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Research Article

Screening and identification of potential probiotic bacteria with cholesterol-lowering property from fermented food products

Sirikhwan Tinrat*, Onnicha Jiraprasertwong

Department of Biotechnology, Faculty of Applied science, King Mongkut's University of Technology North Bangkok, Bangkok, Thailand

ABSTRACT

Hypercholesterolemia is a major risk factor for cardiovascular diseases. The use of probiotic lactic acid bacteria (LAB) from fermented food products with cholesterol-lowering abilities has emerged as a promising dietary strategy for its management. *Lactiplantibacillus plantarum* SM14-2STR and *Weissella cibaria* SE8-2STR were screened and identified by 16S rRNA gene sequencing and were selected for assessment of their probiotic and safety properties, with a focus on their potential antimicrobial activity. Both LAB strains exhibited probiotic potential, sensitivity to multiple antibiotics and γ-hemolytic activity. Moreover, the two selected strains demonstrated remarkable bacteriostatic activity against various pathogenic bacteria (6 out of 9 pathogenic strains) and were capable of surviving under high bile salt concentration (0.3–1%) and low pH conditions (2.0–3.0). *L. plantarum* SM14-2STR significantly exhibited high cell surface hydrophobicity (~74%) and strong auto-aggregation abilities (~61%). It effectively inhibited the adhesion of *Proteus mirabilis* DMST 8212 (~60%), whereas *W. cibaria* SE8-2STR interfered with the growth of *Salmonella enterica* ATCC 13312 (~61%). Notably, *L. plantarum* SM14-4STR (84.07±0.89%) and *W. cibaria* SE8-2STR (39.24±1.19%) significantly showed the highest cholesterol and triglyceride removal capacity in culture media containing 0.3% bile salt ($p \leq 0.05$), and both strains exhibited bile salt hydrolase (BSH) activity towards sodium taurocholate. *L. plantarum* SM14-2STR also significantly inhibited cholesterol uptake in colon epithelial cells by 28.44±0.28%, followed by *L. plantarum* SM16-4STR at 25.95±0.58% ($p \leq 0.05$). These findings indicated that LAB strains derived from fermented meat and Isan sausage may serve as promising probiotics with cholesterol-lowering potential, suitable for applications in the food and health industries.

Keywords: Probiotics; *Lactiplantibacillus plantarum* and *Weissella cibaria*; Fermented foods; Cholesterol-lowering capacity; Bile salt hydrolase

1. INTRODUCTION

Cholesterol is an important substance in the human body, but longstanding elevated levels of blood cholesterol can lead to atherosclerosis and may increase the risk of cardiovascular diseases (CVDs). Hypercholesterolemia (the accumulation of cholesterol in the blood) is a major risk factor for cardiovascular disease and heart attack. The World Health Organization (WHO) reports that cardiovascular disease is responsible for 30% of deaths worldwide, and it is predicted to remain the leading cause of death over the next two decades.

The WHO also states that a diet high in saturated fat, trans fat, cholesterol and salt, and low in fruits, vegetables and fish is unhealthy and increases the risk of cardiovascular disease[1]. Although there are drugs available to lower cholesterol levels, unwanted side

*Corresponding author:
* Sirikhwan Tinrat Email: sirikhwan.t@sci.kmutnb.ac.th

effects can occur in some cases, such as gastrointestinal discomfort. Therefore, finding new approaches to reduce cholesterol levels without relying on medication has gained increasing attention, especially the use of probiotic microorganisms.

Probiotics are "live microorganisms that when administered in adequate amounts confer a beneficial health effect on the host"[2]. Nowadays, probiotics play an important role as a health-promoting food for humans and animals. They are considered safe and can provide an effective barrier against microbial infections[3]. The selection of probiotic microorganisms should be based on the following criteria: non-pathogenic and toxic, resistant to gastrointestinal enzymes, ability to adhere to intestinal mucosal cells, auto- and co-aggregative activities, and non-resistant to antibiotics. Over the years, many studies have investigated the probiotic activities of lactic acid bacteria (LAB), including their roles in improving lactose intolerance, increasing natural resistance to gastrointestinal infections, suppressing cancer and improving digestion[4]. Fermented foods are a good source of lactic acid bacteria as probiotics. Traditional Thai fermented foods are made from meat, fish, beans, vegetables and fruits through a natural fermentation process to preserve and enhance the flavor of the food. These foods are rich in bioactive compounds and probiotics that provide various health benefits. Food-associated lactic acid bacteria (*Lactiplantibacillus plantarum*, *Lacticaseibacillus casei*, *Weissella cibaria,* etc.) have showed great potentials to directly affect host health[5]. Many studies have been conducted to investigate the cholesterol- and triglyceride-lowering effects of LAB probiotics[6]. The isolation of microorganisms from fermented foods, especially naturally fermented foods, is an important approach to obtain novel potential probiotic LAB strains with specific properties for industrial use. To obtain health benefits, a potential probiotic must tolerate low pH and bile salts, must adhere to epithelial cells and colonize the gastrointestinal (GI) tract of human. Antimicrobial activity against pathogenic microorganisms in the GI tract is also a key functional requirement of probiotic strains[5-6].

Lactobacillaceae are well-characterized probiotic bacteria that has shown promising cholesterol-lowering abilities both in *vitro* and in animal/clinical studies[7-8]. *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) had shown to reduce total cholesterol (TC) and LDLc in experimental animal studies[9] and assimilated cholesterol directly from the medium[10]. Few studies have reported the evaluation of *Weissella* strains as potential probiotics[11-12]. Probiotic potential of *W. confusa* MD1 and *W. cibaria* MD2 from fermented batter had showed high bile salt hydrolase activity and reduction in the cholesterol in medium[12]. Several mechanisms have been proposed for cholesterol removal with probiotics, such as co-precipitation of cholesterol with deconjugated bile salts, deconjugation of bile salts via bile-salt hydrolase (BSH) activity, assimilation of cholesterol and production of short-chain fatty acids (SCFAs) during the growth of probiotics[13]. Many probiotic microorganisms are capable of producing the enzyme bile salt hydrolase (BSH)[7-8, 12], which catalyzes deconjugation of bile salts linked to glycine or taurine[14]. However, the triglyceride-lowering ability of LAB had not been widely reported. Moreover, relatively few studies on isolated LAB strains from fermented foods, including fermented meat and vegetables. From the aforementioned, the current study aimed to screen, identify and assess potential cholesterol- and triglyceride- lowering probiotics from various fermented food products for the development of functional probiotics.

2. MATERIALS AND METHODS

2.1 Collection of Sample and Bacterial Isolation

Twenty samples of various fermented products (Pickled cabbage, kimchi, pork sausage, Isan sausage) were collected from local markets in around the Central and Northeastern part of Thailand, specifically in Bangkok (n = 8; Pickled cabbage or *Phak-gard-dong* (PC1-4) and kimchi (KC1-4) and Khon Kaen provinces (n = 8; Spicy fermented meat or *Mum* (SM1-4) and Isan sausage or *Sai-krok Isan* (SE1-4) provinces. All samples were purchased from local markets and were transported to the laboratory in ice box under approximately 4°C. In brief, the samples were serially diluted in sterile 0.85% NaCl solution and were spread on MRS (Himedia, Mumbai, India) agar supplement with 1% (w/v) calcium carbonate and 0.04% (w/v) bromocresol purple (MRS-CB). After incubation at 37°C for 24–48 h under anaerobic condition using an anaerobic jar (BBL, Gas Pack System), the typical colonies were randomly selected and purified. The colonies of acid-producing bacteria on MRS-CB agar were identified and selected by the presence of a yellow zone around each colony. Only Gram-positive and catalase-negative strains were taken as presumptive lactic acid bacteria (LAB) and stored at 4°C in MRS agar plate. The working cultures were kept at 5°C on agar plate for further studies. The pure cultures were maintained in broth with 30% (v/v) glycerol at -20 °C.

2.2 Preliminary identification of LAB

The LAB isolates were initially characterized using cell morphology on agar medium and under

microscopy. Morphological characters (color, shape, margin, elevation and surface) of single colony were observed after growth on MRS agar at 37°C for 24 h under anaerobic condition using an anaerobic jar (BBL, Gas Pack System). The initial identification as lactic acid bacteria based on the Gram-positive and catalase-negative strains.

2.3 Preliminary screening of LAB for their antibacterial activity

The antagonistic effect of LAB was tested with the drop plate technique according to Tinrat and Jiraprasertwong[15] using *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 as indicator strains. In brief, 5 µL of 24–48 h isolated cultures (10^8 CFU/mL; A_{600}=0.5) were spotted on surface of Brain Heart Infusion (BHI, Himedia, Mumbai, India) agar plates containing overnight indicator strains (10^8 CFU/mL; A_{600}=0.5) by spreading. After incubation at 37 °C for 24 h, the isolated strains showing inhibition zones surrounding the bacterial spots (inhibition zone of ≥1 mm) were further studied with the well-diffusion assay. The halos of more than 3 mm were considered positive.

2.4 Antimicrobial assay of isolated strains

The bacteriostatic test was carried out agar well diffusion method against nine pathogenic strains (*Bacillus cereus* DMST 5040, *Enterococcus faecalis* DMST 4736, *Escherichia coli* ATCC 35218, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* DMST 8212, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 13312, *Salmonella* Typhimurium ATCC 13311 and *Staphylococcus aureus* ATCC 10823). In brief, the inoculated BHI plates (1.5% Agar) were covered with 5 mL of soft BHI agar (0.75% agar) containing overnight cultures of indicator strains (A_{600} = 0.5, 10^8 CFU/mL). After waiting for 10–15 min, each well with a diameter of 6 mm was formed on the plate. Then, the the cell-free supernatants (CFSs) of 24-h isolated cultures (100 µL) were added into the wells. The inhibition zones (IZ) were measured after incubation at 37°C for 24 h. Ampicillin (10 µg) was used as positive control[15].

2.5 Phenotypic characterization of LAB isolates

The promising LAB isolates were preliminarily identified using in Bergey's manual of systematic bacteriology basing on cell morphology (shape, arrangement, and Gram reaction) and various biochemical tests (Fermentation patterns with API 20 kits (Biomerieux SA, France), oxidase test, etc.) according to standard procedures.

2.6 Molecular Identification of LAB isolates

The genomic DNA of selected LAB isolates was extracted by Genomic DNA purification kit (QIAGEN, USA) following the manufacturer's protocol. Each genomic DNA was used as the template with the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′) (Macrogen, Seoul, Korea) for the 16S rRNA gene amplification[1] by polymerase chain reaction (PCR) with thermal cycle (Eppendrof, Germany)[16]. Thermal cycling conditions are as follows: initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 60 s, extension at 72°C for 60 s and the final extension at 72°C for 7 min. After amplification, the PCR products were purified using regenerated or fresh columns and buffers from commercial QIAquick PCR purification kit (QIAGEN, USA) and then were quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher) and analyzed by electrophoresis (Bio-rad, USA) in 1.0% agarose (Sigma-Aldrich, USA). The nucleotide sequencing of 16S rRNA was carried out by an automated sequencing. Eventually, sequence homology was aligned and analyzed by using the ClustalX program and BLAST program available online at http://www.ncbi.nlm.nih.gov/genbank for bacterial identification (considering of % homology and GenBank accession number).

