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Isolation and Screening of Lactic Acid Bacteria from the Gut of Blue Swimming Crab, *P. pelagicus*, an *in vitro* Inhibition Assay and Small Scale *in vivo* Model for Validation of Isolates as Probiotics

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ABSTRACT

Blue swimming crab, *Portunus pelagicus* has not anchored the roots in aquaculture due to non availability of commercial seed production. Letdown of seed production is owing to microbial infections. To combat with microbes, study was aimed to isolate and screen probiotics from the gut of female crab for larviculture. Based on characteristics of inhibitory activity against pathogenic *V. harveyi*, *V. parahaemolyticus* and *P. piscicida,* bile, acid, salt tolerances and survival in sea water, isolates were identified as *L. plantarum*, *L. salivarius*, *L. rhamnosus* *W. confusa* and *W. cibaria* and evaluated for probiotics. A new model small scale *in vivo* validation was developed for conformity of the isolates as probiotics for *P. pelagicus* larviculture. The LAB isolates were administrated as water additive at concentrations 10^2, 10^4 and 10^6 cfu mL^{-1} for one day and five days *in vivo* validation experiments and positive control was inoculated with same concentrations of *V. harveyi* while negative control employed with larvae and no inoculation. Highest larval survival achieved at concentration 10^6 cfu mL^{-1} and *L. plantarum, L. salivarius* and *L. rhamnosus* did show significant larval survival. *W. confusa* and *W. cibaria* did not demonstrate as probiotics. *L. plantarum* showed highest survival 49.45±4.80% and 54.44±6.74% in both inoculations, respectively and no survival was observed in five days+ve control. Water quality degradation was not evident but improvement in pH was noticed. Based on results of small-scale *in vivo* test three LAB probiotics, *L. plantarum*, *L. salivarius* and *L. rhamnosus* were selected for larviculture of *P. pelagicus*.

Key words: *Portunus pelagicus*, *in vitro*, *in vivo*, inoculations, probiotic

INTRODUCTION

The gastrointestinal (GI) tract of fish is a complex ecosystem possessing a specific microbiota consisting of aerobic, facultative anaerobic and obligate anaerobic bacteria (Cahill, 1990; Gomez and Balcazar, 2008; Balcazar *et al*., 2008). The gut microbiota could be a "new" possible "internal factor" as it is gradually recognized that it plays very important role in the health and growth of the host (Comstock, 2007; Mazmanian *et al*., 2008; Vine *et al*., 2006). The role of gut

microbiota in fish are poorly understood, in general, the gut microbiota has been suggested to hinder the colonization of pathogenic bacteria (Kennedy *et al*., 1998; Verschuere *et al*., 2000; Spanggaard *et al*., 2001) and antibacterial substances (Sugita *et al*., 1998, 2002).

The term Lactic Acid Bacteria (LAB) is used to describe a broad group of Gram-positive, catalase-negative, non-sporing rods and cocci, usually non-motile. LAB utilise carbohydrates fermentatively and form lactic acid as the sole or major end product (Ringo *et al*., 2001). *Lactobacillus* is a common inhabitant of the gastrointestinal tract of a wide range of animals. The ecological roles played by *Lactobacillus* in the gut include amongst others: production of antimicrobial substances; modulation of the immune system, fermentation of some non-digestible carbohydrates and increase of availability of nutrients (Fuller, 1989). The microbiota of marine fish is usually composed of Gram negative psychrotrophic bacteria and Gram positive such as *Micrococcus, Corynebacterium, Bacillus, Lactobacillus* and *Clostridium* may also be present in variable proportions (Marie-France and Francoise, 2011). Bacteria belonging to the Lactic Acid Bacteria (LAB) appeared to be promising candidates and several species of LAB are part of the indigenous intestinal microbiota of healthy fish. Many genera and species have been reported from fish and shellfish such as *Lactobacillus plantarum, L. paracasei, L. casei, Carnobacterium maltaromaticum, C. divergens, C. gallinarum* and *C. inhibens, Streptococcus* spp., *Leuconostoc* spp., *L. lactis, L. delbrueckii* and *Lc. piscium, Vagococcus salmoninarum, Weissella* spp., etc. (Joborn *et al*., 1999; Ringo and Gatesoupe, 1998; Huber *et al*., 2004; Balcazar *et al*., 2007b; Yang *et al*., 2007; Balcazar *et al*., 2008; Rengpipat *et al*., 2008; Picchietti *et al*., 2009; Valerio *et al*., 2009; Sun *et al*., 2009; Sarkono *et al*., 2010; Askarian *et al*., 2008). Early studies suggested that LAB were not considered to be indigenous in the digestive tract of fish and it is generally accepted that LAB occur among the normal intestinal flora of fish from the first few days of their life and onwards (Ringo *et al*., 1995; Ringo and Gatesoupe, 1998). Although, LAB are not dominant population in fish/shellfish, it has been well documented in several investigations that lactic acid bacteria are a part of the native microbiota of aquatic animals from temperate regions (Ringo, 2004). Antimicrobials have been used increasingly as a primary intervention for inhibition or inactivation of pathogenic microorganisms (Davidson and Zivanovic, 2003). LAB isolates have the ability to survive, grow and produce their antimicrobials both under wide range of acidic and alkaline conditions, cold and warm temperature hence can be applied therapeutics (Bhattacharya and Das, 2010).

In the search for new antimicrobials, common practice adopts initial *in vitro* screening methods. Such methods screen the effectiveness of test isolates in a controlled environment. This has also been witnessed in past research concerning probiotic bacteria for aquaculture (Spanggaard *et al*., 2001; Chythanya *et al*., 2002; Hjelm *et al*., 2004). Therefore, before pilot scale or large scale *in vivo* application of probiotics, these could be validated *in vitro* through antimicrobial assay against indicator pathogens and small scale *in vivo* validation whether they are capable or not for target animal.

