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Probiotics isolated from Thai fermented foods for potential uses against foodborne pathogens

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Abstract

Lactic acid bacteria (LAB) are a group of bacteria showing promising probiotic characteristics conferring health benefits. Several studies have reported that LAB can inhibit pathogenic bacteria in the digestive system, reduce blood cholesterol levels, and improve functions of the immune system. A variety of fermented foods was reported as one of the sources of LAB. This study characterized LAB isolated from local fermented foods and determined the probiotic properties. Three potential probiotic strains, P09, P10, and P11 exhibited high acid and bile salt tolerances. The 16S ribosomal DNA (rDNA) gene analysis revealed that P09, P10 and P11 isolates were all identified as *Lactobacillus plantarum*. Additionally, all isolates showed negative results for hemolytic and deoxyribonuclease (DNase) activities in which they may be safe for applying as food vehicles. *L. plantarum* P10 showed the highest adhesion ability to Caco-2 cells at the level of 4.52%. *L. plantarum* P10 was thus investigated regarding the antimicrobial activity against bacterial pathogens by co-culture assay. The result demonstrated that the strain P10 had a significantly inhibitory effect on the growth of *Escherichia coli* ATCC 25922, *Salmonella typhimurium* DMST 560, *Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* DMST 5040 after co-culturing for 24 h ($p<0.05$). Remarkably, *L. plantarum* P10 showed the highest activity against *S. aureus* with 58.63 % inhibition at 24 h of co-incubation. The results indicated that *L. plantarum* P10 isolated from local fermented fish (Pla-Som) satisfied the criteria for potential probiotics suitable for applying in products against foodborne pathogens.

Keywords: Lactic acid bacteria, Probiotic, Fermented foods, Antibacterial activity

1. Introduction

The Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) define probiotic organisms as live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host [1]. Lactic acid bacteria (LAB) are a type of probiotic which has been the most widely used in food products. LAB are "generally regarded as safe (GRAS)" as they play an essential role in the food fermentation process. Moreover, LAB can produce antimicrobial substances, such as, bacteriocins, lactic acid, acetic acid, and hydrogen peroxide (H_2O_2) [2]. The sources of LAB are diverse, for example, from the natural environment, intestinal tracts of mammals, and fermented foods. Several research studies have isolated and identified LAB from fermented meat products, traditional fermented fish products, and other food products [3]. The screening and isolation of micro-organisms from natural sources have always been the most powerful means for obtaining useful strains for health products. Furthermore, the important factors for considering an appropriate probiotic are its survival through the digestive system, attachment to the intestinal epithelium, maintaining good viability, capable of exerting a beneficial effect, and safety. Many researchers have examined the survivability of probiotics and some LAB strains have been found to be unable to survive

through the gastro-intestinal conditions [4]. Even though several studies tried to isolate and characterize new probiotic strains from variety of natural sources including fermented foods, only a few reports established the test to show the strong inhibitory activity when probiotics and pathogens were co-incubated together.

The present study aimed to isolate and characterize LAB derived from local Thai fermented foods and thus to identify and determine the probiotic properties in term of their functional and safety characteristics. The isolated LABs were primarily screened for antibacterial activity against 6 pathogenic bacteria. The selected LAB strains exhibiting high antibacterial activity were then tested for acid and bile salt tolerances. LABs with the highest survival rates in acid and bile salt conditions were identified and evaluated for ability to adhere to the Caco-2 cells. The LAB isolate displaying the highest adhesion ability to Caco-2 cells was further investigated to determine its property to inhibit pathogenic bacteria by co-culture assay.

2. Materials and methods

2.1 Sampling of fermented foods

Fifty fermented food samples were collected from several local markets and supermarkets in Ubon Ratchathani and nearby provinces. The food samples, including pickled fish, pickled kimchi, pickled crab, fermented minced fish, Nham and sausages were conveyed directly to the laboratory and kept at 4 °C for further analysis.

2.2 Isolation and characterization of LAB

Ten grams of each fermented food sample were aseptically transferred to the sterile tube and 50 mL of saline (NaCl 0.85% w/v) was then added. The preparation was blended for five minutes, and 100 µL of the samples was spread on de Man, Rogosa and Sharpe (MRS) agar and incubated at 37 °C for 24 h. The colonies of the LAB were randomly selected from the MRS agar plates of each sample with the morphology of a white color and convex shape then transferred as a single colony on the MRS agar plates by streak plate method and incubated at 37 °C for 24 h. A single colony of LAB was tested for Gram-staining and catalase test [5].

