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

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Research Article

Keywords: Antimicrobial, TNF- α production, Cholesterol reducing, Lactic acid bacteria

Posted Date: August 31st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-843728/v1>

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Abstract

Fourteen lactic acid bacteria from fermented foods and feces of healthy animals in Thailand were characterized for their potential as probiotics. All isolates could survive in simulated gastrointestinal fluid (pH 2) and bile salt solution (pH 8) more than 70% and 63%, when compare with initial cell concentration, respectively. Adhesion test showed more than 70% adhesive property an *in vitro* experiment. The susceptibility assay showed that all isolates were susceptible to amoxicillin, ampicillin, erythromycin, chloramphenicol, clindamycin, imipenem, kanamycin, norfloxacin, penicillin, tetracycline and vancomycin. Based on phenotypic and genetic characteristics, they belonged to the genera *Lactiplantibacillus*, *Levilactobacillus*, *Capanilactobacillus*, *Pediococcus*, *Enterococcus*, *Limosilactobacillus* and *Lacticaseibacillus*. The isolates exhibited antimicrobial ability against pathogenic bacteria; Gram positive strains (*Staphylococcus aureus* TISTR 1466 and *Listeria monocytogenes* TISTR 2196) and Gram negative (*Escherichia coli* TISTR 780, *Salmonella enteritidis* TISTR 2202 and *Salmonella typhimurium* TISTR 292). *Limosilactobacillus reuteri* MF67.1 and *Companilactobacillus farciminis* R7-1 showed bile salt hydrolase activity. Cell-free culture supernatants of all 14 isolates were screened for immunomodulating effects on Tumor Necrosis Factor Alpha (TNF- α) production. Twelve isolates were able to decrease TNF- α production at different levels, especially *Lactiplantibacillus paraplantarum* R26-3 and *Lacticaseibacillus zeae* M2/5 could high inhibit TNF- α production, showing 34 and 29% reduction, respectively. These results suggested that all 14 strains met the general criteria of probiotics and four strains, including *Lacticaseibacillus zeae* M2/5, *Lactiplantibacillus paraplantarum* R26-3, *Limosilactobacillus reuteri* MF67.1 and *Companilactobacillus farciminis* R7-1, represent interesting candidates for further studies as anti-inflammatory (M2/5, R26-3) or cholesterol reducing agents (MF67.1, R7-1) *in vivo* animal models.

Introduction

Nowadays, diet, stress, and modern medical practices (antibiotics and radiotherapy) play important roles in threatening human health (Dunne et al. 2001). In particular, foods containing beneficial microorganisms such as *Lactobacillus* and *Bifidobacterium* exhibit a pivotal role in enriching health well-being and suppressing disease (Meyer and Stasse-Wolthuis, 2009). Nevertheless, some bacteria such as *Escherichia coli*, *Bacteroides fragilis*, *Fusobacterium nucleatum*, and *Clostridium perfringens* are potential pathogens as disease sources (Skar et al. 1986). Colonic diseases and colon cancer are reported to be 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 (O'Keefe 2008; Abdelazez et al. 2017). Thus, foods and new creative diets have focused on the prevention of chronic diseases and disorders (Salveti and O'Toole 2017). Probiotics are living microorganisms that have beneficial effects on the host when administered in an adequate amount (usually at 10^6 - 10^7 CFU/g of product) by improving digestion, immune modulation, and intestinal function. They are widely used in food, feed, dairy, and the fermentation industry (FAO/WHO, 2006). Several studies revealed that probiotics are associated with anti-inflammatory, cholesterol-lowering, anti-allergic, enzyme inhibition, anti-

hypertensive, lactose intolerance and mood changes (Lorea Baroja et al. 2007; Kumar et al. 2012; Lee et al. 2014; Panwar et al. 2014; Staudacher 2015; Steenbergen et al. 2015). Strains of microorganisms reported as probiotics include lactic acid-producing bacteria (lactobacilli, streptococci, enterococci, lactococci, bifidobacteria), *Bacillus*, *Saccharomyces* and *Aspergillus* (FAO/WHO 2002). These bacterial strains must be easy to consume and inoffensive for ingestion, able to colonize the gut epithelium, adhere to the mucosal membrane, remain stable during storage and survive in the upper GI tract under high acid and bile salt concentration (Verna and Lucak 2010). Several probiotics have received temporary approval from the European Union. Thus, the aim of this study was to identify and characterize the potential of candidate probiotics from natural resources in Thailand such as edible sources, fermented food, soil and fecal samples from healthy animal. The potential probiotic strains were further their functional properties studies and deposited in the TISTR collection to advantage knowledge concerning the sustainable utilization of microorganisms in the supplementary food and animal feed industries.

