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Characterization of high exopolysaccharide-producing *Lactobacillus* strains isolated from mustard pickles for potential probiotic applications

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Summary. The aim of this study was to characterize high exopolysaccharide (EPS)-producing lactic acid bacteria (LAB) isolated from mustard pickles in Taiwan for potential probiotic applications. Among 39 collected LAB strains, four most productive EPS-producing strains were selected for further analysis. Comparative analyses of 16S rDNA genes *rpoA* and *pheS* sequences demonstrated that these strains were members of *Lactobacillus plantarum*-group (LPG). NCD 2, NLD 4, SLC 13, and NLD 16 showed survival rates of 95.83% ± 0.49%, 95.07% ± 0.64%, 105.84% ± 0.82%, and 99.65% ± 0.31% under simulated gastrointestinal conditions, respectively. No cytotoxic effects on macrophage RAW 264.7 cells were observed when they were treated with a low dose (1 μg/ml) of stimulants extracted from the tested LAB strains. The production of nitric oxide in RAW 264.7 cells incubated with various LAB stimulants showed a dose-dependent increase. Among the four strains, SLC 13 showed higher inhibitory activity on growth of *Enterococcus faecalis* (BCRC 12302) and *Yersinia enterocolitica* (BCRC 10807). NLD 4 showed strong inhibitory activity against *Escherichia coli* O157:H7 (ATCC 43894) as compared with the other three strains. In summary, our results suggest that *Lactobacillus pentosus* SLC 13 may be a good candidate for probiotic applications and for development of antibacterial compounds. [**Int Microbiol** 20(2):75-84 (2017)]

Keywords: *Lactobacillus* spp. · exopolysaccharide · probiotics

Introduction

The market of dietary supplements that promote health has increased since 1990s. Among them, probiotics are defined as live microorganisms that could confer a health benefit on the host when adequate amounts are administered [18]. Currently, the genera *Lactobacillus* (a member of the lactic acid bacteria (LAB) group) and *Bifidobacterium* are the most common probiotics used for human nutrition. LAB generally have many useful properties, including tolerance to gastric acid and bile salts, tolerance to antimicrobial agents, improvement of immune responses, gastrointestinal adsorption, and stability during processing [30]. Moreover, fermentation by the LAB group is a natural bioprocessing technology for production of foods such as milk, vegetables, yogurt, cheese, meat, and cocoa beans. LAB-mediated fermentation thus improves nutrition and preserves qualities of food and beverage products for long periods [7].

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The beneficial effects of LAB and its derivative exopolysaccharide (EPS), include the prevention and treatment of diarrheal disease, prevention of infections, antitumor activity, immunomodulation, prevention and treatment of allergies, and alleviation of lactose intolerance [10,22,29,30,31,37]. In addition, the EPS produced by LAB is an important source of natural alternatives to various chemical compounds and plays a critical role in the production of fermented dairy products in Asia, Eastern Europe, and Northern Europe. However, the characteristics and production of EPS by LAB show a great variety, which depends on the type of LAB strains, culture conditions, and composition of the medium [14].

Mustard pickles, a vegetable processing product that is fermented by LAB and yeast, is an important dietary dish in Taiwan. Traditional processing of mustard pickles is as follows: whole mustard vegetables (*Brassica juncea*) are packed in layers with addition of solar salt to each vegetable layer. Followed by placement of a large rock on top of the container, the mustard inside the container is slowly pressed and fermented. In this study, we aimed to isolate and characterize LAB strains with potential probiotic applications from mustard pickles. To our knowledge, this is the first report of characterization of EPS-overproducing LAB strains from mustard pickles in Taiwan.

Materials and methods

Sampling and isolation of lactic acid bacteria. Fifteen mustard pickle samples for LAB isolation were collected in traditional markets (Beipu Township, Hsinchu County, 4 samples and Meinong District, Kaohsiung City, 3 samples) and a mustard pickle production center (Dapi Township, Yunlin County, 8 samples) in Taiwan. The samples were transported to the laboratory at room temperature for isolating LAB in batches within 24 h of acquisition.

MRS (de Man, Rogosa, and Sharpe) agar plates were used for LAB isolation. Each mustard pickle sample was crushed and mixed with phosphate-buffered saline (PBS) buffer. A serial dilution of the suspension was spread onto the surface of MRS agar plates. Samples were then incubated under anaerobic conditions at 37 °C for 48 h. Six colonies were randomly selected from each MRS agar plates (total 90 colonies) and initially tested for acid production, catalase activity, bacterial motility, Gram staining, and cell morphology. Among 90 colonies, a total of 39 LAB strains were isolated from mustard pickles. The selected strains were stored at –80 °C in MRS broth containing 16% glycerol until testing.