2.3 Screening of antibacterial activity of LAB isolates by cylinder-plate method

Six pathogenic bacteria comprising four Gram negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* DMST 4739, *Salmonella enteritidis* DMST 15676, *Salmonella typhimurium* DMST 560), and two Gram positive bacteria (*Bacillus cereus* DMST 5040 and *Staphylococcus aureus* ATCC 25923) were evaluated. The overnight culture of LAB in MRS broth was centrifuged at 8,000 x g for 15 minutes at a temperature of 4 °C, and the supernatant was filter sterilized. The indicator strains were cultured in trypticase soy broth (TSB) for 18-24 h at 37 °C. Then the overnight culture of pathogenic bacteria (10^8 Colony Forming Unit (CFU)/mL) were spread on Mueller Hinton Agar (MHA) by a cotton swab. Then, the wells were made using a clean and sterile stainless steel cylinder cup with an outside diameter of 8 mm (±0.1 mm) and an inside diameter of 6 mm (±0.1 mm) with a length of 10 mm, and 180 µL of cell free culture supernatant (CFCS) was added into the cup. The plates were incubated at a temperature of 37 °C overnight. Finally, the diameter of the zone of inhibition was measured in millimeters (mm) using a Vernier caliper. Oxytetracycline 50 µg/mL was used as the positive control, and MRS broth was used as the negative control.

2.4 Acid and bile salt tolerances

Bacterial cells from the overnight cultures were centrifuged at 4,000 rpm for 10 minutes at 4 °C, then the cell pellets were washed with Phosphate Buffered Saline (PBS) suspended in MRS broth to obtain the cell concentration of approximately 1.5 x 10^8 CFU/mL. One mL of LAB culture was added into 9 mL of PBS that was adjusted the pH value to pH 2.0, 3.0 and 6.5 using 1 M HCl and 3 M NaOH. The viable cell counts were determined after incubation at 37 °C for 0 and 4 h by standard plate count on MRS agar. The ability of the isolates to grow in the presence of bile was determined by adding bacterial suspensions to MRS broth supplemented with 0.15 and 0.30% (w/v) bile salt. The viable cell counts were determined after incubation at 37°C for 0 and 4 h, reflecting the time spent by food in the small intestine. The survivability of the LAB isolates under acidic and bile salt conditions was calculated using the following equation:

$$\% \text{ Survival} = [\log \text{no. of viable cell survived (CFU/mL)} / \log \text{no. of initial viable cell inoculated CFU/mL})] \times 100$$

2.5 Adhesion to the Caco-2 cell line

An *in vitro* adherence assay of LAB isolates to the human colon carcinoma cell line (Caco-2) was performed as previously described by Pennaacchia et al. and Charnchai et al. [6,7]. The Caco-2 cells were grown in a cell culture flask using a Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA), 10% heat-inactivated fetal bovine serum, 100 mL streptomycin, 1% non-essential amino acid, and 100 IU/mL penicillin. The Caco-2 cells were subsequently added into 24-well culture plates at a concentration of $2.5x10^5$ cells per well and allowed to differentiate for three days, while the medium was changed daily. The Caco-2 cells were incubated at a temperature of 37 °C in a 5% CO_2 atmosphere. Overnight cultures of the LAB isolates were centrifuged, washed twice with PBS, and resuspended in the same buffer. After that, the bacterial cell suspension of LAB isolates was added to each well, and the plates were incubated at a temperature of 37ºC for four h. After incubation, the wells were washed 3 times with PBS to remove non-adherent bacterial cells and then 0.1% Triton X-100 solution was added to each well to lyse Caco-2 cells. The mixtures of lysed Caco-2 cells and LAB isolates were serially diluted and spread on the MRS agar plates to determine the number of viable adhered LAB isolates. The agar plates were incubated at a temperature of 37 ºC for 24-48 h. The percentage of the bacterial adhesion on Caco-2 cells was calculated as a percentage of the viable bacteria according to their initial population.

2.6 Safety test

For antibiotic resistance test, the selected LAB isolates were grown in MRS broth at a temperature of 37 °C for 24 h and then adjusted to be 10^8 CFU/mL. Then, bacterial cells were spread onto the MRS agar plate using sterile cotton swab. The antibiotic discs used for the antibiotic resistance assay were sulphamethoxazole 25 µg, norfloxacin 10 µg, tetracycline 30 µg, ampicillin 10 µg, and gentamicin 10 µg, and these were placed on agar and incubated at a temperature of 37 °C for 24 h. The antibiotic susceptibility was reported with sensitivity, intermediate susceptibility, and resistance [8].

Hemolytic activity was determined by inoculating the strains on the Columbia Blood agar plates. After 48 h of incubation at a temperature of 37 °C, the hemolytic reaction was recorded by observation of the clear zone of the hydrolysis around the colonies (beta-hemolysis), partial hydrolysis and the greenish zone (alpha-hemolysis), or no reaction (gamma-hemolysis). *Staphylococcus aureus* ATCC 25923 was used as the positive control [9].

DNase test agar was used for detecting the deoxyribonuclease (DNase) activity of the bacteria. LABs were grown in MRS broth at a temperature of 37 °C for 24 h and were then adjusted to be 10^8 CFU/mL. The LAB culture was dropped on the DNase agar. After incubation at 37 °C for 24 h, the agar plate was flooded with 1 N HCl, and the clear zone around the growth was recorded. *Staphylococcus epidermidis* DMST 15505 was used for the negative control, and *Staphylococcus aureus* ATCC 25923 was used for the positive control [10].

