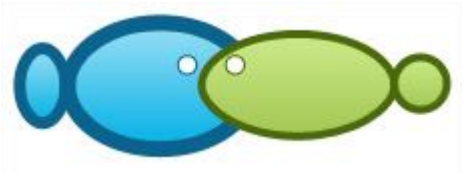

Isolation and selection of lactic acid bacteria from Thai indigenous fermented foods for use as probiotics in tilapia fish *Oreochromis niloticus*

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Abstract. In this study, 119 bacterial strains were isolated from various samples such as healthy tilapia fish (*Oreochromis niloticus*), water and sediment around the culture fish-cages, and several kinds of traditional fermented foods. These bacterial isolates were screened for antibacterial activities against bacterial fish pathogens i.e. *Aeromonas hydrophila, A. caviae* and *Streptococcus agalactiae* using an agar well diffusion assay. The isolate CR1T5 derived from fermented rice showed the highest antibacterial activity against all three fish pathogens tested. It was identified as *Lactobacillus plantarum* by using both conventional and molecular methods. The other probiotic properties were evaluated in vitro which revealed that strain CR1T5 tolerated the simulated gastrointestinal conditions well, showed high capacity to adhere intestinal mucosa and did not lyse red blood cells. The efficiency of *L. plantarum* CR1T5 was also examined in vivo. *O. niloticus* were employed in the feed-trial experiments. Fish fed a diet containing strain CR1T5 (10^8 CFU g^{-1} feed) displayed not only no mortality but also growth improvement. At the end of feed-trial, fish were challenged by intramuscularly injection of *A. hydrophila* ($3.1x10^5$ CFU) The *L. plantarum* CR1T5-fed fish survived (87.5%) better than the fish fed a control diet (12.5%) after a two week-challenge. This study clearly shows that *L. plantarum* strain CR1T5 is a promising probiotic candidate for farmed fish.

Key Words: *Lactobacillus*, *Aeromonas*, fermented food, antibacterial, aquaculture, fish pathogen.

Introduction. Tilapia (*Oreochromis niloticus*) is an African fish that can now be found farmed in many parts of the world including Thailand. It is one of the most consumed freshwater fish in Thailand. Farmers who rear this fish on commercial scales face the problems of fish diseases. Bacterial infection has been reported as one of the major causes of mortality in rearing fish (Toranzo et al 2005; Fečkaninová et al 2017). Prophylactic and therapeutic treatments employing antibiotics have been practiced by farmers. However, these kinds of treatments may leave drug residues in fish meat and can lead to the evolution of resistant strains of bacteria (Smith 2008). Therefore, the development of probiotics as an alternative to antibiotics has gained considerable interest. Probiotics are defined as the dietary supplements of live microorganisms that confer beneficial effects on the host (Fuller 1989).

Microorganisms that have been most reported for use as probiotics are lactic acid bacteria (LAB) especially in the genus *Lactobacillus* (Pérez-Sánchez et al 2011; Giri et al 2013; Beck et al 2015; Dawood et al 2015, 2016; Lim et al 2016). Lactic acid bacteria are gram positive, non-sporeforming rods and cocci. They do not produce catalase but can ferment various kinds of carbohydrates to organic acids-mainly lactate and acetate. In recent years, there have been many reports describing the application of probiotics in aquaculture to enhance fish growth and provide protective effects against fish pathogens (Giri et al 2013; Dawood et al 2015; Pirarat et al 2015; Liu et al 2017). Therefore, this study aimed to isolate and select lactic acid bacteria for use as probiotics in *O. niloticus*.

Material and Method

Bacterial strains, growth media and isolation of lactic acid bacteria. Three bacterial fish pathogens were used as indicator organisms: *Aeromonas hydrophila*, *A. caviae* and *Streptococcus agalactiae*. These indicator bacteria isolated from diseased tilapia fish (Tongpim et al 2009) were grown on blood agar plates, suspended in 20% glycerol and kept at -80ºC until use.

Forty five various samples including the intestine of healthy tilapia fish, water and sediment around the cultured fish-cages, and Thai indigenous fermented foods were used for the isolation of lactic acid bacteria. The samples were ten-fold serially diluted, plated on MRS agar containing 0.5% $CaCO_3$ and incubated at 30ºC for 2 days. Isolated colonies of different appearances and surrounded by a halo were picked up and tested for catalase and Gram reaction. The bacterial isolates showing Gram positive and catalase negative were chosen for determination of antibacterial activity.

