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INHIBITION OF PATHOGENS BY LACTIC ACID BACTERIA AND APPLICATION AS WATER ADDITIVE MULTI ISOLATES IN EARLY STAGES LARVICULTURE OF *P. PELAGICUS* (LINNAEUS, 1758)

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ABSTRACT

In aquaculture practices, to date, probiotics are considered a valid alternative to antibiotics and in particular, in fish larviculture, to prevent high mortality. Looking for innovative ways, Lactic Acid Bacteria (LAB) were isolated from the gut of female *P. pelagicus* and tested as water additive mixture in the *P. pelagicus* larviculture to determine their effects on survival, water quality, and enzymatic activity. The LAB mixture was added to the culture water daily at $1x10^6$, $5x10^6$ and $1x10^7$ cfu mL^{-1}. Control group did not receive any bacteria. All trials were replicated. Probiotic addition improved larval survival significantly in all the trials. Higher larval survival averaged 10.3%, 11.2% and 11.0% respectively was achieved in treatment groups inoculated with a mixture of three LAB including *L. plantarum*, *L. salivarius* and *L. rhamnosus*. The isolates also lowered pH and increased digestive enzyme activity particularly protease and amylase compared to that of control. The data collected provided scientific and technical support for the utilization of probiotics larval rearing of *P. pelagicus* for sustainable culture of this crab species in specific and aquaculture in general.

Key words: *P. pelagicus*, Lactic Acid Bacteria; Larviculture, Digestive enzyme.

INTRODUCTION

Aquaculture of blue swimming crab, *Portunus pelagicus* mostly depend on seed caught from the wild. Indoor production of its seed is still in an experimental stage. *P. pelagicus* has high market value, and its average survival rates during the larval stages are either too low or even zero, which is a major obstacle for the growth of aquaculture industry of this marine shellfish species. High mortalities are occurred due to *V. harveyi* transmitted through mother feces in hatching tanks (Talpur *et al*., 2011a) which are ultimately transferred to larvae through water intake (Olafsen, 2001; Talpur *et al*., 2011a).

In aquaculture, antibiotics are used to prevent infections and as therapeutic agent. Persistent use of antibiotics induce resistance among pathogenic bacteria (Balcázar *et al*., 2006a; Weber *et al*., 1994) *P. pelagicus* are harbouring drug resistant pathogens in the gut which released in hatching tanks through feces and the credibility of antibiotic is questionable in larvae rearing (Talpur *et al*., 2011a,b). Therefore, it is important to control the infections in larviculture; use of environment friendly microorganisms is one option. In the last decade, the researchers warily examined roles and effects of probiotics in aquaculture as a substitute to antimicrobial drugs with positive effects on fish survival (Villamil *et al*., 2002) and health (Balcázar *et al*., 2006a; Silvi *et al*., 2008).

Probiotics that have been examined for use in crustacean aquaculture particularly shrimp include bacteria, yeasts, bacteriophage, and microalgae (Ajitha *et al*., 2004; Balcázar *et al*., 2006b; Irianto and Austin, 2002; Park *et al*., 2000). Lactic acid bacteria, *Bacillus* species were recently employed to improve the aquatic environment in aquaculture (Farzanfar, 2006). *Lactobacillus* species have wielded strong antimicrobial activity against the pathogenic microorganisms (Rossland *et al*., 2003; Sanni *et al*., 1999). Numerous other researchers have reported encouraging results in the application of probiotics in aquaculture (Merrifield *et al*., 2010; Swain *et al*., 2009; Vine *et al*., 2006; Wang *et al*., 2008). Majority of workers have demonstrated probiotics positive effects using a single or two probiotic strains, and just few studies described the effects of a mixture of probiotics in aquaculture (Avella *et al*., 2010a; Balcázar, 2003; Ziaei-Nejad *et al*., 2006). The administration of mixture of three LAB in rainbow trout have led higher growth and developed immune resistance (Bagheri *et al*., 2008; Raida *et al*., 2003). However, *Bacillus* species have been suggested as suitable alternative to the use of antibiotics in shrimp aquaculture (Banerjee *et al*., 2007). Moreover, LAB produces proteases and other enzymes that support natural digestion the host (Ziaei-Nejad *et al*., 2006). Until now, information is scanty on the role of these bacteria in larviculture of *P. pelagicus*.

In the present study, the isolates of three lactic acid bacteria namely *L. plantarum*, *L. salivarius* and *L.*

rhamnosus previously isolated from the gut of female *P. pelagicus* and validated as potential probiotics through small scale *in vivo* model (Talpur *et al*., 2012) were added to larvae rearing water to determine the effect of mixture of LAB probiotic isolates on the survival of *P. pelagicus* larvae.

MATERIALS AND METHODS

Broodstock management and hatching: Study was conducted during the year 2010-2011. Berried females were collected from Strait of Tebrau (1° 22' N and 103° 38' E), Johor, West Malaysia and were transported to marine hatchery of the Institute of Tropical Aquaculture, Universiti Malaysia Terengganu (UMT), Malaysia. Females were disinfected and maintained according to Talpur *et al*., (2011a) and stocked in hatching tanks for breeding, with sand substrate and adequate aeration. Zoea 1 stage of *P. pelagicus* was used for experimental trials.