2.7 The 16S rDNA gene sequences of the selected LAB

The selected LABs were identified by 16S ribosomal DNA (rDNA) gene sequencing (Mahidol University-Osaka University Collaborative Research Center for Bioscience and Biotechnology, Mahidol University). The 16S rDNA sequences were analyzed using the GenBank databases and identification was conducted on the basis of 16S rDNA sequence homology using the BLAST algorithm of the National Center for Biotechnological Information (NCBI), USA through: http://blast.ncbi.nlm.nih.gov.

2.8 Co-culture of probiotic and bacterial pathogens

The co-culture assay was performed with 4 bacterial pathogens including *B. cereus* DMST 5040, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *S. typhimurium* DMST 560. The LAB strain and the pathogenic strains were each incubated at a temperature of 37 °C for 24 h. The cells were removed from the culture broth and washed twice by using 30 mL of 0.85% (w/v) NaCl and centrifuged at 8,000 x g for 15 minutes at a temperature of 4 °C. The cell suspension was determined using a spectrophotometer at 625 nm and diluted to an optical density of 0.08-1.0, the cell suspensions of each pathogenic bacteria at 10^8 CFU/mL were co-cultured with LAB in the modified media containing 1:1 MRS and TSB. All groups were cultivated in an Erlenmeyer flask containing 100 mL of broth incubated at a temperature of 37 °C. The growth was monitored by plating onto selective agars at specific time points (0, 6, 12, 18, and 24 h) over a period of 24 h. The samples of 1 mL were taken and serially diluted in normal saline. A 100 µL aliquot was plated onto MRS agar for the probiotic strain, Phenol Red Egg Yolk Polymyxin (MYP) Agar Base for *B. cereus* DMST 5040, Mannitol Salt Agar (MSA) for *S. aureus* ATCC 25923, MacConkey Agar for *E. coli* ATCC 25922, and *Salmonella Shigella* (SS) Agar for *S. typhimurium* DMST 560. As the controls, 100 µL of each pathogen was used to be inoculated and kept under the same condition. All plates were incubated at a temperature of 37 °C for 48 h for the MRS and selective

agars. One mL was taken from each sample at each time point for the pH measurement (adapted from [11]. The percentage of inhibition after 24 h of co-incubation was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{\text{(CFU/mL of the pathogen alone)-(CFU/mL of the pathogen in co-culture) x 100}}{\text{CFU/mL of pathogen alone}}$$

2.9 Statistical analysis

Values were presented values and standard deviations of triplicate experiments. Significant ANOVA results were tested with Tukey's Multiple Comparison Test and differences were considered statistically significant if $p < 0.05$.

3. Results and discussion

3.1 Isolation and screening of LABs for probiotic properties

Eighty-three LAB isolates from 50 fermented foods were tested for antibacterial activity against six pathogenic bacteria. Ten LAB isolates displayed the strong inhibition zone against each of the pathogenic bacteria were selected for further characterization (Table 1). The inhibition zones of FFPR2 and FFPS1 against *S. aureus* were 21.00±1.41 mm and 21.00±1.00 mm, respectively. The isolate P06 also showed the high inhibition zone against *S. aureus* at 21.00±1.00 mm. The isolates P01 and P02 exhibited the highest inhibition zone against *P. aeruginosa* (18.00±1.00 mm). P03 showed the inhibition zone against *S. typhimurium* at 17.33±0.58 mm. P05 and P11 could inhibit *B. cereus* with the inhibition zone of 18.00±1.00 mm. The isolates P09 and P10 showed the same inhibition zone against *S. enteritidis* at 19.00±1.00 mm. The isolates P10 exhibited the highest inhibitory activity against *E. coli* at the inhibition zone of 18.00±1.00 mm. All 83 LAB isolates obtained in this study demonstrated a strong inhibitory effect against *S. aureus, P. aeruginosa*, *S. enteritidis*, *E. coli*, *S. typhimurium,* and *B. cereus,* and 51 LAB isolates showed inhibitory activity against all six bacterial pathogens (data not shown). Additionally, the isolates FFPR2, FFPS1, P01, P02, P03, P05, P06, P09, P10, and P11 showed the highest inhibition zone against each pathogenic bacteria.

LABs produced many organic acid compounds resulting in the decreasing pH levels that could inhibit unfavorable bacteria, including pathogenic and spoilage bacteria. Inhibition of pathogens is a major probiotic selection criterion and this mechanism is involved in the restoration of gut microbiota balance [12]. Additionally, after neutralizing the pH of cell-free supernatants from 4.0 (±0.5) to the pH 6.5, no inhibition activities of 10 LAB isolates were observed (data not shown). Therefore, inhibitory effects of the strains cannot be explained by bacteriocin action and were most probably due to the production of organic acids along with the low pH. Similarly, a study on screening of lactic acid bacteria from swine origins for multi-strain probiotics showed that the antimicrobial activity was significantly decreased after neutralization, and no inhibition against the pathogens was observed when the pH was adjusted to 6.0 [13].

Table 1 Antibacterial activity of 10 selected LAB isolates determined by cylinder-plate method.

