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Screening and Identification of Probiotic Lactic Acid Bacteria Isolated From *Poo-Khem*, A Traditional Salted Crab

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Abstract. The present of pathogenic bacteria in *Poo-Khem* can cause the direct negative effects on human health. Thus, the use of probiotic lactic acid bacteria (LAB) which possess antimicrobial activity as starter culture may enhance the quality of this product. This study aimed to screen and identify the probiotic LAB with antimicrobial properties isolated from *Poo-Khem*. A total of 306 isolates of LAB were obtained from 30 samples of *Poo-Khem* sold in Songkhla Province, Thailand. Preliminary screening was based on antimicrobial activity against six food-borne pathogenic bacteria (*Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* Typhimurium and *Vibrio cholerae*). A total of 96 isolates which showed inhibitory activity against all food-borne pathogenic bacteria were selected and tested for acid and bile tolerance, anaerobic growth and hydrophobicity. Forty two isolates which showed high survival rates under simulated gastric and intestinal conditions with ability to growth under anaerobic condition were selected for further examination. Only 4 isolates which showed ability to adhere to epithelial cells were identified as *Enterococcus thailandensis* sp. P26-23, *Lactobacillus plantarum* sp. P28-32, *L. fermentum* sp. P26-9 and *L. fermentum* sp. P26-16 by 16S rDNA sequencing analysis. Based on their probiotic properties including their antimicrobial activities, they were considered as potential candidate lactic acid bacteria for use as starter culture in *Poo-Khem* production.

Keywords: Probiotic, Lactic acid bacteria, *Poo-Khem*, Antimicrobial activity

1. Introduction

Poo-Khem is a traditional salted crab widely consumed in Thailand. It is made from crab, salt and water and fermented with the natural microflora [1]. The fermentation process of *Poo-Khem* was based on the natural microflora. The microbial quality of this product were depended on the species and loads of pathogenic bacteria as well as the number of lactic acid bacteria. In the previous study, they found that *Poo-Khem* was contaminated with pathogenic bacteria such as *Bacillus cereus, Escherichia coli, Salmonella* Typhimurium and *Staphylococcus aureus* while LAB were found in a small number [2]. The present of pathogenic bacteria in *Poo-Khem* may cause direct negative effects on human health.

Lactic acid bacteria are widely employed in industrial fermentation processes and as starter cultures in the fermented foods. Especially, LAB with probiotic properties are used as adjuncts in food to provide a wide variety of health benefits. High tolerance at low pH and bile salts considered as important selection criteria for probiotic. Another criterion of the probiotic culture includes the ability to adhere the intestinal epithelium cell and the ability to inhibit the pathogenic bacteria [3]. Probiotic LAB with antimicrobial activity has attracted much attention as they can be used to enhance the hygienic quality of the product by inhibit the growth of pathogenic bacteria. In this study, lactic acid bacteria which isolated from *Poo-Khem* were screened for their antimicrobial activity against 6 food-borne pathogenic bacteria and some probiotic properties such as pH and bile tolerances, ability to growth under anaerobic condition and *in vitro* adhesion. Then selected probiotic LAB were subjected to identify by 16S rDNA sequencing analysis.

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2. Materials and Methods

2.1. Bacterial Strains and Culture Condition

LAB were isolated from 30 samples of salted crab (*Poo-Khem*) collected from local markets in Songkhla, Thailand by plating technique on MRS agar (Himedia, India). LAB were sub-cultured twice in MRS broth at 37°C for 18 h before use and were kept frozen at -20°C in MRS broth supplement with 20% glycerol.

Food-borne pathogenic bacteria used as indicator microorganism in this study were *Bacillus cereus* DMST 5040, *Escherichia coli* DMST 4212, *Staphylococcus aureus* DMST 8840, *Salmonella* Typhimurium DMST 562, *Vibrio cholerae* non O1/non O139 DMST 2873 and *Listeria monocytogenes* ATCC 19115. They were obtained from Department of Medical Sciences, Thailand (DMST) and American Type Culture Collection (ATCC). Bacteria indicator were sub-cultured twice in nutrient broth (NB) at 37°C for 18 h before use and were kept frozen at -20°C in NB supplement with 20% glycerol.