Materials And Methods

Microorganism

Five pathogenic strains (*Escherichia coli* TISTR 780, *Listeria monocytogenes* TISTR 2196, *Salmonella enteritidis* TISTR 2202, *Salmonella typhimurium* TISTR 292, and *Staphylococcus aureus* TISTR 1466) were obtained from the TISTR culture collection (Thailand Institute of Scientific and Technological Research, Thailand).

Chemical and Reagents

Antibiotic discs of amoxicillin (10 µg), ampicillin (10 µg), erythromycin (15 µg), chloramphenicol (30 µg), clindamycin (2 µg), imipenem (10 µg), kanamycin (30 µg), norfloxacin (10 µg), penicillin (10 µg), tetracycline (30 µg) and vancomycin (30 µg) were purchased from Oxoid (Hampshire, UK). De Man Rogosa and Sharpe (MRS), Trypticase Soy Agar (TSA), Nutrient broth (NA), and Blood agar base were received from Merck (Darmstadt, Germany). Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI-1640) were purchased from Gibco (Gibco-Invitrogen, USA) while sodium taurodeoxycholate hydrate (TDCA), NaCl, bile salts and pepsin were purchased from Sigma-Aldrich (MO, USA). Universal primers for 16S rDNA sequencing analysis (27F and 1492R) were obtained from 1st BASE (Malaysia). Reaction agents for DNA extraction and 16S rRNA gene amplification, CaCl_2 and CaCO_3 were purchased from Thermo Fisher (Invitrogen). Dextrose agar and other chemicals were obtained from BD/Difco Laboratories (Franklin Lakes, NJ, USA).

Selection and identification of lactic acid bacteria (LAB)

Samples from Thai-fermented foods and feces from healthy animal (Table 1) were collected and kept in 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 TSA and MRS agar supplemented with 0.3% CaCO_3 and incubated

under the anaerobic condition (anaerobic jar; Anaerocult® System, Merck, Germany) at 37°C for 24–48 h. The isolates were initially identified based on colony morphology, Gram's reaction, catalase activity, and hemolysis (Igarashi et al. 1999). For further study, non-hemolytic strains were selected and kept at -80°C (in 15% v/v glycerol).

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

Source	Isolate no.	Nearest type strain	%Similarity
Feces of calf	AKB2.8	<i>Pediococcus pentosaceus</i>	99.86
Feces of cow	AKB4.1	<i>Lactiplantibacillus pentosus</i>	99.93
Feces of goat	AKM29.1	<i>Lactiplantibacillus plantarum</i>	99.31
Fermented rice flour noodles	M1/2.1	<i>Limosilactobacillus fermentum</i>	99.92
Fermented rice flour noodles	M2/5	<i>Lacticaseibacillus zae</i>	99.43
Fermented pork meat (Nham)	M22-5	<i>Lactiplantibacillus pentosus</i>	98.75
Fermented fish (Pla-som)	M26-10	<i>Lactiplantibacillus paraplantarum</i>	99.94
Fermented fish (Pla-som)	M26-20	<i>Lactiplantibacillus paraplantarum</i>	99.47
Fermented shrimp (Kung-jom)	M29-6	<i>Enterococcus durans</i>	99.63
Feces of cow	MF58.1	<i>Limosilactobacillus reuteri</i>	99.63
Butterfly	MF62.2	<i>Levilactobacillus brevis</i>	100.00
Feces of cow	MF67.1	<i>Limosilactobacillus reuteri</i>	99.72
Fermented fish (Pla-som)	R26-3	<i>Lactiplantibacillus pentosus</i>	99.93
Fermented shrimp (Kung-som)	R7-1	<i>Companilactobacillus farciminis</i>	99.56

Genomic DNA was extracted following Wilson (2001). The 16S rRNA gene sequences were aligned with selected sequences obtained from the EzBioCloud server database (Yoon et al. 2017) and NCBI BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) by using the CLUSTAL X version 1.81 in BioEdit software (Thompson et al. 1997). Phylogenetic trees were constructed based on the neighbour-joining (Saitou and Nei 1987), maximum-likelihood (Kimura 1980), and maximum-parsimony (Nei and Kumar 2000) methods using MEGA 7 software (Kumar et al. 2016). The confidence values of nodes were evaluated using the bootstrap resampling method with 1,000 replications (Felsenstein 1985).