LAB Isolates	Inhibition zone (mm.)					
	Gram negative bacteria				Gram positive bacteria	
	E. coli	*P. aeruginosa*	*S. enteritidis*	*S. typhimurium*	*B. cereus*	*S. aureus*
FFPR 2	11.67±0.58	15.00±1.41	12.00±1.00	15.00±1.41	10.67±0.58	21.00±1.41
FFPS 1	11.33±1.53	14.67±1.53	12.67±0.58	12.00±1.73	-	21.00±1.00
P01	16.33±1.53	18.00±1.00	17.00±1.41	15.50±0.71	14.00±1.73	13.33±1.53
P02	14.67±1.15	18.00±1.00	17.33±0.58	11.00±0.00	16.00±1.41	15.67±1.15
P03	15.67±0.58	15.50±0.71	16.33±1.15	17.33±0.58	13.00±1.00	13.00±1.00
P05	15.67±0.58	14.00±0.00	16.00±1.41	11.00±0.00	18.00±1.00	14.00±1.73
P06	16.33±1.15	15.33±0.58	16.67±1.53	11.33±1.53	12.50±0.71	21.00±1.00
P09	16.33±0.58	16.33±1.53	19.00±1.00	9.67±0.58	14.50±0.71	13.00±0.00
P10	18.00±1.00	16.00±1.00	19.00±1.00	15.00±0.00	15.50±0.71	14.50±0.71
P11	16.33±0.58	17.00±1.41	16.50±2.12	11.50±0.71	18.00±1.00	11.67±0.58
Oxytetracycline (50 µg/mL)	19.56±1.17	21.33±1.53	17.44±2.17	28.56±1.71	33.56±2.22	31.33±1.53

Note: Each value represents the mean of determinations ± standard deviation. (-): no inhibition zone.

3.2 Acid and bile salt tolerances

According to their antibacterial activities, 10 LAB isolates were selected to determine the acid and bile salt tolerances. All 10 isolates showed a high survivability of more than 90.00% (10^6-10^8 CFU/mL) in strong acid conditions at both pH 2.0 and pH 3.0 after incubation for 3 h (Figure 1). At 0.15% w/v of the bile salt, there was a slight effect on viability of the selected LAB, especially, the isolates P09, P10, and P11 that showed a very high tolerance to 0.15% bile salt with a more than 120% survival rate. Additionally, P06, P09, P10, and P11 displayed a high bile salt tolerance with more than 110% survival rate at the 0.30% concentration of bile salt (Figure 2).

Microbial bile tolerance is a recognized criterion for probiotic strain selection [14]. Lee et al. (2016) reported that *Lactobacillus plantarum* C182 survived well after 3 h exposure to 0.30% bile salts with the survival ratio of 58.53% [15]. In this study, the survival rates of all 10 isolates decreased when exposed to 0.3% bile salts except the isolates P06, P09, P10, and P11. The resistance to low pH conditions of LAB isolates is crucial to survive under human gastrointestinal (GI) conditions. The strains considered for use as probiotics must exhibit a high GI tolerance. The strains used in this study showed great resistance to acid condition, thus this may be due to the fact of their origins in which they were isolated from high acid foods (fermented foods). Similar findings were also reported by Turchi et al. (2013) presenting the high survival rate of some strains of *Lactobacillus plantarum* in acid conditions at the % survival rate ranging from 100.81 to 102.87 [16]. According to their strong acid and bile salt tolerances (viability at pH 2.0 more than 100% and survivability at 0.15% bile salt higher than 120%), the 3 LAB isolates P09, P10 and P11 were then selected for further investigation of Caco-2 cells adhesion.

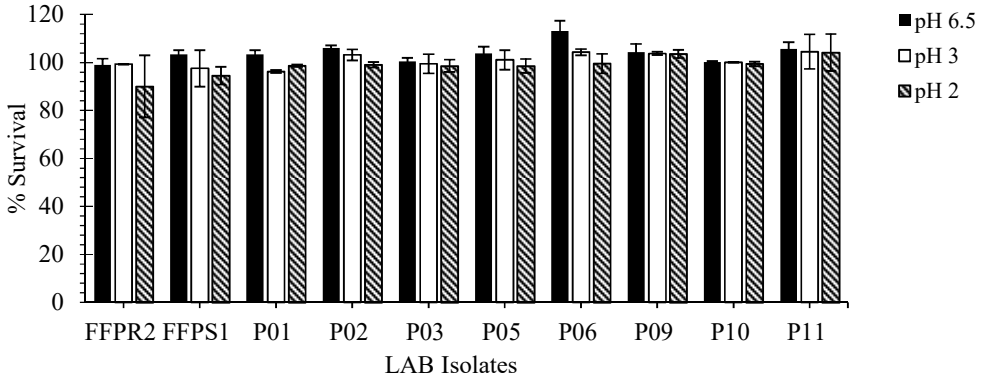

Figure 1 The survivability of 10 LAB isolates under acidic conditions (pH 2 and pH 3) for 3 h. The results showed as a mean ± SD of triplicate experiments.