Antibacterial activity assay. Antibacterial activity was determined using an agar well diffusion technique and tested against three bacterial fish pathogens i.e. *A. hydrophila, A. caviae* and *S. agalactiae*. The LABs isolated were grown in MRS broth at 30ºC for 48 h without shaking to obtain LAB broth cultures. *Aeromonas* spp. and *S. agalactiae* were cultivated in Brain heart infusion broth (BHI) for 24 and 48 h, respectively and their turbidity was adjusted to an equivalent of McFarland standard 0.5. A sterile cotton swab was submerged in a bacterial suspension and excess fluid was removed prior to evenly swabbing 3 planes on the surface of BHI agar plate. An agar well of 6 mm diameter was punched using a cork borer. A 40 µL aliquot of LAB broth culture was added into the agar well. Incubation was done at 35ºC for 18 h. The inhibition zones were measured and compared to determine the potentially effective probiotic LABs.

Identification of probiotic LAB. Taxonomic studies of LAB were carried out as described in Bergey's Manual of Systematic Bacteriology (Hammes & Hertel 2009). The API 50 CHL (BioMerieux) was used to determine carbohydrate fermentation. Molecular characterization was performed according to Tongpim et al (2014). The 16S rRNA gene was amplified through polymerase chain reaction (PCR) using universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5' GGTTACCTTGTTACGACTT-3'). The PCR products were purified by using the QIAquick PCR Purification Kit (Qiagen). Sequence homology was compared with 16S rRNA gene sequences available in the National Center for Biotechnology Information (NCBI) and EzTaxon server (Kim et al 2012). The neighbor-joining method (Saitou & Nei 1987) with Kimura's two-parameter distance correction model was used to construct phylogenetic trees. The sequences obtained were submitted to NCBI and an accession number was obtained.

Survival in simulated gastrointestinal conditions. Tolerance to simulated gastric and intestinal fluids was performed according to the procedures described by Huang & Adam (2004) with some modifications. The LAB cultures were grown overnight at 35ºC in MRS broth. Bacterial cells were harvested by centrifugation at 5000 rpm, 4ºC for 20 min (Himac CR20B2, Hitachi, Japan) prior to inoculation into simulated gastric fluid [3 mg mL^{-1} pepsin (porcine stomach mucosa; Sigma) adjusted to pH 2.5] to obtain the initial cell concentration ~ 1.0 x 10^6 CFU mL^{-1} (OD_{600} = 0.1) and incubated at 30ºC. Bacterial cell counts were performed on MRS agar containing 0.5% $CaCO_3$ at 0, 1, 2, and 3 hours of incubation. Similarly, the overnight grown LAB cultures were incubated in simulated intestinal fluid [1 mg mL^{-1} pancreatin (porcine pancreas; Sigma) and 0.3% bile salts (Oxoid)] adjusted to pH 8]. Aliquots of bacterial cultures were drawn at intervals of 0, 1, 2, 3, and 6 hours for dilution plate count on MRS agar containing 0.5% $CaCO_3$. Percentage survival at each time interval was calculated by comparing to the viable cell number at 0 h.

Haemolysis. LAB isolates were streaked onto blood agar plates and incubated at 30 ºC for 24-48 h. Clear zones around colonies indicated blood haemolysis had occurred.

Mucin binding assay. Adhesion to porcine gastric mucin type III (Sigma, USA) of the chosen LAB isolate was performed following the procedures described by Tallon et al (2007). *Lactobacillus plantarum* strain 299v, a known adhesive strain, was used as a positive control. Assays were performed in four replicates and data were expressed as % adhesion according to the following formula:

% Adhesion = (log CFU of adhered bacteria ÷ log CFU of initial bacteria) x 100

Feed-trial and challenge test. *O. niloticus* were fed basal diet and allowed to acclimatize for 2 weeks. Fish weighing 44±3.2 g were divided into 2 groups and randomly distributed into 60 L tanks (20 fish per aquarium). Fish in the control group were fed the normal commercial diet whereas fish in the test group were fed the commercial diet supplemented with the chosen probiotic LAB (3.6×10^8 CFU g^{-1} feed) twice daily at a feeding rate of 3% body weight. Each aquarium was supplied with compressed air using a low-pressure electric air pump. The remaining feed and fish excrement were siphoned out together with 70% water changing daily throughout the 2-week experimental period. Fish growth was determined by measuring weight and length of the fish at the beginning and end of the experimental period. After growth evaluation, fish were subjected to a challenge test by being injected with 0.1 mL of *A. hydrophila* (3.1×10^6 CFU mL^{-1} in normal saline solution). Fish were observed daily and the symptoms of illness and mortality were recorded for the following 2 weeks.

