

***In vitro* antimicrobial assessment on lactic acid bacteria isolated from common freshwater fishes**

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Abstract. Probiotic is well-known as an effective agent to control and manage diseases in aquaculture. Unlike antibiotics and chemotherapeutic agents, probiotic does not trigger the emergence of antibiotic-/chemo-resistant bacteria. This study was aimed to isolate, identify and evaluate lactic acid bacteria from intestines of three common food fish, i.e. tilapia (*Oreochromis niloticus*), catfish (*Clarias gariepinus*) and rohu (*Labeo rohita*). Thirty-four lactic acid isolates were isolated and screened for inhibitory effect against fish pathogens e.g. *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella enterica*. Positive antagonists were subsequently tested in haemolytic, salt tolerance and bacteriocin-like inhibitory substances (BLIS) assays. Our results showed only three isolates displayed positive inhibitory effect against all four pathogens. These three isolates were classified as γ -haemolytic bacteria. Our results revealed that bacterial isolates (T2.1.2 - *Pediococcus acidilactici* and T2.2.2 - *Lactobacillus fermentum*) isolated from *O. niloticus* (tilapia) showed a better adaptation in the range of 0 - 20 ppt; while, the bacteria isolated from *L. rohita* (R1.1.1 - *P. acidilactici*) could survive up to 35 ppt. These isolates were then identified based on 16S rRNA gene sequences. BLIS data revealed that both *P. acidilactici* and *L. fermentum* isolated from *O. niloticus* and *L. rohita* could suppress the growth of pathogens with cell density as low as 10^4 cfu/ml. Our study shows that *P. acidilactici* and *L. fermentum* have the potential to be further explored as biocontrol/probiotic agents in aquaculture.

Keywords: aquaculture, lactic acid bacteria, pathogen, probiotic

INTRODUCTION

Intensive aquaculture often associated with high frequency of diseases outbreak. Fish pathogens such as *Aeromonas* sp., *Edwardsiella* sp., *Pseudomonas* sp. and *Vibrio* sp. are commonly found to be highly infectious in both freshwater and marine environments (Banerjee and Ray, 2017). The early onset of these symptoms could be associated with fin rot, exophthalmia, and skin ulcers. Infected fish showed a high rate of mortality or stunted growth in the severe stage (Dong *et al.*, 2017;

Fečkaninová *et al.*, 2017).

Antibiotics are the common practices used in many animal productions to manage and control diseases. In aquaculture, antibiotics are administered through the feeds or directly into the water. Although antibiotics are effective in treating fish diseases, the rampant use of an antibiotic can cause negative impacts on the host and the environment (Lazado *et al.*, 2015). Antibiotics increase the emergence of antibiotic-

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resistant bacteria that can tolerate the use of antibiotics, which will in turn, detrimental to the host's health. Therefore, higher concentration or different antibiotics are required to control the infection during the onset of the same disease in the future. Furthermore, antibiotic residues indirectly discharge through excretion, can lead to inevitable stress effects on the environmental microbial community.

Probiotics are live and beneficial microorganisms that modulate intestinal microbial flora in the host. The term probiotic in Greek language "pro bios" means "for life" (Gismondo *et al.*, 1998). Probiotics can be originated from a wide range of bacteria from Gram-negative to Gram-positive species. The important criteria of a group of microbes to be considered as probiotic; it should not be causing any pathogenic effect to the host, and it should be able to enhance the bowel/intestinal health (Banerjee and Ray, 2017). Study by Fečkaninová *et al.* (2017) showed the application of lactic acid probiotics could reduce *Aeromonas* infection in salmonids, the host-associated symbiotic relationship not only contribute to the diversity of microbial flora in the gut, but it also improves digestion, growth, stress tolerance and regulate nutritional factors of the hosts (Wang *et al.*, 2008; Lazado *et al.*, 2015).

The present study aimed to discover the potential of intestinal lactic acid bacteria in probiotic development for aquaculture species. Intestinal microflora was isolated from three common food fish included tilapia (*Oreochromis niloticus*), catfish (*Clarias gariepinus*) and rohu (*Labeo rohita*). The bacteria isolates were screened through agar well diffusion assay against *Salmonella enterica*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Hemolytic activities and salt tolerance test was used to evaluate the pathogenicity of the probiotics. After multi-screening tests, the potential probiotic candidates were identified based on molecular approach. Bacteriocin-like inhibitory substances (BLIS) assay was performed to determine the minimum inhibitory effect against the pathogens.