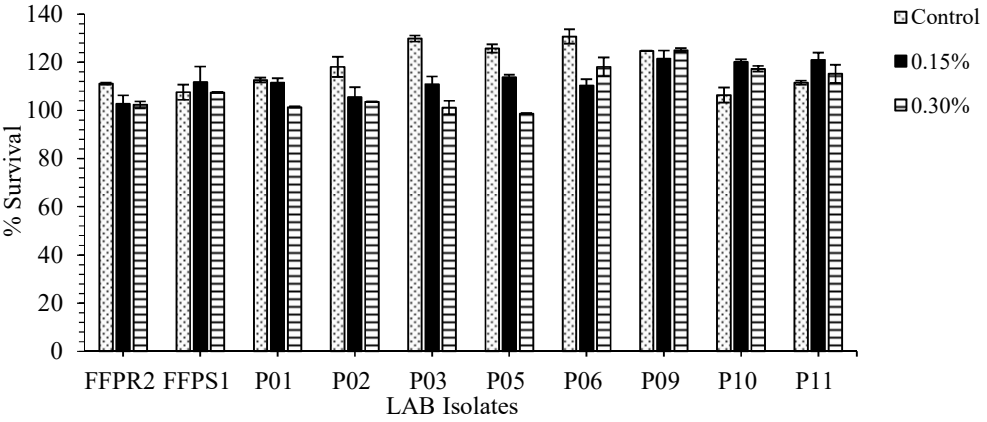

Figure 2 The survivability of 10 LAB isolates in MRS broth treated with 0.15% and 0.30% bile salt and MRS without bile salt as a control group. The results showed as a mean ± SD of triplicate experiments.

3.3 Adhesion of the selected LAB isolates to the Caco-2 cells

Based on the acid and bile salt tolerances evaluation of the selected LAB strains, three isolates (P09, P10 and P11) were chosen for further investigation of Caco-2 cells adhesion. The P10 isolate displayed the highest level of adherence (4.52%) followed by the isolate P11 (2.85%) compared to those of the control strains. P09 showed a significantly lower level than the P10 isolate (1.78) as shown in (Figure 3). Thus, the P10 strain was considered as a candidate probiotic and further investigated for anti-pathogenic bacteria by co-culture assay. The adhesion capacity of LAB to the intestinal mucosa for colonization of the gut for optimal functionality has been considered as an important property of probiotics. This ability may provide beneficial effects such as the exclusion of pathogens. Adhesion levels of LAB to Caco-2 cells depend on the strain, species and genera. Collado et al. (2006) found that adhesion values of *Pediococcus freudenreichii* JS was 0.9%, whereas, 20% Caco-2 cells adhesion of *Lactobacillus rhamnosus* GG was observed. *P. pentosaceus* CIAL-86 presented the high adhesion percentage (12.2%), followed by *L. plantarum* CIAL-121 (7.10%) [17]. Previous research also showed the similar adhesion capability of *Lactobacillus plantarum* to intestinal epithelial cells (HT-29 cells) with an average range of 5.3-7.2 [18]. The adhesion ability is affected by many factors, including the production of exopolysaccharide (EPS).The structure of EPS may promote strain-specific interactions of bacteria with specific receptors and effectors of Caco-2 cells [19]. In addition, bacteria can generate protein on the cell wall (extracellular matrix molecules) such as collagen, fibronectin, and vitronectin, and these proteins can adhere to the mucous membrane on the intestinal wall [20]. Gastrointestinal colonization of probiotic bacteria may provide a good protection against the adhesion of pathogens to epithelium cells [21]. Therefore, the suggestion for further study is to investigate the ability of the candidate probiotic to inhibit the adhesion of pathogens.

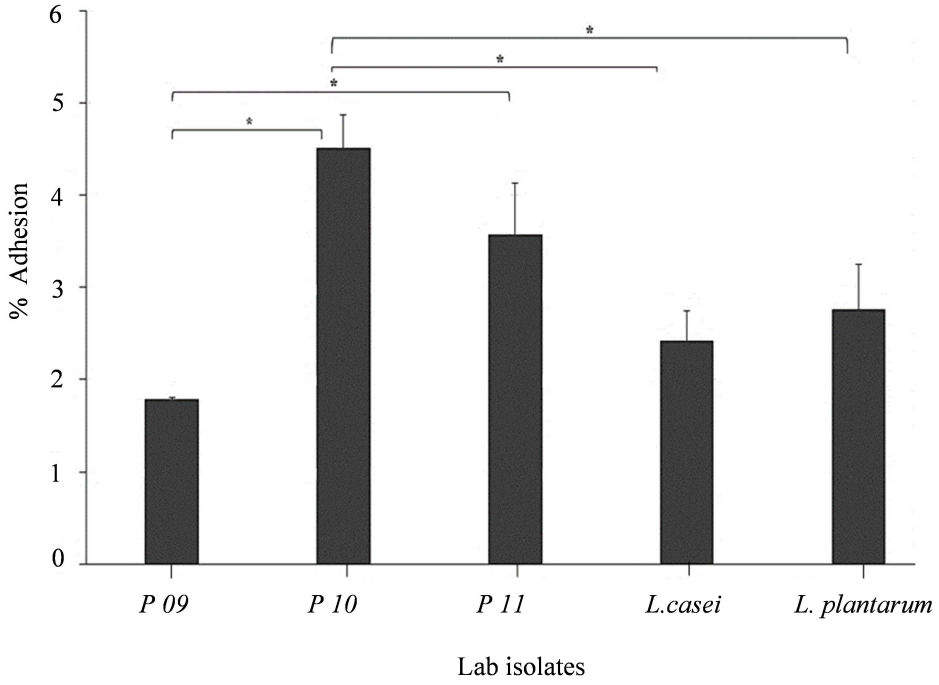

Figure 3 The adhesion ability to Caco-2 cells of the isolates P09, P10, P11, and reference strains (*Lactobacillus casei* TISTR 1341 and *L. plantarum* TISTR 541).
*Represent a significantly different between group (p<0.05) determined by Tukey test.

