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Probiotic characterization and *in vitro* functional properties of lactic acid bacteria isolated in Thailand

Jaruwan Sitdhipol[a,*], Kanidta Niwasabutra[a], Neungnut Chaiyawan[a], Siritorn Teerawet[a], Punnathorn Thaveethaptaikul[a], Sukanya Phuengjayaem[b], Malai Taweechotipatr[c,d], Somboon Tanasupawat[e], Pongsathon Phapugrangkul[a]

[a] Biodiversity Research Centre, Research and Development Group for Bio-Industries, Thailand Institute of Scientific and Technological Research, Pathum Thani 12120 Thailand

[b] Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok 10140 Thailand

[c] Department of Microbiology, Faculty of Medicine, Srinakharinwirot University, Bangkok 10110 Thailand

[d] Center of Excellence in Probiotics, Strategic Wisdom and Research Institute, Srinakharinwirot University, Bangkok 10110 Thailand

[e] Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330 Thailand

[*]Corresponding author, e-mail: jaruwan_s@tistr.or.th

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ABSTRACT: Fourteen lactic acid bacteria isolated from fermented foods and healthy animal feces in Thailand were characterized for their potential as probiotics. The 16S rRNA gene sequence analyses identified them as *Lactiplantibacillus*, *Levilactobacillus*, *Limosilactobacillus*, *Lacticaseibacillus*, *Campanilactobacillus*, *Pediococcus*, and *Enterococcus* genera. All strains survived in simulated gastrointestinal fluid (pH 2) and bile salt solution (pH 8) at over 70% and 63%, compared with initial cell concentration, respectively. *In vitro* adhesion testing showed their adhesive property of over 70%, while the results of antibiotic susceptibility indicated that all strains were susceptible to amoxicillin, ampicillin, erythromycin, chloramphenicol, clindamycin, imipenem, kanamycin, norfloxacin, penicillin, tetracycline, and vancomycin. All strains exhibited antimicrobial ability against *Staphylococcus aureus* TISTR 1466, *Listeria monocytogenes* TISTR 2196, *Escherichia coli* TISTR 780, *Salmonella* Enteritidis TISTR 2202, and *Salmonella* Typhimurium TISTR 292. Moreover, *Limosilactobacillus reuteri* MF67.1 and *Companilactobacillus farciminis* R7-1 showed bile salt hydrolase activity. Cell-free culture supernatants of all 14 strains were screened for immunomodulating effects on Tumor Necrosis Factor alpha (TNF-α) production. *Lactiplantibacillus paraplantarum* R26-3 and *Lacticaseibacillus zeae* M2/5 showed high inhibition of TNF-α production at 34% and 29% reduction, respectively; while the other 12 strains decreased TNF-α production at various lower levels. Results suggested that all 14 strains met the general criteria of probiotics. *Lac. zeae* M2/5, *Lac. paraplantarum* R26-3, *Lim. reuteri* MF67.1, and *Com. farciminis* R7-1 were interesting candidates for further studies as anti-inflammatory (M2/5, R26-3) or cholesterol-reducing agents (MF67.1, R7-1) in *in vivo* animal models.

KEYWORDS: antimicrobial, cholesterol reducing, lactic acid bacteria, probiotics, TNF-α production

INTRODUCTION

Nowadays, human health is threatened by diet, stress, and modern medical practices (antibiotics and radiotherapy) [1]. Foods containing microorganism beneficial to human health, such as *Lactobacillus*, are now reclassified to 23 genera [2]. *Bifidobacterium* exhibits pivotal roles in enriching health well-being and suppressing diseases [3] caused by *Escherichia* (*Esc.*) *coli*, *Salmonella* sp., *Campylobacter* sp. *Bacillus cereus*, and *Clostridium perfringens* [4]. Colonic diseases and colon cancer are directly influenced by the diversity of gut microbiota, while consumption of probiotics can suppress obesity, diabetes, and heart disease by balancing the intestinal microbial composition [5]. Thus, foods and new creative diets now focus on the prevention of chronic diseases and disorders [6]. Probiotics are living microorganisms that have beneficial effects on the host when administered in an adequate amount (usually 10^6–10^7 CFU/g of product) by improving digestion, immune modulation and intestinal function [7]. Probiotics are widely used in food, feed, dairy and fermentation industries [8]. Several studies revealed that probiotics are associated with anti-inflammatory, cholesterol-lowering, anti-allergic, enzyme inhibition, anti-hypertensive, lactose intolerance, and mood changes [9].

Probiotics include lactic acid-producing bacteria, *Bacillus*, *Saccharomyces*, and *Aspergillus*. These bacteria are easy to consume, innocuous for ingestion, and able to colonize on gut epithelium and adhere to mucosal membrane. They remain stable during storage and survive in the upper gastrointestinal (GI) tract under high acid and bile salt concentrations [10]. Several probiotics have received temporary approval from the European Union. The aim of this study was to identify and characterize the potential of candidate probiotics isolated from fermented food and fecal samples of healthy animals. The functional properties of potential probiotic strains were determined, and the strains were

deposited in the Thailand Institute of Scientific and Technological Research (TISTR) culture collection to advance knowledge concerning the sustainable utilization of microorganisms in the supplementary food and animal feed industries.