Statistics. Statistical analysis of data was performed using SPSS 19.0. Student's t-test was used to calculate statistical significance for the results of *in vitro* assays (adhesion test) and in vivo test (feeding experiment). Differences were considered significant when *p*-value was <0.05.

Results and Discussion

Isolation and selection of lactic acid bacteria for their antibacterial activity against bacterial fish pathogens. One hundred and nineteen isolates of LABs were obtained from 45 samples of the intestine of healthy *O. niloticus*, water and sediment around the cultured fish-cages, and Thai indigenous fermented foods. The isolated strains have the typical characteristics of lactic acid bacteria, which are gram-positive, rod shaped, non-sporeforming, non-motile, and catalase negative (Hammes & Hertel 2009).

The 119 LAB isolates were screened for antibacterial activity against fish pathogens i.e. *A. hydrophila, A. caviae* and *S. agalactiae*, using an agar well diffusion technique (Figure 1).

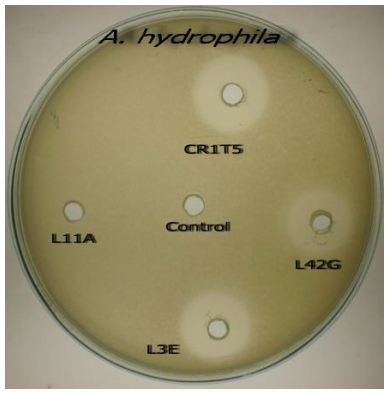

Figure 1. Antibacterial activity of the isolated lactic acid bacteria against *Aeromonas hydrophila* determined by using an agar well-diffusion assay.

There were 25 LAB isolates that exhibited inhibitory effects on the tested fish pathogens, of which 11 isolates displayed satisfactory antibacterial activity (Table 1).

Table 1

Antibacterial activity of 11 effective isolates of LABs against 3 bacterial fish pathogens determined by an agar well diffusion assay

Isolate	*Source*	*Inhibition zones (mm) against fish pathogens*		
		A. hydrophila	*A. caviae*	*S. agalactiae*
FJ2	Fermented fish	10.6	12.8	9.2
VE2	Fermented vegetable	10	12	8.4
MO2	Fermented beef	9.4	9.8	8.6
MO3	Fermented beef	8.6	10.76	10.3
F2	Fermented fish	12	13.2	11.2
CR1T5	Fermented rice	16	14	14.2
P1	Fermented pork	9.2	12.5	11.4
M3	Fermented beef	9.4	16	18
Y1	Fermented milk	11.2	12.8	8.2
3A	Yoghurt	10.6	11.7	12.2
2D	Yoghurt	10.8	12	9.6

The efficiency of antimicrobial activity is one of the major mechanisms through which probiotic organisms function and hence is considered one of the principle criteria for strain selection when screening potential probiotics (Messaoudi et al 2013; Lü et al 2014; Zhang et al 2016). Among the 11 effective LABs, isolate CR1T5 exhibited the best probiotic candidate, as it produced the largest inhibition zones against all three fish pathogens tested. Therefore, isolate CR1T5 was selected for further experiments, including feed-trials and challenge tests.

Fermented foods have been reported as good sources of probiotics (Klayraung et al 2008) which could be beneficially applied in humans and animals (Maragkoudakis et al 2010). Several studies had shown that LABs could produce antibacterial substances against various pathogens such as *Escherichia coli, Salmonella enterica*, *Staphylococcus aureus*, *A. hydrophila* and *Listeria monocytogenes* (Anacarso et al 2014; Leite et al 2015). Interestingly, the antibacterial activity produced by the chosen isolate CR1T5 was a broad spectrum as it displayed inhibitory effects on both gram positive and gram negative bacteria (Gao et al 2010).

Identification of the isolate CR1T5. The isolate CR1T5 was a gram-positive, non-sporeforming and non-motile rod. Its physiological and biochemical characteristics, shown in Table 2, corresponded to *L. plantarum* (Hammes & Hertel 2009).