Preparation of seawater: UV treated seawater for brood stock and larviculture was filtered through a 10 µm net, salinity adjusted to 28‰ and sterilise/disinfected with sodium hypochlorite (50 mg L^{-1}) for 24 h. It eliminated almost all naturally occurring bacteria. The sterilise water neutralized by sodium thiosulphate. The culture water in experimental aquaria exchange 10-12% daily began from the day second, using sterilise seawater.

Bacterial isolates: Three LAB probiotics previously isolated from the gut of female crab, *P. pelagicus* identified as *L. plantarum*, *L. salivarius* and *L rhamnosus* were validated as probiotics through *in vitro* tests against indicator pathogens and small scale *in vivo* model (Talpur *et al*., 2012) were used for the present study.

Experimental design: Total four trials (3 sub-trials under each trial total 12 trials) were conducted in aerated 10 litre aquaria; all aquaria contained 200 larvae (20 larvae L^{-1}). All treatments including controls had three replicates. In experimental set up (Table 1), each aquaria received mixture of two or three LAB bacteria at ratio 1:1 at a final concentration of $1x10^6$ cfu mL^{-1}, 5.0×10^6 cfu mL^{-1} and 1.0×10^7 cfu mL^{-1} daily and one control against each without any probiotic. Each trial lasted for 14 days. The temperature and salinity was maintained within constant range at 28±1°C and 28±0.5‰ respectively. Experimental aquaria were confined in water bath 3' x 5' x 1.5' flat bottom tank which was filled with fresh tap water and supplied with submerged heater in order to maintain the temperature of aquaria water to 28°C. A 12 h dark/12h light photoperiod was maintained during the study period. Dead larvae and debris was siphoned out daily. Water lost during cleaning of aquaria was replaced by same salinity seawater and any live larva escaped during this process was transferred to respective aquaria tank with big bore pipette tip.

Prior to exposure to bacteria (probiotic), healthy larvae were acclimated in sterilise seawater under similar conditions as exercised in hatching tanks and rearing aquaria in order to minimise the bacterial load with larvae adhering from hatching. Larvae were fed on microalgae *Nannochloropsis* sp., rotifers (*Brachionus plicatilis*) and *Artemia* without any enrichment following the procedure of Talpur *et al*., (2011a). Water exchange was started on the day second of experiment and maintained @ 10 -12 % of the total aquarium volume daily. In controls, water was changed 30-40 % daily from day two of the experiment to reduce the pathogenic bacterial risk which multiplying in aquaria or may be ingress via live feed. During the study period, temperature, salinity, dissolved oxygen (DO) and pH were monitored daily using YSI 556 MPS multi meter (USA).

Table 1. Experimental setup for probiotics administration

Trial	Dose cfu mL^{-1}	Mixture of Multi Isolates @ ratio 1:1	Control (Non inoculated)
T-1	$1x10^6$	*L. plantarum* and *L. salivarius*	(Against each)
T-2	$5x10^6$		
T-3	$1x10^7$		
T-4	$1x10^6$	*L. plantarum* and *L. rhamnosus*	(Against each)
T-5	$5x10^6$		
T-6	$1x10^7$		
T-7	$1x10^6$	*L. salivarius* and *L. rhamnosus*	(Against each)
T-8	$5x10^6$		
T-9	$1x10^7$		
T-10	$1x10^6$	*L. plantarum,* *L. salivarius* and *L. rhamnosus*	(Against each)
T-11	$5x10^6$		
T-12	$1x10^7$		

Preparation of probiotic culture: A pure colony from the MRS agar (Merck) slant stock culture of each isolate was aseptically isolated and inoculated in 10 mL screw cap tube containing sterilise MRS broth (Fluka) prepared in 28‰ seawater. Tube was incubated on an orbital shaker at 150 rpm for 24 hours at 37°C and then transferred to 2 litre fresh MRS broth and incubated on shaker at 150 rpm for 24-48 h at 37°C. After incubation, the bacterial cells were harvested by centrifugation (2,000xg for 10 min). Cells were washed three times with 28‰ sterilise seawater and re-suspended in sterilise seawater before use. Cell density was measured in OD_{630nm}, and each aquarium was inoculated until day 13 and the trials were terminated on day 14.

Survival test of pathogens

Culture of bacteria, cell-free supernatants and survival test: For survival test of pathogens, LAB bacteria were grown in sterilise nutrient broth prepared in 28‰ seawater supplemented with 20% glucose and 5%

yeast and incubated on shaker at 150 rpm for 48 h at 37°C. Cell free supernatants were obtained by centrifuging the culture at 2,000xg for 10 min, which was followed by filtering the supernatant through a 0.2 µm-pore filter (Whatman, England). A volume of 20 mL cell-free supernatants (LAB) poured in three 50-mL Erlenmeyer flasks at 37 °C for respective pathogen.