Acid and bile salt tolerance

Simulated gastrointestinal fluid (SGI) was prepared according to the modified method of Hyronimus et al. (2000). Briefly, 0.1% of pepsin 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. (1984). Bile salts at 0.3% were dissolved with MRS broth with 0.05% L-cysteine (pH 8) and sterilized on liquid cycle for 15 minutes. One milliliter overnight cultures in MRS broth were 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 minutes. The viability of bacteria remaining was investigated according to the above methods. Survival rate was calculated as log values of colony-forming units per milliliter (CFU/mL) and calculated according to the following formula:

$$\text{Percentage of survival cell (\%)} = (\text{Log } N_1 / \text{Log } N_0) \times 100$$

where N_1 is the average of viable cell (CFU/mL) after incubation for 180 minutes and N_0 is the average of viable cell (CFU/mL) at initial incubation time (0 minutes).

Antibiotic susceptibility

Antibiotic susceptibility of the selected isolates was evaluated against large spectra of clinically important antibiotics by the disc diffusion method (Clinical and Laboratory Standards Institute or CLSI, 2019). Eleven antibiotics as amoxicillin (10 µg), ampicillin (10 µg), erythromycin (15 µg), chloramphenicol (30 µg), clindamycin (2 µg), imipenem (10 µg), kanamycin (30 µg), norfloxacin (10 µg), penicillin (10 µg), tetracycline (30 µg) and vancomycin (30 µg) were applied. Cell concentration of each bacterial culture was adjusted equal to McFarland No.1 and seeded onto MRS agar using a sterile cotton swab and allowed to stand at room temperature for 15 minutes. The antibiotic discs were plated onto agar under aseptic conditions. *E. coli* TISTR 780, and *S. aureus* TISTR 1466 were used as a positive control. The agar plates were incubated at optimal conditions. Results were measured and compared with the breakpoint value designated by CLSI (2019).

Antimicrobial activity

Bacterial inhibition was determined by agar diffusion assay (Ennahar et al. 2000). Five pathogenic bacteria : *L. monocytogenes* TISTR 2196, *S. aureus* TISTR 1466, *E. coli* TISTR 780, *S. typhimurium* TISTR 292, and *S. enteritidis* TISTR 2202 were used as indicator strains. After 18–24 h cultivation at their optimal growth conditions, each indicator strain was suspended with 0.85% NaCl and cell density was adjusted to McFarland No.1 (10^8 CFU/mL). Cell suspensions of indicator strains were overlaid onto Nutrient agar (NA) by cotton swab and aseptically cut off NA using Cork borer No.3. The overnight isolate cultures were centrifuged for 20 minutes at $10,000 \times g$ 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

$$\text{Antimicrobial index} = [d (\text{clear zone}) - d (\text{cork})] / d (\text{cork})$$

where, d (clear zone) = diameter of clear zone, d (cork) = diameter of cork borer

Bile salt hydrolase (BSH) assay

BSH activity was detected using agar plate assay following the method of Singh et al. (2012). Each of overnight isolated cultures on MRS agar was suspended and adjusted cell concentration to 10^8 CFU/ml in sterile saline solution. 20 μ l of cell suspension was spotted onto MRS agar containing 0.5% (w/v) Sodium taurodeoxy cholic acid (TDCA). After 48–72 h incubation under the anaerobic condition at 37°C. The presence of precipitated bile acid around colonies (called as opaque halo) was considered as a positive reaction. The experiment was performed in triplicate. MRS agar without the supplementation of TDCA were used as a negative control.

