In Vitro Assessment of Probiotic Properties in *Lactobacillus* Strains Isolated from Traditionally Fermented Thai Foods

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Abstract

Traditionally fermented Thai foods are good sources for *Lactobacilli* isolation due to these food types include a wide variety of ingredients. A total of 82 *Lactobacillus* strains were preliminarily isolated from Pla-Som, Pla-Duk-Ra, Pla-Pang-Dang, Kung-Som, and Nham-Hed-Nang-Fa. Their 16S rDNA sequences showed homology to *L. plantarum*, *L. lactis*, *L. amylovorus*, *L. casei*, *L. acidophilus*, *L. farciminis*, and *L. graminis*. Gastrointestinal tract experimental found 10 isolates that can survive at pH 2.5 and 0.9% bile salt concentration. Remaining 4 isolates of *L. plantarum* (LpWP48/12, LpNH48/12) and *L. acidophilus* (LacKS48/15, LacWP48/22) had antagonistic activity against human pathogens including *Staphylococcus aureus* (TISTR 746), *Escherichia coli* (TISTR 527), *Pseudomonas aeroginosa* (TISTR 357), *Salmonella* Typhi, and *Shigella dysentery*. All four isolates were resistant to streptomycin, cefotetan, nalidixic acid, vancomycin, kanamycin, and methicillin. PCR analysis revealed positive identification of the *bsh*, *atpD* and *mapA* genes. The virulence-related genes *cylA*, *ace*, *esp* and *gelE* were found to be absent. As a result, *L. plantarum* (LpWP48/12 and LpNH48/12) and *L. acidophilus* (LacKS48/15 and LacWP48/22) showed potential as probiotic candidates and were safe to use as starter culture.

Keywords: Lactic acid bacteria, Probiotics, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, Traditionally fermented Thai food

Introduction

Lactic acid bacteria (LAB) are one of the major microbial groups involved in the fermentation of different types of food. They play important roles in converting glucose to lactic acid with pH reduction to control other microbials in food for shelf life improvement (Rodgers, 2008; O'Sullivan, Ross, & Hill, 2002). The LAB *Lactobacillus* genus is widespread in many fermented food types. Some species of *Lactobacillus* are probiotics and desirable members of intestinal microflora. These bacteria have "Generally Recognized as Safe" (GRAS) status (European Food Safety Authority, 2013). However, the quality of candidate probiotics should be proven both for its functional and safety properties such as gastric acidity and bile salt resistances, antagonistic effect against pathogenic bacteria, antibiotic resistance, and virulence factor. As virulence-related genes have been shown to increase the severe infective endocarditis in animal model (Chow et al., 1993). The *gelE* (encoding gelatinase) and *cylA* (encoding cytolysin A) genes that have been reported to exacerbate endocarditis (Gutschik, Moller, & Christensen, 1979). The *esp* (encoding cell-wall-associated protein involved in immune evasion) and *ace* (encoding collagen adhesion precursor) genes are related to the microbial colonization and adhesion (Visessanguan, Plengvidhya, Chokensajjawatee, & Baker, 2015).

Many *Lactobacillus* strains have been isolated from traditionally fermented food and characterized as novel probiotics, for example, *L. plantarum* SK119 isolated from fermented pork sausage (Botthoulath, Upaichit, & Thumarat, 2018), *L. brevis* isolated from Japanese pickle (Pavlova et al., 2002), *L. plantarum* and *L. paracasei* isolated from Teff dough, Kocho, and Ergo as traditionally fermented food of Ethiopia (Mulaw, Tessema, Muleta, & Tesfaye, 2019), *L. fermentum* isolated from fermented pork (Klayraung, Viernstein, Sirithunyalug, & Okonogi, 2008) and fermented beverages (Ghosh et al., 2015).

Traditionally fermented Thai foods are good sources for probiotic bacteria isolation, especially *Lactobacillus* species (Botthoulath et al., 2018). These food types have a wide variety of ingredients and raw materials. For example, Pla-Som is fermented fish with rice, garlic and low salt concentration. Pla-Duk-Ra is a dried fermented catfish with high concentrations of salt and sugar. Pla-Pang-Dang is a fermented fish with a red rice yeast and low salt concentration. Kung-Som is a fermented shrimp with low salt concentration and Nham-Hed-Nang-Fa is a fermented oyster mushroom with low salt concentration. Previously, we isolated and characterized 82 isolates of *Lactobacillus* from the 5 fermented foods as mentioned above. Here, we identified the species of isolated *Lactobacillus* spp. and assessed their probiotic properties.