MATERIALS AND METHODS

Sampling of common food fish. Three common food fish ($n=6$) (Table 1) were purchased from a local fish market at Kuchai Entrepreneurs Park, Kuala Lumpur, Malaysia. Prior to the dissection, the surface body of common food fish was disinfected with 70% (v/v) ethanol. The fish were then dissected using a sterile dissecting kit, incision was made from the anus to post-stomach of the fish to remove the intestinal samples for subsequent processes.

Isolation of intestinal microflora from common food fish. Intestinal samples (1 g) from each fish were homogenized using an electric homogenizer (LabGEN®125, Cole-Parmer, USA) in deMan, Rogosa, and Sharpe (MRS) broth and incubated for 24 h at $37\pm 2^\circ\text{C}$. Serial dilution was performed (10^{-1} to 10^{-9}), and 1 mL from each intestinal sample was spread plated onto MRS agar and incubated for 24 h at $37\pm 2^\circ\text{C}$. The MRS plates were then incubated at room temperature ($26\pm 2^\circ\text{C}$) for 24 h. Pure cultures were collected from single colonies and established on corresponding fresh agar plates. Individual colonies were taken based on morphological differences and subsequently streaked on MRS agar. Pure bacteria were obtained after three times of plate streaking.

In vitro screening for antagonistic bacteria. Bacterial isolates were screened for their antagonistic activity against *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. enterica* (UCSI laboratory's collection, Microbiology Lab in South Wing campus, KL) using agar well diffusion method. Supernatants of overnight bacterial culture were collected by centrifugation at 7,500 rpm for 15 min at room temperature using a mini-spin centrifuge (MiniSpin® plus, Eppendorf, China). The supernatant of bacteria was then filtered through 0.45 μm pore size filter (Sortunos, USA) to obtain cell-free supernatant (CFS). An overnight culture of pathogens was added into 20 ml of soft nutrient agar (NA) at 37°C , and mixed before pouring into the Petri dishes. To each agar plate, five wells were prepared using a cork borer with a diameter of 2 cm. Each well was filled with 100 μL of CFS including a blank control of sterile

MRS broth. The plates were then incubated at $37\pm 2^{\circ}\text{C}$ for 24 h. The experiment was performed in triplicates. Antimicrobial activity was expressed as the diameter of the inhibition zones formed around the wells. Inhibitory strength was defined as: zone of inhibition >20 mm=strong inhibition; 10-20 mm=intermediate/moderate inhibition; and <10 mm=low inhibition (Shokryazdan *et al.*, 2014).

Haemolytic activity assay. Bacteria exhibited positive antimicrobial activity were then streaked onto horse blood agars (Thermo Scientific, Malaysia) and incubated at $37\pm 2^{\circ}\text{C}$ for 24 h. Haemolysis was classified as α -haemolysis, β -haemolysis and γ -haemolysis based on red blood cell lysis patterns. Bacteria isolates with only γ -

haemolysis were selected for subsequent study (Loh *et al.*, 2014).

Salt tolerance assay. Salt tolerance was determined by incubating γ -haemolytic bacterial isolates in MRS broth containing a various concentration of sodium chloride (NaCl; Merck, Germany). Sodium chloride was supplemented to MRS broth at a different concentration of 0, 5, 10, 15, 20, 25, 30 and 35 ppt. Ten mL of MRS broth was used to culture the bacterial isolates in 15 mL tubes. The experiment was performed in triplicates. Bacterial culture was incubated for 24 h at $37\pm 2^{\circ}\text{C}$. Optical density was determined at 600 nm using a UV-spectrophotometer (Eppendorf, Malaysia).

Table 1. Specifications of common food fish specimens ($n=6$).