Adhesion assay

The ability of each isolated bacteria was tested for adherence to human epithelial cells according to Jacobsen et al. (1999). Briefly, adenocarcinoma cell line (Caco-2) (ATCC, HTB-37) was cultured in DMEM and seeded into a 24-well cell culture plate at density 2×10^5 cells/well and incubated at 37°C in 5% CO₂ with a humidified incubator for 14 days. Before assay, complete DMEM was replaced with antibiotic-and serum-free DMEM for 16 h. Caco-2 cells were then washed twice with sterile PBS (pH 7.2). An aliquot of 2 mL of DMEM (without serum and antibiotics) was added to each well and incubated at 37°C for 30 minutes. Cultured cells of each isolated bacteria were harvested by centrifugation (4,000 $\times$ g, 10 minutes, 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, 1 mL of each isolate suspension was added to each well and incubated at 37°C in a 5% CO₂ 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. (2006). 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 RPMI-1640 medium (Gibco-Invitrogen, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco-Invitrogen, USA) and incubated at 37°C in 5% CO₂ with a humidified incubator for 3–7 days. Each of isolated LAB cultures was centrifuged at 3,000 $\times$ g for 15 minutes. Cell-free supernatant was collected and dried under vacuum condition (35°C) and then re-suspended with 500 μ L RPMI-1640 and sterile-filtered through a membrane (0.2 μ m, Sigma, USA), the sample called as conditioned media. For bioassay, cell viability was assessed by the trypan blue stain exclusion assay. THP-1 monocytic cells were seeded at density 2.5×10^5 cell/mL into a 96-well cell culture plate and incubated at 37°C in 5% CO₂ for 10 minutes before adding 10 μ L of

conditioned media and 5 µL of 100 ng/ml purified lipopolysaccharide (LPS) from *E. coli* serotype O127:B8 (Sigma, USA). Then, the plates were incubated at 37°C in 5% CO₂ for 4 h. Finally, supernatants were collected by centrifugation at 1000 ×g for 9 min in 4°C for TNF-α measurement. Assays were done three times, in duplicate. TNF-α production was measured using 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 MicroplateReader, USA).

Cell viability assay

Trypan blue exclusion assays were carried out as previously described (Liu et al. 1999). THP-1 monocytic cells (ATCC, TIB 202) (at 2x10⁵ cells/mL) were dispensed into 24-well microplates, in 1 mL of medium/well and incubated overnight at 37 °C, under 5% CO₂. Cells were then treated with conditioned media for 24 h at the final concentration of 10 µl/mL. Control cultures were maintained under the same conditions. After incubation time, enumeration of viable THP-1 cells were measured by immediate microscopic observation, after Trypan blue staining; using a KOVA counting cell (Hycor, VWR, Strasbourg, France). THP-1 cells were stained blue and no longer viable, which had damaged membranes allowed entry of the dye. Viability is expressed as the percentage of cells alive after contact with the conditioned media.

Statistically analysis

Data were statistically 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 tests at a *p*-value of 0.05 (Duncan 1955). The data were expressed as mean values of triplicates ± standard deviation.

Results

Selection and Identification of lactic acid bacteria (LAB)

A total of 14 lactic acid bacteria were isolated and selected from Thai-fermented foods and healthy animal feces. All isolates were preliminarily characterized based on their morphology, Gram staining, and catalase reaction. The results showed there were 2, 8, 3 and 1 isolates in coccus, rod, short rod and long rod shape, respectively. All isolates were Gram positive, while the catalase test result showed all isolates were negative. Non-hemolytic activity was found in all isolates. Results suggested that 14 isolates (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, R7-1) could be considered safe. All isolates were also screened by mimic tests to human intestinal barrier conditions and identified by 16S rRNA gene sequencing analysis.

The 14 isolates were identified based on 16S rRNA gene sequencing analysis. The results revealed that all were lactic acid bacteria (LAB) and they were belong to *Enterococcus*, *Companilactobacillus*,

Pediococcus, *Lacticaseibacillus*, *Levilactobacillus*, *Limosilactobacillus*, and *Lactiplantibacillus* genera, respectively. The 16S rRNA gene sequences were identified as a strain of *Enterococcus durans* (isolate M29-6), a strain of *Companilactobacillus farciminis* (isolate R7-1), a strain of *Pediococcus pentosaceus* (isolate AKB2.8), a strain of *Lacticaseibacillus zeae* (isolate M2/5), a strain of *Levilactobacillus brevis* (isolate MF62.2), a strain of *Limosilactobacillus fermentum* (isolate M1/2.1), two strains of *Limosilactobacillus reuteri* (isolates MF58.1 and MF67.1), a strain of *Lactiplantibacillus plantarum* (isolate AKM29.1), two strains of *Lactiplantibacillus paraplantarum* (isolates M26-10 and M26-20), and three strains of *Lactiplantibacillus pentosus* (isolates AKB4.1, M22-5 and R26-3) at different close similarity. Sample source and identification based on 16S rRNA gene sequences of isolates are summarized in Table 1 and Fig. 1. These 14 isolates were tested for further probiotic characterization.