MATERIALS AND METHODS

Microorganisms

Five pathogenic strains (*Esc. coli* TISTR 780, *Listeria* (*Lis.*) *monocytogenes* TISTR 2196, *Salmonella* (*Sal.*) Enteritidis TISTR 2202, *Sal.* Typhimurium TISTR 292, and *Staphylococcus* (*Sta.*) *aureus* TISTR 1466) were obtained from the TISTR culture collection.

Isolation and identification of lactic acid bacteria (LAB)

Samples from Thai fermented foods and healthy animal feces (Table 1) were collected and kept at 4 °C until the isolation. Ten grams of each sample was suspended in 90 ml of 0.1% peptone water and mixed in a stomacher. One microliter of the suspension was ten-fold serially diluted (10^2–10^3), and 0.1 ml of each diluted sample was spread on De Man Rogosa and Sharpe (MRS) supplemented with 0.3% $CaCO_3$ and Blood Agar Base (Merck, Darmstadt, Germany), and then incubated in anaerobic condition (anaerobic jar; Anaerocult® System, Merck) at 37 °C for 24–48 h.

All isolates were initially characterized by their colony morphology, Gram's reaction, catalase activity, and hemolysis [11]. Non-hemolytic strains were selected for further study and preserved in 15% v/v glycerol at −80 °C. Genomic DNA was extracted following Wilson [12]. The 16S rRNA gene sequences were aligned with selected sequences obtained from the EzBioCloud server database [13] and NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using CLUSTAL X version 1.81 in BioEdit software [14]. Phylogenetic tree was constructed based on the neighbor-joining method of bootstrap resampling with 1000 replications [15], using MEGA 7 software [16].

Acid and bile salt tolerance

Simulated gastrointestinal fluid (SGI) was prepared according to the modified method of Hyronimus et al [17]. Briefly, 0.1% of pepsin (Sigma-Aldrich, MO, USA) was dissolved in MRS broth with 0.05% L-cysteine, adjusted to pH 2 with 1 M HCl and 1 M NaOH, and sterile-filtered through a membrane (0.2 µm, Life Sciences, Ann Arbor, MI, USA). The solution was either used immediately or kept in the fridge until required (not longer than 24 h). Bile salt tolerance was determined according to the method of Gilliland et al [18]. Bile salts (Sigma-Aldrich) at 0.3% were dissolved in MRS broth with 0.05% L-cysteine (pH 8) and sterilized on a liquid cycle for 15 min. A one ml overnight culture in MRS broth was inoculated in 9.0 ml of SGI (pH 2) and 0.3% bile salt solution (pH 8). The sample mixtures were assessed immediately after mixing to determine the viability of candidate probiotics using the pour plate method and then incubated at 37 °C for 180 min. Remaining bacteria viability was investigated according to the above methods. Survival rate was calculated as log values of colony-forming units per milliliter (CFU/ml) according to the following formula:

$$\text{Cell survival percentage}(\%) = (\log N_1 / \log N_0) \times 100$$

where N_1 is the average of viable cells (CFU/ml) after incubation for 180 min, and N_0 is the average of viable cells (CFU/ml) at initial incubation time.

Antibiotic susceptibility

Antibiotic susceptibility of the selected strains was evaluated against spectra of clinically important antibiotics by the disc diffusion method [19] using 11 antibiotics: amoxicillin (AML; 10 µg), ampicillin (AMP; 10 µg), erythromycin (E; 15 µg), chloramphenicol (C; 30 µg), clindamycin (DA; 2 µg), imipenem (IPM; 10 µg), kanamycin (K; 30 µg), norfloxacin (NOR; 10 µg), penicillin (P; 10 µg), tetracycline (TE; 30 µg), and vancomycin (VA; 30 µg) (Oxoid, Hampshire, UK). Cell concentration of each bacterial culture was adjusted to McFarland No. 1 (10^8 CFU/ml), seeded onto MRS agar (Merck) using a sterile cotton swab, and allowed to stand at room temperature for 15 min. The antibiotic discs were placed onto agar under aseptic conditions. *Esc. coli* TISTR 780 and *Sta. aureus* TISTR 1466 were used as a positive control. The agar plates were incubated at 37 °C. Results were measured and compared with the breakpoint value as previously described [19].