Methods and Materials

Bacteria preparation

A total of 82 isolates of *Lactobacillus* spp. were isolated from traditionally fermented Thai foods including Pla-Som 45 isolates, Pla-Duk-Ra 12 isolates, Pla-Pang-Dang 18 isolates, Kung-Som 4 isolates and Nham-Hed-Nang-Fa 3 isolates. The bacterial cells were maintained at -80^{O}C. The control strains containing *E. faecalis* KS-07 and *E. faecalis* KM-16 isolated from intestinal bovine were kindly supplied by the Faculty of Veterinary Medicine, Kasetsart University, and *L. acidophilus* TISTR 2365 was purchased from the Thailand Institute of Scientific and Technological Research (TISTR).

16S rDNA identification

Bacterial genomic DNA (gDNA) was extracted and purified using TIANamp Bacterial gDNA Kit (Ward medic, China) according to the manufacturer's instructions. The quality of gDNA was measured using a Qubit 4.0 Fluorometer (Invitrogen Detection Technologies, Eugene, OR). 16S rDNA amplifications were carried out in a 25 μl mixture including 2X Dream Taq PCR Master Mix (Thermo Fisher Scientific, USA), containing 0.5 U of Taq DNA polymerase, Tris-HCl buffer, 200 μM concentration of dNTP and 20 mM $MgCl_2$, a 0.5 μM concentration of each primer (details shown in **Table 1**) and 20 ng of gDNA. Amplification conditions were as follows: initial denaturation at 95^{O}C for 3 min followed by 30 cycles of denaturation at 95^{O}C for 30 s, annealing at 56^{O}C for 45 s, extension at 72^{O}C for 30 s, and a final extension at 72^{O}C for 7 min. The PCR products were strained with SyBr Gold (Thermo Fisher Scientific, USA), determined on 1.5% agarose gel electrophoresis and visualized using Gel Doc XR+ (BioRad, USA). Sequencing of the 16S rDNA was performed by 1st BASE DNA sequencing service (Malaysia) following the Sanger method and sequences were aligned with the NCBI database using the BLAST method (Botthoulath et al., 2018).

Acid and bile salt tolerance

MRS medium was adjusted to pH 2.5, 3 and 4 with 1 N HCl. Each *Lactobacillus* isolate was performed at approximately 10^8 cfu/ml and inoculated into the MRS medium. The isolates were then incubated at 37^{O}C for 3 h under anaerobic condition, and cells were harvested and washed. Viability for growth of each *Lactobacillus* isolate was determined based on the number of colony-forming units by the plate count method. Ten-fold serial dilutions (up to 10^{-7}) were performed with 0.85% NaCl. Next, 100 µl of 10^{-4} to 10^{-7} dilutions from each sample were spread-plated on MRS (pH 6.2) agar and incubated anaerobically at 37^{O}C for 24 h.

Bile salt (Sigma, USA) was added to MRS broth for final concentrations of 0.3, 0.6 and 0.9% (w/v). Then, approximately 10^8 cfu/ml of each isolate was inoculated and incubated anaerobically at 37^{O}C for 3 h. Viability was determined by the plate count method. Both pH and bile salt tolerances were calculated as percentage of *Lactobacillus* growth compared to the initial bacterial concentration following the equation (Mulaw et al., 2019).

$$\text{Survival rate } (\%) = [\log \text{cfu}(N_1)/\log \text{cfu}(N_0)] * 100$$

where N_1 is the viable count of isolates after incubation and N_0 is the initial viable count. Positive tolerance was indicated when the survival rate was more than 50%.

Table 1 Primers and PCR conditions used for the identification and screening of virulence genes

Primers	Sequences (5' to 3')	Tm (^{O}C)	Size (bp)	References
16S rRNA	27f: AGAGTTTGATCCTGGCTCAG 1492r: AGAAAGGAGGTGATCCAGCC	56	1,500	Gong, Meng, & Wang, 2010
bsh1	bsh-f: ATGGGCGGACTAGGATTACC bsh-r: TGCCACTCTCTGTCTGCATC	54	712	Zago et al., 2011
atpD	atpD-f: GCCAACCTGGTTCGTATGTG atpD-r: ACCACGTCGTCGATCTTACC	54	624	Ventura, anchaya, Sinderen, Fitzgerald, & Zink, 2004
mapA	mapA-f: TGGATTCTGCTTGAGGTAAG mapA-r: GACTAGTAATAACGCGACCG	54	1,024	Todorov et al., 2008
ace	ace-f: AGAGTTTGATCCTGGCTCAC ace-r: GGTTACCTTGTTACGCTT	56	1,000	Martin-Platero, Valdivia, Maqueda, & Martinez-Bueno, 2009
cylA	cylA-f: TGGATGATAGTGATAGGAAGT cylA-r: TCTACAGTAAATCTTTCGTCA	54	517	Tan et al., 2013
gelE	gelE-f: AATTGCTTTACACGGAACGG gelE-r: AGCCATGGTTTCTGGTTGTC	54	547	Tan et al., 2013
esp	esp-f: TTGCTAATGCTAGTCCACGACC esp-r: GCGTCAACACTTGCATTGCCGAA	56	933	Lindenstrauss, Pavlovic, & Bringmann, 2011