Acid and bile tolerance Acid and bile salt tolerance

To consider probiotic properties, all isolates were tested for their survivability in human simulated gastrointestinal fluid (SGI) (pH 2) and 0.3% bile salt solution (pH 8). After incubation of each of the 14 isolates at SGI pH 2 and 0.3% bile salt solution pH 8 for 3 h, percentage of viable bacteria numbers were evaluated. Results indicated that all isolates showed more than 60% survival with tolerance to acid pH 2 and 0.3% bile salt solution pH 8 (Fig. 2). Survival rates of 14 isolates were higher than 70% in SGI (pH 2), while isolates No. M22-5 and AKM29.1 were sensitive to bile salt solution more than the others at 66 and 68% survival cell, respectively. Survival rates of 3 isolates, including M1/2.1, M2/5 and MF67.1 showed higher values than 90% viable cell after incubation in SGI (pH 2) and 0.3% bile salt solution (pH 8) for 3 h. The results suggested that these 14 isolates could successfully pass the human stomach and reach to the intestine with at least 60% of the amount of initial cells.

Antibiotic susceptibility

Isolated lactic acid bacteria should not contain antibiotic resistance gene that could be transferred to human pathogens. Antibiotic susceptibility was determined by the disc diffusion method. Antibiotic susceptibility of 14 isolated LAB were tested with 11 antibiotics. Testing of antibiotic susceptibility of the isolated LAB strains revealed that they are resistance to kanamycin (K; 30 µg), norfloxacin (NOR; 10 µg) and vancomycin (VA; 30 µg) and are susceptible to amoxicillin (AML; 10 µg), ampicillin (AMP; 10 µg), erythromycin (E; 15 µg), penicillin (P; 10 µg), chloramphenicol (C; 30 µg), clindamycin (DA; 2 µg), tetracycline (TE; 30 µg), and imipenem (IPM; 10 µg). Only *Pediococcus pentosaceus* AKB2.8 and *Enterococcus durans* M29-6 are susceptible to vancomycin (VA; 30 µg). Most of LAB species have antibiotic resistance on aminoglycosides group (kanamycin, streptomycin and gentamycin), quinolones group (norfloxacin, ciprofloxacin and nalidixic acid) and vancomycin. This specific pattern of resistance is considered as intrinsic factor which can not transfer this property to other microorganisms. (Ammor et al. 2008; Gueimonde et al. 2013) The important property of probiotic screening, the isolated LAB should not resistant on Ampicillin, Tetracycline, Penicillin G, Chloramphenicol, Amoxicillin and Erythromycin. Because of the resistant property to these antibiotics is considered as acquired resistant which can

transfer this gene or this property to intestinal pathogens (Abriouel et al. 2017). All 14 isolated LAB were sensitive to these antibiotics, therefore the results indicated that all isolates were safe (Table 2).

Table 2
Antibiotics susceptibility of isolates

Isolate no.	Antibiotic susceptibility										
	AML	AMP	E	P	C	DA	IPM	K	NOR	TE	VA
AKB2.8	S	S	S	S	S	S	S	R	R	S	S
AKB4.1	S	S	S	S	S	S	S	R	R	S	R
AKM29.1	S	S	S	S	S	S	S	R	R	S	R
M1/2.1	S	S	S	S	S	S	S	R	R	S	R
M2/5	S	S	S	S	S	S	S	R	R	S	R
M22-5	S	S	S	S	S	S	S	R	R	S	R
M26-10	S	S	S	S	S	S	S	R	R	S	R
M26-20	S	S	S	S	S	S	S	R	R	S	R
M29-6	S	S	S	S	S	S	S	R	R	S	S
MF58.1	S	S	S	S	S	S	S	R	R	S	R
MF62.2	S	S	S	S	S	S	S	R	R	S	R
MF67.1	S	S	S	S	S	S	S	R	R	S	R
R26-3	S	S	S	S	S	S	S	R	R	S	R
R7-1	S	S	S	S	S	S	S	R	R	S	R
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											