To increase the likelihood of identifying probiotic bacteria effective in *P. pelagicus* larviculture, direct screening of test bacterial isolates using *P. pelagicus* larvae was preferred in the initial screening stages. The development of a *in vitro* and small scale *in vivo*, replicable bioassay to enable future screening for probiotics of *P. pelagicus* larvae was investigated. However, isolations of LAB from fish/shellfish in a tropical climate are few but to our knowledge no report on blue swimming crab, *Portunus pelagicus*. The main purpose of the study was to isolate LAB from the

gut of *P. pelagicus* and to validate through *in vitro* antimicrobial activity and to establish small scale *in vivo* bioassay tests to determine the one-on-one effect of bacteria on larvae and to screen out putative probiotics.

MATERIALS AND METHODS

Study site and sampling site: The present study was conducted in the marine hatchery of Faculty of Agrotechnology and Food Sciences and the laboratory of the Institute of Tropical Aquaculture, Universiti Malaysia Terengganu (UMT), Malaysia. The crab samples were collected during the month of January 2010 from the Strait of Tebrau, (1° 22'N and 103° 38'E) Johor, West Malaysia.

Segregation of the gut: Gut was removed from the crab specimen according to Talpur *et al.* (2011), Bacteria isolation were carried out by serial dilution (10-fold). Total seven female crab specimens were sacrificed for the study.

Culture media and growth conditions: Samples were analysed by the dilution pour plate method. deMan, Rogosa and Sharpe (MRS, Merck) culture media prepared in sea water (SW) with salinity 28 ppt was used as selective medium. One mL aliquot of the 10^{-1}, 10^{-2}, 10^{-3} dilutions were plated onto MRS agar (pH 7.6); for isolation and enumeration of LAB. All plates were anaerobically incubated for 2-3 days at 37°C. After the incubation when colonies were evident, the plates with colony forming units (cfu) were selected for enumeration.

Isolation of lactic acid bacteria from culture media and Initial identification: White or milky colour colonies from the MRS agar slants were taken and purified on the same medium. Purity was ensured by repeated streaking and was examined through microscopic observations. All the isolates were checked for colony morphology, Gram reaction and catalase tests and CO_2 production in MRS-Durham tubes and growth in MRS broth.

Catalase test: LAB were known as catalase negative. Hence, in order to confirm catalase status of the isolates, catalase test was performed. For this purpose, overnight cultures of isolates anaerobically grown on MRS agar plates at 37°C for 24 h. Catalase activity was investigated by dropping 5% hydrogen peroxide solution (one drop) onto randomly chosen colony. The formation of gas bubbles therefore indicates the presence of catalase enzyme.

Gas production from glucose: In order to define further homofermentative isolates, CO_2 production from glucose test was performed. For this purpose, citrate lacking MRS broths and inverted Durham tubes were used. Fifty microlitter of overnight cultures measuring OD to 10^6 cfu mL^{-1} were transferred into the 8 mL test media. After incubation for 5 days at 30°C, gas accumulation in Durham tubes was taken as the evidence for CO_2 production from glucose.

Initial antibacterial activity test: Preliminary bacteriocin detection and activity was tested using the disc diffusion Assay according to Ma *et al.* (2009). Cell-free culture supernatants for antibacterial assay was prepared by growing the isolates in deMan, Rogosa and Sharpe (MRS) broth prepared in sea water with salinity 28 ppt incubated at 37°C and centrifuged at 12,000x g for 10 min and sterilised through 0.2 μ (Whatman, England). Using aseptic technique, sterile filter discs were saturated with 40 μL supernatant and placed onto plates inoculated with indicator

pathogenic bacteria. The inoculated plates were incubated for 24 h at 37°C. Based on the zone of inhibition around the filter discs, the effectiveness of antimicrobial activity was determined. Those inhibited less number of pathogen or less inhibition zones in initial test were excluded leaving five candidates from an original nine isolates for further investigation.

Identification of LAB isolates: Those isolates which showed inhibitory activity in preliminary study were identified using 16SrRNA gene sequencing until species level.

Analysis of 16S rDNA sequences

Total genomic DNA extraction: The total genomic DNA of samples were extracted by Wizard® Genomic DNA Purification Kit (Promega, USA). One mL of an overnight culture was taken to a 1.5 mL microcentrifuge tube, centrifuged at 16,000x g for 2 min to pellet the cells. The Supernatant was removed. DNA was extracted according to the instructions of Wizard® Genomic DNA Purification Kit (Promega, USA). The gel electrophoresis was run at 100 voltages (V) for 1 h and bands were visualised in transillumination to determine the quality of DNA.

Polymerase chain reaction (PCR) amplification of 16S rDNA: The 16S rDNA fragments were amplified using Oligonucleotides universal primers pair 27f (5'-AGAGTTTGATCCTGGCTCAG -3') and 1492r (5'- GGTTACCTTGTTACGACTT-3') (Cho and Do, 2006; Lucena *et al*., 2010). The PCR reaction was performed in a Bio-Rad thermal cycler (USA) according to Talpur *et al*. (2011). The final PCR products were then visualised on a 1% agarose gel (molecular grade) using a gel documentation system (Alpha Innotech, UK) after staining with 1 μg mL^{-1} ethidium bromide solution.