Target bacterial cells (*Vibrio harveyi*, *V. parahaemolyticus* and *Pseudoalteromonas piscicida*) previously isolated from the gut of female *P. pelagicus*, were grown in sterilise marine broth prepared in 28‰ seawater for 48 h at 37 °C. Target bacterial cells were harvested by centrifugation at 2,000 ×g for 10 min and supernatants was discarded. Cell pellets washed three times with sterilise seawater and then inoculated to approximately log 10^6 cells mL^{-1} in LAB cell-free supernatants containing Erlenmeyer flasks for respective pathogen. Control contained target bacteria at same concentration (10^6 cells mL^{-1}) in 20 mL sterilise marine broth without LAB inoculation. Viable cells were counted by plating on MRS agar for LAB and marine agar for target pathogens. Data were recorded at 1 h, 2 h, 3 h, 4 h respectively and plotted accordingly. Cell density for supernatants and target cells at $1x10^6$ cfu mL^{-1} was measured in OD_{630nm} using a UV-1800 spectrophotometer (Shimadzu, Japan) according to the standard set previously**.**

Survival of larvae: Alive larvae were counted and then % survival rate was determined by the following mathematical expression;

(Survival rate (%) =Total number of larvae survived/ Initial number of larvae stocked x100)

Bacterial samples: Water samples from larvae rearing tanks (LRT) were collected on day 2, 6, 10, and 14 of the start of trials and were kept in sterilise test tubes for the detection of LAB in multi isolates administration. The presence/absence of probiotics in the larvae was determined only in samples of 14 DAH larvae. Larvae samples were washed thrice with sterilise seawater to remove any adhering particles and microbes. Then larvae samples were disinfected with 10% formalin and again washed with sterilise seawater until formalin smell had totally gone off. Larvae samples were homogenized in seawater using sterilise mortar and pestle to prepare the inoculums. All samples were serially diluted (10 fold) before plating onto culture media (MRS and TCBS). TCBS agar was used to evaluate the *Vibrio* presence. TCBS plates were incubated for 24-48 h at 37°C while MRS plates were incubated for three days at 37°C.

Enzyme activities: Three larvae (megalopa), a day before termination of experiment (13 DAH) were collected from each treatment and control for enzyme assay. All samples were collected after 6 h of feeding. Larvae were washed with sterilise distilled water and immediately frozen at −80 °C in 2.0 mL Eppendorf tubes until enzyme assays were done. Frozen samples were homogenized in buffer solution (10Mm sodium citrate/0.1M NaCl, pH 7.0). Homogenates were centrifuged at 13,000 ×g for 10 min at room temperature. Homogenates (crude or diluted) either were immediately used for enzyme assays or stored at -80°C until start of procedure.

Enzyme assay was performed using assay kits. Protein contents were determined using Bio Rad protein assay Kit (Bio Rad, USA). Protease determination was performed using kit, Protease AZCL-casein solution; (Megazyme, Ireland). Amylase was determined using Amylase Assay Kit (Megazyme, Ireland). Enzyme assay was performed only with larvae taken from T-10, T-11 and T-12, administrated with mixture of three isolates. Enzyme activities were measured as the change in absorbance using a Shimadzu 1800-UV spectro-photometer and expressed as specific activity (U mg^{-1} protein).

Statistical analysis: Percentage survivals of larvae were arc sin square root transformed to approximate normality. The significance of differences was determined using ANOVA, followed by Tukey's test to compare the means of the samples for multi group comparisons, with a statistical software package SPSS 16.0 for windows. Differences were considered significant at $p<0.05$.

RESULTS

Survival of pathogenic bacteria: The survival rates of indicator bacterial pathogens *V. harveyi, V. parahaemolyticus* and *P. piscicida* were examined after 1–4 h of incubation with cell-free supernatants of *L. plantarum, L. salivarius* and *L. rhamnosus* and the results are shown in Fig. 1, Fig. 2 and Fig. 3 respectively. *V. harveyi* when challenged with cell free supernatant of *L. plantarum* did show 4.17 log cfu mL^{-1} survival in 1 h and it showed existence level of 3.84 cfu mL^{-1} in 2 h and eliminated in 3 h. *V. parahaemolyticus* survived with 4.017, 3.50 and 1.85 log cfu mL^{-1} during 1 h, 2 h and 3 h respectively and were eliminated in 4th hour. *P. piscicida* did show survival 3.74 and 3.34 log cfu mL^{-1} during 1 h and 2 h respectively, thus, was eliminated in 3rd hour (Fig.1).