Antibacterial activity

The pathogenic bacteria including *Staphylococcus aureus* (TISTR 746) *Escherichia coli* (TISTR 527) and *Pseudomonas aeruginosa* (TISTR 357) were purchased from the Thailand Institute of Scientific and Technological Research (TISTR), and *S.* Typhi and *S. dysentery* were obtained from the Faculty of Veterinary Medicine, Kasetsart University, Thailand. Ten of the selected *Lactobacillus* strains survived pH 2.5 and 0.9% bile salt concentration and these were investigated for antibacterial activity using the agar well diffusion method (Schillinger & Lucke, 1989) as following; each pathogenic bacterium was prepared using turbidity standard McFarland 0.5. Then, the suspension was swabbed on MHA (Muller Hinton Agar) plates using sterile cotton. Four wells were punched in each inoculated agar medium plate using a sterile cork borer (6 mm diameter). A 50 µl aliquot of each *Lactobacillus* isolate culture grown in MRS for 24 h was pipetted into each well. The plate was incubated at 37°C for 24 h. Diameters of inhibition zones (including the 6 mm well diameter) were measured. All assays were repeat three times.

Antibiotic resistance

Antibiotic resistant testing for the *Lactobacillus* isolate was carried out using the antibiotic disk diffusion method according to (Zhang et al., 2016) with minor modifications. Antimicrobial diffusion disks (Oxoid, Thermo Scientific) were used following gentamycin (10 µg), kanamycin (30 µg), streptomycin (10 µg), chloramphenicol (30 µg), rifampin (5 µg), nalidixic acid (30 µg), ampicillin (10 µg), erythromycin (15 µg), vancomycin (10 µg), cefotetan (30 µg) and methicillin (10 µg). The density of each *Lactobacillus* isolate was adjusted to 0.5 of McFarland standard with sterile 0.85% NaCl, and spread on MRS agar plate. Antibiotic disks were placed on the plate and anaerobically incubated at 37°C for 24 h. Their susceptibility was interpreted according to the diameter of the clear zone and determined using the CLSI standard (Clinical and Laboratory Standard Institute, 2009).

Probiotic and safety characterization

Probiotic marker genes including *bsh* (encodes bile salt hydrolase), *atpD* (encodes neutral pH), *mapA* (encodes adhesion proteins) and virulence-related genes including *cylA* (encodes cytolysin A), *ace* (encodes collagen adhesion precursor), *esp* (encodes cell-wall-associated protein involved in immune evasion) and *gelE* (encodes gelatinase E) were investigated with specific primers (Table 1). The gDNA of *Lactobacillus* isolate and control strains (*E. faecalis* KS-07, KM-16, and *L. acidophilus* TISTR 2365) were used as the template for amplification. The PCR products were determined by 1.5% agarose gel electrophoresis and SyBr staining.

Statistical analysis

The experiments were performed in triplicate. The statistical analysis was performed using excel and presented as means ± standard deviation.

Results and discussion

16S rDNA identification

A total 82 *Lactobacillus* isolates had Gram-positive and rod-shaped, and also showed negative catalase test. The result of 16S rDNA identification in **Figure 1** indicated that species of 82 *Lactobacillus* isolates closely related with *L. plantarum* (35/82, 42.7%), *L. lactis* (22/82, 26.8%), *L. amylovorus* (12/82, 14.6%), *L. casei* (7/82,

8.5%), *L. acidophilus* (3/82, 3.6%), *L. farciminis* (2/82, 2.4) and *L. graminis* (1/82, 1.2%). The amount of *Lactobacillus* and the strain diversity in various fermented products were determined by the raw materials used and process parameters such as inoculum length, salt concentration, and incubation time. (Behera, Ray, & Zdolec, 2018).