Common Food Fish	Species Name	Weight (g)	Length (cm)
Catfish	<i>Clarias gariepinus</i>	334 \pm 10.0	45.2 \pm 3.0
Rohu	<i>Labeo rohita</i>	716 \pm 20.2	63.5 \pm 4.5
Tilapia	<i>Oreochromis niloticus</i>	480 \pm 12.1	47.8 \pm 2.8

16S rRNA characterization of selected bacterial isolates. Selected bacterial isolates were cultured in MRS broth at $37\pm 2^{\circ}\text{C}$ for 24 h prior to DNA extraction. Genomic DNA was extracted using a GF-1 Nucleic Acid extraction kit (Vivantis Tech, Malaysia). The 16S rRNA gene of the extracted DNA was amplified using primers 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') forward primer and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3') reverse primer to recover nearly full length of the 16S rRNA (Loh *et al.*, 2017). The polymerase chain reaction (PCR) process was performed on 25 μL reaction mixtures containing 2.5 μL of extracted DNA, 0.5 μL of 10 μM forward primer, 0.5 μL of 10 μM reverse primer, 2.5 μL of 10X reaction buffer, 0.5 μL of 10 mM dNTPs, 0.5 μL of 5 U polymerase enzyme, 0.5 μL of 25 mM magnesium chloride and 17.5 μL of Milli-Q water. PCR reaction was carried out using the thermal cycler (Eppendorf, Malaysia) with the following parameters: pre-denaturation at 94°C for 5.00 min, followed by 30 cycles of denaturation at 94°C for 0.30 min, annealing at 57°C for 0.45 min, and extension at 72°C for 1.30 min, with a final extension at 72°C for 7.00 min. DNA purification was performed in

50 μL of PCR reaction mixture prepared for each bacteria isolate using MEGAquick-spinTM Total Fragment DNA purification kit (YMS, Korea) prior to bi-directional sequencing using primers 27f and 1492r. After purification, PCR products were sent for sequencing (MyTACG, Malaysia). The results of gene sequences were analyzed using BioEdit 7.0.5.3 and matched with bacterial sequences in the BLAST (NCBI) database.

BLIS assay. The bacteriocin-like inhibitory substances (BLIS) assay was performed to determine the minimum inhibitory effect of bacteria isolates on the pathogens. The identified bacterial isolates and pathogens were cultured separately in 30 mL MRS broth and nutrient broth (NB) and subsequently incubated for 24 h at $37\pm 2^{\circ}\text{C}$. The intestinal bacterial culture was then adjusted to the densities of 10^4 , 10^5 , 10^6 and 10^7 cfu/ml using a microplate reader (Infinite 200 PRO NanoQuant, Switzerland) at OD_{540} . Subsequently, bacterial isolates with various cell densities were streaked onto NA in a single line using a sterile cotton swab. Pathogens at a cell density of 10^5 cfu/ml were streaked perpendicularly to the line of the testing bacterial

isolates of different cell densities (Loh *et al.*, 2014). The inoculated plates were then incubated for 24 h at $37\pm 2^\circ\text{C}$. The experiment was performed in triplicates. Inhibitory lengths were measured and compared with the control (sterile MRS broth).

Data analysis. Data were analyzed using statistical analyzing software SPSS version 23. One-way analysis variance (ANOVA) was used to compare the means and Tukey's ($p < 0.05$) to determine the significance of different bacterial activities in agar well diffusion, salt tolerance, and BLIS assay. Statistical significance was accepted at $p < 0.05$.

RESULTS

A total of 34 bacterial isolates were isolated from the intestinal tract of fish specimens. There were 16 isolates from *Clarias gariepinus* (catfish), 10 isolates from *Labeo rohita* (rohu) and eight isolates from *Oreochromis niloticus* (tilapia). Among these isolates, five isolates showed positive antagonistic activity towards the pathogens tested, with one potential antagonists isolated from *C. gariepinus*; one potential antagonist from *L. rohita* and three potential antagonists from *O. niloticus* (Table 2). Out of the five potential antagonists tested for haemolysis. Only three isolates showed γ -haemolytic activity. The other two bacterial isolates showed either α -or β -haemolytic activity. Only γ -haemolytic activity was selected for salt tolerance test. Out of three γ -haemolytic bacterial isolates, two bacterial isolates were originated from *O. niloticus* and one bacterial isolate was isolated from *L. rohita*. None of the bacterial isolates from *C. gariepinus* showed a positive result (γ -haemolysis) (Figure 1). Salt tolerance test revealed that all three bacterial isolates were able to grow in a wide range of salt-rich environment, i.e. 15, 30 and 35 ppt (Table 3). Bacterial isolates with γ -haemolytic activity were characterized using a molecular approach to determine their species. Bacteria isolated from *O. niloticus* (T2.1.2) and *L. rohita* (R1.1.1) showed 98-100% similarity to *Pediococcus acidilactici*. While bacterial isolate (T2.2.2) obtained from *O. niloticus* showed 99%

