balance of gut microbiota (Thompson et al., 2015). Moreover, Park et al. (2016) examined the production and activity of AI-2 in LAB isolated from kimchi, and they were able to show an association between this molecule and kimchi. Furthermore, Blana et al. (2011) identified AI-2 activity in *Leuconostoc* isolates from minced beef, which is

Table 6. Pepsin (3 mg/mL) and pancreatin (1 mg/mL) resistance tests for lactic acid bacteria isolates[1]

Isolate	Pepsin resistance			Pancreatin resistance		
	0 h (log cfu/mL)	3 h (log cfu/mL)	Survival (%)	0 h (log cfu/mL)	4 h (log cfu/mL)	Survival (%)
B.67	8.384 ± 0.06^{a}	7.077 ± 0.06^{a}	85	8.463 ± 0.05^{a}	7.521 ± 0.03^{d}	89
D.7	8.412 ± 0.05^{a}	7.386 ± 0.09^{a}	88	8.443 ± 0.04^{a}	7.645 ± 0.03^{bcd}	91
I.60	8.389 ± 0.07^{a}	7.355 ± 0.07^{a}	88	8.443 ± 0.04^{a}	7.848 ± 0.02^{a}	93
J.27	8.434 ± 0.08^{a}	7.316 ± 0.06^{ab}	87	8.487 ± 0.05^{a}	7.779 ± 0.02^{ab}	92
M.2	8.516 ± 0.07^{a}	7.328 ± 0.06^{ab}	86	8.549 ± 0.04^{a}	7.752 ± 0.05^{abc}	91
M.21	8.411 ± 0.12^{a}	7.456 ± 0.07^{a}	89	8.387 ± 0.08^{a}	7.607 ± 0.03^{cd}	91
Lactobacillus acidophilus KACC 12419	8.435 ± 0.09^{a}	7.473 ± 0.06^{a}	89	8.535 ± 0.14^{a}	7.656 ± 0.11^{bcd}	90
Lactobacillus paracasei KACC 12427	8.462 ± 0.05^{a}	7.435 ± 0.07^{a}	88	8.562 ± 0.07^{a}	7.744 ± 0.04^{abc}	91

$^{a–d}$For each column, different lowercase superscript letters indicate significant difference according to Duncan's multiple-range test ($P < 0.05$) between different strains.

[1]Values are presented as mean ± SEM (n = 3).

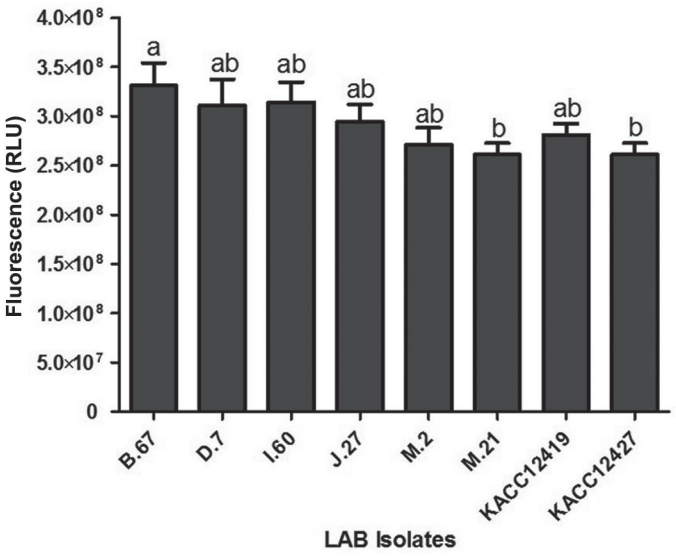

Figure 2. Production of autoinducer-2 (AI-2) by lactic acid bacteria (LAB) isolates at 30°C; RLU = relative light units. Values represent means ± SEM (n = 3). Within each treatment group, the values marked with different letters differ significantly based on Duncan's multiple-range test ($P < 0.05$).

in accordance with the results observed with our *Leuconostoc* isolates. However, the *Lb. sakei* isolates that we examined exhibited AI-2 signaling activity, whereas this was not observed with the *Lb. sakei* isolates of this previous report (Blana et al., 2011).