2.7 Safety issues assessment

2.7.1 Antibiotic Resistance

The antibiotic resistance profile of potential probiotic LAB was investigated by the agar diffusion assay with ten antibiotics, including ampicillin (Amp; 10 µg), penicillin (P; 10 µg), gentamycin (CN: 10 µg), streptomycin (S; 10 µg), erythromycin (E; 15 µg), chloramphenicol (C; 30 µg), tetracycline (TE; 30 µg), clindamycin (CD; 2 µg), norfloxacin (NOR; 10 µg) and ciprofloxacin (CIP; 5 µg). After incubation at 37 °C for 18–24 h, the diameter of the inhibition zones was measured and then compared with the breakpoint values of CLSI for *S. aureus* to categorize the bacterial isolates as susceptible or resistant to the tested antibiotic[17]. The minimum inhibitory concentrations (MICs) of different antibiotics were determined using broth microdilution method according to the protocol ISO 10932:2010. Briefly, the isolated LAB was inoculated in 96 microwell plates containing serial two-fold dilutions of the following antibiotics (ampicillin, penicillin, gentamycin, streptomycin, erythromycin, chloramphenicol, tetracycline and clindamycin). After incubation, the calculated MIC values were compared with antibiotic breakpoint specified by EFSA[18] in *L. plantarum*.

2.7.2 β-Haemolysis Test

Haemolytic reaction was assessed onto a plate containing Tryptic Soy Agar (TSA; Himedia, Mumbai, India) containing 5% sheep blood. Each LAB isolate was streaked on the agar and incubated at 37°C for 48 h. The reaction was read by placing the plates against the light to evaluate the presence of lysis zone surrounding colonies which expressed according to the type of hemolysis including α-haemolysis (green halos around the colonies), β-haemolysis (clear halos around the colonies) or γ-haemolysis (without halos)[19].

2.8 Tolerance to acids and bile salts

The overnight cultures of LAB isolates under anaerobic conditions were centrifuged at 5,000×g, 15 min, 4°C. The obtained pellets were washed twice in phosphate buffered saline (PBS; pH 7.2) and then re-suspended in acidified MRS broth with pH values of 2.0, 3.0, 4.0, 5.0 (assay) and 7.0 (control) at 10^8 CFU/mL (1% v/v; A_{600} = 0.5). For bile salt tolerance ability, the cultures of lactic acid bacterial strains were assessed by monitoring growth in presence of MRS broth supplemented with 0.00% (control), 0.30%, 0.60% and 1.00% bile salt (Oxoid, UK, LP0055)) and incubated at 37°C. The viable cell counts (CFU/mL) for acid and bile salt tolerance abilities were evaluated at an interval of 0, 3, 6 12, 24, 28, 32 and 36 h on MRS agar[15]. The survival rate was calculated using the following;

$$\text{Survival rate (\%)} = (\text{Log N}/\text{Log N}_0) \times 100$$

where Log N_0 and Log N are the logarithm of the number of viable cells before and after exposure to the test condition, respectively.

2.9 Cell surface hydrophobicity assay

The cell surface hydrophobicity (H%) of lactic acid bacterial strains was determined by bacterial adhesion to hydrocarbon (hexadecane). After centrifuging the isolated strains (5,000×g, 15 min), the bacterial cells were washed with twice with phosphate buffer solution (PBS; pH 7.2). Pallet cells were diluted until reaching an absorbance value of 0.9–1.0 at 600 nm (A_1). After that, 1 mL of hexadecane (Merck, Germany) was slowly added on the bacterial suspension (3.0 mL). The mixture solution was vortexed to the emulsion formation for 3 min. The aqueous phase was separated and determined by spectrophotometer at 600 nm after incubation at room temperature for 30 min (A_2). The percent hydrophobicity was calculated as follows:

$$\text{Cell surface hydrophobicity (\%)} = [(A_1 - A_2)/A_1] \times 100$$

The affinity of probiotic to solvent was classified, following hydrophilic (<10%), moderately hydrophilic (10–34%), moderately hydrophobic (35–70%) or highly hydrophobic (71–100%)[20].

2.10 Auto-aggregation assay

Ability of auto-aggregation of bacterial strains was assessed by re-suspending the pellet cells and diluting in PBS (pH 7.0) to reach the absorbance value of 0.9–1.0 at 600 nm (initial absorbance; A_0). After that, bacterial suspension was vortexed for 10 s and the absorbance (600 nm) was measured after incubation for 24 h at room temperature in the upper suspension (A_1)[21]. The percentage of auto-aggregation was calculated as follows:

$$\text{Auto-aggregation\%} = [1 - (A_1/A_0)] \times 100$$

2.11 Co-aggregation assay

The co-aggregation potential of candidate probiotic was evaluated using nine pathogenic strains as the mixed strain as described by de Oliveira Coelho et al.[21]. In brief, the absorbance values of candidate probiotic (A_x) and pathogenic bacteria (A_y) suspensions were separately measured at 600 nm. After blending the suspensions for 30 s, the homogenous mixture was incubated at room temperature for 24 h without agitation and the absorbance value of the mixture solution was read at 600 nm ($A_{(x+y)}$). Finally, co-aggregation was calculated as follows;

$$\text{Co-aggregation (\%)} = \{[((A_x+A_y)/2)) - A_{(x+y)}]/[A_x+A_y)/2]\} \times 100$$

2.12 Adhesion to Caco-2 cell lines

The adhesion ability of the isolated LAB strains was assessed on Caco-2 cell lines. Caco-2 cells (ATCC®HTB-37TM, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS; JR Sciencific, CA) and 1% penicillin-streptomycin (JR Sciencific, CA) in the CO_2 incubator at 37°C with 5% CO_2. Cells CFU/mL) were then seeded into a in 24-well culture plates (SPL Life Science, South Korea) at a density of 2×10^5 cells per well. Next, overnight cultures (10^8 CFU/mL) pathogenic strains (0.5 mL) re-suspended in DMEM medium without serum and antibiotics were added to the Caco-2 monolayer. The monolayer of Caco-2 cells was washed twice with PBS buffer (pH 7.2) after 3 h-incubation to remove non-adhering bacteria and then lysed by Triton-X100 solution (0.1% v/v in PBS). The number of adherent LAB isolates was assessed using the plate count method

on MRS agar. The percentage adhesion of the potential probiotic LAB strains was calculated as followed;

Adhesion capacity (%) = [Adherent cells (log CFU/mL)/Initial cells (log CFU/mL)]×100

Adhesion degrees were classified as non-adhesive (NA: 0–10%), weakly adhesive (W: 10–20%), moderately adhesive (M: 20–50%) and strongly adhesive (S: >50%)[22].

2.13 Anti-adhesion activity

For competition assay, equal quantities of both bacteria (probiotic LAB and pathogenic strains (10^8 CFU/mL)) were simultaneously placed and co-incubated with Caco-2 monolayer in the wells. After incubation at 37°C for 3 h, the unattached bacteria were removed by washing with sterile PBS (pH 7.2) and adherent bacteria (both probiotic and pathogenic strains) was released using Triton-X100 solution (0.1% v/v in PBS). Adherent bacteria were counted by plate count method on MRS agar for LAB isolates and on selective/differential medium (Himedia, Mumbai, India) for pathogen. A specific culture medium was utilized to identify the pathogenic bacteria[15]. The percentage competition of adhesion between the two species was determined as follows;

Competition (%) = (Adhered pathogen combined with LAB /Bounded pathogen in the absence of LAB)×100

2.14 Bile salt hydrolase (BSH) activity

24-h culture of selected LAB strains was spotted on surface of MRS agar-containing 0.37 g/L $CaCl_2$ (MRS-TCA agar) and 0.3% (w/v) taurocholic acid sodium salt (TCA) (Sigma, USA) (MRS-TCA). All plates were then incubated at 37℃ for 48–72 h under anaerobic condition using an anaerobic jar (BBL, Gas Pack System). Finally, the diameter of the precipitation zones was measured. The presence of the precipitated hydrolyzed product of bile salts in or around the colony growth (white opaque) were recorded as indication of BSH activity and the diameter of the precipitation zones was measured[23].

2.15 Cholesterol-lowering activity

In vitro lowering-cholesterol capacity of the lactic acid bacterial strains were performed as according to Iranmanesh et al.[24] with minor modification. In brief, the selected strains were inoculated in MRS supplemented with 0.3% bile salt (bovine cholic acid (Sigma-Aldrich, USA)) + 0.25 g/L cholesterol (Sigma-Aldrich, USA); pH 5.0) (MRS-CHOL) and anaerobically incubated at 37°C for 24 h. After that, 0.2 mL of culture medium and 4.8 mL of anhydrous ethanol were shaken to mix and then set for 5 min. Bacterial cells removed by centrifuging at 3,700×g for 15 min. The remaining cholesterol in broth of was determined by ammonium ferric sulphate method in absorbance at 560 nm. The uninoculated MRS−CHOL medium was used as a control. The percentage of cholesterol assimilation was determined as follows;

Cholesterol assimilation (%) = [(Cholesterol in control – Cholesterol in sample)/Cholesterol in control] ×100

2.16 Triglyceride-lowering activity

Similarly, the triglyceride-Lowering ability of the LAB isolates was assessed in MRS supplemented with 0.3% bile salt (bovine cholic acid (Sigma-Aldrich, USA) + 0.25 g/L triglyceride (Sigma-Aldrich, USA); pH 5.0) (MRS-TRIG) as described by the Iranmanesh et al.[24] with minor modification. The triglycerides degradation was determined using Triglyceride Determination Kit (Sigma-Aldrich, USA; TR0100) at a wavelength of 510 nm. The percentage of triglyceride assimilation was expressed as follows:

Triglyceride assimilation (%) = [(Triglyceride in control – Triglyceride in sample)/ Triglyceride in control] ×100

2.17 Preparation of cholesterol micelles

The cholesterol micelles (1.650 mmol/L sodium taurocholate, 0.250 mmol/L oleic acid, 0.125 mmol/L monoolein and 0.500 mM cholesterol (Sigma-Aldrich, USA)) were prepared and solvents were evaporated under nitrogen gas. The deposit was resuspended in DMEM and sonicated for 1 h. The mixed solution (100 µL mixing sample + 2.0 mL o-phthalaldehyde reagent (0.5 mg/mL) + 1.0 mL concentrated H_2SO_4 were measured cholesterol content before incubating at 25°C for 10 min by a spectrophotometer at wavelength 570 nm. The cholesterol standard calibration curve was generated with concentrations of 0–0.500 mmol/L of cholesterol micellar solution[25].