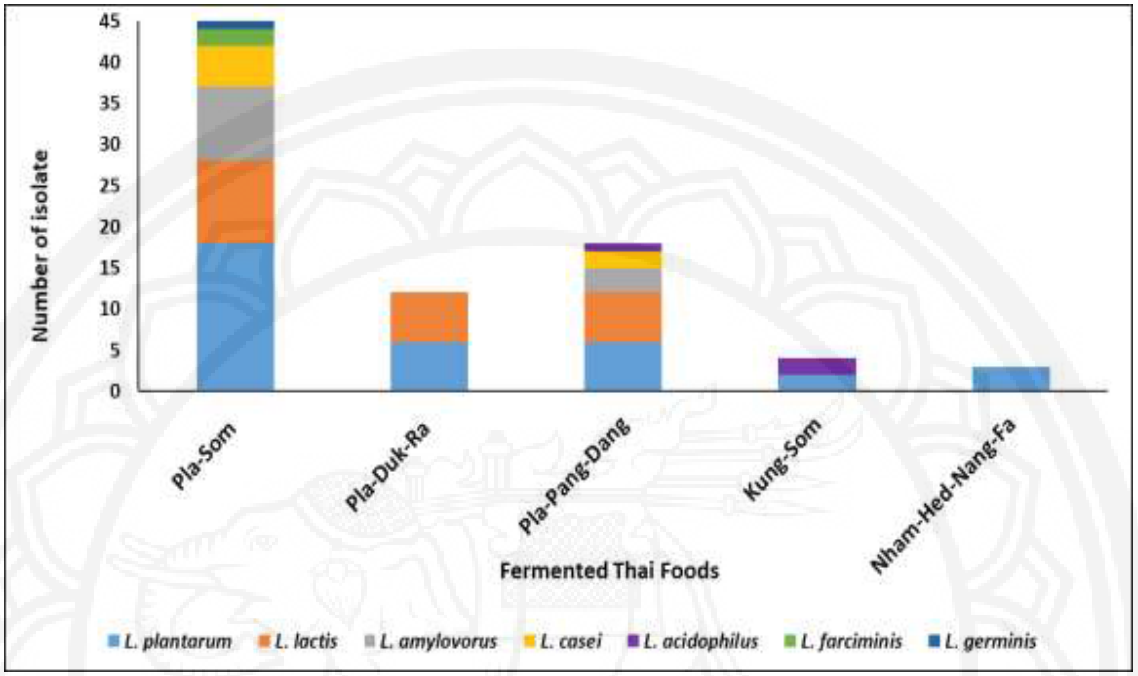

Figure 1 Species of *Lactobacillus* isolates by 16S rDNA identification

Acid and bile salt tolerance

Abilities of acid and bile salt tolerances simulate conditions in the gastrointestinal tract (GIT) as good indicators for survival of a bacterial strain. Thus, these characteristics are often used for preliminary selection of probiotic strains. Normally, values of pH 3 and 0.3-0.6% bile salt concentration are used to investigate *Lactobacillus* strains (Jin, Ho, Abdullak, & Jalaludin, 1998). In this study, 10 isolates were survived at pH values of 2.5 and 0.9% (w/v) bile salt concentration for 3 h, (**Table 2**). Interestingly, our isolates were able to survive for longer periods of time at low pH and high bile salt concentration than those observed in previous studies (Jin et al., 1998; Jacobsen et al., 1999; Liong, & Shah, 2005; Koll, Mandar, Marcotte, Leibur, Mikelsaar, & Hammarstrom, 2008; Sahadeva et al., 2011). Ten isolates belonged to *L. plantarum* (LpWP48/10, LpWP48/12, LpNH48/09, LpNH48/12), *L. lactis* (LlNH24/03, LlPD48/17, LlDR24/01), *L. acidophilus* (LacKS48/15, LacWP48/22), and *L. amylovorus* (LamWP48/16).