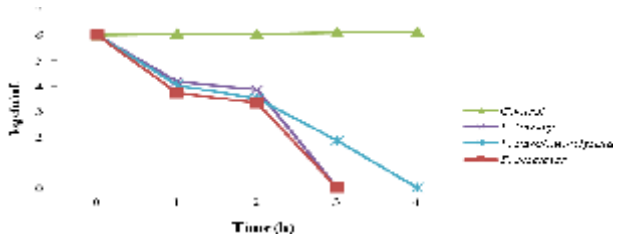

Figure 1. Survival of pathogens after exposure to cell-free supernatants derived from *L. plantarum.*

When *V. harveyi* was challenged against cell free supernatant of *L. salivarius* survival was 4.22 and 3.90 log cfu mL^{-1} in 1 h and 2 h respectively and eliminated in 3 h. *V. parahaemolyticus* survived with 4.30, 3.84 and 2.89 log cfu mL^{-1} during 1 h, 2 h and 3 h respectively and was eliminated in 4th hour. *P. piscicida* did show survival 3.84 and 3.43 log cfu mL^{-1} during 1 h and 2 h respectively, and it was eliminated in 3rd hour (Fig. 2).

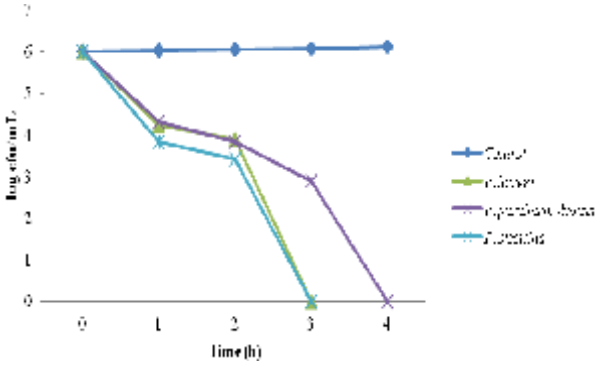

Figure. 2. Survival of pathogens after exposure to cell-free supernatants derived from *L. salivarius.*

When *V. harveyi* was challenged against cell free supernatant of *L. rhamnosus* survival was 4.17 and 3.84 log cfu mL^{-1} in 1 h and 2 h respectively and eliminated in 3 h. *V. parahaemolyticus* survived with 4.11, 3.87 and 2.67 log cfu mL^{-1} during 1 h, 2 h and 3 h respectively and was eliminated in 4th hour. However, *P. piscicida* did show survival 3.79 and 3.30 log cfu mL^{-1} during 1 h and 2 h respectively, and it was eliminated in 3rd hour (Fig. 3). No elimination of pathogens was observed in controls and a growth was enhanced at every hour though it was nominal (Fig. 1, Fig. 2 and Fig. 3). All pathogens were significantly susceptible to the supernatants and were destroyed within 3 h and 4 h of exposure respectively.

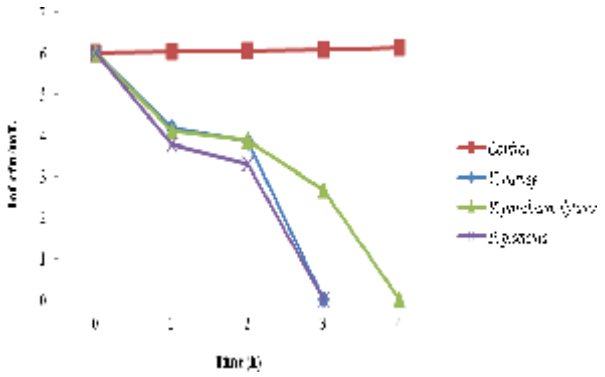

Figure 3. Survival of pathogens after exposure to cell-free supernatants derived from *L. rhamnosus.*

Survival of larvae: Larvae present in LAB administered probiotics were swimming more actively than control until day 14. The colour of larvae was better than control larvae throughout the larval period. Further treated larvae have better survival at the end of the larval period than that of control group.

Larvae which received combined doses of three LAB at ratio 1:1:1 displayed better survival than their counterparts. Highest survivals were observed in T-11 and T-12 superior than control respectively. However, a mixture of *L. plantarum* and *L salivarius* did produce significantly highest survival in T-2 over untreated control. A mixture of *L. salivarius* and *L. rhamnosus* did produce significantly better survival in T-5 and T-6 over the non inoculated control respectively. Moreover, an allowance of *L. plantarum* and *L. rhamnosus* did afford significantly greater larval survival at T-8 over control (Table 2). All the controls showed lowest survival. Survival between groups and within groups of multi isolate dosages of *L. plantarum* and *L. salivarius* were only statistically significant ($p<0.05$).

Table 2. Mean survival (%) of *P. pelagicus* larvae (14DAH) at various doses of multi isolate administration of LAB.

Multi Isolate	Trial	cfu mL^{-1}	Survival (%)	Control (Survival (%)
L. plantarum and *L. salivarius*	T-1	$1x10^6$	9.5± 1.5[a]	2.5± 0.9
	T-2	$5x10^6$	10.8±0.3[a]	2.3± 0.3
	T-3	$1x10^7$	8.3± 1.3[a]	2.7± 0.3
L. salivarius and *L. rhamnosus*	T-4	$1x10^6$	8.0± 0.5	3.2± 0.8
	T-5	$5x10^6$	9.5± 1.0	2.3± 0.8
	T-6	$1x10^7$	9.5± 1.0	3.2± 0.6
L. plantarum and *L. rhamnosus*	T-7	$1x10^6$	9.7± 1.0	2.2± 0.3
	T-8	$5x10^6$	10.7± 1.0	1.8± 0.8
	T-9	$1x10^7$	9.3± 0.8	2.3± 0.3
L. plantarum, L. salivarius and *L. rhamnosus*	T-10	$1x10^6$	10.3±1.0	2.2± 0.3
	T-11	$5x10^6$	11.2±1.2	3.3± 0.6
	T-12	$1x10^7$	11.0±1.3	2.8± 0.6