2.18 Inhibition of cholesterol uptake by Caco-2 cells

Caco-2 cells were used to determine the efficiency of selected LAB strains in cholesterol uptake. Caco-2 cells (2×10^5 cells/well) were sub-cultured into 24-well plates and were incubated for 48 h at 37°C and 5% CO_2. The pellets of bacterial strain were washed with PBS solution (pH 7.2) after centrifugation at 3,700×g for 15 min and

resuspended in 10 mL of DMEM (Sigma-Aldrich, USA). 500 µL of bacterial suspensions (10^8 CFU/mL) were added into 24-well plates containing Caco-2 cells (2×10^5 cells/well). DMEM was added as a control. Cholesterol (0.5 mmol/L) micelles were added to the Caco-2 monolayer and incubated for 6 h at 37°C and 5% CO_2. Finally, the cell-supernatant containing the cholesterol micelles was collected for measurement the remaining cholesterol content according to the above method[25].

2.19 Statistical analysis

All data were expressed as mean ± standard deviation (SD) of triplicate experiments. Significant one-way ANOVA results (level $p \leq 0.05$) were followed up with Tukey's multiple comparisons test and/or paired t-tests in all experiments. Graphical representation was generated using GraphPad Prism 10 (GraphPad Software, USA).

3. RESULTS AND DISCUSSION

3.1 Isolation and preliminary screening of probiotic properties

From the 84 isolates of acid-producing bacteria (yellow zone around each colony) obtained from sixteen fermented food products cultured on MRS-CB agar under anaerobic conditions, a total of 31 isolates (36.90%) exhibited lactic acid bacteria characteristics that were gram-positive bacteria, catalase-negative and lactose-fermenting. Antimicrobial activity is an important criterion for evaluating candidate bacteria for use as probiotics. Only 22 isolates exhibited preliminary inhibitory activity against *E. coli* ATCC 23522 and/or *S. aureus* ATCC 25923 by agar spot method. To assess the antagonistic activity of isolated LAB strains, the CFSs of these isolates were tested bacteriostatic activity against nine pathogenic strains by agar well diffusion. As shown in Table 1, all LAB isolates demonstrated antagonistic activity against all tested bacterial pathogens, except *E. faecalis* DMST 4736 and *S.* Typhimurium ATCC 13311. Two LAB isolates, SM14-2STR and SE8-2STR, exhibited a broad antibacterial spectrum against both Gram-positive and Gram-negative bacteria. These isolates showed remarkable antibacterial activity against 6 out of 9 pathogenic bacteria. Both LAB isolates inhibited *P. mirabilis* DMST 8212 with inhibition zone (IZ) of 14.00±0.00 and 11.00±0.00 mm, respectively. In addition, SM14-2STR and SE8-2STR strains significantly exhibited strong antagonistic activity against *K. pneumoniae* ATCC 35657 (IZ =13.67±0.58 mm) and *P. aeruginosa* ATCC 27853 (IZ =16.00±0.00 mm), respectively ($p \leq 0.05$). The antimicrobial activity

Table 1 Antimicrobial activity of isolated bacteria by agar well diffusion assay

Isolated Strains	Inhibitory Zone (∅ = 6 mm; mm±SD)								
	Gram positive strains			Gram negative strains					
	BC	SA	EF	EC	KP	PM	PA	SE	ST
SM5-1STR	-	8.00 ± 0.00^{bB}	-	8.00 ± 0.00^{aB}	-	10.00 ± 0.00^{cA}	-	-	-
SM9-1STR	-	7.00 ± 0.00^{cB}	-	8.00 ± 0.00^{aB}	-	10.00 ± 0.00^{cA}	-	-	-
SM10-2STR	-	7.00 ± 0.00^{cB}	-	-	-	10.67 ± 0.58^{bcA}	-	-	-
SM14-2STR	8.00 ± 0.00^{bC}	10.00 ± 0.00^{aB}	-	8.00 ± 0.00^{aC}	13.67 ± 0.58^{bA}	-	8.00 ± 0.00^{bC}	7.00 ± 0.00^{cD}	-
SE4-1STR	-	-	-	-	8.00 ± 0.00^{fA}	-	-	-	-
SE5-2STR	-	-	-	-	-	-	-	-	-
SE8-2STR	-	8.00 ± 0.00^{bC}	-	8.00 ± 0.00^{aC}	11.00 ± 0.00^{cB}	11.00 ± 0.00^{bB}	16.00 ± 0.00^{aA}	8.00 ± 0.00^{dC}	-
SE9-2STR	-	-	-	-	8.00 ± 0.00^{fA}	-	-	-	-
SE11-3STR	-	-	-	-	10.00 ± 0.00^{dA}	-	-	-	-
SE12-3STR	-	-	-	-	9.00 ± 0.00^{eA}	7.67 ± 0.58^{cB}	-	-	-
SE13-3STR	-	8.00 ± 0.00^{bC}	-		11.00 ± 0.00^{cB}	10.33 ± 0.58^{bcB}	12.33 ± 0.58^{aA}		-
SE14-3STR	-	8.00 ± 0.00^{bB}	-	-	-	-	-	9.00 ± 0.00^{cA}	-
SE15-4STR	-		-	-	15.00 ± 0.00^{aA}	-	-	-	-
SE16-4STR	-	7.00 ± 0.00^{cA}	-	-		-	-	-	-
KC5-1STR	-		-	-	7.00 ± 0.00^{gB}	-	-	11.00 ± 0.00^{bA}	-
KC6-1STR	-		-	-	7.00 ± 0.00^{gA}	-	-		-
KC8-2STR	9.00 ± 0.00^{aC}	7.00 ± 0.00^{cD}	-	-	10.67 ± 0.58^{cdB}	-	-	13.00 ± 0.00^{aA}	-
KC10-3STR	-	7.00 ± 0.00^{cA}	-	-	7.00 ± 0.00^{gA}	-	-	-	-
KC13-3STR	-	7.00 ± 0.00^{cA}	-	-	7.00 ± 0.00^{gA}	-	-	-	-
PV5-1STR	-	8.00 ± 0.00^{bA}	-	-	-	-	-	-	-
PV9-1STR	-	7.00 ± 0.00^{cB}	-	-	-	10.00 ± 0.00^{cA}	-	-	-
PV10-1STR	-	7.00 ± 0.00^{cB}	-	-	-	10.67 ± 0.58^{bcA}	-	-	-

- = Resistance; ^{abcde} = Values on the same column with different superscripts were significantly different ($p \leq 0.05$); ^{ABCD} = Values on the same row with different superscripts were significantly different ($p \leq 0.05$) BC = *B. cereus* DMST 5040, SA = *S. aureus* ATCC 10832, EF = *E. faecalis* DMST 4736, EC = *E. coli* ATCC 35218, KP = *K. pneumoniae* ATCC 35657, PM = *P. mirabilis* DMST 8212, PA = *P. aeruginosa* ATCC 27853, SE = *S. enterica* ATCC 13312, ST = *S.* Typhimurium ATCC 13311; IZ of Ampicillin against pathogenic bacteria (BC = 8.33±1.15 mm, SA = 40.33±1.15 mm, EF = 31.33±0.58 mm, KP = 10.00±0.00 mm, PM = 27.00±0.00 mm, PA = 25.67±0.58 mm, SE = 25.67±0.58 mm, ST = 28.67±1.15 mm, EC = Resistance)

Table 2. Phenotypic and genotypic characteristics of isolated strain from fermented food products

Character	Observations	
	SM14-2STR	SE8-2STR
Phenotypic characteristics		
Colony morphology / Shape	Circular, cream/Rod	Circular, cream/ Coco-bacilli
Gram stain	Positive	Positive
Cell arrangement	Single and Group	Single and Group
Motility test	-	-
Biochemical Tests		
Haemolytic activity	γ-haemolysis	γ-haemolysis
Catalase	-	-
Oxidase	-	-
Fermentation		
- Glucose	+	+
- Fructose	+	+
- Sucrose	+	+
- Lactose	+	+
- Arabinose	+	+
- Mannitol	+	+
- Sorbitol	+	+
Growth temperature range (°C)	25–55 °C (optimum = 37°C)	25–55 °C (optimum = 37°C)
Growth pH range	3–5 (optimum = 5)	3–5 (optimum = 6)
Genotypic characteristics		
Accession No.	NR_113338.1	NR 036924.1
Phylum/Class/Family	Firmicutes/Bacilli /*Lactobacillaceae*	Firmicutes/Bacilli / *Leuconostocaceae*
Species	*Lactiplantibacillus plantarum* NBRC15891	*Weissella cibaria* IH-59
% identity	99	99

+ = positive; - = negative

observed in LAB is attributed to the production of organic acids such as lactic acid and acetic acids, as well as hydrogen peroxide (H_2O_2) and bacteriocin, which inhibit pathogenic microorganisms and remain active under gastrointestinal conditions[3, 6, 12]. These positive outcomes suggested that SM14-2STR and SE8-2STR isolates (obtained from Two Brothers Shop) are suitable candidates for further identification and evaluation as potential probiotic. However, in *vitro* antibacterial activity does not necessarily guarantee in *vivo* efficacy. The gastrointestinal environment is highly dynamic and involves complex interactions between microbiota and host cells. Therefore, further validation of these strains in animal models or simulated gastrointestinal systems is required to assess their survival, colonization, immunomodulatory effects, and pathogen inhibition under physiological conditions. In this study, in *vitro* gastrointestinal model assessments, including acid and bile salt tolerance tests as well as evaluation of cell surface hydrophobicity were performed as a preliminary approach to evaluate their probiotic properties. Subsequently, studies in Caco-2 monolayers were conducted to assess their ability to adhere to intestinal epithelial cells and to compete with pathogens for adhesion.