Exopolysaccharide production analysis. The EPS production of isolated LAB strains was evaluated according to a previous study with a modification [17]. In brief, the liquid fermented by LAB was centrifuged at 6000 ×*g* for 15 min after incubation for 48 h, and the resulting supernatant was collected carefully. An aliquot of the supernatant (1 ml) was mixed with 4 ml of 95% ethanol, and then incubated at 4 °C for 24 h. The precipitated EPS was centrifuged at 9000 ×*g* for 15 min, and the supernatant was discarded. The precipitate of pure EPS was dried in the oven at 60 °C for 24 h. The production of EPS was analyzed by the phenol-sulfuric method using glucose as a reference standard [35]. Among collected isolates, EPS yields were ranging from 0 to 0.43 ± 0.04 g/l. Four high EPS-producing LAB strains were further identified by means of the API 50 CH (BioMérieux, Marcy l'Etoile, France) strips and gene sequences (16S rDNA, *rpoA*, and *pheS* genes) to the species level [4].

DNA techniques. Mini Qiagen columns and a QiaAmp DNA extraction kit (Qiagen, Valencia, CA) were used for chromosomal DNA extraction. PCR was carried out according to the manufacturer's instruction using Taq polymerase (Promega, Madison, WI, USA).

DNA sequencing and phylogenetic analysis. The sequence of 16S rDNA gene, the housekeeping genes *rpoA* (the gene encoding the DNA-dependent RNA polymerase alpha-subunit), and *pheS* (the gene encoding the phenylalanyl-tRNA synthase alpha-subunit) were used for phylogenetic analysis and species identification. Primers and conditions for PCR amplification of the 16S rDNA, *rpoA*, and *pheS* genes were described previously [4]. To draw a phylogenetic tree of the 16S rDNA, *rpoA*, and *pheS* genes, sequences were first aligned using the CLC Workbench (CLC sequence viewer 7.0, CLC Bio/Qiagen, Aarhus, Denmark), and the aligned file was then subjected to the neighbour-joining method to draw a phylogenetic tree with bootstrap analysis of 1000 replicates.

Random amplified polymorphic DNA (RAPD)–PCR amplification. Three different primers (primers B, 5′-AACGCGCAAC-3′; primer E, 5′-GGCGTCGGTT-3′; and primer F, 5′-GGCCACGGAA-3′) for RAPD–PCR analysis used in this study were described previously [5]. In brief, the PCR mixtures were made in a volume of 50 µl containing 100 ng of DNA, 10 pmol of primer, 0.15 mM each deoxynucleoside triphosphate, reaction buffer with $MgCl_2$, and 1 U of Taq DNA polymerase. The cycling program consisted of 1 cycle of 94 °C for 5 min; 8 cycles of 94 °C for 30 s, 36 °C for 1 min, and 72 °C for 90 s; 35 cycles of 94 °C for 20 s, 36 °C for 30 s, and 72 °C for 90 s; and a final cycle of 72 °C for 3 min. The PCR products were electrophoretically separated in 1% agarose gels. The RAPD profiles were used to discriminate between the different isolates.

Tolerance of simulated gastrointestinal conditions. To evaluate the survival of LAB strains in an environment that mimics *in vivo* human upper-gastrointestinal tract conditions, an in vitro methodology was used. For acid tolerance analysis, 0.1 ml of a LAB culture medium from an overnight culture was added to 9.9 ml of PBS (pH = 3). The mixture was incubated at 37 °C with agitation (80 rpm) for 3 h. One milliliter of the culture medium was mixed with 9 ml of PBS, followed by serial dilution and spreading on MRS agar plates. The number of surviving bacteria was measured by the plate counts after 48-h incubation under anaerobic conditions. The survival rate of LAB strains under acidic conditions was calculated according to the following equation:

Survival rate (%) = $[A_1(\text{Log CFU/ml})/A_0(\text{Log CFU/ml})] \times 100$, where A_1

is the viable count of LAB after 3 h at pH 3.0, and A_0 is the viable count of LAB at 0 h.

For analysis of bile salt tolerance, after acid treatment for 3 h, the surviving LAB were collected by centrifugation (7000 ×*g*, 5 min) and washed two times with PBS (pH 7.2). The bacteria were resuspended in 10 ml of MRS broth with or without 0.3% (w/v) bile salt, and then incubated at 37 °C for 24 h under anaerobic conditions. One milliliter of the culture medium was mixed with 9 ml of PBS, followed by serial dilution and spreading on MRS agar plates. The number of surviving bacteria was determined by the plate counts after 48-h incubation under anaerobic conditions. The survival rate of LAB strains in the culture medium containing 0.3% of bile salt was calculated according to the following formula:

Survival rate (%) = [B_1(Log CFU/ml)/B_0(Log CFU/ml)] × 100, where B_1 is the viable count of LAB in 0.3% bile salt for 24 h, and B_0 is the viable count of LAB cultured without 0.3% bile salt for 24 h.