Note: values with same superscript are statistically significant ($p<0.05$)

Bacterial Study: In multi isolates administration LAB were re-isolated from tank waters on day 2, 6, 10, and 14 and from larvae on day 14 post-hatching when experiment was terminated. , LAB were not detected in

water or larvae from the control treatments (Table 3). *Vibrio* presence was confirmed on TCBS plates. Probiotics however were not identified up to species level just witnessed based on Gram staining and colony morphology observed under microscope. In most cases, *Vibrio* was not detected after day 6 in treated groups.

Table 3. Detection of inoculated LAB as multi isolates in culture water, larvae, and *Vibrio* during probiotic treatments

Treatment	Cfu mL^{-1} inoculated	Trial		Day2	Day6	Day10	Day14	
				W	W	W	W	L
Non-inoculated Control	-		Prob	-	-	-	-	-
			Vib	+	+	+	+	+
L .plantarum &	$1x10^6$	T-1	Prob	+	+	+	+	+
L. salivarius			Vib	+	+	-	-	-
L .plantarum &	$5x10^6$	T-2	Prob	+	+	+	+	+
L. salivarius			Vib	+	+	-	-	-
L .plantarum &	$1x10^7$	T-3	Prob	+	+	+	+	+
L. salivarius			Vib	+	-	-	-	-
Non-inoculated Control			Prob	-	-	-	-	-
			Vib	+	+	+	+	+
L .plantarum &	$1x10^6$	T-4	Prob	+	+	+	+	+
L. rhamnosus			Vib	-	+	-	-	-
L .plantarum &	$5x10^6$	T-5	Prob	+	+	+	+	+
L. rhamnosus			Vib	+	+	-	-	-
L .plantarum &	$1x10^7$	T-6	Prob	+	+	+	+	+
L. rhamnosus			Vib	+	+	-	-	-
Non-inoculated Control			Prob	-	-	-	-	-
			Vib	+	+	+	+	+
L. salivarius &	$1x10^6$	T-7	Prob	+	+	+	+	+
L. rhamnosus			Vib	+	+	-	-	-
L. salivarius &	$5x10^6$	T-8	Prob	+	+	+	+	+
L. rhamnosus			Vib	+	+	-	+	+
L. salivarius &	$1x10^7$	T-9	Prob	+	+	+	+	+
L. rhamnosus			Vib	+	+	-	-	-
Non-inoculated Control			Prob	-	-	-	-	-
			Vib	+	+	+	+	+
L. plantarum,	$1x10^6$	T-10	Prob	+	+	+	+	+
L. salivarius & *L. rhamnosus*			Vib	+	+	-	-	-
L. plantarum,	$5x10^6$	T-11	Prob	+	+	+	+	+
L. salivarius & *L. rhamnosus*			Vib	+	+	-	-	-
L. plantarum,	$1x10^7$	T-12	Prob	+	+	+	+	+
L. salivarius & *L. rhamnosus*			Vib	+	+	-	-	-

Note: Prob, probiotic, Vib, Vibri

Enzyme activities

Protease activity: The highest protease activity 0.20 ± 0.00 U mg^{-1} protein and 0.19 ± 0.01 U mg^{-1} protein, respectively was observed in treatments inoculated at $1x10^7$ and $5x10^6$ (T-12 and T-11) over the control 0.11 ± 0.01 U mg^{-1} protein and 0.11 ± 0.01 U mg^{-1} protein respectively was statistically significant ($p<0.05$). Thus, lowest protease activity 0.16 ± 0.01 U mg^{-1} protein observed in treatment inoculated at $1x10^6$ (T-10) was

statistically significant (p<0.05) but control of the same was not significant (p>0.05) Fig 4.

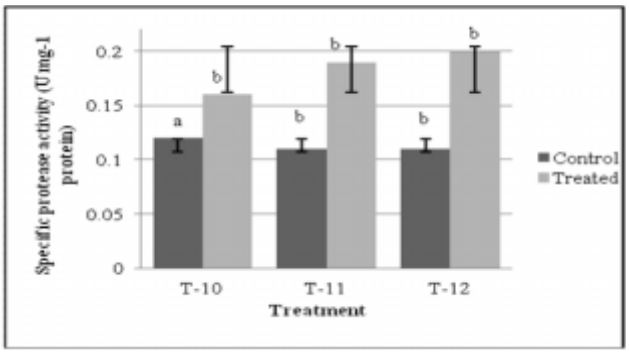

Figure. 4. Specific activity of protease of *Portunus pelagicus* larvae (13DAH) treated with (T-10, T-11 and T-12) or without probiotic (control), a mixture of *L. plantarum, salivarius* and *L. rhamnosus*, as water additives. T-10 (1.0 ×10^6 cfu mL^{-1}), T-11 (5.0×10^6 cfu mL^{-1}) and T-12 (1.0×10^7 cfu mL^{-1}). Bars showing similar words are statistically significant (*P*<0.05)

