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# ANTAGONISTIC ACTIVITY AGAINST FISH PATHOGENS AND *IN VITRO* PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA

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**Abstract**

This study was conducted in order to evaluate the antagonistic activity and probiotic properties of lactic acid bacteria isolated from chicken intestine, shrimp and fish intestine and fermented food. The major properties, including antagonistic activity, aggregation, cell surface hydrophobicity and gastrointestinal tolerance were investigated. Twenty three isolates of lactic acid bacteria were screened for their antagonistic activity against fish pathogens by agar well diffusion method. It was found that three isolates including TISTR 1340, PKWA−2 and PKWA−3 exhibited the highest antagonistic activity against *Aeromonas hydrophila*, *Aeromonas caviae*and *Streptococcus agalactiae*with inhibition zone of >27.10, 20.90 and 13.55 mm, respectively. All these three isolates showed high autoaggregation percentage (>31.42%) after 5 h incubation in toluene and xylene at room temperature and positive coaggregation with the fish pathogens. Of three isolates, PKWA−3 showed the highest cell surface hydrophobicity of > 95.97%. This observation showed that PKWA−2 and PKWA−3 had ability to survive in simulated gastrointestinal juice for 4 h with survival rate of 84.87 and 86.84%, respectively. Based on the results, PKWA−2 and PKWA−3 can be classified as potential probiotics for fish culture and were identified as *Pediococcus pentosaceus* and *Lactobacillus reuteri*, respectively.

**Key** words: lactic acid bacteria, antagonistic activity, probiotics, *Pediococcus pentosaceus*, *Lactobacillus reuteri*

## INTRODUCTION

Aquaculture is currently the fastest growing food producing sector in the world. During 2000 and 2012, aquaculture production had increased morethan twice.By 2050, the production of aquaculture will increase to more than twice of the current amount.The increasing intensification and commercialization of aquaculture production cause disease outbreak. The huge impact of bacterial disease in aquaculture worldwide have reached billions of dollars annually (Aly, Abdel-Galil Ahmed, Abdel-Aziz Ghareeb, & Mohamed, 2008; Martins et al., 2008; Mohideen, Selva, Mohamed, & Hussain, 2010; Welker & Lim, 2011).Many approaches have been implemented for diseases control including antibiotic use(Prem Anand, Chellaram, Kumaran, & Felicia, 2011). However, this method results in development of antibiotic-resistant strain of pathogenic bacteria and efficacy reduction of antibiotic treatment for human and animal diseases (Alderman & Hastings, 1998; MacMillan, 2001; Moriarty, 1997). Therefore, the development of environmentally friendly approach including probiotic microorganisms has been of great interest to confer better health of aquatic animals.

Currently, the application of probiotics has been widely used for inhibiting effect against pathogens, improving immune response and disease resistance as well as growth performances of aquatic animals. In China, over 50,000 tonnes of commercial probiotic products for aquaculture are sold annually with a market value estimated at 50 million euros (Qi, Zhang, Boon, & Bossier, 2009). The criteria for selecting a good probiotic strain have been listed by several authors and include: adherence to the gut epithelial tissue, a lack of pathogenicity, reduction of pathogenic bacteria adherents, survival during gastrointestinal tract, production of inhibitory compounds such as organic acids, hydrogen peroxide, bacteriocins and enhancement of the immune response (Bhutada & Tambekar, 2010; Farzanfar, 2006; Giraffa, Chanishvili, & Widyastuti, 2010).These properties make it possibleto screen and select specific probiotic strains.

Therefore, the aim of the present study was to screen for probiotic strains of lactic acid bacteria. The evaluation was based upon the *in vitro* probiotic properties including antimicrobial activity, adhesion property, and survival during a simulated gastrointestinal tract.

## RESEARCH METHOD

### Bacteria and culture conditions

Twenty-three strains of LAB isolated from chicken intestine, shrimp and fish intestine and fermented food were obtained from the Department of Biotechnology, Faculty of Agro-industry, Kasetsart University, Thailand. *A. hydrophila* DMST 2798 and *A. caviae* DMST 21252 were purchased from the Department of Medical Science, Ministry of Public Health while *S. agalactiae* was obtained from the Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Thailand. LAB were grown in De Man Rogosa and Sharpe (MRS) broth Merck, Darmstadt, Germany) for 18 h at 37$^{o}$C while pathogenic bacteria were grown in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA) for 24 h at 37$^{o}$C.

