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Characterization of Lactic Acid Bacteria Isolated From *Kung-Som*, A Traditional Fermented Shrimp, in Respect of Their Probiotic Properties

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Abstract. The isolation and selection of probiotic lactic acid bacteria (LAB) originating from the products are consider used as starter cultures in fermented foods. This study aimed to isolate and characterize in respect of their probiotic properties LAB from *Kung-Som.* A total of 254 isolates of LAB were obtained from 55 samples of *Kung-Som*. One hundred and fifty five strains colud grown on MRS agar supplemented with 30 g L^{-1} NaCl and 3 g L^{-1} bile salt whereas only 14 strains could survived under gastro-intestinal tract condition. Among them, 3 strains showed hydrophobicity value >50% and could grown well under anaerobe condition. All strains exhibited an antimicrobial activity against six food-borne pathogenic bacteria (*Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium, *Vibrio cholerae* and *Listeria monocytogenes*). They were identified as *Lactobacillus plantarum* sp. K39, *L. plantarum* sp. K50 and *Enterococcus hirae* sp. K34 by 16S rDNA sequence. According to the results, the contribution of the selected probiotic LAB strains to a possible inhibition of food-borne pathogenic bacteria would be of considerable interest to use as starter culture for *Kung-Som* production.

Keywords: Probiotic, Lactic acid bacteria, *Kung-Som*

1. Introduction

Kung-Som is a traditional fermented shrimp widely distributed in the Southern of Thailand. It is made from shrimp, sugar, salt and water and fermented with the natural microbial flora at room temperature for 7 days. The main microorganisms in *Kung-Som* are Lactic acid bacteria (LAB) [1]. LAB strains are important microorganisms used as starter cultures in fermented foods [2]. Their addition may improve safety and stability of the product by extending the shelf life and provides diversity resulting in new sensory properties as well as health benefits by probiotic characteristics [3]. "Probiotic" cultures are, by definition, cultures that after ingestion in sufficient numbers, exert health benefits beyond inherent basic nutrition [4]. 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 to the intestinal epithelium cell and the ability to inhibit the pathogenic bacteria [5].

The isolation and selection of LAB which can be used as starter cultures in fermented food present a considerable challenge to standardization and management of quality of the products. LAB which originating from the products are specially adapted to their ecology during fermentation [6]. It exists a need to select strains of LAB originating from the indigenous microflora of the products on the basis of probiotic properties in order to obtain strains which are well adapted in the microenvironment and could dominate in the microflora of the fermented product [2]. The aim of this study was to screen LAB isolated from naturally fermented *Kung-Som* and the selection of the most suitable strains according to their probiotic properties, including antimicrobial activity against food-borne pathogenic bacteria, to be used as starter cultures.

2. Materials and Methods

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2.1. Bacterial Strains and Culture Condition

LAB were isolated from 55 samples of fermented shrimp (*Kung-Som*) collected from local markets in Songkhla and Nakhon si thammarat, Thailand by dilution plating technique on de Man Rogosa and Sharpe (MRS) agar (Himedia, India) containing 30 g L^{-1} NaCl. LAB were sub-cultured twice in MRS broth containing 30 g L^{-1} NaCl at 37°C for 18 h before use.

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 indicators were sub-cultured twice in nutrient broth (NB) at 37°C for 18 h before use.

2.2. Screening for Bile Resistance LAB

The ability of LAB strains to grown in the presence of bile was determined as described follow. Ten microlitres of LAB from 24 h incubation were spotted on MRS agar contaning 30 g L^{-1} NaCl and 3 g L^{-1} bile salt (HiMedia, India). After incubation at 37°C for 48 h, bile resistance bacteria were indicated by the presence of colonies on plates. Bile resistance LAB ware selected for further analysis.

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 8000 g for 5 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 g L^{-1} bile salt and then 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 [7]. LAB which showed >50% survival rate were selected for further analysis.

2.4. Hydrophobicity

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 optical density at 600 nm of watery phase before and after mixed with n-hexadecane were determined percentage of hydrophobicity was calculated as follows [8]:

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

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

One hundred microliters of 18 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 and overlaid with 15 g L^{-1} agar, followed by incubation at 37°C for 24 h in anaerobic jar [9]. Finally, growth rate was compared between micro-aerobic and anaerobic condition by statistical analysis.