Amylase activity: The highest specific amylase activity 0.38 ± 0.01 U mg^{-1} protein and 0.38 ± 0.01 U mg^{-1} protein respectively was observed in T-12 and T-11 over the control 0.27 ± 0.02 U mg^{-1} protein and 0.25 ± 0.03 U mg^{-1} protein. Moreover, lower amylase activity was observed in treatment T-10 (0.34 ± 0.03 U mg^{-1} protein) and in control (0.26 ± 0.01 U mg^{-1} protein). Amylase activity in treated and controls was statistically significant (*P*<0.05) Fig-5.

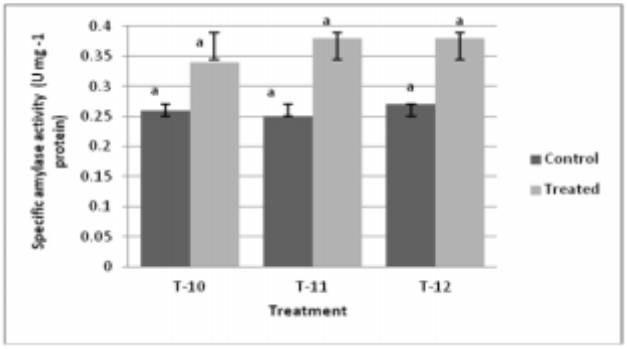

Figure. 5. Specific activity of amylase of *Portunus pelagicus* larvae (13DAH) treated with (T-10, T-11 and T-12) or without probiotic (control), a mixture of *L. plantarum, salivarius* and *L. rhamnosus*, as water additives. T-10 (1.0 ×10^6 cfu mL^{-1}), T-11 (5.0×10^6 cfu mL^{-1}) and T-12 (1.0×10^7 cfu mL^{-1}). Bars showing similar words are statistically significant (*P*<0.05)

Water parameters: Temperature was more or less constant and remained between the ranges 28.16^{o}C to 28.23^{o}C in all groups. Like temperature, the salinity was also constant throughout the trials and it ranged between 28.15‰ to 28.23‰. Dissolved oxygen (DO) level was observed within the range 6.05 - 6.13 mg/L in groups. It was observed that LAB did produce significantly effect on pH in treatment groups. The pH value in treated tanks inoculated with two isolates such as *L. plantarum* and *L. salivarius* ranged from 7.92±0.10 to 7.99±0.25.11. pH value for a mixture of *L. plantarum* and *L. rhamnosus* ranged between 7.93±0.12 to 8.01±0.24 and for *L. salivarius* and *L. rhamnosus* pH range was 8.01±0.25 to 7.93±0.12, while non inoculated control pH ranged between 8.13±0.02, 8.13±0.02 and 8.14±0.03 respectively. However, pH was significantly influenced by the allowance of three LAB probiotics together was 8.02±0.24, 7.93±0.12 and 7.92±0.12 against 10^6, 5x10^6 and 1x10^7 cfu mL^{-1} respectively over the mean pH of control 8.14±0.03 Table-4.

However, data analyse showed that pH was statistically significant among groups (p<0.05), at the same stage, temperature, salinity and DO were not significant (p>0.05) among and between the groups.

Table 4. pH ranges for control and multi probiotic treatments.

Treatments	*L. plantarum* & *L. salivarius*	*L. plantarum* & *L. rhamnosus*	*L. salivarius* & *L. rhamnosus*	*L. plantarum* *L. salivarius* & *L. rhamnosus*
Control	8.13±	8.13±	8.14±	8.14±
cfu mL^{-1}	0.02^a	0.02^b	0.03^c	0.03^{ab}
1x10^6	7.99±	8.01±	8.01±	8.02±
	0.25^b	0.24^{ab}	0.25^a	0.24^a
5x10^6	7.92±	7.93±	7.93±	7.93±
	0.10^{ab}	0.12^a	0.12^{ab}	0.12^b
1x10^7	7.92±	7.94±	7.93±	7.92±
	0.10^c	0.12^c	0.12^a	0.12^c

Note: Values in column not sharing the same letters are statistically significant (p<0.05).

DISCUSSION

To date, probiotics can be considered a valid alternative to the use of antibiotics in aquaculture and in particular, in fish larviculture, to prevent high mortality and to improve welfare and promote growth and survival. In the two last decades, many studies reported promising results using a single beneficial bacterial strain in the culture of many finfish species (Avella *et al*., 2010a) Looking for novel approach, during present study the mixture of LAB were tested as probiotics focusing to improve survival of *P. pelagicus* larvae.