### Antimicrobial activity against fish pathogens

Antimicrobial activity was determined by the agar well diffusion methodas described by Domrongpokkaphan and Wanchaitanawong (2006). An overnight culture of each pathogen (200 µl, $10^8$ CFU.ml$^{-1}$) was poured using a BHI agar plate. Wells (8 mm diameter) were then punched out of the BHI agar. An overnight culture of each test strain (50 µl, $10^8$ CFU.ml$^{-1}$) was added in the well and incubated for 24 h at 37$^{o}$C. The diameter of the inhibition zone was then measured. Control was performed in the same manner using broth media in the well.

### Autoaggregation assay

Autoggregation assay was performed according to Kos et al. (2003). An overnight culture of bacterial isolates was harvested by centrifugation at 8000 rpm for 8 min. The cell pellet was washed twice and resuspended in sterile phosphate buffer saline (PBS) to an $OD_{600}$ of 0.5. The cell suspension (4 ml) was votexed for 10 s. During 5 h of incubation at room temperature, 0.1 ml of the upper suspension was transferred at 1 h interval to another tube containing 3.9 ml of PBS, and $OD_{600}$of the mixture was then measured. Percentage of autoaggregation was calculated by the formula: $\text{autoaggregation (\%)} = 1 - \left(\frac{OD_t}{OD_0}\right) \times 100$,where $OD_t$represents the optical density at time t = 1, 2, 3, 4 or 5 h and $OD_0$ represents optical density at t = 0.

### Coaggregation assay

Coaggregation between test bacteria and pathogen was investigated. The cell suspension was prepared in the same manner as described in the autoaggregation assay. Equal volumes (2 ml) of each testbacterial isolate and each pathogen suspension were mixed together by vortexing for 10 s. Control tube containing each cell suspension (4 ml) was set up at the same time. After incubation at room temperature for 5 h, the $OD_{600}$was then measured. The percentage of coaggregation was calculated according to Handley et al. (1987): coaggregation (%) =

$\left[\frac{((OD_x+OD_y)/2)-OD_{(x+y)}}{(OD_x+OD_y)/2}\right] \times 100$, where x and y represent test bacteria and the pathogen, respectively and x + y represents the mixture of test bacterial isolate and each pathogen.

**Cell surface hydrophobicity assay**

Cell surface hydrophobicity was determined as described by Taheri et al. (2009)with minor modifications. An overnight culture of bacterial isolates was harvested by centrifugation at 8000 rpm for 8 min. The cell pellet was washed twice and resuspended in sterile 0.85% NaCl to give a viable concentration of $10^7$–$10^8$ CFU.ml$^{-1}$. One millilitter of toluene or xylene was added to test tubes containing 3 ml of the cell suspension. The mixture was then mixed by vortexing for 90 s. For separation of the 2 phases, the tube was left to stand for 15 min and $OD_{600}$ of the aqueous phase was then measured. Cell surface hydrophobicity was calculated as the percentage decrease in the $OD_{600}$ of the bacterial suspension due to partitioning of cells into the hydrocarbon layer: $\text{cell surface hydrophobicity (\%)} = \left[\frac{OD_0-OD_t}{OD_t}\right] \times 100$, where $OD_0$ and $OD_t$ are $OD_{600}$ of the bacterial suspension before mixing and $OD_{600}$ of aqueous phase after mixing.

**Survival after sequential exposure to simulated gastric and intestinal juices assay**

The tolerance of test strains to simulated gastrointestinal tract(GIT) condition was determined using a method as described by Musikasang, Tani, H-kittikun, and Maneerat (2009) with modifications. Simulated gastricjuice was prepared by means of suspension of pepsin (P7000, Sigma, St Louis, USA) in sterile 0.5% NaCl to a final concentration of 3 g.l$^{-1}$and adjusted to pH 2.0 and 3.0 with 3 M HCl. Simulated small intestinal juice was prepared by suspension of pancreatin USP(P–1750, Sigma, St Louis, USA) in a sterile 0.5% NaCl to a final concentration of 1 g.l$^{-1}$then added with 4.5% bile salt (Oxoid, Basingstoke, Hampshire, UK)and adjusted to pH 8.0 with sterile 0.6 M NaOH.