Bile tolerance of *Lactobacillus* sp.: The modified method of Arihara *et al*. (1998) was used to determine bile tolerance of LAB probiotics. Before testing for bile tolerance, LAB isolates were grown at 37°C for 24 h in MRS broth without bile. One mL of the culture broth was poured onto MRS agar with bile salt concentrations of 2000, 3000 and 4000 ppm. Bacterial growth was determined after incubation at 37°C for 48 h.

Survival of *Lactobacillus* sp. under acidic conditions: A modified method of Hyronimus *et al*. (2000) was applied to evaluate the survival of LAB isolates under acidic conditions. Aliquots of each active cultures grown in MRS broth prepared in sea water with salinity 28 ppt for 24 h at 37°C where optical density (OD_{680}) was obtained 10^6 cfu mL^{-1} and were adjusted to pH 1.0, 2.0, 2.5 and 3.0 with glacial acetic acid and incubated at 37°C for 3 h. Samples were taken every hour and the viable number of LAB were enumerated by pour plate counts on MRS agar plates, incubated at 37°C for 48 h and compared to the initial bacterial concentration. The results shown were the averages of two replicates.

Acidifying activity (pH change) of isolates: Acid production ability of LAB is one of the most important technological characteristic (Cogan *et al*., 1997; Herreros *et al*., 2003). In order to determine acidifying activity, potentiometric and titeric methods was applied (Sarantinopoulos *et al*., 2001). For this purpose, isolates were activated from agar slant in 5 mL MRS broth for 24 h at 37°C and 0.1 mL overnight cultures was inoculated in 50 mL of sterile MRS broth. Duplicate inoculations were prepared and incubated at 37°C with 150 rpm in shaker. Ten milliliter aliquots were taken aseptically in vial for the procedures from every sample and readings

were taken at 0, 6, 12, 18 and 24 h. For this reason, pH meter with glass electrode was used (Hanna instruments). Before using, pH meter was calibrated in buffer (pH7) and neutralised with sterilised non ionic water. After the calibration, the glass electrode was soaked into each of the samples and pH values were recorded. The acidification values or change in pH values (ΔpH) were expressed as pH decrease, calculated as the difference between the value immediately after inoculation and values at 0, 6, 12, 18 and 24 h. Following formula was used to determine the pH values.

$$\Delta pH = pH_{\text{at time}} - pH_{\text{zero time}}$$

Where:
ΔpH = Change in pH
$pH_{\text{at time}}$ = Sample taken at the time of reading
$pH_{\text{zero time}}$ = Initial pH at the time of inoculation

Growth at different temperatures: A pure colony was streaked on new MRS agar plate from agar slants already grown with LAB. Plates were sealed and incubated anaerobically for 3-5 days at 10, 40 or 45°C. Cells growth at any of these temperatures were detected and recorded.

Characterisation and selection of probiotic

Cell-free culture supernatants: Cell-free culture supernatants for antibacterial assay was prepared by growing the LAB isolates in MRS broth modified, Vegitone (Fluka) prepared with sea water (28 ppt) at 37°C, after 24 h of incubation pH was adjusted to pH 7.6 adding 1 M NaOH. Cells were harvested in 1.5 mL centrifuge tube and centrifuged at 12,000x g for 10 min which was followed by sterilising the supernatant through a 0.2 μm-pore (Whatman, England) filter.

Antagonistic activity via disc diffusion agar assay: Antimicrobial activity of the supernatants against the indicator pathogens was determined with the plate disc diffusion method described by Ma *et al*. (2009). The antimicrobial effects LAB against indicator pathogenic bacteria, *V. harveyi*, *V. parahaemolyticus* and *P. piscicida* previously isolated from the gut of *P. pelagicus* were evaluated. The pathogens were grown in nutrient broth (Merck) for 24 h at 37°C and OD_{630} at 10^6 cfu mL^{-1} was determined as standard was established previously. Using aseptic technique, sterile filter discs were saturated with 40 μL supernatant and placed onto plates inoculated with indicator pathogenic bacteria and incubated for 24 h at 37°C. The diameter of the inhibition zone was measured with callipers (Aslim *et al*., 2005).

Small-scale *in vivo* validation: Further to reduce the number of candidate probiotics those showed inhibition against indicator pathogens *in vitro* used for small-scale *in vivo* studies, those produced higher survival were selected as probiotics for further studies.

Experimental larvae: Blue swimming crab, *P. pelagicus* larvae used in experiments were breed in the marine hatchery of the Faculty of Agrotechnology and Food Science (FASM), University Malaysia Terengganu (UMT), Malaysia. Prior to spawning, broodstock was disinfected and Larvae were fed on a mixture of live prey composed of 30-40 rotifers (*Brachionus* sp.) mL^{-1} with micro algae (*Nanochloropsis* sp.) 8×10^5 cells mL^{-1}.