similarity to *Lactobacillus fermentum* (Table 4). *P. acidilactici* (T2.1.2) and *L. fermentum* (T2.2.2) were subsequently selected for BLIS assay in this study. The growth of *E. coli* (10^5 cfu/ml) was significantly inhibited by *P. acidilactici* at 10^4 cfu/ml and 10^7 cfu/ml. For *K. pneumonia*, the inhibitory effect exerted by *P. acidilactici* at 10^4 cfu/ml was recorded the lowest at 5 ± 0.82 mm and the highest at 10^6 cfu/ml (9.67 ± 0.47 mm). While for *P. aeruginosa*, lowest inhibition was found when treated with 10^4 and 10^5 cfu/ml *P. acidilactici*. There is no significant different ($p > 0.05$) of the inhibitory effect showed by different bacterial cell density against the pathogen. On the other hand, different trend was observed in *S. enterica*, moderate inhibition was recorded when pathogen was treated with 10^7 cfu/ml *P. acidilactici* (Table 5). In the case of *L. fermentum*, low inhibition was recorded in *E. coli* treatment, whereby the pathogen showed rather mild suppression in their growth at all concentration. Moderate inhibition was found when *K. pneumoniae* treated with *L. fermentum* at 10^7 cfu/ml (10.67 ± 0.94 mm), and mild inhibitory effect at 10^4 and 10^5 cfu/ml, respectively. Likewise, *P. aeruginosa* and *S. enterica* shared the similar inhibitory trend as in *K. pneumonia*, where moderate inhibition was found when these pathogens were treated with 10^7 cfu/ml *L. fermentum*, both recorded at 12.33 ± 2.10 and 12.67 ± 1.25 mm, respectively. Nonetheless, mild inhibition of the pathogens was still observable when treated with $< 10^6$ cfu/ml *L. fermentum* (Table 6).

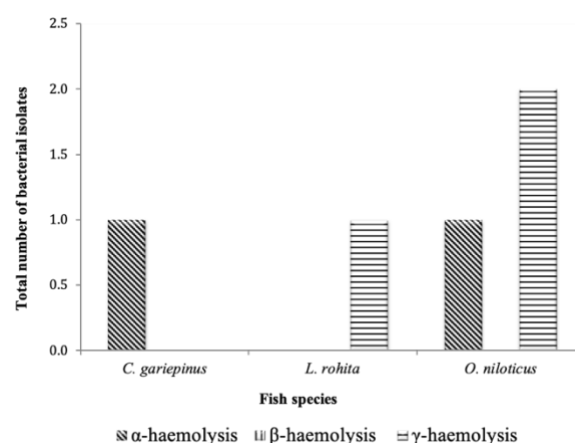


Figure 1. Haemolytic activities of intestinal bacteria isolated from *C. gariepinus*, *L. rohita* and *O. niloticus*.

Table 2. Inhibitory effect of potential antagonists against pathogens.

Pathogens (10 ⁵ cfu/ml)	Annular radius by bacteria isolates (cm)				
	T2.1.2	T2.2.1	T2.2.2	R1.1.1	C1.2.2
<i>E. coli</i>	1.63±0.047 ^a	-	1.83±0.047 ^b	-	-
<i>K. pneumoniae</i>	1.63±0.047 ^a	-	1.57±0.047 ^a	1.13±0.047 ^b	0.93±0.047 ^b
<i>P. aeruginosa</i>	1.77±0.047 ^a	1.07±0.094 ^b	1.6±0.082 ^a	-	-
<i>S. enterica</i>	1.67±0.047 ^a	-	1.63±0.047 ^a	-	-

Means (n=3) ± standard deviation; Values with different letters in the same column are significantly different (p<0.05) among the pathogens; “-” indicates no inhibition. “T” in front of the numbering denotes as Tilapia, “R” denotes as rohu, “C” denotes as catfish.

Table 3. Growth performance of bacterial isolates exposed under various NaCl concentrations.