An overnight culture of each bacterial isolate was harvested by centrifugation at 8000 rpm for 8 min and washed twice with 0.85% NaCl. The cell pellet was then resuspended in the same solution. An aliquot of each cell suspension (0.2 ml) was transferred to a sterile tube, mixed with sterile 0.5% NaCl (0.3 ml) and finally blended with simulated gastric juice (1.0 ml). After incubation for 120 min at 30$^o$C, the supernatant was removed by centrifugation and subsequently resuspended in simulated bile juice (1.0 ml). The suspension was further incubated for 120 min.Viable counts were determined by the standard plate count method at interval 30 min of incubation.

**Statistical analysis**

All the experiments were carried out intriplicate. The results were statistically evaluatedwith one way analysis of variance and statisticalsignificance was considered at the $P < 0.05$level.

## RESULT AND DISCUSSION

Of 23 test isolates, TISTR 1340, PKWA–2 and PKWA–3 showed high inhibitory activity against *A. hydrophila* and *A. caviae* with inhibition zone of 27.10−29.30 and 20.90−23.10 mm, respectively. They also showed inhibition against *S. agalactiae* with inhibition zone of 13.55−16.23 mm, respectively (Table 1). The inhibition ability against *S. agalactiae* of test bacteriawas lower than both *A. hydrophila* and *A. caviae*. These probably result from the different structure cell wall of Gram-positive (*S. agalactiae*) and Gram-negative (*Aeromonas* spp.) bacteria (Purivirojkul & Areechon, 2007).The inhibitory mechanism of the interaction was not characterized in this study. However, previous studies have suggested that the inhibitory effects ofLAB might be due to their secondary metabolites such as bacteriocins

which are active against a wide range of Gram-positive bacteria (Klose, Bayer, Bruckbeck, Schatzmayr, & Loibner, 2010; Lee et al., 2007). The antagonistic activity of LAB also causes by bacteriocin-like substance, sideropheros, lysozyme, protease, hydrogen peroxide, the alteration of pH values, including the production of organic acids and ammonia (Verschuere, Rombaut, Sorgeloos, & Verstraete, 2000).

Based on the high inhibitory activity against *A. hydrophila*, the major diseases pathogenic bacteria for causing hemorrhagic septicemia in freshwater fish farms in Asia and other countries (Gopalakannan & Arul, 2011; Longyant et al., 2008), TISTR 1340, PKWA–2 and PKWA–3 were selected to further evaluate their probiotic properties.

The LAB isolates were further evaluated for their autoaggregation and coaggregation. As shown in Table 2 and Figure 1, after 5 h incubation PKWA–2, PKWA–3 and TISTR 1340 showed the highest autoaggregation of 59.61, 58.08 and 31.42 %, respectively while all pathogens showed lower autoaggregation ability of 7.36−15.17%. These results were in agreement with Collado, Meriluoto, and Salminen (2007) that probiotic strains (*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12) presented higher autoaggregation ability than pathogens (*E. coli* K2 and *Salmonella enterica*). This was an advantage property because bacteria with high autoaggregation capacity showed good adhesion capacity to intestinal epithelium cell adhesion (Del Re, Sgorbati, Miglioli, & Palenzona, 2000).

As shown in Table 2 and Figure 2, TISTR 1340 showed high coaggregation ability with*A. hydrophila* (38.62%) and *A. caviae* (46.24%). While PKWA−3 exhibited high ability with *A. hydrophila* (37.85%), PKWA−2 showed high values with only *S. agalactiae* (40.75). These results indicated that coaggregation ability was depended on specific test strain with pathogen strain(Tulumoglu et al., 2013). Probiotics with high coaggregation property could be interfere an ability of the pathogens to adhere to receptors on the epithelial surface (Kaewnopparat et al., 2013).In addition, inhibitor producing probiotics with high coaggregation ability also have more effective of antimicrobial ability since they interact with pathogen closely (Reid, McGroarty, Angotti, & Cook, 1988).