Experimental setup: All experiments were conducted in 1 L transparent aquaria containing sterilised sea water (SW) with salinity 28 ppt equipped with aeration. Aquaria were provided with gentle aeration. Probionts those showed promising inhibition *in vitro* against indicator pathogens were used in the bioassay. Negative control was employed with no pathogen and positive control was inoculated with pathogen (*V. harveyi*) at same concentration as in treated aquaria with probionts. Day one hatch (1 DAH) energetically swimming larvae were used as an experimental animal in the small scale *in vivo* probiont assay. Prior to exposing to bacteria (probiotic and pathogen), energetic larvae were acclimated in sterilized sea water (SW) with similar parameters as in hatching tanks and treated aquaria. Water from acclimation aquaria was sucked out with a small pipe fixed with 10 μ net at the suction end and new water was poured in with other pipe. This practice was exercised in order to minimise the bacterial load with larvae adhering from hatching tank water. All larvae were washed in this way using five litre-sterilised sea water. *V. harveyi,* bacteria previously isolated from the gut of *P. pelagicus* was cultured in nutrient broth prepared in sea water with salinity 28 ppt at 37°C for 24 h under agitation (75 rpm). Moreover, probionts LAB isolates from the gut of *P. pelagicus* were cultured in MRS broth prepared in sea water with salinity 28 ppt at 37°C for 24 h under agitation (150 rpm) and pH 7.6 was adjusted by 1M NaOH. Bacterial cells were subsequently collected by centrifugation (1200 g, 10 min) rinsed with sterile sea water and re-suspended in sterile sea water. Bacterial suspension was measured to $OD_{630\,nm}$ and administrated as water additive at three concentrations (10^2, 10^4 and 10^6 cfu mL^{-1}) (Triplicate). Treatments inoculated with LAB were not inoculated with pathogen and vice versa. Each aquaria contained 1 L autoclaved sea water with 20 larvae/L. Two experimental set up were established, aquaria inoculated one time in experiment one and daily inoculation in experiment two. All experiments were carried out in triplicate. Survival (%) was evaluated by direct counting of larvae (Total number of larvae survive/Initial number of larvae stocked×100).

Water parameters: During small scale *in vivo* validation test of LAB, the water parameters such as temperature, salinity, DO and pH were recorded daily and measured with YSI 556 MPS (USA). Seven sets of equipments were used one for each inocula. Five for probionts, one for inoculated control and one for non inoculated control to ensure hygiene conditions.

Biochemical characteristics: Each isolate was activated in 50 mL MRS broth for 24 h at 37°C and biochemical characteristics were performed using API 50 CH Kit (bioMerieux, France).

Survival of inoculated LAB in sea water, culture water a salt (NaCl): LAB colonies were inoculated in sterilised sea water for their viability. Reading were taken at 24, 48, 72 and 96 h. Water samples and larvae from small scale *in vivo* experiments were taken for viability and presence of LAB. To examine the salt tolerance of isolates from the gut of *P. pelagicus,* isolates were inoculated onto MRS-agar plates prepared with NaCl at concentration to 0, 2, 5 and 7% salt, respectively. The inoculated plates were incubated at 37°C for 2-3 days under anaerobic conditions in incubator and tolerance was visualised as colonies were grown on each medium.

Detection of LAB and pathogen in larvae: Larvae were disinfected in 10% formalin and were aseptically washed with sterilised sea water until the smell of formalin off. Larvae were

homogenised and samples were serially diluted, plated on MRS agar for detection of LAB and for pathogen, TCBS agar was used. MRS plates were incubated at 37°C for 72 h or until LAB evident while TCBS plates were taken out after 24 or 48 h. Bacteria were isolated from TCBS and streaked for purity and BD BBL crystal identification system (Enteric) was used to identify the pathogenic *V. harveyi*. For LAB confirmation morphological and physiological characters, Gram staining and catalase test was performed.

Growth of LAB: One mL of overnight culture of LAB was aseptically inoculated to 200 mL of fresh MRS broth prepared with sea water (SW) 28 ppt with pH 7.6 and was incubated at 37°C on shaker machine at 150 rpm. Inocula were made in triplicate and control was employed without inoculum. All samples were used for reading at time 0, 2, 4, 6, 8,10, 12, 14, 16, 18. 20, 22, 24, 26, 28, 30, 32, 34 and 36 h, respectively for OD_{630nm} and bacterial counting was determined on agar plates by serial dilutions upto 10^{-8} and plates were inoculated in duplicate incubated at 37°C for 2-3 days and mean was worked out. The cell densities were measured through spectrophotometer Shimadzu UV-1800 (Japan).

Probiotic preservation and maintenance: LAB Isolates were preserved in (MRS) broth medium which contained 30% (v/v) glycerol as frozen stocks at -80°C for long term preservation and for short term in -20°C upto six month. Working cultures were maintained on 20 mL MRS agar slants or as stab culture in vials with agar.

Statistical analysis of data: Means of different concentrations and survival were compared by ANOVA analysis of variance using statistical software (SPSS 16.0 for windows). Post hoc test was carried out using LSD, Tukey's and Duncan tests, correlation two tailed test, if they were significant (confidence level ±95).

RESULTS

Isolation of LAB: Total seven female *P. pelagicus* specimens were sacrificed for the isolation of LAB. Out of seven, LAB were found only in three (42.86%) female guts while in rest of four (57.14%) female guts no LAB was recovered. Average LAB cfu mL^{-1} obtained from the gut of *P. pelagicus* are illustrated in Table 1. The dissection of all seven female specimens was performed from one sample collected for the study.

Table 1: Colony forming units (cfu mL^{-1}) isolated from the gut of female *P. pelagicus* on MRS selective microbiological media

		Carapace size (cm)			
Crab's specimen	B W (gm)	CW	CL	No of plates	cfu mL^{-1}
1	181.20	12.50	5.90	6	2.20×10^{3}
2	137.45	10.70	5.50	6	1.50×10^{3}
3	157.34	11.70	5.70	6	-
4	166.22	13.10	6.10	6	-
5	127.56	11.20	5.40	6	3.33×10^{3}
6	112.76	10.20	5.30	6	-
7	129.12	12.50	5.90	6	-

BW: Body weight, CW: Carapace length, CL: Carapace length

It was fascinating to observe that the cfu mL^{-1} were not proportioned with the Body Weight (BW) carapace width (CW) and carapace length (CL) of the specimen. Highest body weight 181.2 gm female produced 2.20×10^{3} cfu. mL^{-1} of LAB isolates whereas the lowest weight female 127.56 gm resulted in 3.33×10^{3} cfu. mL^{-1} of LAB microbes in the gut (Table 1).