3.2 Identification of probiotic LAB strains

The phenotypic and genotypic characteristics of isolated LAB strains from fermented food products are shown in Table 2. All LAB isolates formed opaque, smooth, circular, entire and a diameter of 0.5–1.0 mm after 24 h of incubation on MRS agar (Figure 1a-b). These strains were gram-positive, rod-shape (Fig. 1a-c), catalase-negative and oxidase-negative. Based on 16S rRNA gene sequencing (~1,400 bp) using the BLAST program, the potential probiotic LAB isolates were identified as members of families *Lactobacillaceae* and *Leuconostocaceae.* The 16s rRNA sequence of isolated MS14-2STR showed 99% similarity to *Lactiplantibacillus plantarum* NBRC15891 (Accession No. NR_113338.1), while the SE8-2STR strain was closely related to *Weissella cibaria* IH-59 (Accession No. NR 036924.1). Thus, the two investigated LAB strains were *Lactiplantibacillus plantarum* SM14-2STR and *Weissella cibaria* SE8-2STR. Both LAB isolates were able to utilize glucose, fructose, sucrose, lactose, arabinose, mannitol and sorbitol. They also grew at temperature ranging from 25–55°C (37°C of optimum growth) and pH values between 3–5 (an optimum pH = 5) (Table 2). *L. plantarum* SM14-2STR strain was isolated from fermented meat (*Mum*), while *W. cibaria* SE8-2STR was isolated from Isan sausage (*Sai-krok Isan*). Techo et al. 2022[26] showed that *Mum* is an important source of *E. faecalis* MP1-3, *Lactiplantibacillus pentosus* MB1-1 and *Lactobacillus amylovorus* MB1-5, while *L. plantarum* (NP4-2 and NP1-4) were isolated from *Nham*. Tanasupawat et al. 2015[27] reported that *W. cibaria* SS55-3 was isolated from fermented meat (*Sai-krog-prieo*), which is also considered an important source of

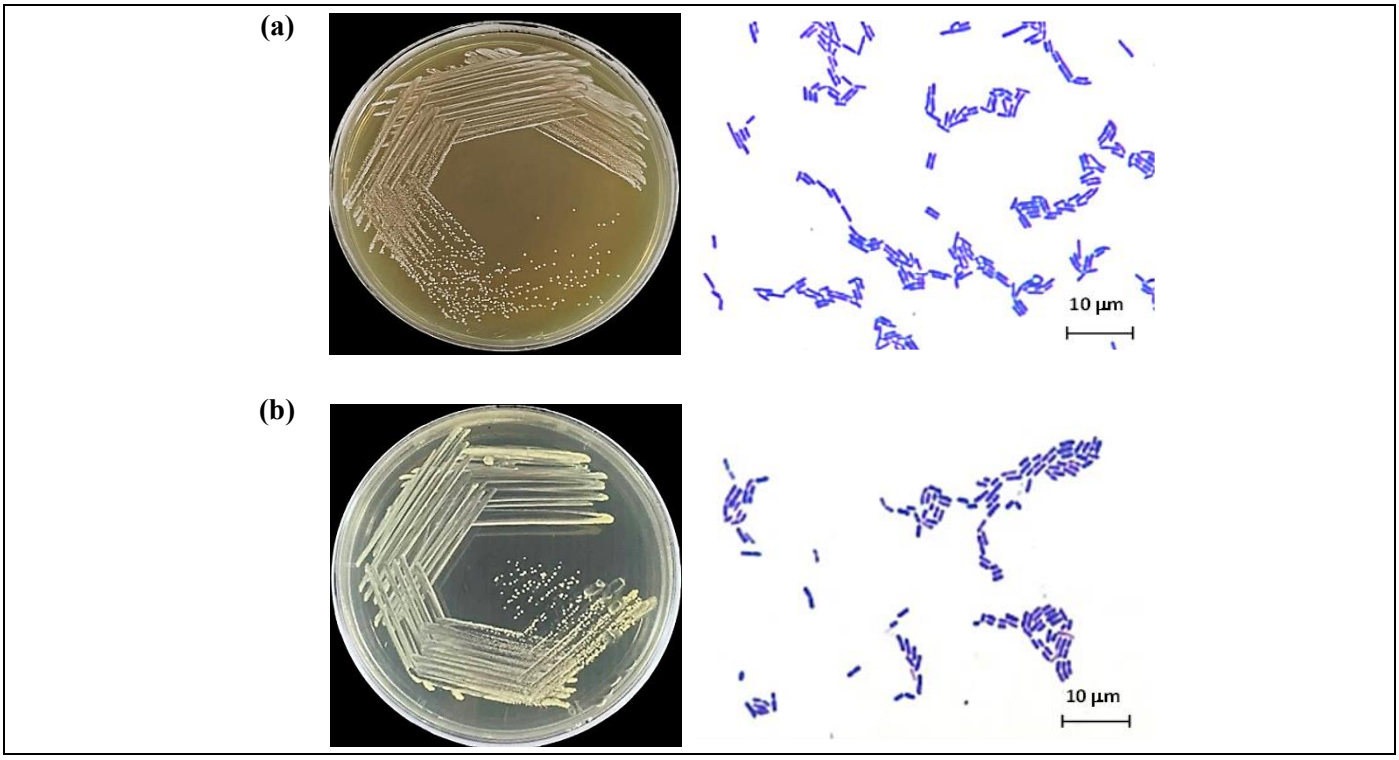


Figure 1. Morphologies of selected LAB isolates from fermented food products on MRS agar and under 100× microscope (Olympus CX21 Microscope, Japan); (a) *L. plantarum* SM14-2STR and (b) *W. cibaria* SE8-2STR

bacteria belonging to the genus *Lactobacillus*. Similar results were previously observed, reporting that *L. plantarum* strains exhibited inhibitory activity against *E. coli*, *S. aureus* and *S. enterica*[28]. *Weissella cibaria* MD2 exhibited antimicrobial activity against *E. coli*, *S. enterica*, *S.* Typhi and *S. aureus*[12], but *W. cibaria* SE8-2STR in this study showed antagonistic activity towards *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 35657, *P. aeruginosa* ATCC 27853, *P. mirabilis* DMST 8212 and *S. enterica* ATCC 13312. The production of antibacterial compounds (organic acids, bacteriocins and short-chain fatty acids) is one of the mechanisms by which probiotics inhibit the growth of pathogens[29]. Additionally, exopolysaccharides derived from *W. cibaria* have been shown to promoted the growth and enhance the antibacterial activity *L. rhamnosus* against *L. monocytogenes* and *S. aureus)*, supporting its probiotic potential[30].

3.3 Tolerance of pH and Bile salt

To provide health benefits to the host, the candidate probiotic strains were evaluated the survival in stomach by culturing in different acidic conditions (pH 2.0, 3.0, 4.0 and 5.0) compared with control (pH 7.0). In the present study, both LAB isolates exhibited good growth in MRS broth at pH 4.0, 5.0 and 7.0 (control), whereas their growth significantly declined at pH 2.0 and 3.0 of incubation. The selected LAB strains differently responded to gastric and intestinal stress conditions depending on the strains. Both potentially probiotic LAB strains showed varying levels of growth at pH 4.0 (4.15×10^{10}–5.93×10^{10} CFU/mL) and 5.0 (4.49×10^{10}–4.59×10^{11} CFU/mL) after 24-h incubation periods, compared with the initial cell of 1.45×10^{9} CFU/mL (Figure 2a-b). *L. plantarum* SM14-2STR and *W. cibaria* SE8-2STR in this study were shown to be acid-tolerant, as they were able to withstand acidic conditions during gastric phase exposure (pH 3 after 3 hours). The numbers of survival cells of all potential probiotic LAB isolates decreased by only 1-log (range from 9.82×10^{8} – 9.85×10^{8} CFU/mL), which significantly expressed survival rate of up to 68% after the first 3 h incubation at pH 3.0 (gastric condition) ($p\leq0.05$). The acid-tolerance of all LAB strains significantly trend continued to decrease until the end of the test at 24 h ($p\leq0.05$). *L. plantarum* SM14-2STR and *W. cibaria* SE8-2STR displayed the short adaptation time of less than two hour with 2-log reduction at pH 2. On the contrary to the findings of Wang et al. 2018[28] who recorded that *L. plantarum* PIC42, *L. plantarum* CB10 and *W. cibaria* CB12 exhibited adaptation time of less than 1 hour. Our results are in consistent with previous studies, which showed that *Lactobacillus* and *Weissella* from fermented food were able to retain tolerance when exposed to pH ranges from 2.0 to 3.0[31-32].

For bile tolerance assay, the ability of these LAB strains to survive at 0.3% (v/v) bile salt has been proposed previously[33]. A microorganism is considered to have a good tolerance if it able to tolerate at 0.3% (v/v)

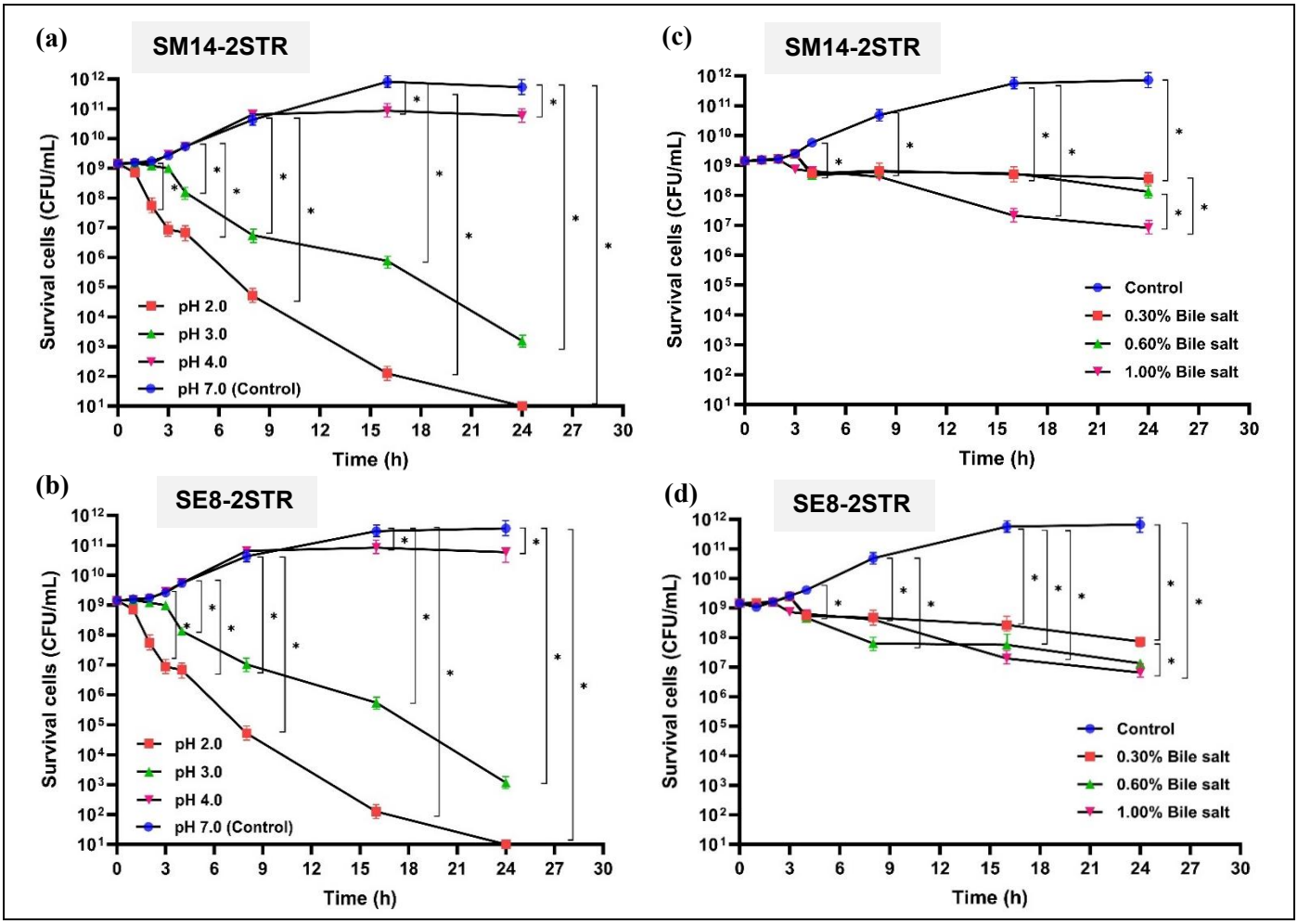


Figure 2. Bile salts and pH tolerance of selected strains with survival cells of selected LAB strains at: (a-b) different pH levels and (c-d) bile salt levels (* = Statistically significant differences ($p \leq 0.05$) between pathogenic strains by paired t-test when compared with control group)