Antimicrobial activity

Bacterial inhibition was determined by the agar diffusion assay [20], with five pathogenic bacteria as indicator strains: *Lis. monocytogenes* TISTR 2196, *Sta. aureus* TISTR 1466, *Esc. coli* TISTR 780, *Sal.* Typhimurium TISTR 292, and *Sal.* Enteritidis TISTR 2202. After 18–24 h cultivation at 37 °C, each indicator strain was suspended with 0.85% NaCl (Sigma-Aldrich), and cell density was adjusted to 10^8 CFU/ml. Cell suspensions of the indicator strains were overlaid onto Nutrient agar (NA) using a cotton swab and aseptically cut off from the NA using Cork borer No. 3. The overnight cultures were centrifuged for 20 min at 10 000×*g* and 70 µl of cell-free supernatant were placed in each well. After incubation for 24 h at 37 °C, the diameters of the inhibition zones were measured. The antimicrobial index was calculated as:

$$\text{Antimicrobial index} = \frac{d(\text{clear zone}) - d(\text{Cork borer})}{d(\text{Cork borer})}$$

where d = diameter.

Table 1 Source, strain number, and nearest type strain based on 16S rRNA gene sequence similarity.

Source	Strain no.	Nearest type strain	% Similarity
Feces of cow	AKB4.1	*Lactiplantibacillus pentosus* JCM 1558^T	99.93
Feces of goat	AKM29.1	*Lactiplantibacillus plantarum* DSMZ 20174^T	99.31
Fermented rice flour noodles	M1/2.1	*Limosilactobacillus fermentum* CECT 562^T	99.92
Fermented rice flour noodles	M2/5	*Lacticaseibacillus zeae* ATCC 15820^T	99.43
Fermented pork meat (Nham)	M22-5	*Lactiplantibacillus pentosus* JCM 1558^T	98.75
Fermented fish (Pla-som)	M26-10	*Lactiplantibacillus paraplantarum* DSM 10667^T	99.94
Fermented fish (Pla-som)	M26-20	*Lactiplantibacillus paraplantarum* DSM 10667^T	99.47
Feces of cow	MF58.1	*Limosilactobacillus reuteri* JCM 1112^T	99.63
Feces of cow	MF67.1	*Limosilactobacillus reuteri* JCM 1112^T	99.72
Butterfly	MF62.2	*Levilactobacillus brevis* ATCC 14869^T	100.00
Fermented fish (Pla-som)	R26-3	*Lactiplantibacillus pentosus* JCM 1558^T	99.93
Fermented shrimp (Kung-som)	R7-1	*Companilactobacillus farciminis* JCM 1097^T	99.56
Feces of calf	AKB2.8	*Pediococcus pentosaceus* DSM 20336^T	99.86
Fermented shrimp (Kung-jom)	M29-6	*Enterococcus durans* NCFB 596^T	99.63

Bile salt hydrolase (BSH) assay

BSH activity was detected using the agar plate assay following the method of Singh et al [21]. Each of the overnight strains on MRS agar was suspended, and cell concentration was adjusted to 10^8 CFU/ml in sterile saline solution. The cell suspension, 20 µl, was spotted onto MRS agar containing 0.5% (w/v) sodium taurodeoxy cholic acid (TDCA) (Sigma-Aldrich). After 48–72 h incubation under anaerobic condition at 37 °C, the presence of precipitated bile acid around the colonies (called opaque halo) was considered a positive reaction. All experiments were performed in triplicate. MRS agar without supplementation of TDCA was used as a negative control.

Adhesion assay

The ability of each strain was tested for adherence to human epithelial cells as described [22]. Briefly, adenocarcinoma cell line (Caco-2) (ATCC, HTB-37) was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Gibco-Invitrogen, USA), seeded into a 24-well cell culture plate at 2×10^5 cells/well and incubated at 37 °C in 5% CO_2 in a humidified incubator for 14 days. Before assay, the complete DMEM was replaced with antibiotic-free and serum-free DMEM for 16 h. The Caco-2 cells were washed twice with sterile PBS (pH 7.2) and 2 ml of DMEM (without serum and antibiotics) was added to each well and incubated at 37 °C for 30 min. Cells of each strain were harvested by centrifugation (4000×g, 10 min, 4 °C) and washed twice with sterile PBS. Cell density was adjusted with DMEM (without serum and antibiotics) to 1×10^9 CFU/ml. Then, one ml of suspension was added to each well and incubated at 37 °C in a 5% CO_2 atmosphere for 1 h. Finally, Caco-2 cells were washed three times with sterile PBS to remove non-adherence cells according to Roselli et al [23]. Cells from monolayers were detached by 1% Triton-X-100. The bacterial cell suspension was serially diluted with sterile saline solution and spread on MRS agar. After incubation for 24–48 h at 37 °C, adhesion ability was determined using the following formula:

$$\text{Percentage of Caco-2 cell adhesion} = (N_1/N_0) \times 100$$

where N_1 = number of bacterial colonies after incubation, and N_0 = number of initial bacterial colonies added as a control.