Table 2

Characterization of the isolate CR1T5, an effective probiotic bacterium

Acid production from:	*Result*	*Growth at:*	*Result*
Glucose	+	10ºC	+
Maltose	+	15ºC	+
Mannitol	+	37ºC	+
Rhamnose	-	45ºC	+
Trehalose	+	Growth in 2% NaCl	+
Lactose	+	Growth in 3% NaCl	+
Galactose	+	Growth in 4% NaCl	+
Sucrose	+	Growth in 6.5% NaCl	+
Fructose	+	Catalase	-
Sorbitol	+	Oxidase	-
Mannose	+	Strictly aerobe	-
Melibiose	+	Facultative anaerobe	+
Raffinose	+	Esculin hydrolysis	+
Xylose	-	Starch hydrolysis	-
Arabinose	+	Nitrate reduction	-
Melizitose	+	Gelatinase	-
Cellobiose	+	Casein hydrolysis	-

The nearly complete 16S rRNA gene sequence (1482 bp) of the strain CR1T5 was determined which displayed 100% sequence similarity to the type strain *L. plantarum* DKO22. A phylogenetic tree was then constructed using the Neighbour-Joining method and Mega 7 software as shown in Figure 2. The 16S rDNA sequence of *L. plantarum* strain CR1T5 was deposited in GenBank under the accession number KY784648.

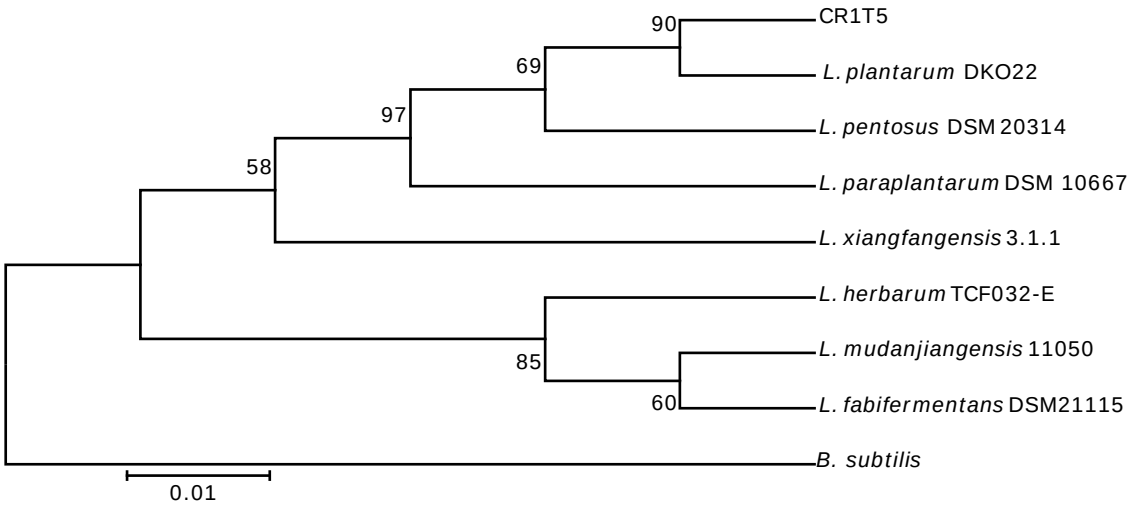

Figure 2. A neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain CR1T5.

Tolerance to gastrointestinal tract conditions. Tolerance to acid and bile is one of the primary requirements of probiotics as ingested strains need to survive acid conditions in the stomach and bile in the small intestine (Reale et al 2015; Klindt-Toldam et al 2016). In this study, *L. plantarum* strain CR1T5 displayed pronounced tolerance to simulated gastric fluid (for 3 h) (Figure 3A) and simulated intestinal fluid (for 6 h) (Figure 3B) with 78.0 and 58.9 percentage survival, respectively.