In addition, the survival rate of the analyzed LAB strains under simulated gastrointestinal conditions (pretreatment with acid conditions before bile salt treatment) was calculated according to the following formula:
Survival rate (%) = [C_1(Log CFU/ml)/C_0(Log CFU/ml)] × 100. where C_1 is the viable count of LAB under simulated gastrointestinal conditions, and C_0 is the viable count of LAB at 0 h.

Antibiotic sensitivity testing. Sensitivity to 15 selected antimicrobial agents was determined by the disk diffusion method on MRS agar plates as described elsewhere [39]. BD BBL Sensi-Discs (Becton Dickinson, Sparks, MD, USA) were used. We tested the antimicrobial agents ampicillin, cefalotin, chloramphenicol, cloxacillin, erythromycin, gentamicin, kanamycin, novobiocin, penicillin G, polymyxin B, rifampicin, tetracycline, neomycin, streptomycin, and vancomycin. All measurements were performed in triplicate. The interpretation of resistance to these antimicrobial agents was in accordance with the recommendations published elsewhere [1,13,38].

Cell culture. RAW 264.7 (mouse macrophage cell line, BCRC 60001) and HT-29 (Human colon colorectal adenocarcinoma cell line, ATCC HTB-38) were purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan) and maintained in the DMEM complete medium and McCoy's 5A (modified) medium, respectively, supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), and 10% of fetal bovine serum at 37 °C with 5% of CO_2 supplemented in a humidified incubator.

Bacterial adhesion assay. Bacterial adhesion assay was performed according to a previousd study with a modification [34]. HT-29 cells (1×10^5/well) were grown overnight in 12-well culture dishes to approximately 80% confluence. Bacteria were added to the wells at a multiplicity of infection (MOI) of 100 without centrifugation and were incubated for 1 h. Each dish with HT-29 *Lactobacillus* co-culture was washed twice with prewarmed PBS buffer to remove unbound bacteria. Adhered *Lactobacillus* cells were quantified by lysing the cells for 2 min with 1% Triton X-100-containing PBS buffer, followed by serial dilution and spreading on MRS agar plates. The number of adhered bacteria was measured by the plate counts after 3 days incubation. The percentage of bacterial adhesion was calculated according to the following formula:

Adhesion ability (%) = (adhered bacteria)/(total bacteria) × 100

Cell viability testing and nitric oxide (NO) production. For the treatment of RAW 264.7 cells, LAB stimulants were prepared from an overnight LAB culture. The culture medium was separated by centrifugation, and the pellet was washed with deionized water three times. The LAB stimulants from the pellet were further sterilized, freeze-dried, mixed with a small amount of deionized water, and sterilized again. Cell viability was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 cell cultures grown overnight at the density of 10^5 cells/ml were incubated with various concentrations of LAB stimulants (1, 10, 50, 100 µg/ml) or lipopolysaccharide (LPS, 0.5 µg/ml) in 100 µl of the DMEM medium for 24 h. After removal of the supernatant from the plate, the cells were incubated at 37 °C with 30 µl of MTT (5 mg/l) for 3 h. The medium was aspirated, and the crystals were dissolved in 200 µL of dimethylsulfoxide (DMSO). Absorbance of each well at 570 nm was measured using a microplate reader. Cell viability was calculated using the following equation:

Cell viability (%) = ($A_{sample}/A_{control}$) × 100, where A_{sample} is the absorbance of the cells that were incubated with the DMEM medium containing various concentrations of LAB stimulants or LPS (0.5 µg/ml), and $A_{control}$ is the absorbance of the cells alone.

The NO production assay was performed according to a previous study with a modification [16]. RAW 264.7 cells incubated overnight were subcultured in 96-well plates at the density of 10^5/ml in 100 µl of the DMEM medium containing various concentrations of LAB stimulants (1, 10, 50, 100 µg/ml) or LPS (0.5 µg/ml) for 24 h. The culture supernatants were collected, and the NO production was measured by means of the Griess reagent. In brief, the supernatants were mixed with an equal volume of the Griess reagent (1:1 mixture of 0.1% N-1-naphthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid), and the samples were inoculated at room temperature with incubation for 15 min. The absorbance of each sample at 540 nm was measured using a microplate reader. NO production was calculated on the basis of a standard curve prepared using sodium nitrite.

The antibacterial-activity assay. The agar well diffusion method as described previously was applied to detect and determine the antibacterial activities of the isolated LAB strains [36]. *Staphylococcus aureus* (BCRC 10908), *Enterococcus faecalis* (BCRC 12302), *Streptococcus mutans* (BCRC 15254), and *Listeria monocytogenes* Scott A [3] were used as Gram-positive indicator strains, and *Yersinia enterocolitica* (BCRC 10807), *Escherichia coli* (BCRC 11634), *Escherichia coli* O157:H7 (ATCC 43894), and *Salmonella enterica* sv. Typhimurium (BCRC 10747) served as Gram-negative indicator strains. All indicator strains were incubated in Luria-Bertani (LB) broth overnight and then diluted to 10^7 CFU/ml with LB broth. The LB agar plates were prepared with five wells of 6 mm in diameter. A hundred microliters of the indicator strains in LB broth was spread on the surface of an LB agar plate, followed by placing 50 µl of an overnight LAB culture medium into the wells. The LB agar plates were incubated at 37 °C for 24 h. The antibacterial activities of LAB strains were assessed by the diameter of the inhibition zones. The interpretation of antibacterial activities of these LAB strains was as follows: < 10 mm (–) no antibacterial activity; 10–15 mm (+) weak antibacterial activity; > 15 mm (++) strong antibacterial activity. The antibacterial activity of each strain were evaluated in three independent experiments.