Salinity (ppt)	Absorbance (nm) of bacterial isolates		
	T2.1.2	T2.2.2	R1.1.1
0	2.592 ± 0.189 ^{bc}	2.392 ± 0.037 ^a	2.295 ± 0.032 ^{ab}
5	2.653 ± 0.029 ^c	2.495 ± 0.038 ^a	2.231 ± 0.013 ^{ab}
10	2.503 ± 0.017 ^{abc}	2.417 ± 0.010 ^a	2.216 ± 0.094 ^{ab}
15	2.536 ± 0.006 ^{abc}	2.519 ± 0.027 ^a	2.312 ± 0.070 ^b
20	2.587 ± 0.031 ^{bc}	2.512 ± 0.045 ^a	2.247 ± 0.044 ^{ab}
25	2.415 ± 0.024 ^{abc}	2.488 ± 0.077 ^a	2.190 ± 0.013 ^{ab}
30	2.339 ± 0.017 ^a	2.416 ± 0.022 ^a	2.151 ± 0.013 ^a
35	2.372 ± 0.004 ^{ab}	2.419 ± 0.020 ^a	2.232 ± 0.009 ^a

Means (n=3) ± standard deviation; Values with different letters in the same column are significantly different (p < 0.05) among different salinities. “T” if front of the numbering denotes as Tilapia, “R” denotes as rohu, “C” denotes as catfish.

Table 4. Bacterial identification through 16S rRNA partial sequences genes.

No.	Isolate	Host	Bacterial species	Strain	Max. identity (%)
1.	T2.1.2	<i>O. niloticus</i>	<i>Pediococcus acidilactici</i>	ML96	100%
2.	T2.2.2	<i>O. niloticus</i>	<i>Lactobacillus fermentum</i>	NWAFU1463	99%
3.	R1.1.1	<i>L. rohita</i>	<i>Pediococcus acidilactici</i>	JFP1	98%

Table 5. Inhibitory effect of *Pediococcus acidilactici*.

Cell density of bacteria (cfu/ml)	Length of inhibition against pathogens (mm)			
	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>S. enterica</i>
0 (Control)	-	-	-	-
10 ⁴	3.67±0.471 ^a	5±0.816 ^a	7±1.414 ^a	6.33±0.471 ^a
10 ⁵	4.67±1.247 ^a	8±1.633 ^{ab}	7±0.816 ^a	7±0.816 ^a
10 ⁶	6.67±1.886 ^{ab}	9.67±0.471 ^b	10.33±2.055 ^a	8.67±1.247 ^{ab}
10 ⁷	9.67±0.943 ^b	9.33±1.700 ^b	11.33±0.943 ^a	10±0.816 ^b

Means (n=3) ± standard deviation; Values with different letters in the same column are significantly different (p < 0.05) among the bacterial cell densities. All pathogenic concentration was set at 10⁵ cfu/ml. Zone of inhibition >20 mm = strong inhibition; 10-20 mm = intermediate/moderate inhibition; and <10 mm = low inhibition.

Table 6. Inhibitory effect of *Lactobacillus fermentum*.

Cell density of bacteria (CFU/ml)	Length of inhibition against pathogens (mm)			
	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>S. enterica</i>
0 (Control)	-	-	-	-
10 ⁴	5±0.816 ^{ab}	4.67±2.357 ^a	6.67±0.943 ^a	6.33±0.471 ^a
10 ⁵	4.67±0.943 ^a	4.67±0.943 ^a	6.33±0.471 ^a	6.33±0.943 ^a
10 ⁶	8.33±1.247 ^{bc}	7.33±0.471 ^{ab}	7.67±0.471 ^a	9.67±1.247 ^b
10 ⁷	9.67±1.247 ^c	10.67±0.943 ^b	12.33±2.055 ^b	12.67±1.247 ^b

Means (n=3) ± standard deviation; Values with different letters in the same column are significantly different (p < 0.05) among the bacterial cell densities. All pathogenic concentration was set at 10⁵ cfu/ml. Zone of inhibition >20 mm = strong inhibition; 10-20 mm = intermediate/moderate inhibition; and <10 mm = low inhibition.

DISCUSSION

Among 34 bacteria isolates, only five isolates showed weak to intermediate inhibitory activity towards pathogens i.e. *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. enterica*. Although catfish had the highest number of bacteria isolates, however, only one bacteria isolate was found to have weak inhibitory activity towards the pathogens. On the other hand, tilapia possessed the highest number of bacteria isolates that showed an intermediate inhibitory effect against the pathogens.