AI-2 Detection in LAB by HPLC-FLD

The AI-2 metabolic precursor DPD is secreted by several different bacterial species (Pereira et al., 2013; Mizan et al., 2017). Detection and analysis of AI-2 levels secreted by bacterial species are of considerable relevance in light of the important roles of this molecule in QS. We used the experimental method of Song et al. (2014) to determine the level of AI-2 excreted by LAB. Representative chromatograms derived from LAB supernatants are displayed in Figure 3. A distinct peak occurred at a retention time of ~9.32 min (Figure 3). Song et al. (2014) observed a distinct chromatographic peak at a retention time of ~9.25 min when they analyzed the supernatants of *Escherichia coli* MG1655 and *V. harveyi* BB 120, thereby confirming secretion of DPD from these bacterial strains. The presence of a chromatographic peak at ~9.32 min in the supernatants of the LAB isolates is solid proof of the presence of DPD. Overloading of the sample, temperature fluctuations in the HPLC column, and other factors are commonly responsible for minor variations in retention times between runs for biological samples. In light of this, the detection of a peak at a retention time of ~9.46 min in the supernatants of *Vibrio parahaemolyticus* isolates, *V. harveyi* BB170, and *E. coli* ATCC 43889 can be presumed to be a clear indication of the presence of DPD (Mizan et al., 2017, 2018). The HPLC-FLD method needs to be validated to minimize the noise and to identify the precise AI-2 peak, which is lacking in the current study. For further study, to get a precise AI-2 peak, the HPLC-FLD method validation can be followed as according to Song et al. (2014). To date, few methods such as *V. harveyi* luminescence bioassays, which is a test with biosensors derived from AI-2 receptor proteins (Zhu and Pei, 2008), GC-MS (Thiel et al., 2009), and HPLC-MS/MS (Xu et al., 2017) have been used to measure AI-2 activity. Thiel et al. (2009) have proposed to identify and quantify AI-2 in biological samples. However, given the simplicity, sensitivity, and low cost of HPLC-FLD, this is an effective way to quantify the presence of AI-2 in bacterial samples, and this approach allows the effects of pH, nutrients, and medium on AI-2 analysis to be avoided (Song et al., 2014).

Metabolite Compositions of CFS

The metabolite profiles of the CFS from *Lb. sakei*, *Lb. plantarum*, and *Lb. acidophilus* KACC 12419 (as a reference) strains are showcased in Table 7. These *Lactobacillus* strains carry different types of metabolites. Within identified metabolites, different types and amounts of organic acids, amino acids, sugars, and others compound were abundance. A total of 9 organic acids were found, including lactic acid, acetic acid, boric acid, acrylic acid, succinic acid, glyceric acid, methyl succinic acid, aminomalonic acid, and citric acid. Among the identified organic acid metabolites, lactic acid and acetic acid were produced in higher amounts compared with other detected organic acid metabolites. The secretion of organic acids, mostly lactic and acetic acid, is one of the vital properties of LAB. However, acrylic acid, glyceric acid, methyl succinic acid, and aminomalonic acid concentrations were detected in very low amounts compare with other organic acids, whereas citric acid was not detected in CFS from *Lb. sakei*. On other side, 6 amino acids were detected, including L-alanine, glycine, L-valine, L-threonine, tyramine, and lysine. Among the detected amino acids metabolites, L-alanine and L-valine displayed the highest concentrations of the amino acid metabolites. In addition, glucose, sucrose, xylitol, and L-altrose were confirmed as sugar metabolites. Some other compounds, such as phosphoric acid, glycerol-3-phosphate, myo-inositol, stearic acid, and phosphine,