Initial identification of isolates: Different typical colonies based on colour either creamy or white from MRS agar were taken from the second lowest countable dilution and purified on the same medium. Purity was examined by microscopic observations. All the isolates were checked for morphology, Gram reaction and catalase tests and CO_2 production in MRS-Durham tubes and growth MRS broth. Results are given in Table 6.

Initial antibacterial activity test: Out of nine LAB isolates only five showed inhibitory zones against all three indicator pathogens. While other four showed mixed inhibitory activity either inhibiting one or two pathogens, eliminated from the study. Based on inhibition activity isolates were identified via 16S rRNA gene sequencing through PCR protocol.

Identification of LAB through 16S rRNA sequences: Results of 16S rRNA gene sequencing of five LAB isolates up to species level found in NCBI online sequence citation http://blast.ncbi.nlm.nih.gov are illustrated in Table 2.

Given the high similarity found between *W. confusa* and *W. cibaria* 16S rRNA gene 99.2% (Bjorkroth *et al.*, 2002). Nevertheless, we found the similarity 100% in PCR protocol for identification of both species of *Weissella* (Table 2).

Bile salt tolerance: All five isolates of LAB were tested for their abilities to grow at the bile salt levels of 1000, 2000 and 3000 ppm in order to select bile-tolerant isolates. It was found that all five tested isolates were bile tolerant.

Survival of *Lactobacillus* isolates under acidic condition: The gut tract of fish or shellfish has varying acid levels. Stomach and the regions after stomach have the highest acidity and the pH of these areas may differ pH values upto to 3 or 4. In order to be used as beneficial LAB, must be able to survive these harsh conditions and colonise in the gut. Each isolate was inoculated at 6 log cfu mL^{-1} to each pH/acidic condition to figure out the survival result. The survival of five different LAB isolates under acidic conditions (pH 1.0, 2.0, 2.5 and 3.0) are illustrated in Table 3. In general, the number of survivors of all cultures during 3 h of incubation decreased at all pH conditions. The viable count (log) cfu mL^{-1} was substantial decreased especially at pH 2.0 and lower. All five isolates showed the highest viability and have shown moderate activity even at pH 2.5 and pH 3.0.

Table 2: Identification of lactic acid bacteria (LAB) strains isolated from the gut of *Portunus pelagicus* females with partial sequences of 16S rRNA genes

Isolate	Max identity (%)
Lactobacillus plantarum	100
Lactobacillus salivarius	100
Lactobacillus rhamnosus	99-100
Weissella confusa	100
Weissella cibaria	100

Table 3: Survival of LAB isolates under different pH (acidic condition) log value (log cfu mL^{-1})

Isolate	Time (h)	pH 1.0	pH 2.0	pH 2.5	pH 3.0
L. plantarum	1	0	3.954	4.934	5.025
	2	0	3.699	3.954	4.556
	3	0	2.602	2.954	3.447
L. salivarius	1	0	3.903	4.913	4.982
	2	0	3.544	3.845	4.447
	3	0	2.477	2.954	3.322
L. rhamnosus	1	0	3.954	5.009	5.140
	2	0	3.732	4.146	4.662
	3	0	2.820	3.025	3.428
W. confusa	1	0	3.903	4.954	5.009
	2	0	3.643	3.924	4.505
	3	0	2.477	2.881	3.342
W. cibaria	1	0	3.934	4.964	5.000
	2	0	3.653	3.914	4.531
	3	0	2.663	2.851	3.255

L. plantarum showed highest viability 5.025 log cfu mL^{-1} at 1h pH 3.0 and lowest at pH 2.0 (log cfu mL^{-1} 2.602 at 3 h). Similar results for *L. salivarius* were obtained with highest log cfu mL^{-1} 4.982 at 1 h pH3.0 and lowest 2.477 log cfu mL^{-1} at 3 h pH 2.0. *L. rhamnosus* showed better result with highest log cfu mL^{-1} 5.14 at pH 3.0 followed by 5.009 log cfu mL^{-1} at pH 2.5 at time 1 h and lowest at pH2 at 3 h, respectively. Comparatively the viability of *L. rhamnosus* was highest in all pH and time intervals. *W. confusa* showed highest viability 5.009 log cfu mL^{-1} at 1 h pH 3.0 and lowest at pH 2.0 (log cfu mL^{-1} 2.477 at 3 h). *W. cibaria* showed highest viability 5.00 log cfu mL^{-1} at 1h pH 3.0 and lowest log cfu mL^{-1} 2.663 at pH 2.0 at 3 h. Results of the study showed that all five isolates of LAB were acid tolerance at pH 2.0, 2.5 and 3.0. A decline in viability was noticed at each interval of time. One common and interesting thing in all isolates was observed that they did not show any viability at pH 1.0.

Acidifying activity (pH change) of isolates: Acid production properties of LAB are main technological characteristics for (Beresford *et al*., 2001). For this aim, pH was monitored for five isolates. All LAB species showed the highest acidifying capacity and it was observed that all LAB were producing acid by decreasing pH values monitored at intervals. Resulted obtained are shown in Fig. 1. Rapid acid production abilities indicated that they were the most suitable candidates for *in vitro* as well as *in vivo* probiotic challenges.

Growth at different temperatures: All LAB were grown at 20 and 40°C and were not grown at temperature 50°C (Table 6).