Statistical analysis. The data were expressed as mean of three replicates ± SD. Duncan's multiple-range test was used to identify the significant differences ($P < 0.05$) between means.

Results

Isolation and characterization of LAB strains from mustard pickles. A total of 39 LAB strains were isolated from mustard pickles. Among them, 9, 23, and 7 strains were isolated from Beipu Township, Dapi Township, and Meinong District, respectively. All isolates that showed characteristics of acid production and were catalase-negative, immotile, Gram-positive, and rod-shaped were assumed to be LAB. Among them, four high ESP-producing LAB strains were selected for further analyses (Table 1). The EPS production of strains NCD 2, NLD 4, and NLD 16 isolated from mustard pickle samples from the Beipu Township was 0.34 ± 0.04, 0.32 ± 0.02, and 0.35 ± 0.01 g/l, respectively (Table 1). The strain SLC 13 isolated from mustard pickles from the Meinong District produced the largest amount of EPS (0.43 ± 0.04 g/l) among the 39 collected LAB isolates (Table 1). LAB strains isolated from mustard pickles of the Dapi Township showed little or no EPS production.

Identification of LAB from 16S rDNA, *rpoA*, and *pheS* sequences. Four isolated strains were further identified using the API 50 CHL species identification system, and the results showed that strains NCD 2 and SLC 13 belonged to *Lactobacillus plantarum*, whereas strains NLD 4 and NLD 16 were *Lactobacillus pentosus*. Boyd et al. showed that the use of the current API 50 CH database for identification of

Table 1. Characteristics, EPS production, acid and bile salt tolerance, and antibiotic sensitivity of the isolated LAB strains

	LAB strain[a]			
	NCD 2	NLD 4	SLC 13	NLD 16
Sources in Taiwan	Beipu Township, Hsin Chu	Beipu Township, Hsin Chu	Meinong District, Kaohsiung	Beipu Township, Hsin Chu
16S rDNA, *rpoA*, and *pheS* genes identification	*Lactobacillus plantarum*	*Lactobacillus pentosus*	*Lactobacillus pentosus*	*Lactobacillus pentosus*
EPS (g/l)[b]	0.34 ± 0.04[b]	0.32 ± 0.02[b]	0.43 ± 0.04[a]	0.35 ± 0.01[b]
Survival, pH 3.0 (% ± SD)[b]	73.73 ± 0.32[c]	88.32 ± 1.03[b]	99.37 ± 0.45[a]	87.36 ± 1.06[b]
Survival, 0.3% bile salt (% ± SD)[b]	97.57 ± 0.74[a]	97.40 ± 1.23[a]	98.94 ± 0.31[a]	97.97 ± 0.62[a]
Survival, pH 3.0 + 0.3% bile salt (% ± SD)[b]	95.83 ± 0.49[c]	95.07 ± 0.64[c]	105.84 ± 0.82[a]	99.65 ± 0.31[b]
Antimicrobial susceptibility[c]				
Penicillin G	R	I	R	I
Ampicillin	S	S	S	S
Cephalothin	R	S	I	S
Cloxacillin	R	S	R	S
Erythromycin	S	S	S	S
Novobiocin	R	S	R	I
Vancomycin	R	R	R	R
Polymyxin B	R	R	R	R
Chloramphenicol	S	S	S	S
Rifampicin	R	S	I	S
Tetracycline	I	S	R	S
Kanamycin	R	R	R	R
Gentamicin	R	R	R	S
Neomycin	R	R	R	I
Streptomycin	R	R	R	R

[a] Four isolated LAB strains showed characteristics of acid production, catalase negativity, a rod shape, immotility, and Gram-positive.
[b] Data are the mean of three replicates ± SD. Different letters indicate statistically significant differences at $P < 0.05$.
[c] S, sensitive; I, intermediate resistance; R, resistant.