Haemolytic assay further tested the suitability of the bacterial isolates and their haemolytic properties. Haemolysis is a resultant of red blood cells breakdown caused by pathogenic bacteria (Sowemimo-Coker, 2002). In the present study, of the five bacterial isolates, only three isolates showed γ -haemolysis. Two isolates showed α -haemolysis and none of the bacterial isolate showed β -haemolysis. The haemolytic assay is a safety measure that can reduce/eliminate suspected pathogens during the screening process in probiotic development. Any bacteria displayed α -/ β -haemolysis activity would not be considered as they may potentially to cause immunological diseases or malfunction in the defence system of fish (Loh *et al.*, 2014).

The salt tolerance level of three bacterial isolates was further tested at 0 - 35 ppt. The assay aimed to understand survivorship of the bacteria in the presence of salt content. The outcome of the assay could further elucidate the feasibility of these probiotic's application in the marine environment setting. The tolerance level of selected potential probiotics was determined through salt tolerance assay in the study. An increment of turbidity signifying a growing number of bacteria in this case (Walter and Deborah, 1952). According to Wong and Rawls (2012), the intestinal environment of a host is largely dependent on its ambient living condition; which implies that intestinal microbiota composition of fish could be significantly influenced by their living habitat. However, our result showed that microflora isolated from freshwater fish's intestines could also survive in a broad range of salinity. Two bacterial isolates were identified as *P. acidilactici* (Strain ML96: *O. niloticus*; Strain JFP1: *L. rohita*), and one bacterial isolate

was *L. fermentum* (Strain NWAUFU1463: *O. niloticus*) through 16S rRNA sequence identification. In our case, *Pediococcus acidilactici* (T2.1.2) isolated from *O. niloticus* (tilapia) showed a better adaptation in the range of 0-20 ppt; however, *Lactobacillus fermentum* (T2.2.2) isolated from the same species, survived the best at the range of 5 - 20 ppt. On the other hand, *P. acidilactici* (R1.1.1) isolated from *Labeo rohita* (rohu) could adapt from 0 - 35 ppt (Table 3). Our result showed that *P. acidilactici* originated from different host's intestines could thrive at a different level of salinity. It is interesting to note that, these bacterial isolates have no doubt to survive in the environment of higher salinity. Our findings somehow were in the agreement with the findings of Wong and Rawls (2012), where intestinal bacteria could tolerate different salinities like their hosts. For instances, *O. niloticus* survive in a wide range of salinity e.g. 0 - 25 ppt (Watanabe *et al.*, 1985) while *L. rohita* survive <12 ppt (Islam *et al.*, 2014). Some literates also indicated that certain lactic acid bacteria (LAB) able to tolerate different temperatures, salt contained environment and a wide range of pH (Papadimitriou *et al.*, 2016). According to Justé *et al.* (2014), *Tetragenococcus* spp. could survive in the extreme high salt environment (25% (w/v) or 250 ppt). However, high salt concentration could induce osmotic stress in certain LABs e.g. *L. delbrueckii* subsp. *lactis*, which can kill the bacteria through autolytic activity (Koch *et al.*, 2007). Thus, it is important to understand the salt tolerance level of proposed probiotic candidates to ensure the effectiveness of its application in various fish species, especially to species inhabiting at intertidal zones or estuaries (Cahill, 1990; Balcázar *et al.*, 2008; Das *et al.*, 2008).

BLIS assay results showed that *P. acidilactici* and *L. fermentum* were able to suppress the growth of *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. enterica*, treated with LAB's cell density as low as 10^4 cfu/ml within 24 hours of application. Pathogenic concentration was set at 10^5 cfu/ml in this study. The infectious concentration of pathogen to its host is varying among host's species, likewise, it also depends on pathogenic species. In general, pathogens that causing fish disease could be as low as 10^5 cfu/ml (Schmid-Hempel and Frank, 2007). Other BLIS producing bacteria e.g. *Bacillus subtilis*, *B. methylotrophicus*, *Enterococcus faecium*, *L. fermentum* and *L. plantarum* are abundantly found in