LAB were individually tested for probiotic capability against target pathogens via cell free supernatants. The survival rates of indicator bacterial pathogens *V. harveyi, V. parahaemolyticus* and *P.*

piscicida were examined after 1–4 h of incubation with cell-free supernatants. *V. Parahaemolyticus. V. harveyi* and *P. piscicida*, were significantly susceptible to the supernatants and were destroyed within 3-4 h of exposure. The pathogenic bacteria tested against LAB in this study have been isolated from the gut of *P. pelagicus* and are reported as pathogens of crabs, shrimp, eels, catfish, and higher vertebrates (Beckers *et al*., 1985; Farzanfar, 2006; Meyer, 1991; Muroga, 2001). *V. harveyi, V. parahaemolyticus* and *P. piscicida* are virulent to larvae of *P. pelagicus* (Talpur *et al*., 2011b). In this work, cell-free supernatants from LAB exhibited effective removal of pathogenic bacteria that cause disease in fish/shellfish. A one thing common was observed that capability to kill the pathogen was similar in all LAB isolates. Pathogens killed in cell-free supernatants of LAB isolates may be due to bacteriocin produced by the isolates.

Addition of the candidate LAB probiotics significantly increased survival of *P. pelagicus* larvae, and it was demonstrated that these bacterial isolates were not adverse and could have a positive effect on *P. pelagicus* larvae. LAB and other probiotics have been shown to be beneficial for aquaculture in terms of growth when compared with normal controls (Lara-Flores *et al*., 2003; Macey and Coyne, 2005; ten-Doeschate and Coyne, 2008) However, wide range research on probiotics has been done on health benefits of organisms against pathogenic molest (Chabrillón *et al*., 2006; Lategan *et al*., 2004). During the present study, addition of LAB resisted against pathogen attacks and improved survival of larvae in all treated groups when compared with non inoculated (control) confirms as a prophylactic agents for bacterial infection prevention in *P. pelagicus* larviculture. Application of probiotics to fish larviculture in particular has yielded to positive effects, mainly in survival and growth rates (Avella *et al*., 2010b; Carnevali *et al*., 2006; 2004; Gatesoupe, 2008; Wang *et al*., 2008). Moreover, probiotic addition had a noticeable effect on *P. pelagicus* swimming behaviour and colouration, which was most evident in the first few days following hatching until the end of experiment. It was possible the LAB probiotics might have controlled the pathogens in water or with larvae, in turn, water condition could be more favourable compared to usual hatchery environment in lieu resulted better health and significant survival.

This study reveals that the application of LAB probiotic via the water had beneficial effects on the survival rate of *P. pelagicus* larvae. The previous study showed that supplementation of the commercial lactic acid producing *Bacillus* probiotic significantly increased the survival rate of Indian white shrimp (*Fenneropenaeus indicus*) in the treatments over the controls (Ziaei-Nejad *et al*., 2006). In *Penaues monodon,* a probiotic *Bacillus*, was able to colonize in both the culture water and the shrimp digestive tract, thereby increasing the black tiger shrimp survival (Rengpipat *et al*., 1998). However, Shariff *et al*. ((2001) found that treatment of *P. monodon* with a commercial *Bacillus* probiotic did not significantly increase survival. Furthermore, the probiotic, *Bacillus coagulans* SC8168, supplemented as water additive could significantly increased survival rate of shrimp *Penaeus vannamei* larvae (Zhou *et al*., 2009). Outstanding survival results were obtained when probiotics were used as water additive in rearing of larvae of green shell muscle *Perna canaliculus* (Kesarcodi-Watson *et al*., 2010). In the present study, LAB administrated in mixture as water additive did produce significant survival in all treatments. From the results, it has been depicted that the dose allowance at $5x10^6$ cfu mL^{-1} was unique in all treatments. Thus, mixture of two LAB in treatments T-2 and T-8 were more effective, in first case *L. plantarum* was mixed with *L. salivarius* and in other case with *L. rhamnosus.* On other hand dose allowance of two LAB mixture at $1x10^6$ and $1x10^7$ cfu mL^{-1} in T-1, T-3, T-4, T-6, T-7 and T-9 produced different survival rates were comparatively less then T-2 and T-8. In mixture of three LAB at $5x10^6$ and $1x10^7$ cfu mL^{-1} did produce highest survival in all treated groups. This indicates that the quantity of probiotics was only one of the factors promoting the survival rate of *P. pelagicus* larvae.

The results of this study indicates that a combination of the LAB probiotics did provide supplementary support against the unfavourable conditions or pathogen attacks during *P. pelagicus* larval rearing, in turn, increased significant survival. Other studies previously demonstrated enhanced protection with multi-species probiotics (Timmerman *et al.,* 2007; Zoppi *et al*., 2001), based on the theory that multiple species-specific benefits possessed broaden spectrum of probiotic effect. Indeed, three LAB probiotics were effective against *Vibrio harveyi*, *V. parahaemolyticus* and *Pseudoalteromonas piscicida* in an *in vitro* assay (Talpur *et al*., 2012) and in present study.

Detection of LAB isolates in culture water and larvae, after the addition, illustrated its ability for retention to the water and larvae. This is in agreement with the previous observation of their presence in water and larvae after addition into larval tanks (Talpur *et al*., 2012). Determination of the probiotic in the water and larvae could be an added benefit by extending the protective effect as a consequence of diminution of pathogens from the culture water and larvae.