**Table 1** Antimicrobial activity of test strains against fish pathogens by agar well diffusion

| Test strains | Inhibition zone (mm) ± S.D. | | |
|---|---|---|---|
| | *A. hydrophila* | *A. caviae* | *S. agalactiae* |
| TISTR1338 | 25.13 ± 4.55 | 21.25± 1.76 | 17.53 ± 0.72 |
| TISTR1339 | 25.85 ± 3.04 | 21.20± 1.13 | 16.23 ± 2.59 |
| TISTR1340 | 29.30 ± 1.55 | 21.10 ± 1.40 | 16.23 ± 0.55 |
| TISTR1341 | 24.60 ± 5.37 | 19.63 ± 0.63 | 15.53 ± 0.96 |
| TISTR1342 | 19.60 ± 0.00 | 21.30 ± 2.26 | 15.66 ± 1.05 |
| PKWA–1 | 22.40 ± 2.08 | 15.76 ± 2.20 | 14.43 ± 0.97 |
| PKWA–2 | 27.10 ± 4.66 | 20.90 ± 0.42 | 14.95 ± 1.48 |
| PKWA–3 | 27.65 ± 2.76 | 23.10 ± 3.39 | 13.55 ± 3.32 |
| PKWA–4 | 16.45 ± 2.89 | 18.40 ± 3.39 | 13.30 ± 1.82 |
| PKWA–5 | 18.00 ± 0.00 | 18.25 ± 0.49 | 11.35 ± 0.49 |
| PKWA–6 | 22.65 ± 0.77 | 14.75 ± 0.78 | 13.63 ± 0.60 |
| PKWA–7 | 19.60 ± 0.70 | 16.40 ± 3.02 | 12.90 ± 0.00 |
| PKWA–8 | 16.90 ± 0.00 | 15.50 ± 0.00 | 13.00 ± 1.27 |
| PKWC–1 | 13.00 ± 1.27 | 16.70 ± 0.12 | 12.75 ± 1.48 |
| PKWC–2 | 20.00 ± 0.00 | 14.50 ± 0.84 | 8.00 ± 0.00 |
| PKWC–3 | 23.90 ± 3.81 | 12.90 ± 0.70 | 15.43 ± 0.06 |
| PKWC–4 | 19.60 ± 0.14 | 18.00 ± 0.00 | 16.23 ± 0.20 |
| PKWC–5 | 17.15 ± 1.76 | 18.10 ± 0.98 | 14.25 ± 0.07 |
| PKWC–6 | 24.55 ± 2.61 | 14.30 ± 3.39 | 17.50 ± 0.43 |
| PKWC–7 | 18.10 ± 0.61 | 13.10 ± 0.56 | 16.36 ± 1.73 |

| | | | |
|---|---|---|---|
| PKWC–8 | 23.33 ± 2.60 | 23.43 ± 0.58 | 16.70 ± 0.00 |
| PKWC–9 | 23.20 ± 1.91 | 12.77 ± 0.11 | 14.16 ± 1.42 |
| PKWC–10 | 14.20 ± 3.87 | 13.06 ± 1.15 | 15.63 ± 1.00 |

**Table 2.** Autoaggregation and coaggregation of test strains and pathogenic bacteria

| Bacterial strains | Autoaggregation | Coaggregation (% ± S.D.) | | |
|---|---|---|---|---|
| | (% ± S.D.) | *A. hydrophila* | *A. caviae* | *S. agalactiae* |
| TISTR1340 | 31.42 ± 4.79[b] | 38.62 ± 4.50[a] | 46.24 ± 0.00[a] | 25.52 ± 1.17[c] |
| PKWA–2 | 59.61 ± 4.37[a] | 14.91 ± 1.75[b] | 27.37 ± 1.93[b] | 40.75 ± 0.00[a] |
| PKWA–3 | 58.08 ± 1.47[a] | 37.85 ± 0.00[a] | 0.73 ± 0.00[c] | 30.90 ± 0.00[b] |
| *A. hydrophila* | 15.71 ± 5.30[c] | – | – | – |
| *A. caviae* | 7.36 ± 0.00[d] | – | – | – |
| *S. agalactiae* | 8.13±0.15[d] | – | – | – |

– = not determined.
Means values within the same column with different letterswere significantly different at $P < 0.05$.