Characterization and small-scale *in vivo* validation

Inhibition assay: For screening approaches, the classical agar disc diffusion assay was used. The analysis showed that all five isolates of the LAB were able to inhibit growth of all indicator pathogens *V. harveyi, V. parahaemolyticus* and *P. piscicida*. The *Lactobacillus* isolates were able to inhibit the growth of pathogen due to their ability to produce the lactic acid. Zone of inhibitions by LAB against indicator pathogens are shown in Fig. 2. *L. plantarum* did produce inhibition 14.40±0.14 mm against *P. piscicida* followed by *V. harveyi* 12.05±0.49 mm and were less

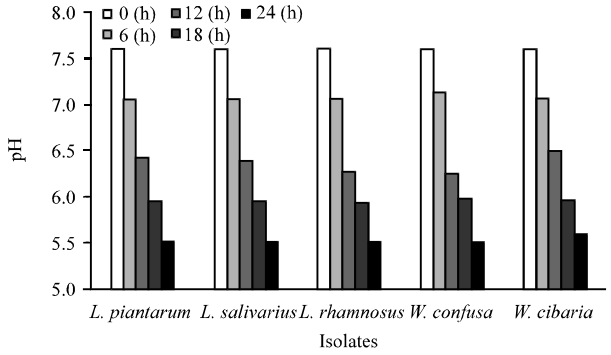

Fig. 1: Change in pH of isolates over a period of 24 h. pH decreased because LAB produced acid in MRS broth

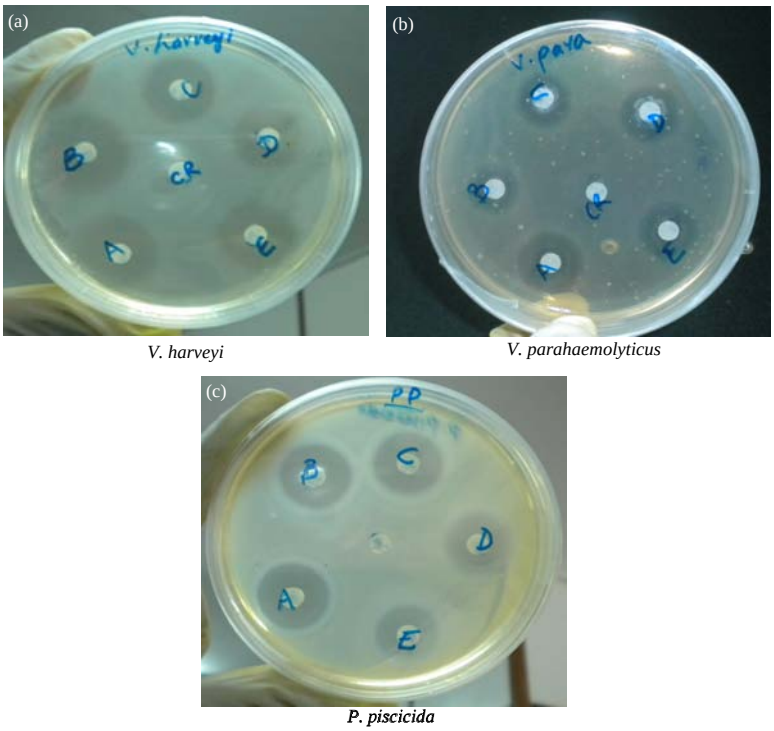

Fig. 2(a-c): Zone inhibition by LAB isolated from the gut of *P. pelagicus*. A-*L. plantarum*, B-*L. salivarius*, C- *L. rhamnosus*, D. *W. confusa* and E- *W. cibaria*

against *V. parahaemolyticus* 9.75±0.64 mm. *L. salivarius* did show highest inhibition against *P. piscicida* showing clear zone 12.85±0.49 mm, followed by *V. harveyi* 11.85±0.92 mm and *V. parahaemolyticus* 7.85±0.49 mm, respectively. *L. rhamnosus* cleared maximum zone towards *P. piscicida* 13.05±0.21 mm, followed by *V. harveyi* 11.71±1.13 mm and *V. parahaemolyticus* 9.40±1.27 mm, respectively. *W. confusa* showed highest inhibition against *P. piscicida* 7.9±0.57 mm followed by *V. harveyi* 9.75±0.07 mm and *V. parahaemolyticus* 7.75±0.35 mm, respectively. However, *W. cibaria* showed more inhibition toward *V. harveyi* 9.75±0.64 mm secondly towards *P. piscicida* 8.45±0.21 mm and lesser to *V. parahaemolyticus* 7.65±0.21 mm. Diameter of inhibition zone (mm) caused by antimicrobial activity of LAB strains against indicator microbes is elaborated in Table 4.

Table 4: Diameter of inhibition zone (mm) caused by antimicrobial activity of LAB isolates against indicator microbes

	Inhibition interpretation (mm) Disc diffusion assay		
Isolates	*V. harveyi*	*V. parahaemolyticus*	*P. piscicida*
L. plantarum	12.05±0.49	9.75±0.64	14.40±0.14
L. salivarius	11.85±0.92	7.85±0.49	12.85±0.49
L. rhamnosus	11.71±1.13	9.40±1.27	13.05±0.21
W. confusa	9.75±0.07	7.75±0.35	7.90±0.57
W. cibaria	9.75±0.64	7.65±0.21	8.45±0.21

Table 5: Small scale *in vivo* results showing survival of *P. pelagicus* larvae at inoculation one time and daily