THP-1 cell culture and TNF-α measurement

THP-1 monocytic cells (ATCC, TIB 202) were cultured in Roswell Park Memorial Institute medium (RPMI-1640) (Gibco, Gibco-Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco-Invitrogen) and incubated at 37 °C in 5% CO_2 in a humidified incubator for 3–7 days. Each strain was centrifuged at 3000×g for 15 min. Cell-free supernatant was collected and dried under vacuum condition (35 °C), then re-suspended with 500 µl RPMI-1640 and sterile-filtered through a membrane (0.2 µm, Sigma). The sample was called conditioned media. For bioassay, cell viability was assessed by the trypan blue stain exclusion assay. THP-1 monocytic cells (2.5×10^5 cells/ml) were seeded at into a 96-well cell culture plate and incubated at 37 °C in 5% CO_2 for 10 min before adding 10 µl of conditioned media and 5 µl of 100 ng/ml purified lipopolysaccharide (LPS) from *Esc. coli* serotype O127:B8 (Sigma, USA). Then, the plates were incubated at 37 °C in 5% CO_2 for 4 h. Finally, supernatants were collected by centrifugation at 1000×g for 9 min at 4 °C for TNF-α measurement. Assays were performed three times, in duplicate. TNF-α production was measured using the sandwich enzyme-linked immunosorbent assay (ELISA) technique according to the manufacturer's instructions (R&D Systems, USA). Recombinant human TNF-α was used as standard. Absorbance was measured at 450 nm using a BioTek® Synergy™ HT (Multi-Detection Microplate Reader, USA).

Cell viability assay

Trypan blue exclusion assays were carried out as previously described [24]. THP-1 monocytic cells (ATCC, TIB 202) (at 2×10^5 cells/ml) were dispensed into 24-well microplates at 1 ml of medium/well and incubated overnight at 37 °C under 5% CO_2. Cells were then treated with conditioned media for 24 h at a final concentration of 10 µl/ml. Control cultures were maintained under the same conditions. After incubation, enumeration of viable THP-1 cells was measured by immediate microscopic observation after trypan blue staining using a KOVA counting cell (Hycor, VWR, Strasbourg, France). THP-1 cells-stained blue were no longer viable, with damaged membranes allowing entry of the dye. Viability was expressed as the percentage of cells alive after contact with the conditioned media.

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS, Statistics version 24.0.0.0) with one-way analysis of variance (ANOVA), while grouping was assessed by Duncan's multiple range test at a p-value of 0.05 [25]. The data were expressed as mean values of triplicates ± standard deviation.

RESULTS AND DISCUSSION

Isolation and identification of lactic acid bacteria (LAB)

Fourteen LAB selected from 850 isolates of Thai fermented foods and healthy animal feces (a total of 262 samples) were obtained. All 14 strains were preliminarily characterized based on their morphology, Gram staining, and catalase reaction. Twelve strains were rod-shaped and two were spherical-shaped. All strains were Gram positive, non-spore forming, and catalase negative.

Non-hemolytic activity was found in all strains. Results suggested that the 14 strains (AKB2.8, AKB4.1, AKM29.1, M1/2.1, M2/5, M22-5, M26-10, M26-20, M29-6, MF58.1, MF62.2, MF67.1, R26-3, and R7-1) could be considered safe. All strains were also screened by replicating human intestinal barrier conditions and identified by 16S rRNA gene sequence analysis.

The 14 strains were identified based on 16S rRNA gene sequence similarity (99.31–100%). Strains MF58.1 and MF67.1 were identified as *Limosilactobacillus* (*Lim.*) *reuteri*; strains M26-10 and M26-20 as *Lactiplantibacillus* (*Lac.*) *paraplantarum*; strains AKB4.1, M22-5, and R26-3 as *Lactiplantibacillus pentosus*; while strains M2/5, MF62.2, M1/2.1, AKM29.1, R7-1, AKB2.8, and M29-6 were identified as *Lacticaseibacillus zeae*, *Levilactobacillus* (*Lev.*) *brevis*, *Limosilactobacillus fermentum*, *Lactiplantibacillus plantarum*, *Companilactobacillus* (*Com.*) *farciminis*, *Pediococcus* (*Ped.*) *pentosaceus*, and *Enterococcus* (*Ent.*) *durans*, respectively (Table 1 and Fig. 1).

LAB are mostly considered Generally Recognized as Safe (GRAS) and widely used in food, feed, dairy, and fermentation industries [8]. In addition to its probiotic qualities, LAB also provide other advantages. *Lev. brevis*, *Lac. pentosus*, *Lac. plantarum*, and *Lim. fermentum* produce Gamma-aminobutyric acid (GABA) which directly influences personality and stress control working as an antidepressant in various physiological functions [26, 27]. *Lim. fermentum* LF33 in fermented lemon juice could convert the substrates in that broth into GABA and others, contributing calming effect on ganglia and significantly reducing ovalbumin induced IgE antibody levels resulting in stabilizing heart rate and reducing allergic effect of ovalbumin in sensitized BALB/c mice [28]. Although Thai fermented foods and healthy animal feces are increasingly considered as reservoir, they are uncharacterized probiotic strains [29].