3.4 LAB identification by 16S rDNA gene analysis

The molecular identification of the selected LAB strains was investigated by 16S rDNA gene analysis. The isolate P09 showed 94.79% *Lactobacillus plantarum* strain CIP 103151, P10 showed 95.52% *Lactobacillus plantarum* strain CIP 103151, and P11 showed 97.78% *Lactobacillus plantarum* strain CIP 103151. All three selected strains were identified as *Lactobacillus plantarum*. Therefore, these 3 strains were named *L. plantarum* P09, *L. plantarum* P10 and *L. plantarum* P11 respectively. Paludan-Müller and colleagues (2002) also reported that *Lactobacillus plantarum* had been isolated from Thai fermented fish [22]. *L. plantarum* as members of probiotic strains are generally regarded as safe and they have a long history of use.

3.5 Safety test

The safety characteristics of three LAB isolates including *L. plantarum* P09, *L. plantarum* P10 and *L.plantarum* P11 were preliminary evaluated by antibiotics susceptibility test, hemolytic and DNase tests according to safety criteria on the human host [23,24]. The susceptibility of LAB to various antibiotics are variable, depending on the species. One thing to be cautious about in the selection and characterization of a probiotic is antimicrobial resistance. It is generally known that fermented foods containing pathogenic microbes transfer their resistant genes to other microbes existing in the gut of human microflora. Therefore, it is necessary to examine antibiotic susceptibility before using probiotics for health relating products. The susceptibility and resistance of LAB to various antibiotics are variable depending on the species [14]. Five antibiotics representing each class of common antibiotic used in clinical were chosen for the antibiotic susceptibility test. The antibiotic susceptibility profiles of the 3 selected LAB isolates to 5 common antibiotics tested by disc diffusion are summarized in Table 2. The result found that all 3 LAB isolates were resistant to norfloxacin. It may be likely indicated that these isolates could be used as the probiotic supplement in the patients who are using norfloxacin as antibiotic therapy. The resistance of *Lactobacillus plantarum* to norfloxacin is consistent with previous reports [25,26]. All isolates showed an intermediate sensitivity to tetracycline, whereas the susceptibility to ampicillin, gentamicin and sulphamethoxazole were different among isolates. *L. plantarum* P09 showed resistance to gentamicin and sulphamethoxazole. *L. plantarum* P10 showed high sensitivity to ampicillin, intermediate sensitivity to tetracycline and gentamicin, and sensitive to sulphamethoxazole. Additionally, *L. plantarum* P11 was susceptible to ampicillin but showed intermediate sensitivity to tetracycline, gentamicin, and sulphamethoxazole. There is the possibility of antibiotic resistant genes transferring to other pathogenic bacteria, but the norfloxacin resistance in lactobacilli has been reported to be intrinsic, chromosomally encoded and not transferable [27]. Therefore, those probiotic strains with intrinsic antibiotic resistance could be restoring the intestinal microbiota after antibiotic treatment. The antibiotic resistance of the strain *L. plantarum* P09 to gentamicin and sulphamethoxazole may require further investigation to investigate the possibility of resistant gene transfer.

According to FAO guidelines, microbial strains to be used as probiotics are recommended to be safe in the host. The selection and application of strains devoid of hemolytic and DNase activities can be used as probiotics. Results showed that all three isolates did not show any red blood cell digestion and DNase activity. Therefore, all isolates may be considered as a safe prerequisite for the selection of the probiotic strain used in food matrices. Similar results indicated that most LAB strains are non-hemolytic [28,29]. LAB was also reported to lack deoxyribonuclease enzymes [30].

Table 2 Safety properties of 3 selected LAB isolates.

Properties	Isolates		
	P09	P10	P11
Antibiotic susceptibility			
Norfloxacin (10 µg)	R	R	R
Ampicillin (10 µg)	I	H	S
Tetracycline (30 µg)	I	I	I
Gentamicin (10 µg)	R	I	I
Sulphamethoxazole (25 µg)	R	S	I
Hemolytic activity	ND	ND	ND
DNase activity	ND	ND	ND

Note: Highly sensitive (H) >31.00 mm, Sensitive (S) 21.00-30.00 mm, Intermediate (I) 10.00-20.00 mm, Resistant (R) to antibiotics <10.00 mm; ND: not detected