2.6. Antimicrobial Activity

An agar spot test was used for detection of antimicrobial activity. Briefly, overnight cultures of the LAB strains were spotted onto the surface of MRS agar containing 30 g L^{-1} NaCl and incubated at 37°C for 18 h to allow colonies to develop. One milliliter of bacteria indicators (10^7 CFU ml^{-1}) were inoculated into 9 ml of nutrient soft agar (7.5 g L^{-1} agar) and poured over the plate on which the LAB strains were grown. After incubation for 18 h at 37°C, the plates were examined for inhibition zones. Antimicrobial activity was reported as the width of the clear zone around the colonies.

2.7. 16S rDNA Sequencing

DNA was extracted and purified from their whole cells by the phenol method. DNA sequence analysis was performed according to the dideoxy-mediated chain termination method [10] using automated DNA sequencer. Homology searches of the 16S rDNA sequences were performed in the GenBank with the Blastn program. 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). Gaps and ambiguous bases were eliminated from the calculations and the distance matrices for the aligned sequences were calculated by the two-parameter method. A neighbour-joining phylogenetic tree was constructed as described by Saitou and Nei using the program MEGA (version 2.1). The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis based on 1000 samplings [11].

2.8. Statistical Analysis

All results are shown as the average from three independent experiments while variation is expressed as standard deviation. Differences in means were analysed by two-tailed paired *t*-test using SPSS software (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Screening of Bile Resistance LAB

Two hundred and fifty four isolates of LAB obtained in this study were composed of cocci (94 isolates) and short rods or rods (160 isolates) shape bacteria. Bile resistance was used as a preliminary screening in this study. It was found that only 155 isolates showed ability to growth on MRS agar plate containing 30 g L^{-1} NaCl and 3 g L^{-1} bile. These isolates were cocci (61 isolates) and short rods or rods (94 isolates) shape bacteria. The relevant physiological concentrations of human bile is range from 0.3% to 0.5%. In Gram-positive bacteria, the toxicity pattern of bile acids resembles that of detergents such as SDS. However, the susceptibility or resistance of LAB to bile is species as well as strain specific [7].

3.2. Survival Under Simulated Gastro-Intestinal Tract Condition

The gastro-intestinal transit tolerance of 155 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 which mimicking the stomach condition. Then cells were further examined for pancreatin and bile salt tolerance. Under this condition, only 14 isolates showed survival rate more than 50%.

About 2.5 L of gastric juice at a pH of approximately 2.0-3.5 is secreted each day in the stomach [12], which causes the destruction of most microorganisms ingested. The protonated (non-dissociated) form of the acid exhibits toxicity through the same mechanism as organic acids, by causing intracellular acidification and collapse of the proton motive force, which in turn results in inhibition of nutrient transport. The survival of bacteria in the gastric juice depends on their ability to tolerate at low pH condition. Moreover, the transit time can be ranged from <1 h to 3–4 h depending on the individual, the diet and other reigning conditions [13]. However it was not clear whether the decrease of viability was caused by high acidity alone or by synergy with pepsin.

In the previous study, they found that most LAB were susceptible to bovine and porcine bile *in vitro*. However, they were resistant to human bile which correlated with the survival in the human gastro-intestinal tract [14]. Moreover, 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 [7].

3.3. Hydrophobicity

Among the former, only three LAB isolates, namely, K34, K39 and K50, showed hydrophobicity value >50% (74.24, 83.11 and 93.01%, respectively). There are several mechanisms 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 [8, 15].

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

All three LAB isolates were able to growth well under micro-aerobic and anaerobic condition and was not significantly different ($P < 0.05$). 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. However, screening of probiotic bacteria with oxygen tolerance ability could ensure high cell count in aerobic condition [9].

3.5. Antimicrobial Activity

Three isolates of LAB were subjected to assess the antimicrobial activity against 6 pathogenic bacteria by the agar spot test. It was found that all isolates showed inhibitory activity against all pathogenic bacteria as shown in Table 1. The primary antimicrobial effect exerted by LAB is the production of lactic acid and the reduction of pH. Another presumptive is LAB may produce some antimicrobial compounds such as hydrogen peroxide, carbon dioxide, diacetyle, reuterine and bacteriocins [16].

Table 1 Antimicrobial activity of LAB against food-borne pathogenic bacteria using agar spot test.

Strain	Radius of inhibition zone (mm)					
	Gram-negative indicator bacteria			Gram-positive indicator bacteria		
	EC	ST	VC	BC	LM	SA
K34	5.75±0.18	5.00±1.38	2.65±0.36	8.22±1.59	3.18±0.40	3.80±1.69
K39	7.60±0.98	6.95±1.42	3.70±0.41	9.65±0.58	4.43±0.33	6.93±1.47
K50	6.88±1.29	8.05±1.43	3.20±0.35	10.50±0.50	5.05±0.55	3.38±1.08

Values are given as mean ± standard deviation (n=3).