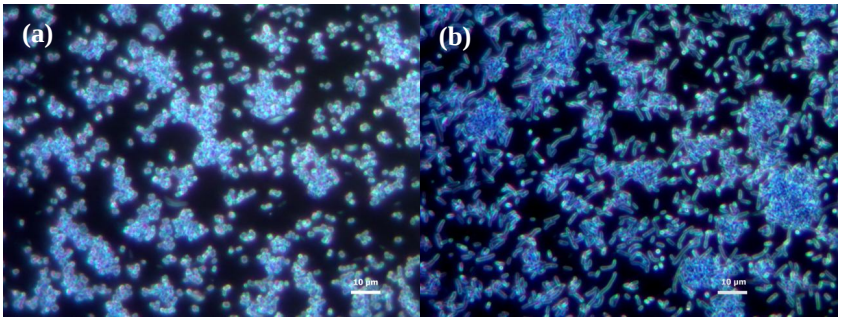


**Figure 1** Dark–field microscopy pictures of autoaggregation of (a) PKWA–2 and (b) PKWA–3

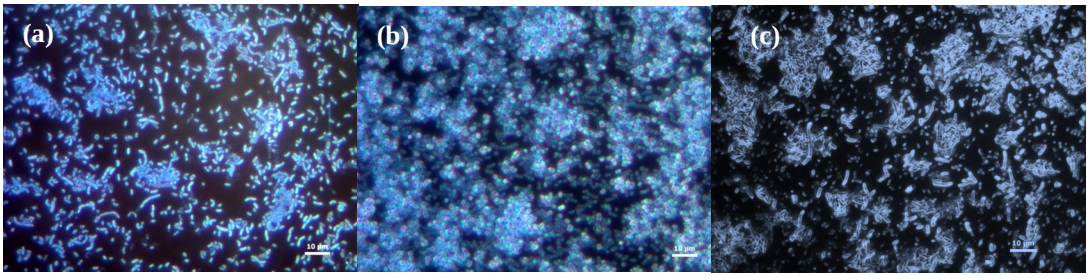


**Figure 2** Dark–field microscopy pictures of coaggregation between (a) TISTR1340 and *A. hydrophila* (b) PKWA–2 and *S. agalactiae* (c) PKWA–3 and *A. hydrophila*

The cell surface hydrophobicity of test LAB and fish pathogens to toluene and xylene was shown in Table 3. PKWA−3 exhibited the greatest cell surface hydrophobicity ranging from 94.37−95.97% and 94.09−96.13% in toluene and xylene, respectively, which was higher than that of *A. caviae* (75.46 and 62.43%), *A. hydrophila* (46.75 and 52.32%) and *S. agalactiae* (20.09 and 18.02%). This suggested that the ability of these strains to adhere to epithelial cell

was greater than that of all pathogens (Garriga, Pascual, Monfort, & Hugas, 1998; Sabir, Beyatli, Cokmus, & Onal-Darilmaz, 2010; Taheri et al., 2009).

**Table 3** Cell surface hydrophobicityof test strains and pathogenic bacteria

| Bacterial strains | Cell surface hydrophobicity (% ± S.D.) | |
|---|---|---|
| | toluene | xylene |
| TISTR1340 | $7.96 \pm 2.58^{e}$ | $30.73 \pm 1.46^{c}$ |
| PKWA–2 | $17.56 \pm 0.55^{de}$ | $29.14 \pm 0.42^{c}$ |
| PKWA–3 | $95.97 \pm 3.88^{a}$ | $96.13 \pm 0.13^{a}$ |
| *A. hydrophila* | $46.75 \pm 7.71^{c}$ | $52.32 \pm 8.74^{b}$ |
| *A. caviae* | $75.46 \pm 5.11^{b}$ | $62.43 \pm 0.73^{b}$ |
| *S. agalactiae* | $20.09 \pm 8.18^{d}$ | $18.02 \pm 4.85^{d}$ |