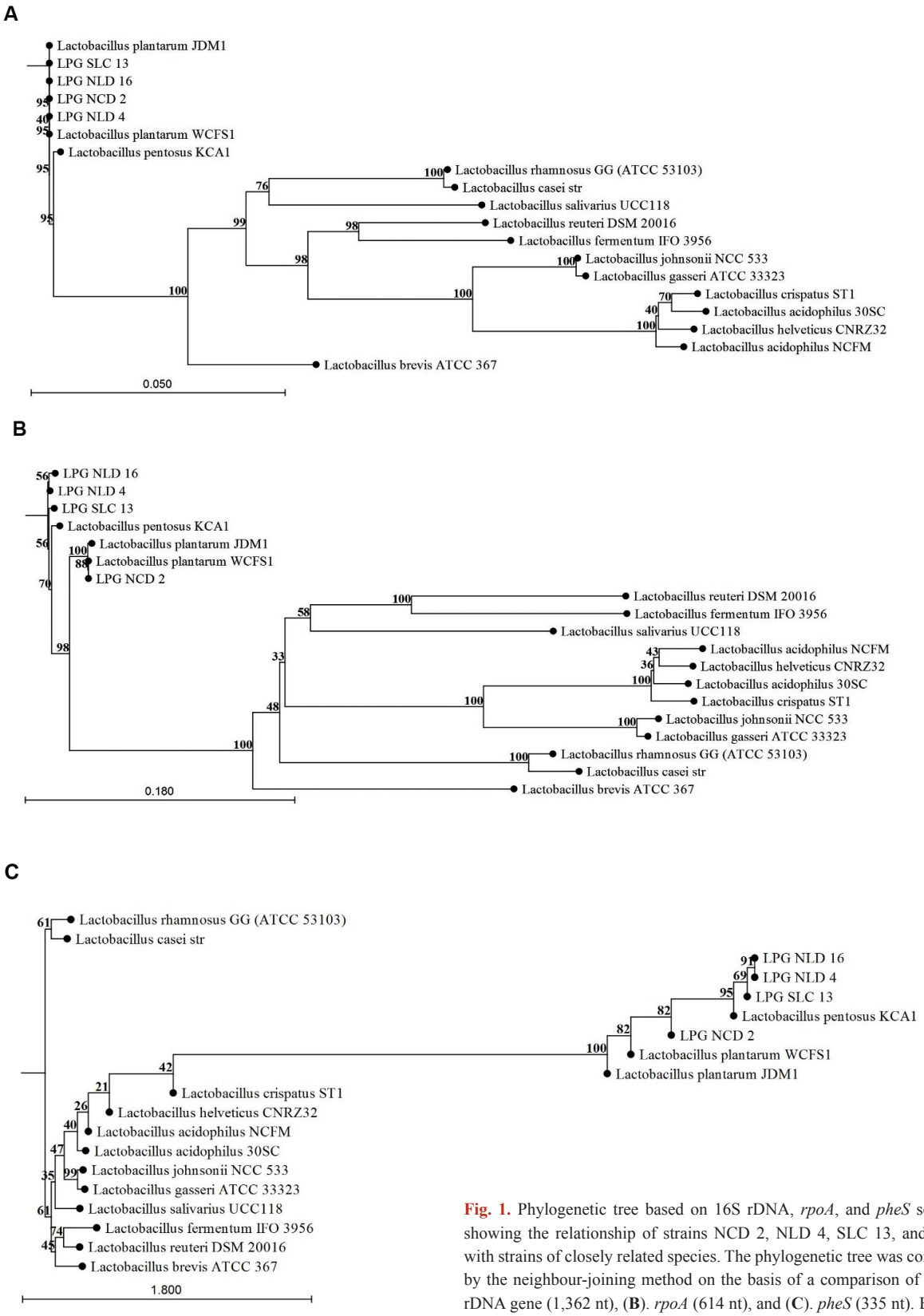

Fig. 1. Phylogenetic tree based on 16S rDNA, *rpoA*, and *pheS* sequences showing the relationship of strains NCD 2, NLD 4, SLC 13, and NLD16 with strains of closely related species. The phylogenetic tree was constructed by the neighbour-joining method on the basis of a comparison of (**A**). 16S rDNA gene (1,362 nt), (**B**). *rpoA* (614 nt), and (**C**). *pheS* (335 nt). Bootstrap values (%) based on 1000 replications are given at nodes. Bar, % sequence divergence.