Acid and bile tolerance

For probiotic properties, all strains were tested for their survivability in human SGI (pH 2) and 0.3% bile salt solution (pH 8). Each of the 14 strains in SGI pH 2 and 0.3% bile salt solution pH 8 was incubated for 3 h, and percentage of viable bacterium numbers was evaluated. Results indicated that all strains showed more than 60% cell survival in acid pH 2 and 0.3% bile salt solution pH 8 (Fig. 2). Survival rates of all 14 strains were higher than 70% in SGI (pH 2), while strains M22-5 and AKM29.1 were more sensitive to bile salt solution than the others at 66 and 68% cell survival, respectively. Survival rates of the three strains M1/2.1, M2/5 and MF67.1 were higher than 90% after incubation in SGI (pH 2) and 0.3% bile salt solution (pH 8) for 3 h. All LAB were more tolerant of SGI pH 2.0 than 0.3% bile salt solution, except for *Ent. durans* M29-6, *Lev. brevis* M62.2, and *Lac. pentosus* R26-3. Results suggested that these 14 strains successfully passed through the human stomach and reached the intestine with at least 60% of the initial cell number. The Gastrointestinal tolerance (GIT) capability of bacterial strains is an important criterion in the selection of potential probiotics.

Antibiotic susceptibility

Antibiotic susceptibility of the 14 strains was determined by disc diffusion method and tested with 11 antibiotics. Test results revealed that the strains were resistant to K, NOR, and VA; and susceptible to AML, AMP, E, P, C, DA, TE, and IPM. Only *Ped. pentosaceus* AKB2.8 and *Ent. durans* M29-6 were susceptible to VA. Most LAB species have antibiotic resistance to the aminoglycosides group (K, streptomycin, and gentamycin), quinolones group (NOR, ciprofloxacin, and nalidixic acid), and VA. This specific pattern is considered intrinsic resistance, and the resistant genes are not transferable to other microorganisms [30].