The effect of GIT condition on the viability of LAB was showed in Figure 3. After sequentially exposured to GIT condition (gastric pH 3), survivals of TISTR 1340, PKWA−2 and PKWA−3 were found of 97.13, 96.46 and 94.80%, respectively while survivals of these LAB decreased to 34.09, 84.87 and 86.64%, respectively after exposured to GIT condition (gastric pH 2). This suggested that GIT condition espectially, very low pH of gastric juice influenced on survival of LAB. This result indicated that PKWA−2 and PKWA−3weretolerant to acid and bile conditions while TISTR 1340 was sensitive to these conditions. Therefore, the capacity of LAB to survive at low pH was variable. The variation in the acid tolerance of bacteria probably related to the difference in $H^{+}$−ATPase activity and on the composition of the cytoplasmic membrane (Musikasang et al., 2009). The higher $H^{+}$−ATPase activity normally belong to bacteria that tolerance to acid condition because they are well to discharge $H^{+}$ from the cell by this enzyme activity resulting in a maintained pH homeostasis(Matsumoto, Ohishi, & Benno, 2004; Ventura, Canchaya, van Sinderen, Fitzgerald, & Zink, 2004).The difference of bacterial bile tolerance might relate to the specific enzyme activity of bile salt hydrolase (BSH). It was found that this enzyme helps the hydrolysis of conjugated bile resulting in a reducing of its toxic effects (Du Toit et al., 1998; Tannock, Bateup, & Jenkinson, 1997). On the contrary, the bile salts at high concentrations can rapidly dissolve membrane lipids and cause dissociation of integral membrane proteins resulting the leakage of cell contents and cell death for bile salt sensitive bacteria (Musikasang et al., 2009).

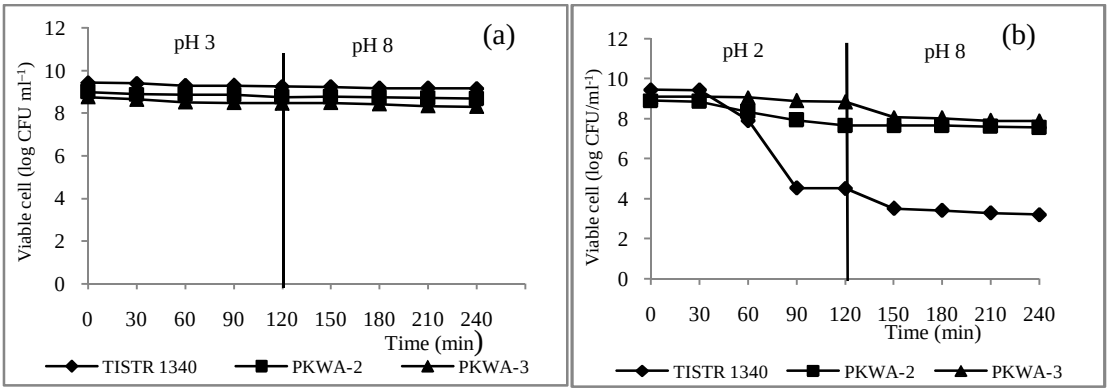


**Figure 3** Viability of LAB strains during exposure to simulated gastric juice and subsequently to simulated intestinal condition (a) simulated gastric juice (pH 3.0),and (b) simulated gastric juice (pH 2.0)

## CONCLUSION AND SUGGESTION

PKWA−2 and PKWA−3 showed potential probiotic effect by inhibiting fish pathogens. Moreover, they showed adhesion property and the capability to survive throughthe stimulatedgastrointestinal tract, by tolerating acid and bile undersimulated stomach and small intestine conditions,respectively.They were further identified using 16s rDNA.The result showed that PKWA−2 and PKWA−3 were *Pediococcus pentosaceus* and *Lactobacillus reuteri*, respectively. However, the *in vitro* study may be different as compared to *in vivo*. Therefore, these bacteria should be evaluated their *in vivo* probiotic properties further.

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