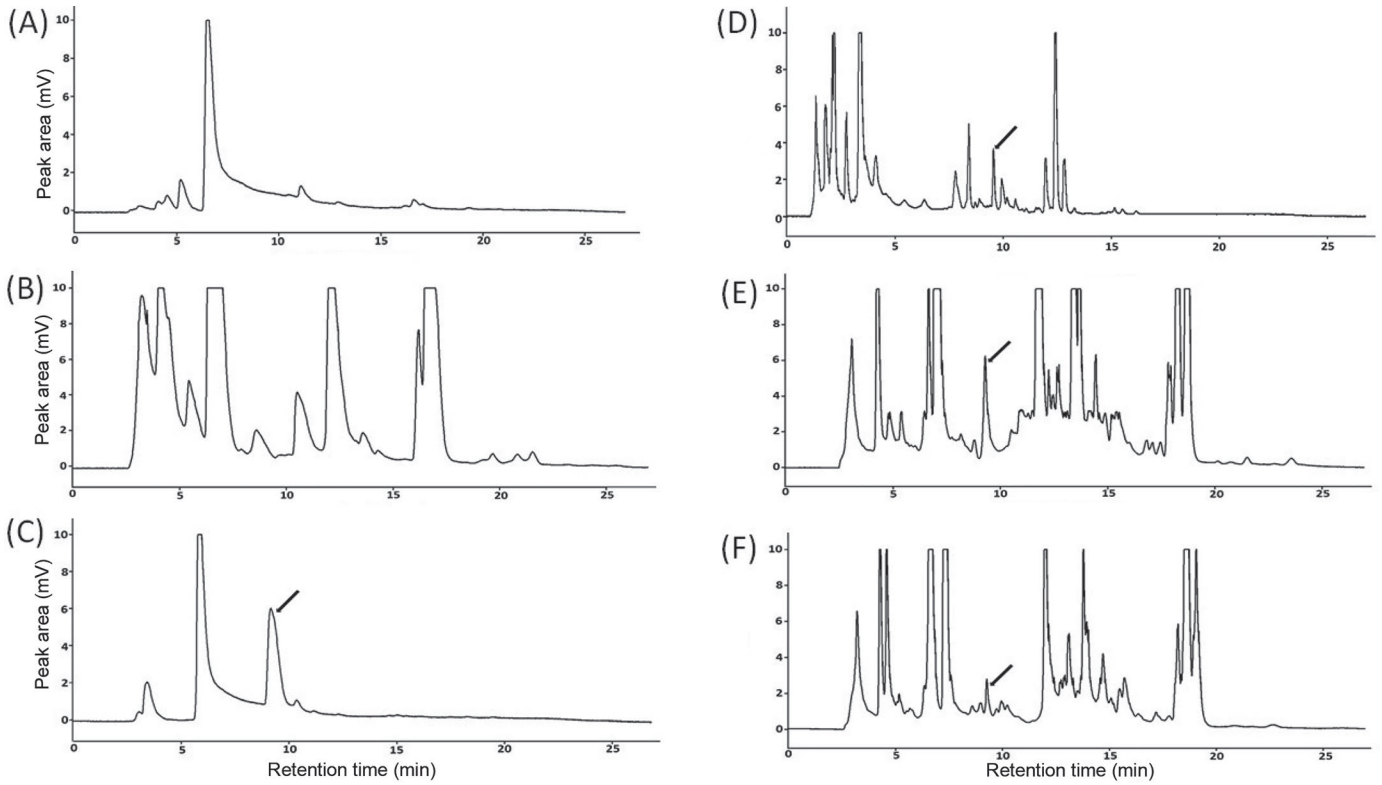

Figure 3. Typical chromatogram of autoinducer bioassay medium (A); de Man, Rogosa, and Sharpe medium (B); standard solution of 4,5-dihydroxy-2,3-pentanedione (DPD) (C); *Vibrio harveyi* BB120 supernatant (D). Typical chromatograms from lactic acid bacteria (LAB) supernatants showing the derivatization of autoinducer-2 (AI-2) with 2,3-diaminonaphthalene (DAN). Representative chromatograms from the LAB isolates that produced AI-2: (E) isolate J.27, (F) isolate B.67. Arrows indicate the products corresponding to a retention time of 9.32 min.