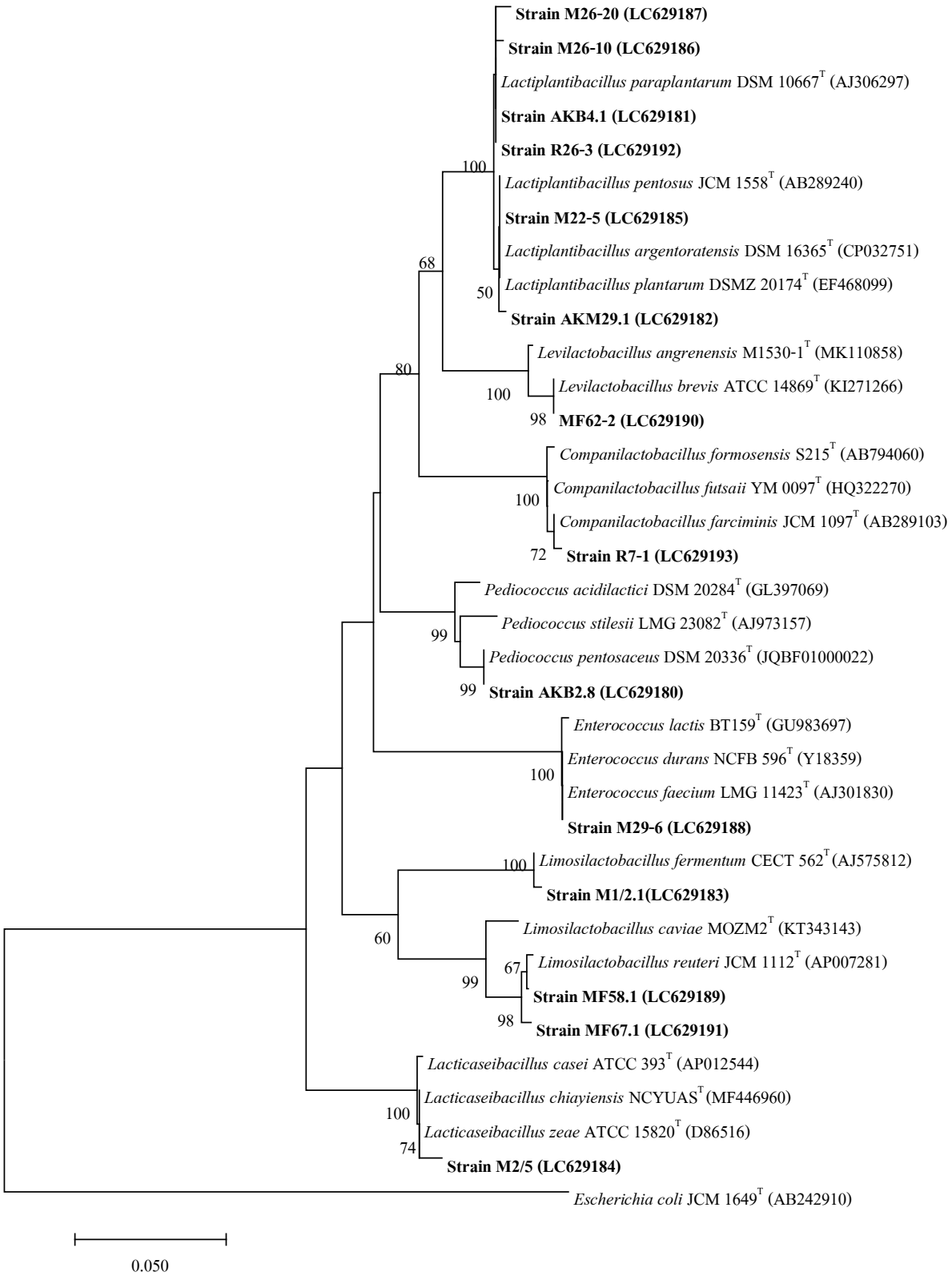

Fig. 1 Neighbor-joining tree of representative strains based on the 16S rRNA gene sequences. Bootstrap values are shown as percentages of 1000 replications; only values > 50% are indicated. Bar, 0.01 substitutions per nucleotide position.

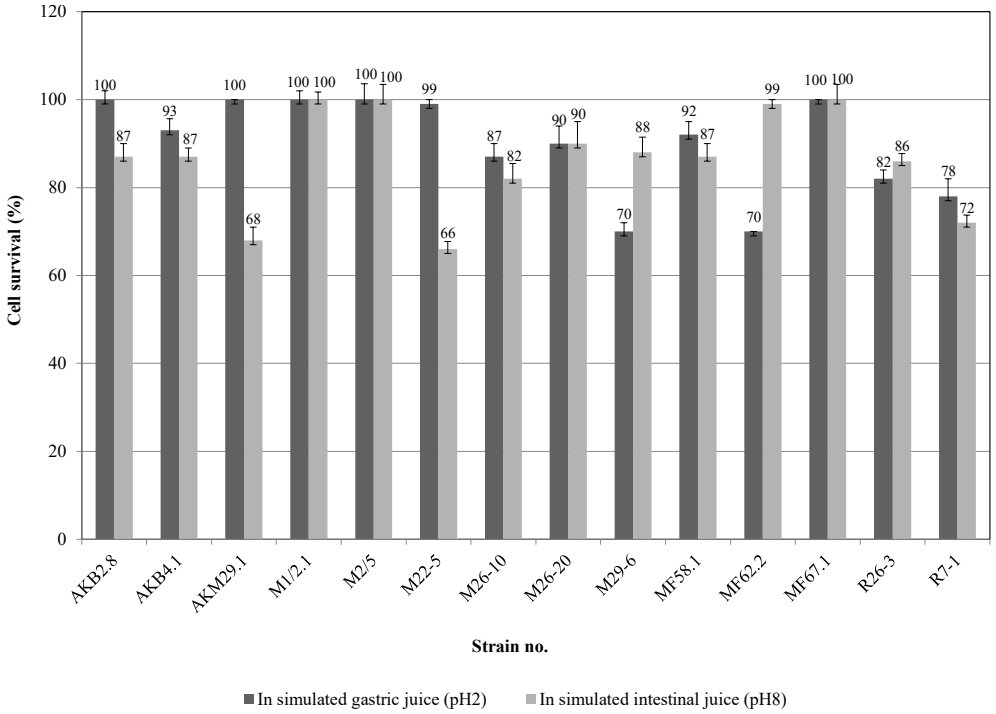

Fig. 2 Viability and survival percentage of strains incubated in simulated gastric juice (pH 2) and simulated intestinal juice (pH 8).

Table 2 Antibiotic susceptibility of the 14 strains.

Strain no.	Antibiotic susceptibility										
	AML	AMP	E	P	C	DA	IPM	K	NOR	TE	VA
Lac. pentosus AKB4.1	S	S	S	S	S	S	S	R	R	S	R
Lac. plantarum AKM29.1	S	S	S	S	S	S	S	R	R	S	R
Lim. fermentum M1/2.1	S	S	S	S	S	S	S	R	R	S	R
Lac. zeae M2/5	S	S	S	S	S	S	S	R	R	S	R
Lac. pentosus M22-5	S	S	S	S	S	S	S	R	R	S	R
Lac. paraplantarum M26-10	S	S	S	S	S	S	S	R	R	S	R
Lac. paraplantarum M26-20	S	S	S	S	S	S	S	R	R	S	R
Lac. reuteri MF58.1	S	S	S	S	S	S	S	R	R	S	R
Lim. reuteri MF67.1	S	S	S	S	S	S	S	R	R	S	R
Lev. brevis MF62.2	S	S	S	S	S	S	S	R	R	S	R
Lac. pentosus R26-3	S	S	S	S	S	S	S	R	R	S	R
Com. farciminis R7-1	S	S	S	S	S	S	S	R	R	S	R
Ped. pentosaceus AKB2.8	S	S	S	S	S	S	S	R	R	S	S
Ent. durans M29-6	S	S	S	S	S	S	S	R	R	S	S

AML, amoxicillin 10 μg; AMP, ampicillin 10 μg; E, erythromycin 15 μg; C, chloramphenicol 30 μg, DA, clindamycin 2 μg; IPM, imipenem 10 μg; K, kanamycin 30 μg; NOR, norfloxacin 10 μg; P, penicillin 10 μg; TE, tetracycline 30 μg; VA, vancomycin 30 μg; R, resistant; S, sensitive.