		Inoculation one time			Inoculation daily (day1 to day5)		
Concentration (cfu mL^{-1})	Bacteria	Survival (treatment)	Survival (control+)	Survival (control -)	Survival (treatment)	Survival (control+)	Survival (control -)
10^2	*L. plantarum*	46.67±2.89	15.00±5.00	45.00±5.00	48.33±2.89	0	43.33±2.89
10^4		46.67±2.89	0	45.00±0.00	53.33±2.89	0	43.33±2.89
10^6		55.00±5.00	0	46.67±2.89	61.67±2.89	0	45.00±0.00
Total mean survival		49.45±4.80	5.00±8.96	45.56±0.96	54.44±6.74	0	43.89±0.96
10^2	*L. salivarius*	45.00±5.00	13.33±2.87	45.00±5.00	46.67±2.89	0	45.00±5.00
10^4		48.33±2.89	0	45.00±0.00	53.67±2.89	0	45.00±0.00
10^6		51.67±2.89	0	45.00±5.00	56.67±2.89	0	46.67±2.89
Total mean survival		48.33±3.33	4.44±7.7	45.00±0.00	52.22±5.09	0	45.56±0.96
10^2	*L. rhamnosus*	45.00±5.00	15.00±5.00	43.33±2.89	46.67±5.77	0	45.00±5.00
10^4		46.67±2.89	0	48.33±2.89	53.33±2.89	0	48.33±2.89
10^6		53.33±2.89	0	46.67±2.89	61.67±2.89	0	45.00±5.00
Total mean survival		48.33±4.41	5.00±8.66	46.11±2.55	53.89±7.52	0	46.11±1.94
10^2	*W. confusa*	45.00±2.89	16.67±2.89	43.33±2.89	46.67±2.89	0	43.33±2.89
10^4		46.67±2.89	0	46.67±2.89	48.33±2.89	0	46.67±2.89
10^6		46.67±2.89	0	45.00±0.00	48.33±2.89	0	45.00±0.00
Total mean survival		46.11±0.96	5.56±9.62	45.00±1.67	47.78±0.96		45.00±1.67
10^2	*W. cibaria*	45.00±0.00	16.67±5.77	46.67±2.89	46.67±2.89	0	46.67±2.89
10^4		46.67±2.89	0	45.00±0.00	50.00±5.00	0	45.00±0.00
10^6		46.67±2.89	0	45.00±0.00	46.67±2.89	0	45.00±0.00
Total mean survival		46.11±0.96	5.56±9.62	45.56±0.96	47.78±1.92	0	45.56±0.96

+: Control inoculated with *V. harveyi* at same cfu as probionts, -: Control with no inoculation

Small-scale *in vivo* validation of probiotic bacteria: Based on total mean survival of larvae against inoculated concentrations (10^2 cfu mL^{-1}, 10^4 cfu mL^{-1} and 10^6 cfu mL^{-1}) of candidate probiotics, the best three out of five were screened as probiotics for further *in vivo* validation for larviculture of *P. pelagicus* crab. *L. plantarum* showed promising mean survival results both in one day and five days inoculations, 49.45±4.80 and 54.44±6.74% survival, respectively over+control survival 5.00±8.96 and 0% while-ve control produced 45.56±0.96% survival. *L. rhamnosus* was second with 48.33±4.41 and 53.89±7.52% mean survival. *L. salivarius* was third with 48.33±3.33 and 52.22±5.09% for one and five days inculcations. No survival was evident in five days inoculated control. *W. confusa* and *W. cibaria* produced slightly better results then the control. They did produce mean survival 46.11±0.96, 46.11±0.96 and 47.78±0.96%, 47.78±1.92 for one and five days inoculations, respectively. Maximum larval survival achieved at concentration 10^6 cfu mL^{-1}. Detail results are shown in Table 5.

Water parameters: All LAB isolates showed highest decrease in pH at inoculation of 10^6 cfu mL^{-1}. *L. plantarum* showed significantly decrease in mean pH 8.06, followed by *L. rhamnosus* 8.07, *L. salivarius* 8.08, *W. cibaria* 8.11 and *W. confusa* 8.14 against control 8.18, 8.18, 8.22, 8.20 and 8.17, respectively. Temperature and pH were found statically significant ($p<0.05$) and salinity and Dissolved Oxygen (DO) were statistically different ($p>0.05$) during experimental trials. Results are shown in Fig. 3 (pH) and Fig. 4 (mean pH), respectively.

Morphological, physiological and biochemical characteristics of LAB isolates: Physiological, morphological studies were carried out during initial identification of isolates and biochemical tests of LAB isolates which shown probiotic characteristics *in vitro* were carried out using API strips (bioMerieux) and results are shown in Table 6.

Survival of LAB inoculated in sea water, culture water and salt: The cells of isolates after releasing to sea water were discovered/re-isolated until day three (72 h) and onward no incidence was found, this is might be due to non availability of nutrients for growth and survival.

The isolates (LAB and pathogenic *V. harveyi*) inoculated to culture water were recovered either from water or larvae inoculated daily during the small scale *in vivo* validation of probiotics are shown in Table 7. Pathogenic *V. harveyi* was frequently observed both in water and larvae either

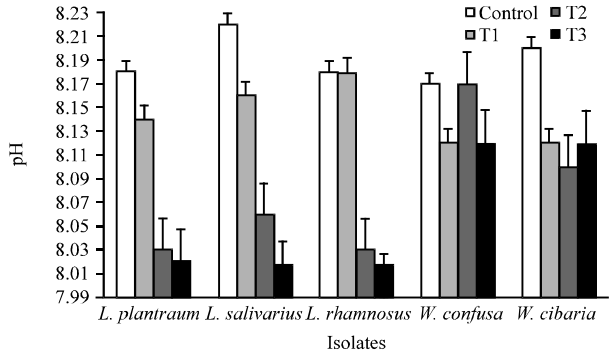

Fig. 3: pH values during treatment in small-scale *in vivo* experiment (T1 = 10^2, T2 = 10^4 and T3 = 10^6 cfu mL^{-1})