bile salts, and thus this concentration is often used to screen potential probiotics for bile tolerance[34]. In this study, the potential bile salt tolerance of selected LAB isolates exhibited concentration-dependent manner (Figure 2c-d). *L. plantarum* MS14-2STR strain significantly revealed higher bile salt tolerance than *W. cibaria* SE8-2STR ($p>0.05$). Both LAB strains showed notable growth to 0.3% and 0.6% bile salt with growth rates of up to 170% after first 3-h incubation periods whereas isolates *L. plantarum* PIC42 and CB10 and *W. cibaria* CB12 displayed the shortest adaptation time of less than one hour[28]. *L. plantarum* SM14-2STR and *W. cibaria* SE8-2STR were able to tolerate 0.3% and 0.6% bile salt after 24-h exposure periods with viability of $1.33–5.29\times10^8$ CFU/mL and $1.35–7.34\times10^7$ CFU/mL (an initial cell of 1.45×10^9 CFU/mL), but revealed the decreased survival rate of 1-log CFU/mL after 3-h incubation periods under 1.0% bile salts ($7.25 – 7.39\times10^8$ CFU/mL). The bile salt-tolerance ability of all potential probiotic LAB strains significantly decreased after 4-h exposure and continued to decline until the end of the 24 h experiment ($p\leq0.05$). However, the dynamic nature and multiple factors of the unique intestinal environment (fluctuating pH, enzyme activities and host-microbe interactions) should be further investigated using simulated gastrointestinal models and animal studies to confirm the viability and functional activity of probiotic strains under physiological conditions.

3.4 Characterization of antibiotic susceptibility and haemolytic activity

For safety features assessment, antibiotic susceptibility and haemolytic activity of the LAB isolates were investigated. The antibiotic susceptibility of both LAB isolates was assessed through determination of agar disc diffusion (CLSI guideline) and broth microdilution (EFSA guideline) with 10 and 7 antibiotics, respectively (Table 3). According to the antibiotic breakpoints established by CLSI (2014 and 2017) and EFSA (2012), a microorganism is defined as susceptible to a specific antimicrobial if it is inhibited at or below the breakpoint level. Conversely, if the zones of inhibition or minimum inhibitory concentrations (MICs) exceed the breakpoint, the microorganisms considered resistant. Based on the

CLSI guideline[17], both candidate LAB strains were sensitive to gentamycin, streptomycin, erythromycin, chloramphenicol, clindamycin and ciprofloxacin, but resistant to penicillin, tetracycline and norfloxacin. Sánchez et al.[35] reported that *L. plantarum* and *W. cibaria* isolated from Peruvian Amazonian fruits were sensitive to penicillin and tetracycline. In contrast, Senjaliya and Georrge[36] found that *L. plantarum* CMGC2 and CMJC7 were resistant to ampicillin, which contradicts the findings of the present study. However, the results of antimicrobial susceptibility assay by agar disc diffusion method may be affected by the limited diffusion of antibiotics on agar medium[18]. The potential LAB isolates were susceptible toward all tested conventional in this study, supporting their suitability for application in food and pharmaceutical products and confirming their safety. However, inconsistencies between the disk diffusion test and the broth micro-dilution assay (as per EFSA guideline) highlight the need to establish specific cut-off values for *L. plantarum* and *W. cibaria*. To assess the haemolytic activity, both selected LAB strains were evaluated on blood agar plates. The results showed γ-haemolysis (no haemolysis), indicating a lack of haemolytic activity in the present study, which is consistent with the findings reported by Teixeira et al.[37]. Although a study by Abriouel et al.[38] reported that *W. cibaria* strains possess genes encoding hemolysin or hemolysin-like proteins, these genes are not associated with the expression of the hemolytic phenotype. Further studies should be conducted to investigate the potential for horizontal gene transfer of antibiotic resistance genes as well as the presence of virulence factors in the probiotic isolates for insights into their safety.

3.5 Cell surface hydrophobicity

Normally, the bacteria must possess a hydrophobic cell surface to adhere to the intestinal epithelial cells and thus this assay was carried out in this study. The physicochemical properties of the cell surface of the LAB isolates were assesses by adhesion to hydrocarbon (hexadecane) and results are presented in Table 4. *L. plantarum* MS14-2STR (73.79±0.29%; highly hydrophobic) had a significantly higher affinity to hexadecane than *W. cibaria* SE8-2STR (65.75±0.28%; moderately hydrophilic) ($p \leq 0.05$). All pathogenic strains exhibited a modulate adhesion percentage to hexadecane which ranged from 48.30±0.51% to 55.75±0.35%. Among of pathogenic strains, *P. aeruginosa* ATCC 27853 (55.75±0.35%) exhibited the highest adhesions to hexadecane. Bacterial strains with high hydrophobicity have better ability to bind to epithelial cells[40].

3.6 Auto-aggregation and co-aggregation ability

The role of auto-aggregation and co-aggregation as barriers to pathogen adhesion and colonization was also considered in this study. *W. cibaria* SE8-2STR (~65%) exhibited a significantly higher auto-aggregation ability than *L. plantarum* MS14-2STR (~61%) after incubation at room temperature for 24 h ($p \leq 0.05$), as shown in Table 4. Pathogenic strains significantly showed lower auto-aggregation than LAB isolates, ranging from 37.58±0.20% to 48.81±0.17% ($p \leq 0.05$). The lowest and highest auto-aggregation abilities were observed in *E. faecalis* DMST 4736 and *E. coli* ATCC 35218, respectively. Auto-aggregation is influenced by the binding of surface components such as proteins, lipids

Table 3. Antibiotic susceptibility profiles of isolated LAB strains

Antibiotic names	Inhibition zone (IZ; mm)		Antibiotics pattern (Breakpoints for interpretation)[a]	EFSA MICs (mg/L)		ECOFF values (mg/L)
	SM14-2STR	SE13-2STR		SM14	SE13	
Group I						
- Amp	30.00±0.00 (S)	23.67±0.58 (R)	S = ≥29 mm	1	1	2
- P	23.00±0.00 (R)	27.00±0.00 (R)	S = ≥29 mm	-	-	-
Group II						
- CN	21.33±0.58 (S)	15.33±0.58 (S)	S = ≥15 mm	2	2	16
- S	15.00±0.00 (S)	16.00±0.00 (S)	S = ≥15 mm	1	1	n.r.
- E	38.67±0.58 (S)	25.00±0.00 (S)	S = ≥23mm	0.125	0.25	1
- C	35.33±1.15 (S)	24.00±0.00 (S)	S = ≥17 mm	2	1	8
- TC	16.67±0.58 (R)	16.00±0.00 (R)	S = ≥19 mm	4	1	32
- CD	23.67±0.58 (S)	29.00±0.00 (S)	S = ≥19 mm	0.25	0.125	1
Group III						
- NOR	18.00±0.00 (R)	17.00±0.00 (R)	S = ≥17 mm	-	-	-
- CIP	21.00±00.00 (S)	21.33±0.58 (S)	S = ≥21 mm	-	-	-

No clinical breakpoints for food-related *Lactiplantibacillus plantarum* are registered in EUCAST or CLSI; [a]CLSI break point for *Staphylococcus aureus*; Amp = Ampicillin; P = Penicillin; CN = Gentamycin; S = Streptomycin; E = Erythromycin; C = Chloramphenicol; TC = Tetracycline; CD = Clindamycin; NOR = Norfloxacin; CIP = Ciprofloxacin; S = Sensitive; R = Resistance; EFSA = European Food Safety Authority; ECOFFs = EUCAST epidemiological cut-off values; - = not determined; n.r. = not required

Table 4. Percentage of hydrophobicity, auto-aggregation, co-aggregation and adhesion capacity of selected LAB strains

Bacterial strains	Cell-surface Hydrophobicity (%)	Auto-aggregation (%)	% of coaggregation	
			SM14-2STR with	SE8-2STR with
MS14-2STR	73.79±0.29^{b}	61.08±0.25^{b}	-	-
MS16-4STR	80.46±0.30^{a}	64.92±0.12^{a}	-	-
SE8-2STR^{d}	65.75±0.28^{c}	64.00±0.24^{a}	-	-
***S. aureus* ATCC 10832**	48.30±0.51^{f}	48.11±0.17^{c}	56.09±0.15^{aB}	57.55±0.35^{aAB}
***E. faecalis* DMST 4736**	52.84±0.57^{e}	37.58±0.20	52.32±0.20^{bB}	52.42±0.34^{bB}
***E. coli* ATCC 35218**	55.60±0.21^{d}	48.81±0.17^{c}	49.20±0.48^{dA}	48.41±0.34^{dA}
***E. cloacae* ATCC 13047**	48.98±0.45^{f}	40.53±0.17^{f}	50.51±0.25^{cA}	47.80±0.31^{deB}
***K. pneumoniae* ATCC 35657**	49.14±0.53^{f}	43.06±0.14^{e}	46.74±0.32^{efB}	48.54±0.21^{dA}
***P. aeruginosa* ATCC 27853**	55.75±0.35^{d}	45.42±0.18^{d}	45.99±0.14^{fA}	46.74±0.43^{eA}
***P. mirabilis* DMST 8212**	55.51±0.37^{d}	42.19±0.24^{e}	47.67±0.33^{eB}	50.08±0.38^{cA}
***S. enterica* ATCC 13312**	54.64±0.41^{d}	40.81±0.14^{f}	44.84±0.20^{gB}	47.07±0.26^{eA}
***S.* Typhimurium ATCC 13311**	54.95±0.50^{d}	39.83±0.18^{f}	43.85±0.21^{gB}	45.40±0.31^{fA}

^{abcdef} = Values on the same column with different superscripts were significantly different ($p \leq 0.05$);
^{ABCD} = Values on the same row with different superscripts were significantly different ($p \leq 0.05$)

and carbohydrates[39]. This ability is one of the mechanisms by which probiotics bind to pathogenic bacteria and eliminate from the digestive tract through the producing antimicrobial compounds[40].