Importantly, the bacterial strain should be susceptible to AMP, TET, P, C, AML, and E because these antibiotic resistant properties are considered as acquired resistance, and these genes can be transferred to other normal flora or pathogenic bacteria [31]. All 14 strains were sensitive to these antibiotics (Table 2). Similarly, Sharma et al [32] found that LAB were usually sensitive to C, AMP, IPM, meropenem, and E. VA was the first glycopeptide antibiotic used clinically, and our results showed that 12 strains were resistant to VA, similar to Tulini et al [33] and Zhang et al [34]; while *Ent. durans* M29-6, *Ped. pentosaceus* AKB2.8, and *Com. farciminis* R7-1 were sensitive. Some strains of *Enterococcus* may possess virulence, especially those that display high levels of resistance to VA [9]. If this resistance is present, a transfer of genes to other microorganisms may occur, thereby enhancing the pathogenesis of such recipients.

Table 3 Antimicrobial activity of the 14 strains.

Strain no.	*Escherichia coli* TISTR 780	*Listeria monocytogenes* TISTR 2196	*Salmonella* Enteritidis TISTR 2202	*Salmonella* Typhimurium TISTR 292	*Staphylococcus aureus* TISTR 1466
Lac. pentosus AKB4.1	++	++	++	++	++
Lac. plantarum AKM29.1	++	+	++	+++	++
Lim. fermentum M1/2.1	+	++	++	++	++
Lac. zeae M2/5	++	++	++	++	−
Lac. pentosus M22-5	++	++	++	++	++
Lac. paraplantarum M26-10	++	++	++	++	+
Lac. paraplantarum M26-20	++	+	++	++	++
Lim. reuteri MF58.1	+	+	+	++	−
Lim. reuteri MF67.1	−	+	+	+	−
Lev. brevis MF62.2	+	+	++	+	+
Lac. pentosus R26-3	++	++	+	+	++
Com. farciminis R7-1	+	+	+	++	+
Ped. pentosaceus AKB2.8	++	++	++	++	++
Ent. durans M29-6	−	+	+	+	−

Antimicrobial index: −, 0; +, ⩽1; ++, ⩽2; +++, ⩽3.

Antimicrobial activity

Antimicrobial activities of the 14 strains against *Esc. coli* TISTR 780, *Lis. monocytogenes* TISTR 2196, *Sal. Enteritidis* TISTR 2202, *Sal.* Typhimurium TISTR 292, and *Sta. aureus* TISTR 1466 were shown in Table 3. Most strains exhibited inhibitory activity against *Lis. monocytogenes*, *Sta. aureus*, *Esc. coli*, *Sal.* Typhimurium, and *Sal.* Enteritidis; while *Lac. plantarum* AKM29.1 showed the highest potency against *Sal.* Typhimurium TISTR 292 (Antimicrobial index >2).

Bile salt hydrolase (BSH) activity

The hydrolysis of bile salts is another functional health characteristic for probiotic selection. BSH activity helps bacteria to grow and colonize in the intestine by deconjugating bile salts [35]. Strains that have BSH activity show a precipitate around other colonies on MRS agar containing 0.5% TDCA. The BSH activity test showed that only two strains (MF67.1 and R7-1) were BSH positive (Table 4). These strains were identified as close to *Lim. reuteri* and *Com. farciminis* at 99.72% and 99.56% similarity, respectively. LAB are the most often utilized probiotics due to their important role in disease resistance and their assessment as GRAS [36]. Probiotics are well documented for their prophylactic and therapeutic benefits. For instance, LAB strains may also have BSH activity that is effective in lowering blood cholesterol levels in hypercholesterolemic patients as well as preventing hypercholesterolemia in healthy people [37]. The probiotic properties of these 14 strains should be further investigated by *in vivo* studies.

Adhesion assay

The Caco-2 cell line has been extensively used as a reliable *in vitro* system to study the adhesion capacity of candidate probiotics [38]. Our 14 LAB showed adhesion rates between 73 and 100% (Table 4). Strains AKM29.1, MF58.1, M22/5, and R7-1 showed the highest adhesion rates at 100%, while MF62.2 showed the lowest at 73%. Results indicated that the 14 strains displayed a high level of adhesion at more than 70%. Consequently, these strains showed ability for adhesion, establishment, and colonization within the GIT, increasing their potential for survival. However, co-culture with pathogen strains and *in vivo* experiments are required for antagonistic assessment activity against pathogens.