3.6 Co-culture of the isolate P10 and bacterial pathogens

According to previous experiments, *L. plantarum* P10 is a candidate probiotic strain showing antibacterial activity with high acid and bile salt tolerance and the highest ability for Caco-2 cell adhesion (Figure 3). Therefore, the strain *L. plantarum* P10 was selected for evaluation of anti-pathogenic bacteria by co-culture assay. The results of co-culture assay demonstrated that *L. plantarum* P10 exhibited significant antibacterial activity as shown in Table 3 and Figure 4. The isolate P10 showed anti-*E. coli* activity by a significant decrease in the growth of *E. coli* to 7.84 ± 0.16 log CFU/mL when compared with the *E. coli* in the control group (8.91 ± 0.01 log CFU/mL) with 12.01% inhibition at 24 h of incubation time (Table 3 and Figure 4). The isolate P10 co-cultured with *S. typhimurium* also displayed a significant decrease in the growth of *S. typhimurium* to 6.34±0.03 log CFU/mL when compared with the *S. typhimurium* in the control group (8.41 ± 0.47 log CFU/mL) with 24.49% inhibition at 24 h of incubation time (Table 3 and Figure 4). For anti-*B. cereus* (Table 3 and Figure 4), the strain *L. plantarum* P10 co-cultured with *B. cereus* showed a significant decrease in the growth of *B. cereus*

to 7.25±0.04 log CFU/mL when compared with the *B. cereus* control group (7.54±0.02 log CFU/mL) with the lowest level of reduction at 3.85 % inhibition at 24 h of co-incubation. *L. plantarum* P10 showed the promising inhibitory activity to *S. aureus* by co-culturing this probiotic strain with *S. aureus* (Table 3). The result revealed a significant decrease in the growth of *S. aureus* to 2.47±0.07 log CFU/mL when compared with the *S. aureus* control group (5.97±0.02 log CFU/mL). Remarkably, *L. plantarum* P10 showed the highest activity against *S. aureus* with 58.63% inhibition at 24 h of co-incubation (Figure 4). Moreover, the growth of *L. plantarum* P10 was not affected by the pathogenic bacteria over 24 h of interaction (Table 3). The antibacterial activity of *L. plantarum* P10 against pathogenic bacteria after 24 h tended to be continually affected by decreasing numbers of pathogens until no detection level. This was supported by the work of Botthoulath et al. (2018) that reported the inhibitory activity of *L. plantarum* subsp. *plantarum* SKI19 in co-cultivation with *E. coli*, *S. aureus* and *Listeria monocytogenes*. This study demonstrated the complete inactivation of pathogens after 36 h incubation [31].

There was a significant decrease ($p<0.05$) in the pH value in the group of *L. plantarum* P10 co-cultured with *E. coli* (4.82±0.01) when compared with the *E. coli* in the control group (4.98±0.01) after 24 h incubation. Similarly, the significant reduction ($p<0.05$) of the pH value was detected in *L. plantarum* P10 co-cultured with *S. typhimurium* (3.97±0.01) compared with the *S. typhimurium* culture in the control group (4.92±0.00) after 24 h. For *B. cereus*, the pH value in the co-culture of *L. plantarum* P10 with *B. cereus* (4.08±0.05) significantly decreased ($p<0.05$) when compared with the *B. cereus* culture in the control group (4.94±0.01) after 24 h incubation. In addition, a significant decrease ($p<0.05$) of the pH value was determined in the culture of *L. plantarum* P10 co-cultured with *S. aureus* (3.87±0.02) when compared with the *S. aureus* in the control group (4.86±0.01) at 24 h incubation.

This co-culture assay showed the suppression of both grams negative (*E. coli* and *S. typhimurium*) and gram positive (*B. cereus* and *S. aureus*) pathogenic bacteria after 24 h of incubation. This indicated that *L. plantarum* P10 presented the potential in reducing the growth of tested pathogenic bacteria with a strongest inhibitory activity against *S. aureus* as shown in Table 3. It is likely that the antimicrobial activity of probiotics is highly pathogen-specific and varies among probiotic species and even strains. Additionally, the starting pH of 6.84 decreased over 24 h to pH 4.86 in *S. aureus* alone and decreased from 6.93 to 3.87 in co-culture with *L. plantarum* P10 (data not shown). Antibacterial activity was even enhanced when the pH of fermentation broth was lower after 18 h of incubation (data not shown). Inhibitory activities against pathogenic bacteria were not observed in the neutralization of cell-free supernatant (data not shown). It was likely that antimicrobial activity against all tested pathogens may be due to an effect of organic acids produced during the growth of *L. plantarum* P10. The production of organic acids by LAB was proven in that it can increase efficacy of inhibitory agents including bacteriocin excreted into the culture, or an additive effect of the inhibitory agent plus suboptimal pH. LAB produce many organic acids such as lactic, acetic and propionic which play an important role in antimicrobial activity [32].

The co-culture study by Kumar et al., (2018) showed that 73% growth of *Salmonella enteric* serovar Typhi was inhibited by *Lactococcus lactis* MTGG-440 due to lactic acid production [33]. The anti-*S. aureus* activity of bacteriocin extracted from *L. plantarum* PKLP5 has been reported [34]. However, the present study did not detect protein or bacteriocin in cell free supernatant. The study showed the promising activity of the strain *L. plantarum* P10 on planktonic *S. aureus*, and it is anticipated that the inhibitory activity against biofilm *S. aureus* is also interesting to evaluate. Further studies on the mechanism of inhibition and identification of antibacterial substances also need investigation.