To quantify cell-cell adhesions, the co-aggregation ability of isolated LAB strains was investigated with nine pathogenic bacteria and the results shown in Table 4. Both LAB strains displayed significantly co-aggregation with the tested pathogens ($p \leq 0.05$) and seemed to be species dependent with ranging from 43.85±0.21% to 58.68±0.27%. *L. plantarum* SM14-2STR significantly exhibited co-aggregation (>50%) with *S. aureus* ATCC 10832, *E. faecalis* DMST 4736 and *E. cloacae* ATCC 13047. However, *W. cibaria* SE8-2STR showed the highest co-aggregation with *S. aureus* ATCC 10832, followed by *E. faecalis* DMST 4736 and *P. mirabilis* DMST 8212, respectively. *Weissella cibaria* fermented Idli batter showed the lowest and highest coaggregation with *L. monocytogenes* MTCC 657 (30.04%) and *E. coli* MTCC 1687 (34.18%), respectively[41]. Numerous factors (cell surface charge, cell surface components, bacterial cell size, and environmental conditions) may affect the aggregative ability of probiotic LAB[37].

3.7 Adhesion capacity and competition assay on Caco-2 cells

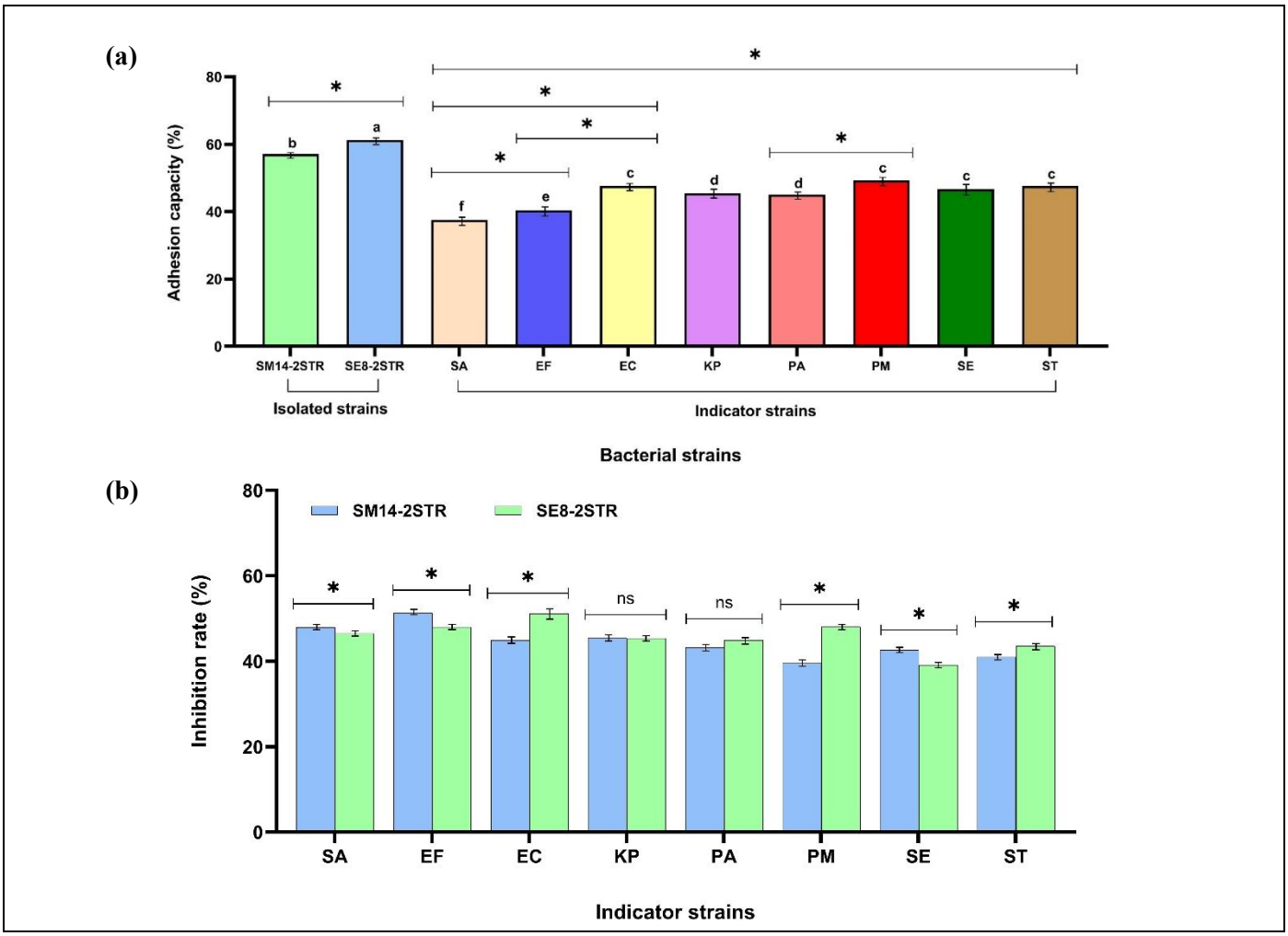
Adhesion capability helps probiotics extend their survival in the gastrointestinal tract and enhance their interactions with the host. In this regard, both LAB isolates were examined the Caco-2 cell-binding ability to assess probiotic potential using an in *vitro* model. The results indicated that *W. cibaria* SE8-2STR (61.24±0.64%) expressed significantly higher adherence than *L. plantarum* SM14-2STR (57.07±0.36%) (Figure 3a) ($p \leq 0.05$). Both potential probiotic LAB isolates showed significantly higher adhesion ability than all tested pathogenic strains ($p \leq 0.05$) and was classified as strongly adhesive. According to competition adhesion assay on Caco-2 monolayer, the inhibition of pathogenic adherence by both LAB isolates ranged from 48.74±0.47%–60.86±0.55% (Figure 3b) and LAB isolates exhibited the inhibition rate of pathogen adhesion depending pathogenic species. Importantly, the *W. cibaria* SE8-2STR (60.86±0.55%) was the most efficient in inhibiting *S. enterica* ATCC 13312 (causing causes foodborne infections) to adhesion on Caco-2 monolayer, while *L. plantarum* SM14-2STR (60.44±0.44%) significantly showed the highest competition abilities against *Proteus mirabilis* DMST 8212 (causing urinary tract infections) ($p \leq 0.05$). Although, the hydrophobicity assay is a preliminary test of the adhesion ability of probiotic bacteria to epithelial cells in this study, the relationship between adhesion and hydrophobicity has been investigated in several studies as well as this present work, often with conflicting results. Because cell adhesion properties are influenced by several factors such as culture medium composition, temperature and pH[42]. Based on antimicrobial activity and adhesion on Caco-2 cells by competition assay, *L. plantarum* SM14-2STR and *W. cibaria* SE8-2STR were able to inhibit the growth of *E. faecalis* DMST 4736 and *S.* Typhimurium ATCC 13311 by agar well diffusion, likely due to biofilm formation act as a defense mechanism[43]. However, both LAB strains were able to inhibit the adhesion of these pathogenic strains to Caco-2 cells under competition conditions, with 48–58% of inhibition rate. These results indicated that the selected LAB strains still have the potential to reduce the pathogenicity of the *E. faecalis* DMST 4736 and *S.* Typhimurium ATCC 13311 by inhibiting their adhesion or invasion to the intestinal epithelial cells.

In this study, the assessment of cell surface hydrophobicity, auto-aggregation, and co-aggregation provided useful information on the potential probiotic strains for colonization and interaction with pathogens tested under steady-state conditions. However, further

studies under dynamic conditions (intestinal peristalsis and the presence of gut mucosa should be performed to gain a more comprehensive understanding of host-microbe interactions.

3.8 Bile salt hydrolase (BSH) activity

The bile salt hydrolase (BSH) activity of probiotic bacteria is often associated with their cholesterol-lowering ability. The selected LAB strains were assessed for BSH activity by qualitative direct plate assay. The results showed that the potential probiotic LAB strains showed notable BSH activity against sodium taurocholate by presenting the thick white opaque colonies on MRS-TCA agar, which is consistent with study of Hernández-Gómez et al.[14] who reported that *L. plantarum* DGIA1 from a double cream cheese showed BSH activity against sodium taurocholate on MRS agar. *W. confusa* 12 MD1 and *W. cibaria* MD2, isolated from a fermented batter, showed high bile salt hydrolase activity towards sodium taurocholate[12], while *W. cibaria* SCCB2306 from Korean fermented Soya Beans showed BSH activity as indicated by the presence of precipitated zones of deoxycholic acid[44]. A study by Moser and Savage[45] revealed that *L. plantarum* BCCM 18021 from milk and *L. plantarum* BCCM 18027 from Laban Rayeb expressed taurocholic acid hydrolase activity, whereas *L. plantarum* BCCM 18035 from milk and *L. plantarum* BCCM 18028 from Laban Rayeb had no taurocholic acid hydrolase activity. Bile salt hydrolase (BSH) activity is one of mechanisms by which probiotics mediated cholesterol reduction in liquid culture media. This involves the enzymatic deconjugation or lysis of bile salts, leading to increased excretion and decrease reabsorption of bile acids. The BSH enzyme produced by bacteria breaks down bile salts to release glycine or taurine along with free bile salts, which in turn reduces cholesterol levels. Furthermore, the degradation of bile salts reduces the formation of cholesterol micelles, which ultimately decreased cholesterol absorption[46].



SA = *S. aureus* ATCC 10832; EF = *E. faecalis* DMST 4736; EC = *E. coli* ATCC 35218; KP = *K. pneumoniae* ATCC 35657; PA = *P. aeruginosa* ATCC 27853; PM = *P. mirabilis* DMST 8212; SE = *S. enterica* ATCC 13312; ST = *S.* Typhimurium ATCC 13311

Figure 3. Adhesion capacity and competition assay on Caco-2 cells of selected LAB strains; (a) Adhesion capacity and (b) Competition adhesion test on Caco-2 cell of LAB strains with pathogenic strains (Bars with the different alphabet are significantly different ($p \leq 0.05$); * = Statistically significant differences ($p \leq 0.05$) between pathogenic strains by paired t-test; ns = not significant ($p>0.05$))

3.9 *In vitro* cholesterol and triglyceride degradations

Co-precipitation of cholesterol with bile salts is one method to study the efficiency of bacteria in removing cholesterol. The cholesterol and triglyceride degradations by potential probiotic LAB strains grown in MRS−CHOL medium containing 0.3% bile salts under pH 5.0 condition. The results indicated that all potential probiotic LAB were able to reduce cholesterol after 24 h of incubation in MRS broth containing cholesterol, demonstrating their cholesterol-lowering capacity. *L. plantarum* SM14-2STR (84.07±0.89%) significantly revealed higher cholesterol degrading ability than *W. cibaria* SE8-STR (61.30±0.82%) ($p \leq 0.05$) (Figure 4a). Ma et al.[47] found that *L. plantarum* CAAS18010 from corn silage showed cholesterol removal rate of 55.2%. Additionally, *L. plantarum* SM14-2STR significantly displayed maximum triglyceride degradation (42.05±1.05%) in MRS-TRIG medium ($p \leq 0.05$), whereas *W. cibaria* SE8-STR showed reduced triglyceride levels by 39.24±1.19% (Figure 4a). Wu et al.[48] found that lactic acid bacteria ZL010 from Chinese pickle displayed triglyceride degradation rate of 5.10%. The lower ability to remove cholesterol from the broth may be related to certain bacterial cell components (fatty acids) that prevent cholesterol from adhering[7]. It has been reported that the fatty acid composition of the cell membranes of probiotics cultured in the presence of cholesterol significantly changed, indicating cholesterol removal through adsorption or desorption ($p \leq 0.05$)[7]. Although, the in *vitro* triglyceride-lowering ability has been rarely demonstrated, there are few studies available on the triglyceride-lowering ability of lactic acid bacteria in animal model[8].