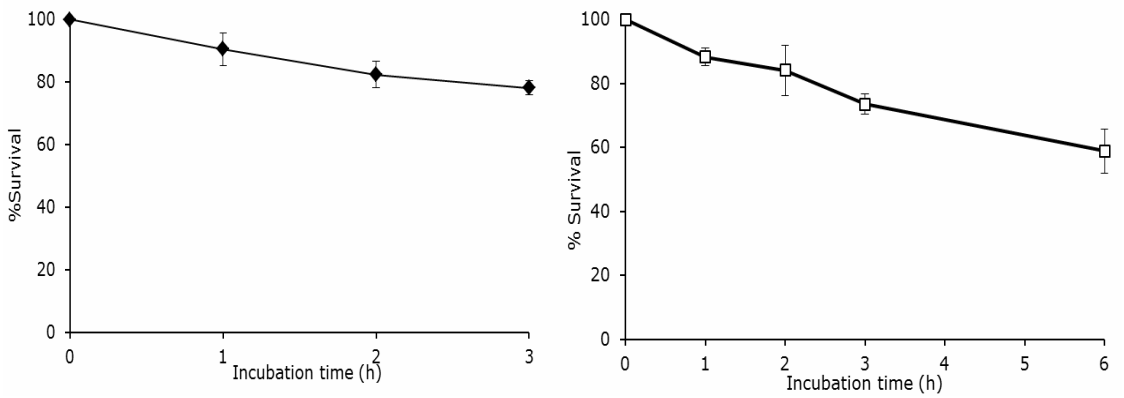

Figure 3. Effect of simulated gastric fluid (3A) and intestinal fluid (3B) on the viability of *L. plantarum* strain CR1T5.

Mucin adhesion and haemolysis. Another important criterion for selecting potential probiotic strains is the adhesion ability to intestinal epithelium (Ouwehand et al 1999). *L. plantarum* strain CR1T5 strongly adhered to porcine gastric mucin at 84.8% while *L. plantarum* 299v, a reference adherent strain, displayed 97.6%. Adhesion to intestinal mucosa is a prerequisite for bacterial colonization of the host gut. For haemolysis assay, the strain CR1T5 did not lyse red blood cells. Absence of haemolysin is one of the preliminary requirements for the selection of probiotic strains (FAO/WHO 2002) as it means less chance of that strain being pathogenic to the host.

Feed-trial and challenge tests. Growth performances of *O. niloticus* were determined based on body weight and length. The results in Table 3 show that weight and length gain increased significantly ($p<0.05$) when tilapia fish were fed a diet containing *L. plantarum* CR1T5 (3.6×10^8 CFU g^{-1} fish feed). Survival rates of the fish after challenge with *A. hydrophila* (3.1×10^6 CFU mL^{-1}, 0.1 mL intramuscularly injection) during a 2 week-period was found to be significantly higher in the test group (87.5%) than in the control group (12.5%). The obtained results demonstrate that *L. plantarum* CR1T5 not only protected tilapia fish from bacterial infection but also enhanced fish growth. The protective effects of probiotic LABs have previously been reported in *Gadus morhua, Oncorhynchus mykiss* and *O. niloticus* (Gildberg & Mikkelsen 1998; Nikoskelainen et al 2001; Pirarat et al 2015). Therefore, *L. plantarum* CR1T5 is a promising bacterial probiotic for use as an alternative to antibiotics and as a growth promoter in fish farming.

Table 3

The effect of *L. plantarum* CR1T5 on growth performances and survival of *Oreochromis niloticus* in feed-trial and challenged test

Parameters	*Control group*	*Test group*
Initial weight (g)	46.29±2.01	41.50±2.17
Final weight (g)	58.86±3.25	71.48±3.46*
Weight gain (g)	12.57±0.04	29.98±0.07*
Initial length (cm)	13.8±0.05	13.3±0.04
Final length (cm)	15.1±0.07	16.1±0.08
Length gain (cm)	1.3±0.02	2.8±0.03*
Survival (%)	12.5	87.5

Values were given as mean ± SD for two treatment groups. Means at the same sampling day with different asterisks are significantly different ($p<0.05$).

Conclusions. *Lactobacillus plantarum* strain CR1T5 isolated from fermented rice displayed the greatest potential for use as probiotic in *O. niloticus* culture. This bacterium displayed a broad-spectrum antibacterial activity against all three fish pathogens tested i.e. *A. hydrophila, A. caviae* and *S. agalactiae*. Strain CR1T5 survived the simulated gastrointestinal conditions well, showed high tendency in adhesion to intestinal mucosa and did not lyse red blood cells. Feed-trial and pathogen challenge tests performed in *O. niloticus* revealed that this bacterium significantly stimulated fish growth and had a protective effect against bacterial infection. It was clearly seen that *L. plantarum* strain CR1T5 displayed remarkable in vitro and in vivo probiotic properties and thus should be considered as a potential probiotic for use as feed supplement in aquaculture.

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