Table 2 Viability of *Lactobacillus* strains (isolates) after 3 h exposure to different pH and bile salt concentrations

Species	Total number of isolate	Number of isolate (%)					
		pH			Bile salt (%)		
		2.5	3	4	0.3	0.6	0.9
L. plantarum	35	4(11.4)	16(45.7)	27(77.1)	15(42.9)	11(31.4)	4(11.4)
L. lactis	22	3(13.6)	10(45.4)	19(86.4)	12(54.5)	8(36.4)	3(13.6)
L. amylovorus	12	1(8.3)	7(58.3)	10(83.3)	7(58.3)	2(16.7)	1(8.3)
L. casei	7	0(0)	2(28.6)	5(71.4)	3(42.8)	2(28.6)	0(0)
L. acidophilus	3	2(66.7)	3(100)	3(100)	3(100)	3(100)	2(66.7)
L. farciminis	2	0(0)	0(0)	1(50)	2(100)	0(0)	0(0)
L. graminis	1	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)

Antibacterial activity

Antibacterial activity against intestinal pathogens is important for potential probiotic strains. Antimicrobial substances or metabolites include organic acids (especially lactic acids), hydrogen peroxide, and bacteriocins that are the most common products of probiotic strains (Suskovic, Blanzenka, Beganovic, Pavunc, Habjanic, & Motosic, 2010). The mean and standard deviations of inhibition zone diameters are shown in **Table 3**. Results indicated that all *Lactobacillus* isolates showed antibacterial activity against *S. aureus, E. coli,* and *S.* Typhi, eight isolates against *P. aeroginosa* and 4 against *S. dysentery*. Remaining four isolates displayed activity against all five tested pathogens, namely *L. acidophilus* (LacKS48/15 and LacWP48/22) and *L. plantarum* (LpWP48/12 and LpNH48/12). Antimicrobial activities of *L. acidophilus* and *L. plantarum* have been previously published. For example, Neal-McKinney et al. (2012) found that *L. acidophilus* produced organic acid against six strains of *Campylobacter jejuni*, while Shokryazdan et al. (2014) who reported that nine isolates of *L. acidophilus, L. fermentum, L. buchneri, and L. casei* had antagonistic effects against *S. sonnei* (ATCC 25931), *S. aureus* (S244) and *E. coli* (ATCC 29181). Hu, Ren, Zhou, and Ye (2019) isolated *L. plantarum* strains (P1, S11, and M7) from Chinese traditional dairy products. These strains showed strong antimicrobial activity against *S. aureus* ATCC12600, *E. coli* ATCC35128 and *Salmonella* ASI.1174. Zhao et al. (2016) reported that a novel bacteriocin, designated as plantaricin JLA-9, was produced from *L. plantarum* JLA-9 isolated from Suan-Tsai, a traditional Chinese fermented cabbage. This plantaricin exhibited broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria.

Table 3 Antimicrobial activities as average inhibition zone diameters of *Lactobacillus* strains against some food-borne pathogens

	Inhibition zone diameter (mm)				
Isolate	***S. aureus***	***E. coli***	***S.* Typhi**	***S. dysentery***	***P. aeruginosa***
LpWP48/10	16.4±0.6	17.1±1.1	9.6±0.7	-	7.1±0.6
LpWP48/12	19.0±0.3	20.4±0.9	13.8±0.3	8.3±0.6	12.3±0.6
LpNH48/09	13.6±1.1	14.4±0.6	8.7±0.3	-	-
LpNH48/12	21.4±0.6	17.3±0.8	11.8±0.6	7.5±0.3	11.4±0.8
LlNH24/03	18.1±0.6	12.7±0.9	9.7±0.6	-	-
LlPD48/17	17.3±0.3	14.2±0.6	10.3±0.3	-	7.2±0.6
LlDR24/01	16.2±1.2	17.7±1.0	8.1±0.6	-	9.9±0.6
LamWP48/16	17.5±0.9	15.7±0.6	7.3±0.6	-	10.4±0.4
LacKS48/15	14.3±0.9	18.1±0.8	7.8±0.6	9.1±0.6	7.6±0.6
LacWP48/22	16.2±0.6	19.1±0.9	10.9±0.7	7.3±0.6	9.3±0.4

Antibiotic resistance

Antibiotic susceptibility is one approach for quality control assessment of probiotic strains. Most lactic acid bacteria show the ability to resist various antibiotics and, subsequently, exhibit profitable effects on the health of the host (Lim, 2009). As **Table 4** showed that the four selected *Lactobacillus* strains (LpWP48/12, LpNH48/12, LacKS48/15, and LacWP48/22) resisted streptomycin, cefotetan, nalidixic acid, vancomycin, kanamycin and methicillin, and were sensitive to rifampin, chloramphenicol, gentamycin, ampicillin and erythromycin. Thus, they were not affected by some antibiotic therapies, and might help to maintain the balance of intestinal bacteria during antibiotic treatments (Salminen et al., 1998). Results agreed with Pisano et al. (2014) who reported that three isolates of *L. plantarum* (11/20966, 19/20711, and 4/16868) from milk resisted vancomycin, streptomycin and kanamycin and were also sensitive to ampicillin, chloramphenicol, erythromycin and gentamycin. The intrinsic antibiotic resistance of *Lactobacilli* has been previously reported (Klein et al., 2000; Danielsen & Wind, 2003),