2.2. Screening for Antimicrobial Activity

An agar spot test was used for detection of antimicrobial activity. Briefly, fleshly prepare cultures of the LAB strains were spotted onto the surface of MRS agar plates and incubated at 37°C for 18 h to allow colonies to develop. One milliliter of indicator strains, approximately 10^7 CFU mL^{-1}, were inoculated into 9 ml of nutrient soft agar (0.75% agar) and poured over the plate on which the LAB strains were grown. After incubation for 24 h at 37°C, the plates were examined for inhibition zones. Antimicrobial activity was scored positive if the width of the clear zone around the colonies of the strains was 0.2 mm or larger**.**

2.3. Survival Under Simulated Gastro-Intestinal Tract Condition

The survival under simulated gastro-intestinal tract condition of LAB was described as follow. Bacterial cells were harvested by centrifuging at 9500 g for 10 min at 4°C. Cells were washed twice with Phosphate Buffer Saline (PBS) pH 7.2 before resuspended in PBS pH 2.0 containing 3 mg mL^{-1} pepsin (Fluka, USA). After incubation at 37°C for 3 h, they were centrifuged and washed twice with PBS pH 7.2 then resuspended in PBS pH 8.0 which contain 1 mg mL^{-1} pancreatin (Sigma, USA) and 5 mg mL^{-1} bile salt (Himedia, India) and incubated at 37°C for 4 h. Survival rate was assessed in terms of viable colony counts before and after exposed to simulated gastro-intestinal tract condition [4]. LAB which showed >50% survival rate were selected for further analysis.

2.4. Effect of Micro-Aerobic and Anaerobic Condition on the Growth of LAB

One hundred microlitres of 24 h incubation of selected LAB were inoculated into 10 mL MRS broth and 10 mL MRS broth containing 0.5 mg mL^{-1} l-cysteine which overlaid with liquid paraffin to generate the micro-aerobic and anaerobic condition, respectively. After incubated at 37 °C without shaking for 24 h, cells from micro-aerobic condition were dropped on MRS agar and further incubated at 37°C for 24 h. While cells from anaerobic condition were dropped on MRS agar containing 0.5 mg mL^{-1} l-cysteine (Sigma, USA) and overlaid with 15 g L^{-1} agar, followed by incubation at 37°C for 24 h in anaerobic jar [5]. Finally, growth rate was compared between micro-aerobic and anaerobic condition by statistical analysis.

2.5. Hydrophobicity

The ability of LAB to adhere to hydrocarbon as a measure of their hydrophobicity, was determined according to method of Vinderola and Reinheimer [6]. Briefly, selected LAB cells were harvested by centrifuging at 8000 g for 5 min at 4°C and then washed twice with PBS. Cell suspension in PBS was added to an equal volume of n-hexadecane (Sigma, USA) and mixed thoroughly for 2 min. The phases were allowed to separate at room temperature for 30 min and then one ml of the watery phase was taken to measure the optical density at 600 nm of watery phase before ($OD_{initial}$) and after (OD_{final}) mixed with n-hexadecane. Percentage of hydrophobicity was calculated as follows:

$$\text{Hydrophobicity (\%)} = [(OD_{initial}-OD_{final})/OD_{initial}] \times 100$$

2.6. 16S rDNA Sequencing Analysis

DNA was extracted and purified from their whole cells by the phenol method [7]. The phylogenetic position of the isolates was studied by the standard analysis based on the 16S rRNA gene sequence. 16S

rRNA gene sequencing was carried out as described by Sumpavapol *et al*. [8]. The sequence obtained was aligned to reference 16S rRNA gene sequences available in the GenBank/EMBL/DDBJ databases by using the program CLUSTAL_X (version 1.81) [9]. Gaps and ambiguous bases were eliminated from the calculations and the distance matrices for the aligned sequences were calculated by the two-parameter method [10]. A neighbour-joining phylogenetic tree was constructed as described by Saitou and Nei [11] using the program MEGA (version 2.1) [12]. The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis [13] based on 1000 samplings.

2.7. Statistical Analysis

Experiments were replicated as least three times. Means and standard deviations were calculated using SPSS software (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Screening for Antimicrobial Activity

Three hundred and six isolates of LAB obtained in this study were subjected to assess the antimicrobial activity against 6 pathogenic bacteria by the agar spot test. It was found that only 96 isolates showed inhibitory activity against all 6 pathogenic bacteria (*E. coli, S.* Typhimurium*, V. cholerae, B. cereus, Sal. aureus* and *L. monocytogenes*) as shown in Table 1. These isolates were cocci (55 isolates), short rods (34 isolates) and rods (7 isolates) shape bacteria.