Antimicrobial activity

Antimicrobial activity of 14 strains against *Escherichia coli* TISTR 780 (Ec), *Listeria monocytogenes* TISTR 2196 (Lm), *Salmonella enteritidis* TISTR 2202 (Se), *Salmonella typhimurium* TISTR 292 (St) and *Staphylococcus aureus* TISTR 1466 (Sa) were shown in Table 3. All strains exhibited inhibitory activity against *L. monocytogenes* TISTR 2196, *S. enteritidis* TISTR 2202, and *S. typhimurium* TISTR 292. *Lactiplantibacillus plantarum* AKM29.1 showed highest potency against *S. typhimurium* TISTR 292 (Antibiotics Index > 2).

Table 3
Antimicrobial activity of isolates

Isolate no.	<i>Escherichia coli</i> TISTR 780	<i>Listeria monocytogenes</i> TISTR 2196	<i>Salmonella Enteritidis</i> TISTR 2202	<i>Salmonella Typhimurium</i> TISTR 292	<i>Staphylococcus aureus</i> TISTR 1466
AKB2.8, AKB4.1, M22-5	++	++	++	++	++
	++	+	++	+++	++
AKM29.1	+	++	++	++	++
M1/2.1	++	++	++	++	-
M2/5	++	++	++	++	+
M26-10	++	+	++	++	++
M26-20	-	+	+	+	-
M29-6, MF67.1	+	+	+	++	-
	+	+	++	+	+
MF58.1	+	+	+	++	+
MF62.2	++	++	+	+	++
R7-1					
R26-3					
Antibiotics Index 0, -; <1, +; <2, ++; <3, +++					

Bile salt hydrolase activity

The ability of probiotic strains to hydrolyze bile salts was also considered for health functional characteristic of probiotics selection. Bile salt hydrolase (BSH) activity helps bacteria to grow and colonize in the intestine by deconjugating bile salts (Begley et al. 2006). The isolate which has BSH activity will show precipitate around other colonies on MRS agar containing 0.5% TDCA. The BSH activity test showed that only 2 isolates (MF67.1 and R7-1) were BSH positive (Table 4). These strains were identified as closely to *Lactobacillus reuteri* and *Lactobacillus farciminis* at 99.66 and 99.56% similarity, respectively. According to the probiotics, LAB are Generally Recognized as Safe (Rolfe, 2000) and most commonly used because of their significant role to the disease prevention or reduction. These LAB isolates could exhibit BSH activity that may be useful to reduce serum cholesterol levels in the patient with hypercholesterolemia and also prevent hypercholesterolemia in normal people (Chae et al. 2013). However this probiotic properties of the isolates should be done for further study in *vivo*.

Table 4

Summary of potential probiotic strains on bile salt hydrolase activity, Caco-2 cell adhesion, and inhibition of TNF- α production.

Isolate no.	Bile salt hydrolase activity	Caco-2 cell adhesion (%)	TNF- α inhibition (%)
AKB2.8	-	94.00 $\pm$ 3.46 ^{a,b}	22.04 $\pm$ 0.46 ^e
AKB4.1	-	88.00 $\pm$ 2.00 ^{b,c}	19.05 $\pm$ 0.58 ^f
AKM29.1	-	100.00 $\pm$ 0.00 ^a	18.07 $\pm$ 0.25 ^g
M1/2.1	-	100.00 $\pm$ 0.00 ^a	-8.09 $\pm$ 0.10 ^k
M2/5	-	73.00 $\pm$ 1.73 ^d	29.09 $\pm$ 0.11 ^b
M22-5	-	86.00 $\pm$ 5.29 ^c	22.02 $\pm$ 0.53 ^e
M26-10	-	90.00 $\pm$ 4.00 ^{b,c}	25.05 $\pm$ 0.92 ^d
M26-20	-	90.00 $\pm$ 2.00 ^{b,c}	26.00 $\pm$ 0.05 ^c
M29-6	-	100.00 $\pm$ 6.08 ^a	6.03 $\pm$ 0.05 ^j
MF58.1	-	88.00 $\pm$ 1.41 ^{b,c}	13.07 $\pm$ 0.31 ^h
MF62.2	-	84.00 $\pm$ 3.61 ^c	-17.08 $\pm$ 0.14 ^l
MF67.1	+	96.00 $\pm$ 3.46 ^a	6.06 $\pm$ 0.10 ^j
R7-1	+	100.00 $\pm$ 0.00 ^a	7.02 $\pm$ 0.03 ⁱ
R26-3	-	87.00 $\pm$ 4.58 ^c	34.08 $\pm$ 0.33 ^a
Note: For Bile salt hydrolase assay: -, refers to no have BSH activity; +, refers to have BHS activity.			
For TNF- α inhibition assay; -, refers to reduced TNF- α production; +, refers to 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 descending order.			