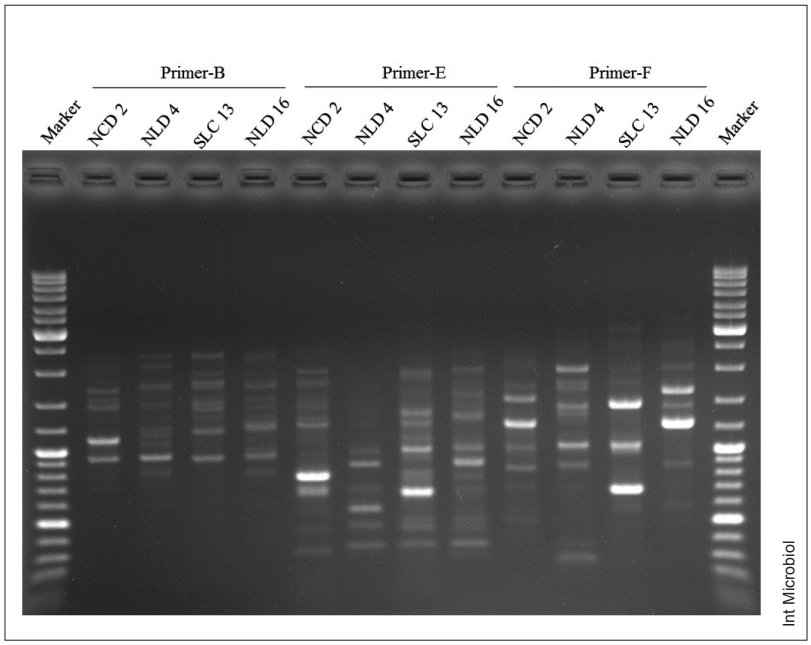

Fig. 2. Genetic relatedness among the four LPG isolates by using RAPD-PCR analysis. Three primers with random sequences (B, E and F) were used. Marker, Thermo Scientific GeneRuler 1 Kb DNA ladder.

commensal *Lactobacillus* species led to misidentification or uninterpretable results [2]. Therefore, 16S rDNA gene sequence of four isolates was used for species identification. The results showed that identical 16S rDNA gene sequence was observed between four isolated strains, *L. plantarum* JDM1 and *L. plantarum* WCFS1 (Fig. 1A). Currently, five different subspecies of *L. plantarum*-group (LPG) have been identified and are found to be closely related, which includes *L. plantarum* (subsp. *plantarum* and subsp. *argentoratensis*), *Lactobacillus pentosus*, *Lactobacillus paraplantarum*, *Lactobacillus xiangfangensis*, and *Lactobacillus fabifermentans* [6]. Therefore, the sequences of *rpoA* and *pheS* genes of 4 isolated LPG strains were analyzed for subspecies identification. The results showed that high *rpoA* and *pheS* gene sequence similarities were observed between strains NLD4, SLC 13, NLD 16, and *L. pentosus* KCA1 (Fig. 1B and 1C). For discrimination of the isolates, RAPD fingerprinting was performed, and the results showed that four examined strains had different RAPD profiles (Fig. 2).

Survival under simulated gastrointestinal conditions. To test the survival of the selected LPG strains under simulated gastrointestinal conditions, we used *in vitro* analysis which mimics the gastrointestinal conditions in the body according to other studies [9,32]. PBS at pH 3.0 was used to simulate gastric juice and the 0.3% bile salt was used to simulate intestinal conditions. All four selected strains were resistant to bile salts, but only SLC 13 showed high resistance to pH 3.0 (Table 1). The results of survival testing under simulated gastrointestinal conditions were as follows: strains NCD 2, NLD 4, SLC 13, and NLD 16 showed survival rates of 95.83% ± 0.49%, 95.07% ± 0.64%, 105.84% ± 0.82%, and 99.65% ± 0.31%, respectively (Table 1).

Antibiotic susceptibility of the LPG strains. To examine the antibiotic sensitivity of the four LPG strains, we selected 15 antimicrobial agents for testing. All strains were resistant to penicillin G, vancomycin, polymyxin B, kanamycin, neomycin, and streptomycin but sensitive to ampicillin, erythromycin, and chloramphenicol (Table 1). The SLC 13 strain showed resistance to 12 selected antimicrobial agents but not to ampicillin, erythromycin, and chloramphenicol (Table 1).

Adhesion ability of isolated LPG strains. The adhesion rates to HT-29 cells varied depending on the tested strains and ranged from 0.04 to 1.69% (Fig. 3). Among four examined strains, NLD 16 showed significantly better binding rates. The adhesion rates of strain NLD 4 were significantly lower than those of other strains (Fig. 3).

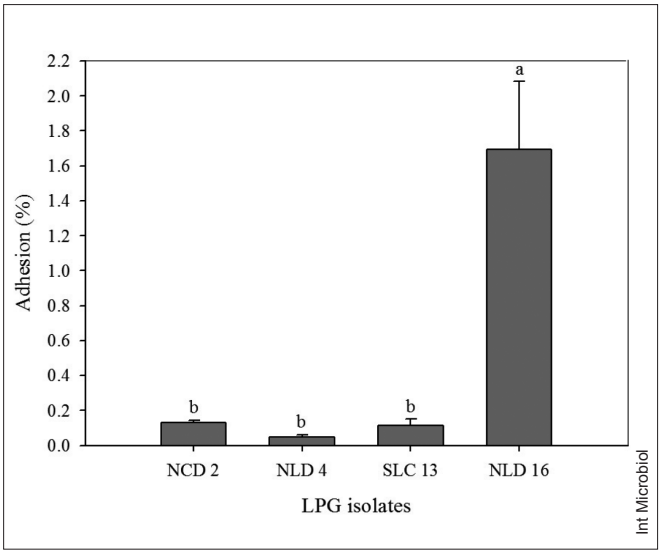

Fig. 3. Adhesion ability of four LPG isolates. The adhesion of LPG to HT-29 cells with MOI 1 : 100 in four examined strains. Values are the average of at least three independent biological replicates. Each vertical bar represents mean ± SD (n ≥ 3). Different letters indicate statistically significant differences ($P < 0.05$).