The primary antimicrobial effect exerted by LAB is the production of lactic acid and the reduction of pH. The relative efficacy of lactic acid against Gram-negative indicator was high while Gram-positive was not affected. Because the Gram-positive indicator bacteria have a thick mesh-like cell wall which made of peptidoglycan (50-90% of cell wall) while Gram-negative indicator bacteria have a thinner layer cell wall. Another presumptive is LAB may produce some antimicrobial compounds such as hydrogen peroxide, carbon dioxide, diacetyle, reuterine and bacteriocins [14].

Table 1 Inhibitory activity of LAB against food-borne pathogenic bacteria.

Inhibitory activity	No. of isolate which showed inhibitory activity against					
	Gram-negative indicator bacteria			Gram-positive indicator bacteria		
	E. coli	*S.* Typhimurium	*V. cholerae*	*B. cereus*	*S. aureus*	*L. monocytogenes*
+	60	80	80	52	80	78
++	36	16	16	38	16	18
+++	0	0	0	6	0	0

+, zone of inhibition between 0.2 and 5 mm in diameter (week)
++, zone of inhibition between 5 and 10 mm in diameter (good)
+++, zone of inhibition larger than 10 mm in diameter (strong)

3.2. Survival Under Simulated Gastro-Intestinal Tract Condition

The gastro-intestinal transit tolerance of 96 LAB isolates was determined by exposing washed cell suspensions to simulated gastric juice (pH 2.0) containing pepsin (3 mg mL^{-1}) at 37 °C for 3 h. Under this condition, forty two LAB isolates showed survival rate more than 50%.

The survival of bacteria in the gastric juice depends on their ability to tolerate at low pH condition. The transit time can be from <1 h to 3–4 h depending on the individual, the diet and other reigning conditions [15]. However it was not clear whether the decrease of viability was caused by high acidity alone or by synergy with pepsin.

LAB which survived from simulated gastric juice were further examined for pancreatin and bile salt tolerance. It was showed that all forty two LAB isolates exhibited a survival rate more than 50% at pH 8.0 containing 5 mg mL^{-1} bile salt and 1 mg mL^{-1} pancreatin.

Most studies have shown that the majority of the strains survived well under such conditions, suggesting a potential recuperation of the initial levels during the passage of the small intestine However, the susceptibility or resistance of probiotic cultures to bile is species as well as strain specific [4].

3.3. Effect of Micro-Aerobic and Anaerobic Condition on the Growth of LAB

In the present study, the growth of 42 LAB isolates under micro-aerobic and anaerobic condition was not significantly different ($P > 0.01$). Because probiotic bacteria generally grow and colonize at the small intestine under strictly anaerobic condition, thus oxygen toxicity is a major problem in the survival of probiotic bacteria. Moreover, screening of probiotic bacteria with oxygen tolerance ability could ensure high cell count in aerobic condition [5].

3.4. Hydrophobicity

In this study, it was found that only four LAB isolates, namely, P26-9, P26-16, P26-23 and P28-32, showed hydrophobicity value > 50% (ranged from 66.72-92.67).

Several mechanisms are involved in the adhesion of microorganisms to intestinal epithelial cells. The hydrophobic nature of the outermost surface of microorganisms has been implicated in the attachment of bacteria to host tissue. This property could confer a competitive advantage, important for bacterial maintenance in the human gastrointestinal tract. The determination of microbial adhesion to hexadecane as a way to estimate the ability of a strain to adhere to epithelial cells is a valid qualitative phenomenological approach [6, 16].