3.10 Suppression of cholesterol uptake in CaCo-2 cells by LAB isolates

To investigate the suppression of cholesterol uptake, Caco-2 cells were used to demonstrate the cholesterol absorption. the reductions in cholesterol uptake by Caco-2 cells treated with potential probiotic LAB isolates is shown in Figure 4b. The results showed that the cholesterol uptake by Caco-2 cells incubated with viable LAB isolates (59.85±0.47–64.88±0.38%) was significantly lower than that of the non-treated control (89.30±0.27%) after 6-h incubation with cholesterol micelles ($p \leq 0.05$). *L. plantarum* SM14-2STR (28.44±0.28%) significantly greater inhibition of cholesterol uptake in colon epithelium cells than *W. cibaria* SE8-2STR (23.41±0.31%), compared to the control after 6-h treatment ($p \leq 0.05$). Likewise, *L. plantarum* has been found to remove cholesterol[49]. *L. plantarum* NR74 is able to control the cholesterol absorption by down-regulation of NPC1L1 expression in Caco-2 cells, thereby reducing cholesterol uptake[50]. In addition, *L. plantarum* FB003 from salted- fermented shrimp exerted cholesterol- lowering effects in Caco-2 cells by downregulating Niemann-Pick C1-like 1 (NPC1L1)[49]. *W. cibaria* SE8-2STR (~23%) showed the inhibitory effect on cholesterol uptake in colon epithelium cells, contributing to cholesterol reduction. This effect was greater than that of *W. cibaria* SCCB2306, isolated from Korean fermented soya beans, which reduced cholesterol levels (20.5±0.5%) by

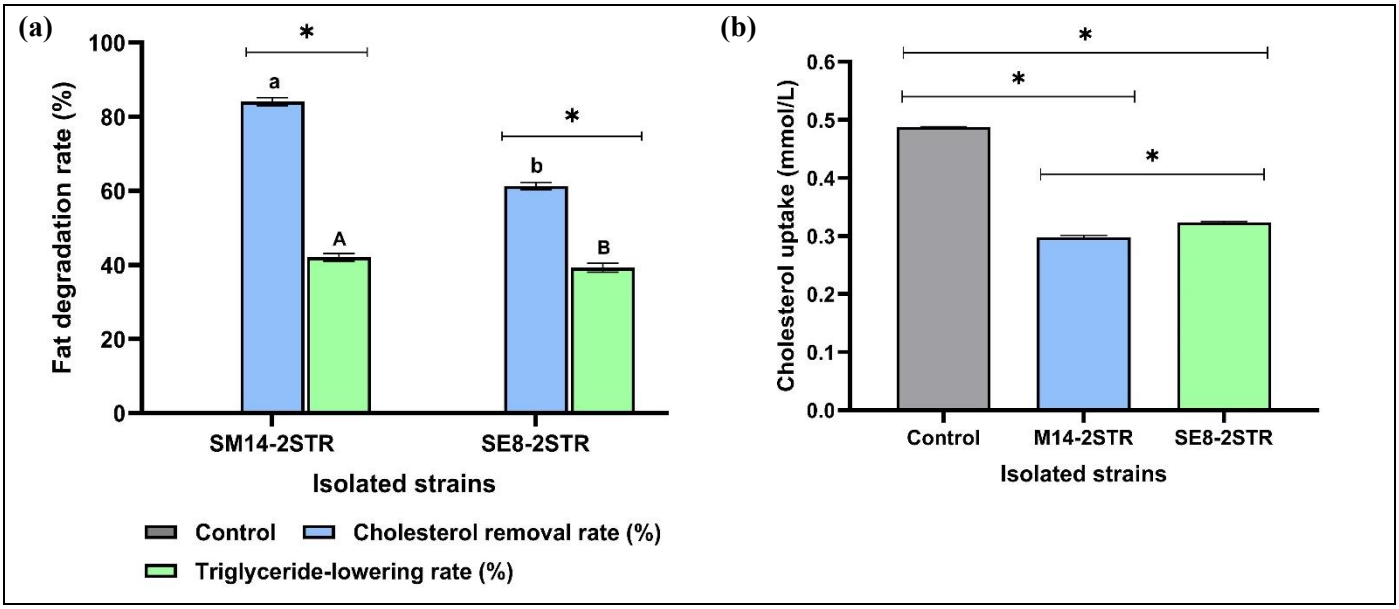


Figure 4. Cholesterol- and triglyceride-lowering activities and cholesterol uptake by isolated LAB strains; (a) degrees of cholesterol/ triglyceride and (b) percentage Caco-2 colon epithelial cholesterol uptake (Bars with the different alphabet are significantly different ($p \leq 0.05$) and * = Statistically significant differences ($p \leq 0.05$) between bacterial strains by paired t-test)

deconjugating bile salts and incorporating cholesterol into its cell membranes[44]. Singh et al.[51] reported that *W. cibaria* 28 from fermented dosa batter showed cholesterol reduction besides preventing LPS-induced pro-inflammatory stress in human colonic epithelial cells (Caco-2). The cell wall of LAB probiotics has also been found to play an important role in their cholesterol lowering potential[52]. Choi and Chang[24] found that cell wall fractions of *L. plantarum* EM and *L. acidophilus* ATCC 43121 from kimchi were able to remove cholesterol in a concentration-dependent manner. One proposed mechanism involves the attachment of cholesterol to the bacterial cell surface and its incorporation into the phospholipid bilayer of the bacterial membranes. This interaction reduces the amount of cholesterol available for absorption in the intestine[24]. Thus, the finding of this study suggested that the cholesterol-assimilating capability of probiotics represent another important mechanism for lowering elevated cholesterol levels, consistent with previous studies[53].

Interestingly, while many studies have investigated the cholesterol-lowering efficacy of *L. plantarum* and *W. cibaria* from various fermented foods such as pickled vegetables, fermented fish and fermented pork sausage[26], there have been very few reports on the cholesterol- and triglyceride-lowering potential of *L. plantarum* isolated from fermented meat (*Mum*) and *W. cibaria* isolated from Isan sausage (*Sai-krok Isan*). Thus, this is the first to report the isolation of *L. plantarum* from fermented meat (Mum) and *W. cibaria* isolated from Isan sausage (Sai-krok Isan) and to demonstrate their cholesterol-lowering potential. While the inhibition of cholesterol absorption in Caco-2 cells in this study indicates the potential of these probiotic strains to interfere with intestinal cholesterol uptake, further investigations into the optimal timing of administration, effective probiotics dose, and the influence of bacterial growth phase on their efficacy in suppressing cholesterol absorption should be conducted in the future.

4. CONCLUSIONS

In summary, the lactic acid bacteria isolates *L. plantarum* SM14-2STR and *W. cibaria* SE8-2STR from (Thai) fermented meat and Isan sausage are considered as cholesterol-lowering probiotic strains due to their effectiveness in reducing cholesterol and triglyceride as well as their bile salt hydrolase (BSH) activity in *vitro* and inhibition of cholesterol uptake by colon epithelium (Caco-2) cells. Both candidate probiotic LAB strains also exhibited antimicrobial activities against both Gram-negative and Gram-positive pathogenic bacteria, strong tolerance to low pH condition (gastric phase) and various bile salts concentrations, All the potential LAB strains demonstrated notably high hydrophobicity, auto-aggregation ability, co-aggregation capability with bacterial pathogens and good adhesion ability to Caco-2 cells. Importantly, the potential probiotic LAB strains were considered to be safe due to their non-haemolytic activity and sensitive antibiotics. These attributes make *L. plantarum* SM14-2STR and *W. cibaria* SE8-2STR promising candidate for use as a potential probiotic with cholesterol-lowering effects. However, the LAB isolates from fermented foods in this study requires further in *vivo* and/or animal experiments to assess their health-promoting effects and potential applications in food and pharmaceutical industries.

Author contribution

Sirikhwan Tinrat: Conceptualization; Methodology; Formal analysis and investigation; Writing - original draft preparation; Writing - review and editing; Funding acquisition; Resources; Supervision. Onnicha Jiraprasertwong: Formal analysis and investigation; Writing - review and editing.

Conflict of interest (If any)

None to declare.

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REFERENCES

1. Maki KC, Dicklin MR, Kirkpatrick CF. Saturated fats and cardiovascular health: Current evidence and controversies. J Clin Lipidol. 2021;15(6):765–72.
2. FAO/WHO. Guidelines for the Evaluation of Probiotics in Food. Food and Agriculture Organization of the United Nations/World Health Organization. London, Ontario; Food and Agriculture Organization of the United Nations/World Health Organization; 2002.
3. Jan T, Negi R, Sharma B, Kour D, Kumar S, Rai AK, et al. Diversity, distribution and role of probiotics for human health: Current research and future challenges. Biocatal Agric Biotechnol. 2023;53:102889.
4. Chandrasekaran P, Weiskirchen S, Weiskirchen R. Effects of probiotics on gut microbiota: An overview. Int J Mol Sci. 2024;25(11):6022.