TNF-α inhibition

TNF-α inhibitory activities of the 14 strains were investigated in THP-1 cells, with TNF-α production measured using the ELISA method. Results revealed that *Lac. zeae* M2/5 and *Lac. pentosus* R26-3 showed the reduction values compared with the control of 29% and 34%, respectively. *Lim. fermentum* M1/2.1 and *Lev. brevis* MF62.2 showed slightly stimulated TNF-α production with increases of 8% and 17% compared with the control, respectively (Table 4). The other strains reduced TNF-α production by 6 to 26% of the control. The viability of THP-1 cells after exposure to the conditioned medium of all 14 strains was more than 80%.

TNF-α suppression was important in alleviating inflammation in a murine model of inflammatory bowel disease [39], while BSH activity was one of the mechanisms involved in cholesterol reduction by bacteria [40]. Results indicated that *Lac. pentosus* R26-3 gave the highest TNF-α inhibition in macrophages.

Our results revealed that the 14 LAB in this study showed potential probiotic properties because of their tolerance to SGI (pH 2) and 0.3% bile salt solution (pH 8), with antimicrobial capability and high adherence to cell lines. The 14 LAB also showed strain-specific activity on anti-allergic, enzyme inhi-

Table 4 Bile salt hydrolase activity, Caco-2 cell adhesion, and inhibition of TNF-α production of the 14 strains.

Strain no.	BSH activity	Caco-2 cell adhesion (%)	TNF-α inhibition (%)
Lac. pentosus AKB4.1	−	$88.00 \pm 2.00^{b,c}$	19.05 ± 0.58^{f}
Lac. plantarum AKM29.1	−	100.00 ± 0.00^{a}	18.07 ± 0.25^{g}
Lim. fermentum M1/2.1	−	$90.00 \pm 4.00^{b,c}$	-8.09 ± 0.10^{k}
Lac. zeae M2/5	−	$90.00 \pm 2.00^{b,c}$	29.09 ± 0.11^{b}
Lac. pentosus M22-5	−	100.00 ± 0.00^{a}	22.02 ± 0.53^{e}
Lac. paraplantarum M26-10	−	$88.00 \pm 1.41^{b,c}$	25.05 ± 0.92^{d}
Lac. paraplantarum M26-20	−	84.00 ± 3.61^{c}	26.00 ± 0.05^{c}
Lim. reuteri MF58.1	−	100.00 ± 6.08^{a}	13.07 ± 0.31^{h}
Lim. reuteri MF67.1	+	86.00 ± 5.29^{c}	6.06 ± 0.10^{j}
Lev. brevis MF62.2	−	73.00 ± 1.73^{d}	-17.08 ± 0.14^{l}
Lac. pentosus R26-3	−	87.00 ± 4.58^{c}	34.08 ± 0.33^{a}
Com. farciminis R7-1	+	100.00 ± 0.00^{a}	7.02 ± 0.03^{i}
Ped. pentosaceus AKB2.8	−	$94.00 \pm 3.46^{a,b}$	22.04 ± 0.46^{e}
Ent. durans M29-6	−	96.00 ± 3.46^{a}	6.03 ± 0.05^{j}

For BSH assay: −, not having BSH activity; +, having BHS activity.
For TNF-α inhibition assay: +, reduced TNF-α production; −, stimulated TNF-α production compared with the control.
The superscript showed significantly different results by multiple comparison using Duncan's method (p-value less than 0.05). The maximum value is represented by the letters 'a' and in descending order.

bition, anti-hypertensive, lactose intolerance, antioxidant, and anti-cancer activities, while reducing cholesterol, lipid, sugar, including other metabolic substances.

CONCLUSION

The results of our study suggested that the absence of hemolysis of all 14 strains isolated from fermented foods and animal feces indicated resistance to biological barriers (acid and bile salts). The 14 LAB exhibited over 70% survival rate in SGI and more than 60% in 0.3% bile salt solution. All of them showed antibacterial activity against *Lis. monocytogenes* TISTR 2196, *Sal.* Enteritidis TISTR 2202, and *Sal.* Typhimurium TISTR 292. They were susceptible to AML, AMP, P, C, DA, TE, and IPM that are commonly used in human and veterinary medicines. Furthermore, they showed good adhesive properties in *in vitro* experiments of over 70% adhesion. However, functional probiotic properties were strain specific. Strains *Lim. reuteri* MF67.1 and *Com. farciminis* R7-1 showed positive BSH activity (associated with cholesterol-lowering), while *Lac. pentosus* R26-3 and *Lac. zeae* M2/5 displayed highest TNF-α inhibition in macrophages (anti-inflammatory) at 34% and 29% reduction compared with the control, respectively. Results indicated that the 14 strains had probiotic properties and could be suitable for applications in functional foods and health supplements. However, further *in vivo* studies are required to determine their efficacy before they can be incorporated in foods or dietary supplements on a large scale.

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