Effects of LPG stimulants on cell viability and NO production. On the basis of the observations that LPG stimulants have the ability to activate macrophages, we determined the potential effects of LPG stimulants on the cell viability and NO production of RAW 264.7 cells. Clearly, treatment with LPS or a high dose of the stimulants significantly inhibited proliferation of these cells. In addition, stimulants from SLC 13 showed a stronger inhibitory effect (Fig. 4A). Nevertheless, no cytotoxic effects on macrophages were observed when the cells were treated with a low dose (1 μg/ml) of the stimulants (Fig. 4A).

RAW 264.7 cells were further incubated with different doses of LPG stimulants, and the amount of NO in the culture supernatant was determined. RAW 264.7 cells in DMEM alone without either LPS treatment or bacterial stimulants served as a negative control. The results showed that when RAW 264.7 cells were incubated with various bacterial stimulants, the production of NO increased in a dose-dependent manner (Fig. 4B). The stimulants from strain SLC 13 appeared to have a stronger effect on NO production than did the stimulants from the other three strains (Fig. 4B).

Antibacterial activity of the LPG strains against bacterial pathogens. Eight indicator pathogenic strains (four Gram-positive strains and four Gram-negative strains) were selected to evaluate the antibacterial activity of the four LAB strains. The results revealed that strains NCD 2, NLD 4, and SLC 13 had higher antibacterial activities (Table 2). Among them, SLC 13 showed higher inhibitory activity against the growth of *E. faecalis* and *Y. enterocolitica*. NLD 4 strain showed strong inhibitory activity against *E. coli* O157:H7 compared with the other three strains (Table 2).

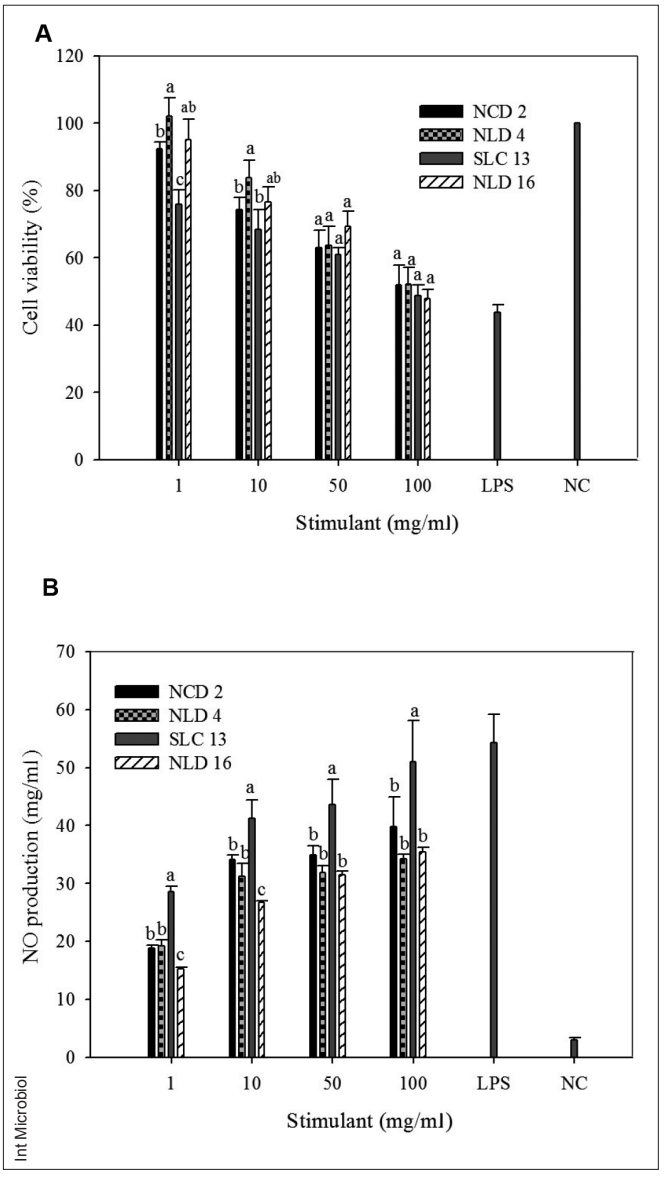

Fig. 4. Cell viability and NO production of RAW 264.7 cells treated with various concentrations of LPG stimulants for 24 h. (A). Cell viability. (B). NO production. Each vertical bar represents mean ± SD (n = 3). Cells treated with 0.5 μg/ml LPS served as a positive control. NC, negative control (DMEM medium only). Different letters indicate statistically significant differences ($P < 0.05$).