5. Maftei NM, Raileanu CR, Balta AA, Ambrose L, Boev M, Marin DB, et al. The potential impact of probiotics on human health: An update on their health-promoting properties. Microorganisms. 2024;12(2):234.
6. Gao Y, Li D (2018) Screening of lactic acid bacteria with cholesterol-lowering and triglyceride-lowering activity in vitro and evaluation of probiotic function. Ann Microbiol. 2018;68:537–45.
7. Miremadi F, Ayyash M, Sherkat F, Stojanovska L. (2014) Cholesterol reduction mechanisms and fatty acid composition of cellular membranes of probiotic *Lactobacilli* and *Bifdobacteria*. J Funct Foods. 2014;9:295–305.
8. Frappier M, Auclair J, Bouasker S, Gunaratnam S, Diarra C, Millette M (2022) Screening and characterization of some *Lactobacillaceae* for detection of cholesterol-lowering activities. Probiotics Antimicrob Proteins. 2022;14(5):873–83.
9. Kim DH, Choi MR, Hong JE, Lee SI, Lee SI, Jung SH, et al. Effect of mixture of *Lactobacillus plantarum* CECT 7527, 7528, and 7529 on obesity and lipid metabolism in rats fed a high-fat diet. J Korean Soc Food Sci Nutr. 2014;43:1484–90.
10. Bosch M, Fuentes MC, Audivert S, Bonachera MA, Peiró S, Cuñé J. *Lactobacillus plantarum* CECT 7527, 7528 and 7529: Probiotic candidates to reduce cholesterol levels. J Sci Food Agric. 2014;94(4):803–9.
11. Fhoula I, Rehaiem A, Najjari A, Usai D, Boudabous A, Sechi LA, et al. Functional probiotic assessment and *in vivo* cholesterol-lowering efficacy of *Weissella* sp. associated with arid lands living-hosts. Biomed Res Int. 2018;2018:1654151.
12. Lakra AK, Domdi L, Hanjon G, Tilwani YM, Arul V. Some probiotic potential of *Weissella confusa* MD1 and *Weissella cibaria* MD2 isolated from fermented batter. LWT - Food Sci Technol. 2020;125:109261.
13. Shehata MG, El-Sahn MA, El Sohaimy SA, Youssef MM. Role and mechanisms lowering cholesterol by dietary of probiotics and prebiotics: A review. J Appl Sci. 2019;19:737–46.
14. Hernández-Gómez JG, López-Bonilla A, Trejo-Tapia G, Ávila-Reyes SV, Jiménez-Aparicio AR, Hernández-Sánchez H. In *vitro* bile salt hydrolase (BSH) activity screening of different probiotic microorganisms. Foods. 2021;10(3):674.
15. Tinrat S, Jiraprasertwong O. Isolation and assessment of probiotic potential of *Acidipropionibacterium acidipropionici* C03B-STR from goat milk with cholesterol-lowering capability. Folia Microbiol (Praha). 2024.
16. Dos Santos HRM, Argolo CS, Argôlo-Filho RC, Loguercio LL. A 16S rDNA PCR-based theoretical to actual delta approach on culturable mock communities revealed severe losses of diversity information. BMC Microbiol. 2019;19(1):74.
17. CLSI: Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing, CLSI supplement M100-S17. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.
18. European Food Safety Authority (EFSA). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J. 12012;0(6):2740.
19. Gerhardt P, Murray RGE, Wood WA, Krieg NR. Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D. C: ASM Press; 1994.
20. Ben Taheur F, Kouidhi B, Fdhila K, Elabed H, Ben Slama R, Mahdouani K, et al. Anti-bacterial and anti-biofilm activity of probiotic bacteria against oral pathogens. Microb Pathog. 2016;97:213–20.
21. de Oliveira Coelho B, Fiorda-Mello F, de Melo Pereira GV, Thomaz-Soccol V, Rakshit SK, de Carvalho JC, et al. In *vitro* probiotic properties and DNA protection activity of yeast and lactic acid bacteria isolated from a honey-based kefir beverage. Foods. 2019;8(10):485.
22. Śliżewska K, Chlebicz-Wójcik A, Nowak A. Probiotic properties of new *Lactobacillus* strains intended to be used as feed additives for monogastric animals. Probiotics Antimicrob Proteins. 2021;13(1):146–62.
23. Choi EA, Chang HC. Cholesterol-lowering effects of a putative probiotic strain *Lactobacillus plantarum* EM isolated from kimchi. LWT - Food Sci Technol. 2015;62(1):210-7.
24. Iranmanesh M, Ezzatpanah H, Mojgani N. Antibacterial activity and cholesterol assimilation of lactic acid bacteria isolated from traditional Iranian dairy products. LWT - Food Sci Technol. 2014;58(2):355–9.
25. Tomaro-Duchesneau C, Saha S, Malhotra M, Jones ML, Rodes L, Prakash S. *Lactobacillus fermentum* NCIMB 5221 and NCIMB 2797 as cholesterol-lowering probiotic biotherapeutics: In *vitro* analysis. Benef Microbes. 2015;6(6):861–869.
26. Techo S, Kuncharoen N, Tanasupawat, S. Diversity and lipolytic activity of lactic acid bacteria isolated from fermented foods and plant materials in Thailand. Thai J Pharm Sci. 2022;46(4):12.
27. Tanasupawat S, Phoottosavako M, Keeratipibul S. Characterization and lipolytic activity of lactic acid bacteria isolated from Thai fermented meat. J App Pharm Sci. 2015;5(3):6–12.
28. Wang J, Yang K, Liu M, Zhang J, Wei X, Fan M. Screening for potential probiotic from spontaneously fermented non-dairy foods based on in *vitro* probiotic and safety properties. Ann Microbiol. 2018;68:803–13.
29. Latif A, Shehzad A, Niazi S, Zahid A, Ashraf W, Iqbal MW, et al. Probiotics: Mechanism of action, health benefits and their application in food industries. Front Microbiol. 2023;14:1216674.
30. Park S, Saravanakumar K, Sathiyaseelan A, Han K-S, Lee J, Wang M-H. Polysaccharides of *Weissella cibaria* act as a prebiotic to enhance the probiotic potential of *Lactobacillus rhamnosus*. Appl Biochem Biotechnol. 2022;2022:1–13.
31. Argyri AA, Zoumpopoulou G, Karatizas KA, Tsakalidou E, Nychas GJ, Paganou EZ, et al. Selection of potential probiotic lactic acid bacteria from fermented olives by in *vitro* tests. Food Microbiol. 2013;33:282–91.
32. Anandharaj M, Sivasankari B, Santhanakaruppu R, Manimaran M, Rani RP, Sivakumar S. Determining the probiotic potential of cholesterol-reducing *Lactobacillus* and *Weissella* strains isolated from gherkins (fermented cucumber) and south Indian fermented koozh. Res Microbiol. 2015;166(5):428–39.
33. Abdel Tawab FI, Abd Elkadr MH, Sultan AM, Hamed EO, El-Zayat AS, Ahmed MN. Probiotic potentials of lactic acid bacteria isolated from Egyptian fermented food. Sci Rep. 2023;13(1):16601.
34. Charteris WP, Kelly PM, Morelli L, Collins JK. Development and application of an in *vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J Appl Microbiol. 1998;84:759–68.
35. Sánchez J, Vegas C, Zavaleta AI, Esteve-Zarzoso B. Predominance of *Lactobacillus plantarum* strains in peruvian amazonian fruits. Pol J Microbiol. 2019;68(1):127–37.
36. Senjaliya DP, Georrge JJ. Functional characterization of *Lactobacillus plantarum* isolated from cow milk and the development of fermented coconut and carrot juice mixed beverage. Curr Microbiol. 2023;80(5):139.
37. Teixeira CG, Belguesmia Y, da Silva Rodrigues R, Lucau-Danila A, Nero LA, de Carvalho AF, et al. Assessment of safety and in situ antibacterial activity of *Weissella cibaria* strains isolated from dairy farms in Minas Gerais State, Brazil, for their food application. Braz J Microbiol. 2024;55(1):699–710.
38. Abriouel H, Lerma LL, Casado Muñoz Mdel C, Montoro BP, Kabisch J, Pichner R, et al. The controversial nature of the *Weissella* genus: technological and functional aspects versus whole genome analysis-based pathogenic potential for their application in food and health. Front Microbiol. 2015;6:1197.
39. Yang SJ, Lee JE, Lim SM, Kim YJ, Lee NK, Paik HD. Antioxidant and immune enhancing effects of probiotic

Lactobacillus plantarum 200655 isolated from kimchi. Food Sci Biotechnol. 2019;28:491–9.
40. Bajaj BK, Claes IJJ, Lebeer S. Functional mechanisms of probiotics. J Microbiol Biotechnol Food Sci. 2015;4:321–27.
41. Unnikrishnan S, Sreeja V. Evaluation of probiotic potential, anticancer, antioxidant, and cholesterol assimilation activity of *Weissella cibaria* strain isolated from fermented Idli batter. Int J Adv. Biochem Res. 2025;9(1S):816–22.
42. García-Cayuela T, Korany AM, Bustos I, de Cadinanos LPG, Requena T, Peláez, C, et al. (2014) Adhesion abilities of dairy *Lactobacillus plantarum* strains showing an aggregation phenotype. Food Res Int. 2014;57:44–50.
43. Li Q, Liu L, Guo A, Zhang X, Liu W, Ruan Y. Formation of multispecies biofilms and their resistance to disinfectants in food processing enviroments: A review. J Food Prot. 2021;84(12):2071–83.
44. Daliri EB, Kim Y, Do Y, Chelliah R, Oh DH. In *vitro* and in *vivo* cholesterol reducing ability and safety of probiotic candidates isolated from Korean fermented soya beans. Probiotics Antimicrob Proteins. 2022;14(1):87–98.
45. Moser SA, Savage DC. Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in lactobacilli. Appl Environ Microbiol. 2001;67(8):3476–80.
46. Foley MH, O'Flaherty S, Barrangou R, Theriot CM. Bile salt hydrolases: Gatekeepers of bile acid metabolism and host-microbiome crosstalk in the gastrointestinal tract. PLOS Pathog. 2019;5(3):e1007581.
47. Ma C, Zhang S, Lu J, Zhang C, Pang X, Lv J. Screening for cholesterol-lowering probiotics from lactic acid bacteria isolated from corn silage based on three hypothesized pathways. Int J Mol Sci. 2019;20(9):2073.
48. Wu H-H, Niu F, Zhong Q, Yang D. Separation, identification and functional analysis of cholesterol-lowering and triglyceride-lowering lactic acid bacteria. Sci. Technol. Food. 2019;40(14):157–62.
49. Le B, Yang SH. Identification of a novel potential probiotic *Lactobacillus plantarum* FB003 isolated from salted-fermented shrimp and its effect on cholesterol absorption by regulation of NPC1L1 and PPARα. Probiotics Antimicro Prot. 2019;11:785–93.
50. Yoon HS, Ju JH, Kim HN, Park HJ, Ji Y, Lee JE, et al. Reduction in cholesterol absorption in Caco-2 cells through the down-regulation of Niemann-Pick C1-like 1 by the putative probiotic strains *Lactobacillus rhamnosus* BFE5264 and *Lactobacillus plantarum* NR74 from fermented foods. Int J Food Sci Nutr. 2013;64(1):44–52.
51. Singh S, Bhatia R, Singh A, Singh P, Kaur R, Khare P, et al. Probiotic attributes and prevention of LPS-induced pro-inflammatory stress in RAW264.7 macrophages and human intestinal epithelial cell line (Caco-2) by newly isolated *Weissella cibaria* strains. Food Funct. 2018;9(2):1254–64.
52. Bhat B, Bajaj BK. Multifarious cholesterol lowering potential of lactic acid bacteria equipped with desired probiotic functional attributes. 3 Biotech. 2020;10(5):200.
53. Ding W, Shi C, Chen M, Zhou J, Long R, Guo X. Screening for lactic acid bacteria in traditional fermented Tibetan yak milk and evaluating their probiotic and cholesterol-lowering potentials in rats fed a high-cholesterol diet. J Funct Foods. 2017;32:324–32.