freshwater fish's intestines (e.g. common carp, catla, snakehead and tilapia). They are able to suppress the growth of many pathogenic bacteria in aquaculture such as *A. hydrophila*, *P. aeruginosa* and *S. putrefaciens* (Cai *et al.*, 1999; Del'Duca *et al.*, 2013; Mukherjee and Ghosh, 2014). A recent study by Loh *et al.* (2018), showed that incorporation of LAB e.g. *Lactococcus lactis* subsp. *lactis* in live feed (e.g. *Artemia*) could significantly reduce the occurrence of edwardsiellosis in fish farming. This evidence further emphasizes the need for probiotic to improve the immune system of fish larvae, in order to reduce the mortality rate during the early stages.

For the past few decades, probiotic has been using for disease prophylactic control and management in aquaculture. Among the probiotic candidates, LABs are the most common probiotics used in fish farming to improve overall health status and to reduce the outbreak of diseases in fish. Two probiotic candidates proposed in the present study i.e. *P. acidilactici* and *L. fermentum* can be considered as a potential probiotic in aquaculture. It is also interesting to note that, *P. acidilactici* was recently suggested as a potential probiotic in the food industry (Barbosa *et al.*, 2015). While, solid shreds of evidence showed that *L. fermentum* isolated from snakehead fish displayed positive inhibitory activity against several fish pathogens such as *A. hydrophila*, *P. aeruginosa* and *S. putrefaciens* (Allameh *et al.*, 2013). Our preliminary results support the fact that the inclusion of *P. acidilactici* and *L. fermentum* as functional foods in a dietary formulation is viable for the aquaculture industry.

CONCLUSION

In conclusion, *P. acidilactici* and *L. fermentum* isolated from food fish able to control against human–fish pathogens with the cell density as low as 10⁴ cfu/ml. Overall, our results indicate that *P. acidilactici* and *L. fermentum* could be potentially used in fish diet formulation. However, *in vivo* tests are still required to validate the efficacy of its application on living hosts.

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REFERENCES

- Allameh, S., Yusoff, F., Daud, H., Ringø, E., Ideris, A., & Saad, C. 2013. Characterization of a probiotic *Lactobacillus fermentum* isolated from Snakehead, *Channa striatus*, stomach. *Journal of the World Aquaculture Society* 44(6): 835-844.
- Balcázar, J., Vendrell, D., de Blas, I., Ruiz-Zarzuela, I., Muzquiz, J., & Girones, O. 2008. Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. *Aquaculture* 278(1-4): 188-191.
- Banerjee, G. & Ray, A. 2017. The advancement of probiotics research and its application in fish farming industries. *Research in Veterinary Science* 115: 66-77.
- Barbosa, J., Borges, S., & Teixeira, P. 2015. *Pediococcus acidilactici* as a potential probiotic to be used in food industry. *International Journal of Food Science & Technology* 50(5): 1151-1157.
- Cahill, M. 1990. Bacterial flora of fishes: A review. *Microbial Ecology* 19(1): 21-41.
- Cai, Y., Suyanandana, P., Saman, P., & Benno, Y. 1999. Classification and characterization of lactic acid bacteria isolated from the intestines of common carp and freshwater prawns. *The Journal of General and Applied Microbiology* 45(4): 177-184.
- Das, S., Ward, L., & Burke, C. 2008. Prospects of using marine actinobacteria as probiotics in aquaculture. *Applied Microbiology and Biotechnology* 81(3): 589-589.
- Del'Duca, A., Cesar, D., & Abreu, P. 2013. Bacterial community of pond's water, sediment and in the guts of tilapia (*Oreochromis niloticus*) juveniles characterized by fluorescentin situ hybridization technique. *Aquaculture Research* 46(3): 707-715.
- Dong, H., Taengphu, S., Sangsuriya, P., Charoensapsri, W., Phiwsaiya, K., Sornwatana, T., Khunrae, P., Rattanaojpong, T., & Senapin, S. 2017. Recovery of *Vibrio barveji* from scale drop and muscle necrosis disease in farmed barramundi, *Lates calcarifer* in Vietnam. *Aquaculture* 473: 89-96.
- Fečkaninová, A., Koščová, J., Mudroňová, D., Popelka, P., & Toropilová, J. 2017. The use of probiotic bacteria against *Aeromonas* infections in salmonid aquaculture. *Aquaculture* 469: 1-8.
- Gismondo, M., Drago, L., & Lombardi, A. 1998. Review of probiotics available to modify gastrointestinal flora. *Antimicrobics and Infectious Diseases Newsletter* 17(10): 79.
- Islam, M., Ahsan, D. A., Mandal, S. C., & Hossain A. 2014. Effects of salinity changes on growth performance and survival of rohu fingerlings, *Labeo rohita* (Hamilton, 1822). *Journal of Coastal Development* 17: 379.
- Justé, A., Lievens, B., Rediers, H., & Willems, K. 2014. The genus *Tetragenococcus*. In: *Lactic Acid Bacteria: Biodiversity and Taxonomy*. Holzappel, W.H. and Wood, J.B.B. (eds). John Wiley & Sons, Ltd. pp. 213-227.