Table 2. Antibacterial activity of the four isolated LPG against eight indicator pathogenic strains

Pathogenic bacteria	Inhibition level[a]			
	NCD 2	NLD 4	SLC 13	NLD 16
Gram-positive				
Staphylococcus aureus (BCRC 10908)	++	+	++	++
Enterococcus faecalis (BCRC 12302)	–	+	++	–
Streptococcus mutans (BCRC 15254)	+	+	+	–
Listeria monocytogenes Scott A [3]	+	+	+	+
Gram-negative				
Yersinia enterocolitica (BCRC 10807)	+	+	++	+
Escherichia coli (BCRC 11634)	+	+	+	–
Escherichia coli O157:H7 (ATCC 43894)	+	++	+	+
Salmonella enterica sv. Typhimurium (BCRC 10747)	+	+	+	+

[a]Inhibition level was determined by the diameter of the inhibition zone: (–) < 10 mm; (+) 10–15 mm; (++) > 15 mm

Discussion

Previous studies showed that the EPS production of LAB strains was highly variable and could be influenced by the genotype and environmental conditions, such as pH, temperature, incubation time, and the medium [11,21,35]. In this study, we isolated four high EPS-producing LPG strains (Table 1). The factors that affect EPS production of selected LPG strains would be worth investigating for probiotic applications in the future.

Jiang et al. showed that EPS production was reduced in the variant strains compared with wild-type *B. longum* BBMN68, causing a reduction in the acid resistance of the variant [15]. The EPS production also contributes to bile tolerance in *L. brevis* strains [33]. In the present study, we isolated four high EPS-producing LPG strains showing high tolerance to simulated gastrointestinal conditions (Table 1). These results suggest that higher EPS production of selected LPG strains may protect bacteria from a harsh environment, and these strains may be used for probiotic development in the future. Mongkolrob et al. showed a possible correlation between antibiotic resistance and biofilm formation in *Burkholderia pseudomallei*, in which the EPS was supposed to be the main component in creation of the diffusion barrier for the antibiotics [27]. As a result, whether the EPS production is associated with the antibiotic resistance in our four LPG strains remains to be verified. In addition, the composition of EPS of our selected strains is still unclear and thus worth investigating.

Here, we showed the adhesion rates to HT-29 cells varied depending on the tested strains and ranged from 0.04 to 1.69% (Fig. 3). A previous study showed that *Lactobacillus* strains isolated from human fecal samples exhibited higher HT-29 cell adhesion ability compared to the strain isolated from cheese [8]. Martin et al. showed that *Lactobacillus* strains isolated from human intestinal origin presented higher affinity to HT-29 cells [24]. These results suggest that *Lactobacillus* strains isolated from human source have strong adhesin-receptor interaction for adapting to human cells.

In the presence of a pathogen, macrophages can engulf microorganisms, produce NO to kill the pathogens, present antigens to helper T cells, and further secrete proinflammatory cytokines such as IL-6 and TNF-α to activate the immune response. In the present work, SLC 13 stimulants induced significant NO production in RAW 264.7 cells. These findings suggest that the possible mechanism of NO upregulation in macrophages treated with SLC 13 stimulants may be mediated by the L-arginine pathway [8]. Nonetheless, the cellular component(s) of SLC 13 that activates RAW 264.7 cells has

yet to be identified. At the physiological level, NO plays a protective and critical role in human defense systems, whereas overproduction of NO during inflammation may lead to cytotoxicity [28]. As a result, the use of the selected LPG strains that can induce macrophages producing appropriate levels of NO may potentially enhance an immune response of the host.

Here, we showed that strains NCD 2, NLD 4, and SLC 13 had higher antibacterial activities (Table 2). *L. plantarum* has been demonstrated to produce bacteriocins, such as plantaricin NC8, plantaricin 35d, plantaricin W, plantaricin A, plantaricin C, and plantacin B, and thus to prevent the growth of some pathogenic bacteria [12,23,25,26]. Currently, whole genome sequences of *L. plantarum* LZ206 and *L. paraplantarum* L-ZS9 strains were used to identify potential gene cluster, which is responsible for bacteriocins biosynthesis and could be associated with its broad-spectrum antimicrobial activity [19,20]. The comparative genome analysis might facilitate probiotic applications to protect food products from pathogens' contamination in the dairy industry. Therefore, whether the LPG strains isolated in this study can synthesize antimicrobial peptides/compounds for inhibition of growth of multidrug-resistant pathogens is worth clarifying.

In conclusion, the present study indicates that four isolated high EPS-producing LPG strains show good tolerance to simulated gastrointestinal conditions. Among them, SLC 13 shows the highest EPS production, stress tolerance, antibacterial activity, and immunopotentiating effect. Thus, we believe that *Lactobacillus pentosus* SLC 13 may be a good candidate for development of probiotics. Further in vivo verification of our findings is necessary.

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