BC: *Bacillus cereus*; EC: *Escherichia coli*; LM: *Listeria monocytogenes*; SA: *Staphylococcus aureus*; ST: *Salmonella* Typhimurium and VC: *Vibrio cholera*e.

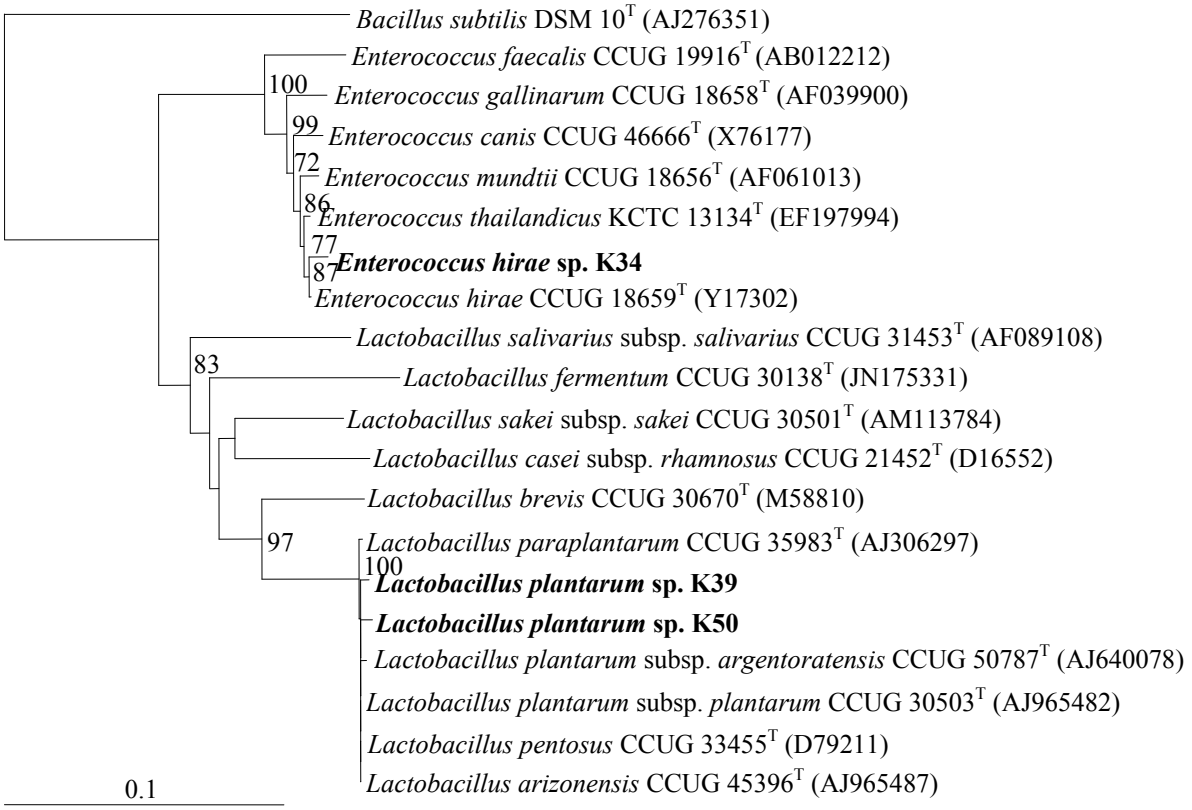

Fig. 1: Neighbour-joining tree comprising 16S rRNA gene sequences of strain K34, K39, K50, recognized *Enterococcus* species, recognized *Lactobacillus* species and related taxa. Bootstap values expressed as percentages of 1000 replications (greater than 60% are shown at the branch points). Bar, 0.01 substitution per nucleotide position.

3.6. 16S rDNA Sequencing Analysis

The 16S rDNA sequencing analysis indicated that the strain K34 belongs to the genus *Enterococcus* and grouped most closely with *E. hirae* CCUG 18659^T as shown in Fig. 1. The similarity between strain K34 and *E. hirae* CCUG 18659^T were 99.5%. While the 16S rRNA gene sequence of strain K39 and K50 indicated that they belong to the genus *Lactobacillus* as shown in Fig 1. Strain K39 and K50 was grouped most closely with *L. plantarum* subsp. *plantarum* CCUG 30503^T with 99.7 and 99.8% similarity, respectively.

4. Conclusion

As cultures originating from *Kung-Som* itself are better adapted to the ecology of their fermentation, those LAB with potential probiotic properties and antimicrobial activity in this study may use as starter culture to provide significant health benefits and contribute to enhance the hygienic quality of the products.

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