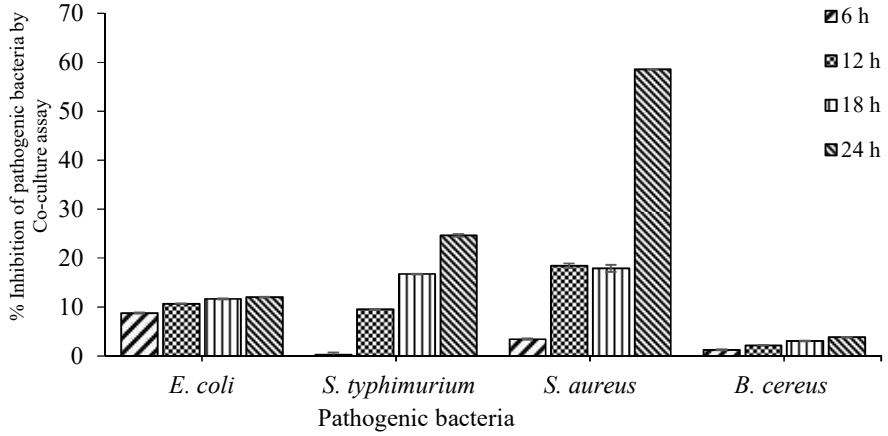

Figure 4 The percentage of inhibition of pathogenic bacteria by *L. plantarum* P10 tested by co-culture assay.

Table 3 Inhibition effect of bacterial pathogens by P10 with co-culture assay.

Co- culture of P10 with gram negative bacteria								
Incubation time (h)	P10 co-culture with *E. coli* (log CFU/mL)				P10 co-culture with *S. typhimurium* (log CFU/mL)			
	P10	P10 co-culture	*E. coli*	*E. coli* co-culture	P10	P10 co-culture	*S. typhimurium*	*S. typhimurium* co-culture
0	5.47±0.07	5.68±0.26	6.46±0.09	6.45±0.08	5.59±0.04[a]	5.22±0.05[b]	4.56±0.02	4.55±0.02
6	9.25±0.02	9.09±0.07	9.25±0.25[*]	8.44±0.01[#]	8.33±0.11	8.27±0.16	7.87±0.16	7.85±0.75
12	9.48±0.01	9.20±0.38	10.06±0.01[*]	8.98±0.04[#]	8.39±0.05	8.37±0.03	8.23±0.02[*]	7.44±0.00[#]
18	8.77±0.06[a]	7.97±0.26[b]	9.05±0.04[*]	8.00±0.21[#]	8.45±0.01	8.42±0.01	8.73±0.05[*]	7.27±0.14[#]
24	9.30±0.22	8.73±0.23	8.91±0.01[*]	7.84±0.16[#]	7.62±0.00[a]	7.12±0.00[b]	8.41±0.47[*]	6.34 ±0.03[#]
	P10 co-culture with *S. aureus* (log CFU/mL)				P10 co-culture with *B. cereus* (log CFU/mL)			
	P10	P10 co-culture	*S. aureus*	*S. aureus* co-culture	P10	P10 co-culture	*B. cereus*	*B. cereus* co-culture
0	3.48±0.05	3.98±0.65	3.42±0.14	3.41±0.18	3.66±0.05	3.73±0.04	3.60±0.09	3.56±0.04
6	5.97±0.70	5.47±0.54	5.96±0.03	5.75±0.30	7.57±0.05[a]	7.06±0.09[b]	6.66±0.12	6.58±0.08
12	8.65±0.16	8.40±0.46	8.21±0.97	6.70±0.02	9.68±0.03[a]	8.87±0.06[b]	8.38±0.01[*]	8.20±0.01[#]
18	7.92±0.10	7.78±0.10	6.11±0.02	5.02±1.40	10.80±0.05[a]	10.19±0.05[b]	8.80±0.05[*]	8.53±0.05[#]
24	7.05±0.03	6.83±0.13	5.97±0.02[*]	2.47±0.07[#]	9.84±0.02[a]	9.04±0.06[b]	7.54±0.02[*]	7.25±0.04[#]

Note: Each value is viable cell count (log CFU/mL) represent the mean of determinations ± standard deviation. Statistical analysis was performed separately for activity of P10 against each pathogen. The different superscripts in a row at the same time period of P10/P10 co-culture and pathogen/pathogen co-culture indicate statistically significant differences ($p < 0.05$) as determined by Independent t-test.

4. Conclusion

This study successfully isolated and identified LAB isolates from fermented foods that demonstrated potential probiotic properties. The results obtained from this study indicated that *L. plantarum* P10 isolated from local fermented fish (Pla-Som) satisfied the property criteria for potential probiotics and may be suitable for applying in many food matrices since it possesses no hemolytic and DNase activities. In addition, the co-culture assay showed the greatest inhibitory activity of *L. plantatrum* P10 against *S. aureus*. The findings in this study may aid the development of new anti- *S. aureus* strategies for the prevention or treatment of foodborne diseases or application as a bio-preservative in regard to anti-food spoilage.

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