Table 4 Antibiotic susceptibility profile of candidate probiotic strains

	Antibiotic[a] (mm)										
Isolates	**RD**	**AMP**	**S**	**CTT**	**C**	**MET**	**CN**	**E**	**NA**	**VAN**	**KAN**
LpWP48/12	S	S	R	R	S	R	R	S	R	R	R
LpNH48/12	S	S	R	R	S	R	R	S	R	R	R
LacKS48/15	S	S	R	R	S	R	S	S	R	R	R
LacWP48/22	S	S	R	R	S	R	S	S	R	R	R

[a] RD, rifampin; AMP, ampicillin; S, streptomycin; CTT, cefotetan; C, chloramphenicol; MET, methicillin; CN, gentamycin; E, erythromycin; NA, nalidixic acid; VAN, vancomycin; KAN, kanamycin, S: susceptible; R: resistance

while vancomycin resistance in *Lactobacilli* is well understood. Bacteria alter the cell wall construction of the peptidoglycan precursor, the target for vancomycin activity, via synthesis of a depsipeptide d-alanine-d-lactate instead of dipeptide d-alanine-d-alanine (Handwerger, Pucci, Volk, Liu, & Lee, 1994). However, glycopeptide resistance in *Lactobacillus* strains is not of the transmissible type (Klein et al., 2000).

Probiotic and virulence genes

Genotypes of LpWP48/12, LpNH48/12, LacKS48/15, and LacWP48/22 were characterized as shown in **Table 5**. They were positively detected the *bsh* and *atpD* genes with 712 and 624 bp, respectively. This evidence supported their acid and bile salt tolerance abilities. The *bsh* gene encodes bile salt hydrolase (BSH) and plays an

Table 5 Molecular identification of probiotic and virulent genes of *Lactobacillus* strains

	Genes[a]						
Isolate	***bsh***	***atpD***	***mapA***	***cylA***	***ace***	***esp***	***gelE***
LpWP48/12	+	+	+	-	-	-	-
LpNH48/12	+	+	+	-	-	-	-
LacKS48/15	+	+	+	-	-	-	-
LacWP48/22	+	+	+	-	-	-	-
L. acidophilus TISTR 2365	+	+	+	-	-	-	-
E. faecalis isolate KS-07	+	-	-	+	+	-	+
E. faecalis isolate KM-16	+	-	-	+	-	+	+

[a] *bsh*, bile salt hydrolase; *atpD*, neutral pH; *mapA*, adhesion proteins; *cylA*, cytolysin A; *ace*, collagen adhesion precursor; *esp*, cell-wall-associated protein involved in immune evasion; *gelE*, gelatinase E

important role in catalyzing conjugated bile salts to unconjugated bile salts. Conjugated bile salts are more efficient than unconjugated molecules in the emulsification of dietary lipids and formation of micelles (Begley, Hill, & Gahan, 2006). The *atpD* gene encodes neutral pH as a key requisite for acid tolerance (Duary, Batish, & Grover, 2010). These four isolates also positively detected the *mapA* gene with 1,024 bp. This encodes adhesion protein and plays an important role in binding probiotic bacteria to intestinal cells such as HT29 cells and Caco-2 cells (Buck, Altermann, Svingerud, & Klaenhammer, 2005). To guarantee the safety of our probiotic candidate strains, the virulence-ralated genes (*cylA*, *ace*, *esp* and *gelE)* were not detected, in agreement with Botthoulath et al. (2018), who found none of these virulence-related genes in *L. plantarum* strains. By contrast, *E. faecalis* KS-07 and *E. faecalis* KM-16 as expected PCR products were observed in the positive control.

Conclusion and Suggestions

Our results showed that *L. acidophilus* (LacKS48/15 and LacWP48/22) and *L. plantarum* (LpWP48/12 and LpNH48/12) have probiotic potential. They had interesting probiotic properties such as excellent pH and bile tolerance, inhibition of intestinal pathogen growth, and ability to display the gene coding for adhesion proteins. They also lacked virulence genes and sensitive to variety of clinically effective. As a result, they were safe *Lactobacillus* candidates and could be used as starters for probiotic products. However, further *in vivo* confirmation is required to consolidate these results.

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