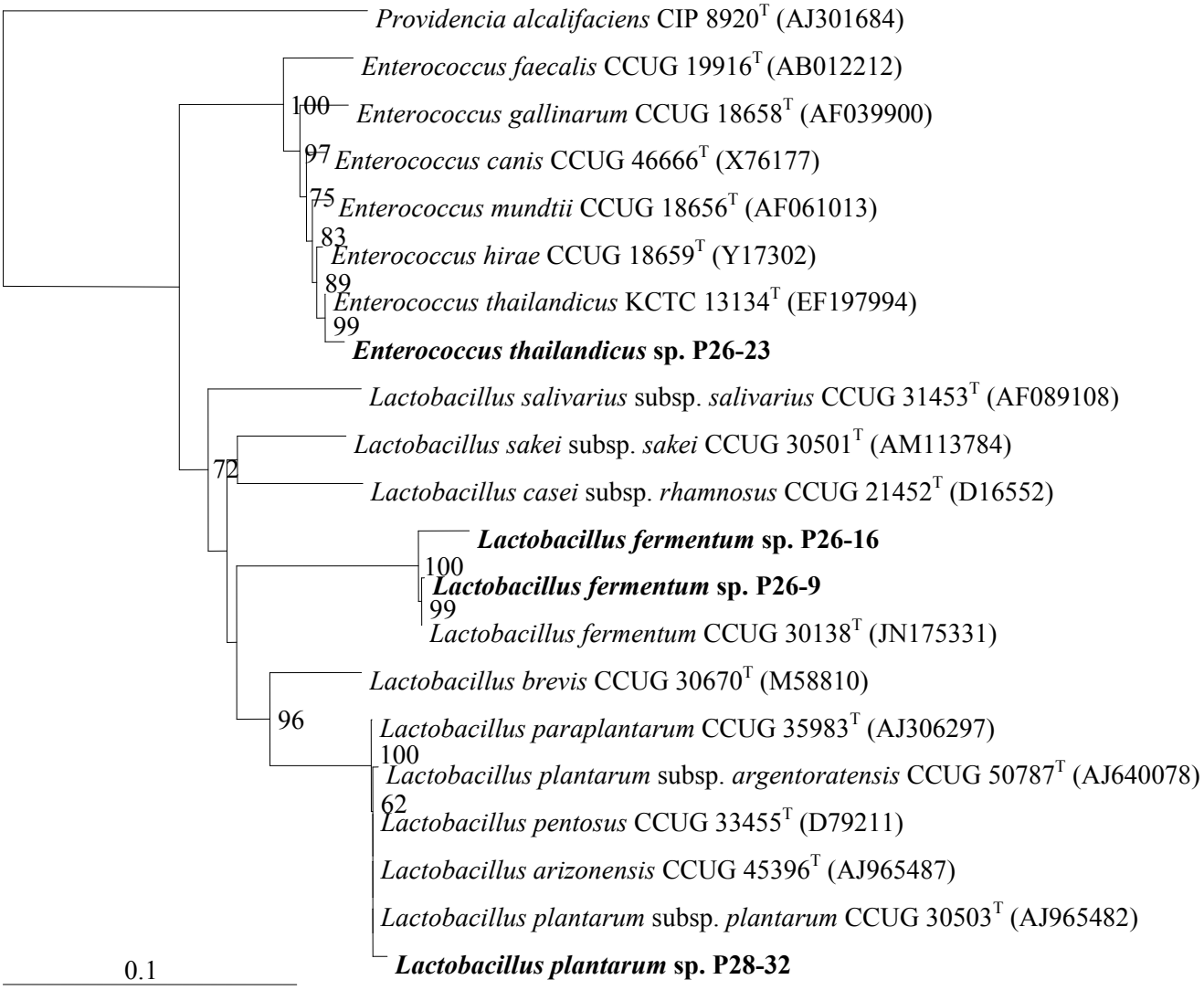

Fig 1: Neighbour-joining tree comprising 16S rRNA gene sequences of strain P26-9, P26-16, P26-23, P28-32, recognized *Enterococcus* species, recognized *Lactobacillus* species and related taxa. Bootstap values (expressed as percentages of 1000 replications) greater than 60% are shown at the branch points. Bar, 0.01 substitution per nucleotide position.

3.5. 16S rDNA Sequencing Analysis

The 16S rDNA sequencing analysis indicated that the strain P26-23 belongs to the genus *Enterococcus* and grouped most closely with *E. thailandensis* KCTC 13134^T as shown in Fig 1. The similarity between strain P26-23 and *E. thailandensis* KCTC 13134^T were 99.5%.

In addition, the 16S rRNA gene sequence analysis indicated that the strain P26-9, P26-16 and P28-32 belongs to the genus *Lactobacillus* as shown in Fig 1. Strain P26-9 and P26-16 were grouped most closely with *L. fermentum* CCUG 30138^T (98.5-99.9% similarity) while strain P28-32 was grouped most closely with *L.plantarum* subsp. *plantarum* CCUG 30503^T (99.6% similarity).

4. Conclusion

LAB obtained in this study presenting some probiotic properties and exhibited broad spectrum of antimicrobial activity against 6 food-borne pathogenic bacteria. The contribution of the selected isolates to a possible inhibition of 6 pathogenic bacteria would be of considerable interest to enhance the hygienic quality of the product. Base on the results of this study, selected LAB should be further studied in challenge experiments to explore their antimicrobial efficiency. Moreover, the chemical and microbial quality of this product affected by those LAB should be explored.

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