were also found in CFS. Within other compounds, phosphoric acid was produced in higher amounts compare with other detected metabolites. The amounts of organic acids, amino acids, sugars, and other detected metabolites fluctuated between tested and reference CFS. The discrepancies between individual components of bacterial CFS seem to be related to strain properties, including the bacterial type, the nature of the bacteria producing the CFS, and metabolite outlining methods such as GS-MS or HPLC (Moradi et al., 2019). Rodríguez et al. (1997) noted that the presence of hydrogen peroxide and oxygen metabolites within CFS could affect the antimicrobial action of LAB, so, in our study, antimicrobial capability linked with hydrogen peroxide is fully removed from the CFS, due to the use of the lyophilization process. According to Lim and Chua (2018), analysis method plays a vital role in the type and number of individual metabolites. The results of a study by Moradi et al. (2019) showed the presence of some organic acids, except for lactic acid, in the CFS of LAB, and identified a biosurfactant compound known as laurostearic acid, which was absent in our tested CFS. Some compounds previously confirmed in the CFS of LAB but not identified in our research include 3,4,5,8-tetrahydroxyhexahydro-2H-pyranol[2,3-*d*][1,2] dioxin-2-one (Kavitha et al., 2020), 2-hydroxy indole-3-propenamide (Jeevaratnam et al., 2015), 3-phenyl lactic acid, benzoic acid, octadecanoic acid, propionic acid, succinamic acid, cyclopentane, and pyrrolo[1,2-*a*] pyrazine-1,4-dione (Arasu et al., 2013; Sharma et al., 2014; Moradi et al., 2019), and 5-oxadodecanoic acid, 3-hydroxy decanoic acid, and 3-hydroxy-5-dodecenoic acid (Sjögren et al., 2003; Ryu et al., 2014).

Challenge Study in Whole Milk (Food) Model

Live *Lactobacillus* spp. cells have limitations for direct use in food because bacterial growth and persistence is influenced by exterior factors, such as food type, temperature, and pH (Moradi et al., 2019). In addition, bacteriocins, as known antimicrobial agents against LAB, usually have narrow-spectrum antibacterial action and are costly, which limits their application for prolonging the shelf life of food (Landete, 2017). A novel approach we investigated in our research is the preparation of CFS of *Lactobacillus* spp. The CFS of

Table 7. Metabolite profiles of the *Lactobacillus acidophilus* Korean Agricultural Culture Collection (KACC) 12419, *Lactobacillus sakei* D.7, and *Lactobacillus plantarum* I.60 (μg/mL)[1]

Metabolite	*Lb. acidophilus* KACC 12419	*Lb. sakei* D.7	*Lb. plantarum* I.60
Organic acid			
Lactic acid	9,930 ± 84	6,580 ± 50	8,460 ± 110
Acetic acid	8,750 ± 139	9,160 ± 100	8,680 ± 150
Boric acid	4,052 ± 16	236 ± 26	654 ± 8
Acrylic acid	21 ± 8	11 ± 2	13 ± 3
Succinic acid	210 ± 29	131 ± 31	185 ± 42
Glyceric acid	17 ± 3	6 ± 1	6 ± 0
Methyl succinic acid	25 ± 6	3 ± 0	29 ± 3
Aminomalonic acid	45 ± 4	8 ± 1	43 ± 1
Citric acid	1,744 ± 178	ND	2,730 ± 222
Amino acid			
L-Alanine	2,780 ± 123	2,437 ± 81	2,889 ± 299
Glycine	1,180 ± 27	823 ± 5	1,208 ± 35
L-Valine	2,786 ± 105	2,613 ± 79	3,174 ± 125
L-Threonine	980 ± 37	908 ± 64	1,237 ± 23
Tyramine	1,074 ± 87	971 ± 69	1,005 ± 135
Lysine	1,250 ± 174	1,402 ± 128	2,213 ± 140
Sugar			
Glucose	5,672 ± 70	9,160 ± 140	3,620 ± 50
Sucrose	460 ± 5	340 ± 10	180 ± 0
Xylitol	343 ± 45	363 ± 13	554 ± 74
L-Altrose	4,981 ± 150	6,918 ± 750	3,923 ± 233
Other compounds			
Phosphoric acid	3,363 ± 235	3,058 ± 185	3,430 ± 439
Glycerol-3-phosphate	153 ± 17	136 ± 11	143 ± 13
Myo-inositol	56 ± 6	61 ± 4	77 ± 0
Stearic acid	36 ± 8	71 ± 0	27 ± 3
Phosphine	85 ± 2	112 ± 7	101 ± 5