Adhesion assay

Fourteen LAB showed adhesion rates between 73–100% (Table 4). Isolates AKM29.1, MF58.1, M22/5, and R7-1 showed the highest adhesion rate at 100% but MF62.2 showed the lowest adhesion rates at 73%. From the result suggested that the 14 strains had the ability for adhesion, establishment, and colonization within the gastrointestinal tract (GIT) and this increased their potential for survival. However, further co-culture with pathogens (for inhibition of pathogen adhesion testing) and studies in animal models are required.

TNF-inhibition

TNF- α inhibitory activity of 14 isolated LAB were investigated in THP-1 cells. TNF- α production was measured using the ELISA method. The results revealed that *Lactocaseibacillus zeae* M2/5 and *Lactiplantibacillus pentosus* R26-3 showed the highest values at 29 and 34% reduction from the control, respectively. *Limosilactobacillus fermentum* M1/2.1 and *Levilactobacillus brevis* MF62.2 was slightly stimulated TNF- α production by increasing 8 and 17% from the control, respectively (Table 4). Nevertheless, other isolates reduced TNF- α production at 6–26% from the control. The viability of THP-1 cell after exposure to the conditioned medium of all 14 strains was more than 80 percent.

Discussion

LAB are considered Generally Recognized as Safe (GRAS) and widely used in food, feed, dairy, and fermentation industries (FAO/WHO 2001). Fourteen isolates were selected from Thai-fermented foods and feces of healthy animal, which are also increasingly considered as reservoir uncharacterized probiotic strains (Ranadheera et al. 2010; Guantario et al. 2018). Morphological and 16S rRNA gene sequencing analysis revealed that all isolates were LAB, Gram positive, non-spore forming, and catalase negative. There were 13 isolates of *Lactobacillus* and 1 isolates of *Enterococcus*. Acidity and bile salt tolerance tests suggested that all LAB were more tolerant of SGI pH 2.0 than 0.3% bile salt solution, except for *Enterococcus durans* M29-6, *Levilactobacillus brevis* M62.2, and *Lactiplantibacillus pentosus* R26-3. The high GIT tolerance capabilities of the 14 strains were an important criterion in the selection of potential probiotics. Antimicrobial activity, antibiotics resistance, and potential adhesive properties of candidate probiotics were investigated for a reliable *in vitro* system. Results indicated that all 14 strains had the ability against both Gram positive and Gram negative pathogens and were susceptible to amoxicillin (AML), ampicillin (AMP), penicillin (P), chloramphenicol (C), clindamycin (DA), tetracycline (TE), imipenem (IPM) and erythromycin (E). Similarly, Sharma et al. (2017) found that LAB is usually sensitive to chloramphenicol, ampicillin, imipenem, meropenem, and erythromycin. Vancomycin was the first glycopeptide antibiotic used clinically, and this study showed that 12 isolates were resistant to vancomycin, similar to Tulini et al. (2013) and Zhang et al. (2016), while *Enterococcus durans* M29-6, *Pediococcus pentosaceus* AKB2.8 and *Lactobacillus farciminis* R7-1 were sensitive. Some strains of *Enterococcus* may possess virulence, especially strains that can display a high level of resistance to vancomycin (Shlaes et al. 1989; FAO/WHO 2001). If this resistance is present, transfer to other microorganisms may occur and this could enhance the pathogenesis of such recipients (Noble et al. 1992; Leclercq and Courvalin 1997). Thus, these results suggested that *Enterococcus durans* M29-6 and other strains can be considered safe. The Caco-2 cell line has been extensively used as a reliable *in vitro* system to study the adhesion capacity of candidate probiotics (Lievin-Le Moal et al. 2002). Results indicated that the 14 strains displayed a high level of adhesion at more than 70%. However, co-culture with pathogen strains and *in vivo* experiments are required for antagonistic assessment activity against pathogens. Furthermore, some beneficial bioactivities such as cholesterol reduction, pro-inflammatory cytokine suppression were evaluated. TNF- α suppression is important in alleviating inflammation in a