Current study examined the activity profiles of enzyme assays particularly protease and amylase in the larvae (13DAH) of *P. pelagicus*. Since the *P. pelagicus* larvae were so small therefore, gut could not be removed, whole larvae were used for enzyme assay. It was observed that enzymatic activities in treated groups were much higher compared to those of non inoculated controls. This could be due to probiotics effective role which enhanced enzymes particularly protease and

amylase. Several probiotics in aquaculture organisms can enhance supplemental digestive enzymes, higher growth; feed efficiency, prevention of intestinal disorders (Thompson *et al*., 1999., Verschuere *et al*., 2000). It has been reported that during transition stage probiotics develop in intestine using carbohydrates for their growth and produce a range of relevant digestive enzymes (amylase, protease and lipase) (El-Haroun *et al*., 2006). However, in aquaculture, probiotics can be administered either as a food supplement or as an additive to the water (Moriarty, 1998). In the current study we administered LAB probiotics in water environment, thus, it was clearly determined where probiotics worked effectively in terms of survival and digestive enzyme activities in the provided environments (culture water). Nevertheless, highest enzyme activity was seen in mixture of three LAB probiotics at the concentration of $1x10^7$ cfu mL^{-1} followed by $5x10^6$ cfu mL^{-1}. In the present study live feed (rotifers and *Artemia*) were fed to *P. pelagicus* larvae without bioencapsulation. It was possible that live feed (rotifers and *Artemia*) ingested probiotic bacteria from the inoculated rearing water and those were taken as feed by the larvae or larvae directly ingested probiotic bacteria from culture water, might have enhanced the immune system and digestive enzymes. The digestive system of *P. pelagicus* larvae is activated particularly in the early stages of larval development where the probiotics LAB, do secrete a wide range of exoenzymes (Moriarty, 1998). Specific activities of larval enzymes, protease and amylase were significantly different in all experimental groups. Enzymatic profile of Exp. T-12 and Exp. T- 11 demonstrated remarkably better activities than the other experiments and control groups. It is likely that probiotics influences digestive processes by enhancing the population of beneficial microorganisms, microbial enzyme activity; improving the intestinal microbial balance, consequently improving the digestibility and absorption, finally resulted better survival rate owing to enhanced capability of larvae to cope with pathogenic attack. The LAB probiotic, *Bacillus coagulans* SC8168, supplemented as water additive at a certain concentration could significantly increased survival rate and some digestive enzyme activities of shrimp *Penaeus vannamei* larvae (Zhou *et al*., 2009). Similar results were found during the present study, therefore, the present study are in match of findings previously mentioned.

In the present study, the aquaria tanks that were treated with probiotics was abundant with LAB probiotics did show improvement in pH and this result may be explained by the good water quality *in vivo* conditions in this study. The possibility of using LAB for the simultaneous removal of pathogenic bacteria and improvement of water quality in shrimp farms, was explored using two LAB spp. JK-8 and JK-11 in shrimp ponds (Ma *et al*., 2009). The probiotic, *Bacillus coagulans* SC8168, supplemented as water additive could significantly influence the pH of treated waters (Zhou *et al*., 2009). Previous studies confirms that the lactic acid bacteria probiotics are able to play a vital role in growth, survival and disease resistance of the animal by maintaining good water quality parameters . During the present study besides with significant survival in larvae, enhancement in enzymes and change in water chemistry particularly in pH was observed, therefore, results of present offer similar findings with those of previous researchers.

In the present study, it was hypothesise that the mixture of LAB isolates supplied during early stages development of blue swimming crab, *P. pelagicus*, acted optimistically on larviculture by improving enzymatic activity and influencing pH to rearing conditions following increase in larval survival.

In fact, probiotic administration to rearing water may have positively acted with the beneficial effects as observed in all treated groups. Not too many differences observed in treated groups but in general, higher survival was archived in groups treated with mixture of three LAB, this may be related with the decrease of pathogenic bacteria load in the water and larvae which finally influenced the survival.

Conclusion: Findings of this study demonstrate that addition of probiotic to rearing water could significantly enhance survival, digestive enzyme activities and influenced pH of rearing water. Results of the study demonstrated that LAB are able to effect the larivculture and possibly be deemed as residents within the intestinal tract and rearing water. This is significant because LAB bacteria were not derived from other source, but rather may wield a unique favourable relationship with larvae and rearing conditions. Moreover, multi species isolates probiotic administration might be a good way to encounter uncertainties in survival and pathogen attack particularly from *Vibrio*. It could finally be concluded that this is the first study to investigate the effects of mixture LAB sp. probiotics in *P. pelagicus* larviculture focusing on survival, enzymatic activities and water chemistry particularly pH of rearing water.

Recommendations: Based on the results of the current study LAB bacteria isolated from the gut of *P. pelagicus,* are recommended for larval rearing practices. They might control opportunistic pathogenic bacteria, improving larval survival and rearing water.

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