[1]Values are mean ± SD; ND = not detected.

probiotics comprises almost all postbiotics (i.e., antimicrobials and antioxidants), which subsequently acts as an alternative, replacing whole bacteria or their particular efficient metabolites (Moradi et al., 2019). The purpose of this research was to investigate the possibility of using CFS of LAB for bioprotection. To achieve this, we explored the antimicrobial activity of lyophilized CFS of *Lactobacillus* spp. against *L. monocytogenes* and its inhibitory effect against *L. monocytogenes* in a food model. The minimum end point concentration of an agent to inhibit the growth of a pathogen in a food commodity is known as the MEC of that agent. The MEC of *Lb. sakei* and *Lb. plantarum* freeze-dried CFS on *L. monocytogenes* in milk and TSB broth challenge were determined (Table 8). In this experiment, *Lb. plantarum* CFS showed strong antibacterial activity (40 mg/mL) in the food model (whole milk) compared with *Lb. sakei* CFS, which showed weak activity (55 mg/mL). The MEC indices were higher in the food model than in culture media. Previous studies have also noted that the antimicrobial activity of *Lactobacillus* CFS is lower in food models than in culture broth media (Gänzle et al., 1999) because of some factors, including solubility, complexity of commodity, adsorption of CFS to food matrix, and interactions of CFS components with food ingredients (Gálvez et al., 2007; Moradi et al., 2019). Hartmann et al. (2011) noted a lower MEC index in culture broth compared with milk for *Lactobacillus* spp. CFS against *L. monocytogenes*. Moreover, the anti-listerial activity of some *Lactobacillus* spp. CFS in milk were investigated and showed a higher MEC range (25–35 mg/mL) compared with culture broth (15–25 mg/mL; Moradi et al., 2019). Koo et al. (2015) reported the efficacy of CFS from *Leuconostoc* against *E. coli* in ground beef and noted their antibacterial activity linked with organic acid production. Additionally, Hamad et al. (2017) concluded that antimicrobial activity of CFS from different LAB could be linked with organic acid composition. According to metabolite profiles analysis, the anti-listerial capability of 2 *Lactobacillus* spp. CFS in milk and broth challenge

Table 8. Minimum effective concentration (mg/mL) of *Lactobacillus sakei* D.7 and *Lactobacillus plantarum* I.60 freeze-dried cell-free supernatant (CFS) against *Listeria monocytogenes* in whole milk and culture medium (tryptic soy broth, TSB) at 4°C over 6 d