murine model of IBD (Pena et al. 2005), while bile salt hydrolase (BSH) activity is one of the mechanisms involved in cholesterol reduction by bacteria (Taranto et al. 1997). Results indicated that *Lactiplantibacillus pentosus* R26-3 gave the highest TNF- α inhibition in macrophages. Although lactic acid bacteria are 'Generally Recognized as Safe' (GRAS) Thus, this studies revealed that the 14 LAB had potential probiotic properties such as tolerance to SGI (pH 2.0) and 0.3% bile salt solution. These results investigated that they have been produced with antimicrobial capability against, antibiotics susceptibility, and high adherence to cell lines, with increasing interest for strain-specific activity on anti-allergic, enzyme inhibition, anti-hypertensive, lactose intolerance, antioxidant, anti-cancer activities. In addition, they can also reduce cholesterol, lipid, sugar or other metabolic substances reducing.

Conclusion

A total of 14 strains of bacteria were obtained from dairy, Thai-fermented foods, edible sources and feces animals. The absence of hemolysis was tested for resistance to biological barriers (acid and bile salts). Fourteen isolates with more than 70% survival rate in SGI and more than 60% survival in 0.3% bile salt solution were selected for further probiotics requirements. All isolates were lactic acid bacteria and had the ability against both Gram positive (*L. monocytogenes* TISTR 2196) and negative (*S. enteritidis* TISTR 2202 and *S. typhimurium* TISTR 292) pathogens. The antimicrobial assay showed that all isolates were susceptible to amoxicillin (AML), ampicillin (AMP), penicillin (P), chloramphenicol (C), clindamycin (DA), tetracycline (TE), and imipenem (IPM) that are commonly used in human and veterinary medicine. Furthermore, the 14 candidate probiotics showed good adhesive properties *in vitro* experiments (more than 70% adhesion). However, functional probiotic properties were specific to strain. The strains; *Limosilactobacillus reuteri* MF67.1 and *Companilactobacillus farciminis* R7-1 showed positive bile salt hydrolase activity (associated with cholesterol-lowering) while *Lactiplantibacillus pentosus* R26-3 and *Lactocaseibacillus zae* M2/5 displayed the highest TNF- α inhibition in macrophages (anti-inflammatory) showing 34 and 29% reduction from the control, respectively. The results indicated that the 14 isolates have probiotic properties and are suitable applications in functional foods and health supplements. However, *in vivo* studies are required to determine their efficacy before they can be incorporated in foods or dietary supplements in further study.

Declarations

Acknowledgements This research was supported by grants from the National Research Council of Thailand (NRCT). We express our thanks to the Biodiversity Research Center, Thailand Institute of Scientific and Technology Research (TISTR) for providing laboratory and instruments. Also many thanks to all my colleagues, especially to Miss Kamonsri Nuankhum, Miss Ratthanattha Nuwha, Dr. Porntipha Vitheejongjaroen and Dr. Boonyarut Ladda who work as lab assistants in some part of these experiments.

Funding Information This work was supported by the Ministry of Science and Technology Scholarship of Thailand.

Availability of data and material Not applicable

Conflicts of interest All authors declare that they have no conflicts interest.

Ethics approval and consent to participate This article does not contain any studies performed by any of the authors with humans or animals participants.

Consent for publication Not applicable

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Figures

Figure 1

Neighbor-joining tree of the representative isolates based on the 16S rRNA gene sequences. Bootstrap values are showed as percentages of 1,000 replications; only values >50% are indicated. Bar 0.01 substitutions per nucleotide position.

Figure 2

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