- Koch, S., Oberson, G., Eugster-Meier, E., Meile, L., & Lacroix, C. 2007. Osmotic stress induced by salt increases cell yield, autolytic activity, and survival of lyophilization of *Lactobacillus delbrueckii* subsp. *lactis*. *International Journal of Food Microbiology* 117(1): 36-42.
- Lazado, C., Caipang, C., & Estante, E. 2015. Prospects of host-associated microorganisms in fish and penaeids as probiotics with immunomodulatory functions. *Fish and Shellfish Immunology* 45(1): 2-12.
- Loh, J. Y., Kay, G. L., & Ting, A. S. Y. 2018. Bioencapsulation and colonization characteristics of *Lactococcus lactis* subsp. *lactis* CF4MRS in *Artemia franciscana*: a biological approach for the control of *Edwardsiella tarda* in larviculture. *Marine Biotechnology* 20(3): 353-362.
- Loh, J. Y., Lim, Y. Y., Harmin, S. A., & Ting, A. S. Y. 2014. *In vitro* assessment on intestinal microflora from commonly farmed fishes for control of the fish pathogen *Edwardsiella tarda*. *Turkish Journal of Veterinary and Animal Sciences* 38: 257-263.
- Mukherjee, A. & Ghosh, K. 2014. Antagonism against fish pathogens by cellular components and verification of probiotic properties in autochthonous bacteria isolated from the gut of an Indian major carp, *Catla catla* (Hamilton). *Aquaculture Research* 47(7): 2243-2255.
- Papadimitriou, K., Alegría, Á., Bron, P., de Angelis, M., Gobetti, M., Kleerebezem, M., Lemos, J., Linares, D., Ross, P., Stanton, C., Turrioni, F., van Sinderen, D., Varmanen, P., Ventura, M., Zúñiga, M., Tsakalidou, E., & Kok, J. 2016. Stress Physiology of Lactic Acid Bacteria. *Microbiology and Molecular Biology Reviews* 80(3): 837-890.
- Schmid-Hempel, P. & Frank, S. 2007. Pathogenesis, virulence, and infective dose. *PLoS Pathogens* 3(10): 147.
- Shokryazdan, P., Sieo, C., Kalavathy, R., Liang, J., Alitheen, N., Faseleh Jahromi, M., & Ho, Y. 2014. Probiotic potential of lactobacillus strains with antimicrobial activity against some human pathogenic strains. *BioMed Research International* 2014: 1-16.
- Sowemimo-Coker, S. 2002. Red blood cell hemolysis during processing. *Transfusion Medicine Reviews* 16(1): 46-60.
- Walter, K. & Deborah, K. 1952. Turbidimetric estimation of number of bacteria. *American Journal of Clinical Pathology* 22 (12): 1181-1185.
- Wang, Y., Tian, Z., Yao, J., & Li, W. 2008. Effect of probiotics, *Enterococcus faecium*, on tilapia (*Oreochromis niloticus*) growth performance and immune response. *Aquaculture* 277(3-4): 203-207.
- Watanabe, W.O., Kuo, C-M., & Huang, M-C. 1985. Salinity tolerance of Nile tilapia fry (*Oreochromis niloticus*), spawned and hatched at various salinities. *Aquaculture* 48(2): 159-176.
- Wong, S. & Rawls, J. 2012. Intestinal microbiota composition in fishes is influenced by host ecology and environment. *Molecular Ecology* 21(13): 3100-3102.