CFS	Whole milk	TSB
Lb. sakei D.7	55	30
Lb. plantarum I.60	40	20

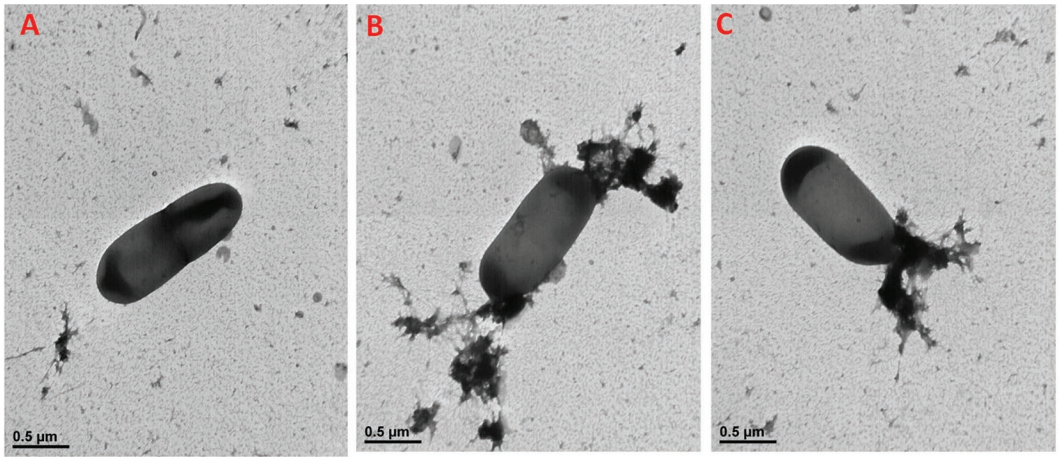

Figure 4. Transmission electron micrographs of untreated *Listeria monocytogenes* cells (A) and cells treated with cell-free supernatants from *Lactobacillus* spp. (B and C).

is due to production of some organic acids and possibly a few unknown bacteriocin-like elements.

TEM Analysis

Morphological changes of *L. monocytogenes* by effect of *Lactobacillus* CFS were investigated via TEM, as shown in Figure 4. Without CFS treatment, the bacterial cells appeared intact, with uniform distribution of cytoplasm, distinct integrity, and smooth surfaces (Figure 4A). Following treatment with CFS (pH 3.5), pore formation and cytoplasmic leakage, plasmolysis, loss of cytoplasmic constituents, and distortion from original structure and shape were observed (Figure 4B and C). Based on morphological changes, TEM results indicate the rupture of the cell membrane, as well as cell membrane permeability. We consider that the CFS facilitates lysing of the cells by working on the bacterial cell membrane. Lactic acid bacteria generate antimicrobial substances, such as bacteriocins and bioactive peptides, which could be effective at damaging bacterial DNA (Khan and Kang, 2016). The antimicrobial action of lactic acid is mostly responsible for the antimicrobial activity of LAB. Organic acids exert an antibacterial effect as a result of pH reduction and production of undissociated molecular structures. Ammor et al. (2006) suggested that the low outer pH triggers acidification of the cell cytoplasm, whereas the undissociated organic acid, being lipophilic, can diffuse passively across the membrane. The undissociated organic acid acts by inhibiting the electrochemical proton gradient or by changing the cell membrane permeability, which results in the interruption of substrate transport systems. Moreover, alterations of the bacterial cell membrane structure may increase potassium ion release and interfere with cell metabolism, thereby inducing cell death (Cox et al., 2001). In earlier research, the inhibitory effects of LAB CFS have been shown to be involved in potassium ion efflux and rupture of the cell membrane (Bajpai et al., 2016).

CONCLUSIONS

Lactic acid bacteria isolates exhibited several probiotic properties, including resistance to the circumstances encountered in the GIT of humans, absence of virulence genes, and hemolytic activity, as well as susceptibility to some antibiotics. Nevertheless, further research is required to confirm their beneficial health effects and applications. Rapid genetic fingerprint typing via REP-PCR was used to classify the LAB isolates. Moreover, AI-2 has not previously been detected in LAB using HPLC-FLD through its derivatization with DAN. The latter is a sensitive, simple, and suitable method for analysis of AI-2 in supernatants from several bacterial strains. Different types of antimicrobial and antioxidant metabolites were detected in the CFS of *Lactobacillus* spp. According to in vitro testing, TEM analysis, and food challenge experiment, our findings indicate that the CFS of *Lb. sakei* and *Lb. plantarum* isolates have potential as effective biopreservatives to control the *L. monocytogenes* pathogens in milk, as well as in the dairy industry. Overall, we highlight the use of LAB as probiotic strains and CFS for their anti-listerial activity in food applications.

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