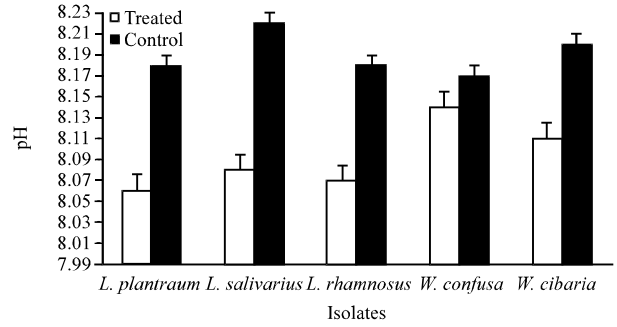

Fig. 4: Mean pH values of treated and control during treatment in small-scale *in vivo* experiment

Table 6: Morphological, physiological and biochemical tests of LAB isolates (probiotics) from the gut of female *P. pelagicus*

Strain Characteristics	*L. plantarum*	*L. salivarius*	*L. rhamnosus*	*W. confusa*	*W. cibaria*
Colony morphology	Round, creamy colour, size 0.5 to 6.0 mm	Oval, Off white or creamy white, Size 0.7-1.3 mm	Round or oval, light cream colour, Size 0.7-1.1 mm	Oval round, Pinkish white, Size 0.2 to 1.0 mm	Oval round, creamy white, Size 0.2 to 1.0 mm
Cell morphology	Rod shaped	Rod shaped	Rod shaped	Oval cocci	Oval cocci
Gram staining	+	+	+	+	+
Catalase	-	-	-	-	-
Gas production	-	-	-	-	-
Growth at					
20°C	+	+	+	+	+
40°C	+	+	+	+	+
50°C	-	-	-	-	-
L-Arabinose	+	-	+	+	+
D-Ribose	+	+	+	+	+
D-Galactose	+	+	+	+	-
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+	+	+		
L-Rhamnose	+	-	+		-
D-Mannitol	+	-	+		
D-Sorbitol	+	+	+		
Methyl-D-Mannopyranoside	+	+	+	+	+
Methyl-D-Glucopyranoside	-	-	-	-	-
N-Acetylglucosamine	+	+	+	+	+
Amygdaline	+	-	+	-	+
Arbutine	+	-	+		
Esculine citrate de fer	+	+	+	+	+
Salicine	+	+	+	+	+
D-Cellobiose	+	+	+		
D-Maltose	+	-	+	+	-
D-Lactose	+	+	+	+	+
D-Melibiose	+	-	-	+	+
D-Saccharose	+	-	+	+	+
D-Trehalose	+	+	+	+	+
D-Melezitose	+	-	-	+	-
D-Raffinose	-	-	-	-	-
Amidon	-	-	-	-	-
Glycogene	-	-	-	-	-
Gentiobiose	+	+	+	+	+
D-Turanose	-	-	-	-	-
D-Tagatose	-	-	-	-	-
D-Aarabitol	+	-	-	+	+
Potassium-Gluconate	-	-	-	-	-

Table 7: Detection of tested (LAB and pathogen *V. harveyi*) isolates from treatments during probiotic combination experiments

		Day											
		1		2		3		4		5		6	
Treatment		W	L	W	L	W	L	W	L	W	L	W	L
Non-inoculated Control	Prob	N/D	N/D	-	-	-	-	-	-	-	-	-	-
	Path	N/D	N/D	-	+	+	+	+	+	+	+	+	+
Inoculated control (10^2)	Prob	N/D	N/D	-	-	-	-	-	-	-	-	-	-
	Path	N/D	N/D	+	+	+	+	+	+	+	+	+	
Inoculated control (10^4)	Prob	N/D	N/D	-	-	-	-	-	-	-	-	-	-
	Path	N/D	N/D	+	+	+		+		+		+	
Inoculated control (10^6)	Prob	N/D	N/D	-	-	-	-	-	-	-	-	-	-
	Path	N/D	N/D	+	+	+		+		+		+	
L. plantarum (10^2)	Prob	N/D	N/D	-	-	+	-	+	-	+	+	+	+
	Path	N/D	N/D	-	+	-	+	+	+	+	+	+	+
L. plantarum (10^4)	Prob	N/D	N/D	+	-	+	+	+	+	+	+	+	+
	Path	N/D	N/D	-	+	-	+	+	+	+	+	+	+
L. plantarum (10^6)	Prob	N/D	N/D	+	-	+	+	+	+	+	+	+	+
	Path	N/D	N/D	-	+	-	+	+	+	+	+	+	+
L. salivarius (10^2)	Prob	N/D	N/D	-	-	-	-	-	-	-	+	+	+
	Path	N/D	N/D	-	+	-	+	+	+	+	+	+	+
L. salivarius (10^4)	Prob	N/D	N/D	+	-	+	+	+	+	+	+	+	+
	Path	N/D	N/D	-	+	-	+	+	+	+	+	+	+
L. salivarius (10^6)	Prob	N/D	N/D	+	-	+	+	+	+	+	+	+	+
	Path	N/D	N/D	-	+	-	+	+	+	+	+	+	+
L. rhamnosus (10^2)	Prob	N/D	N/D	-	-	-	-	-	+	+	+	+	+
	Path	N/D	N/D	-	-	-	-	+	-	+	+	+	+
L. rhamnosus (10^4)	Prob	N/D	N/D	+	-	+	+	+	+	+	+	+	+
	Path	N/D	N/D	-	+	+	+	+	+	+	+	+	+
L. rhamnosus (10^6)	Prob	N/D	N/D	+	-	+	+	+	+	+	+	+	+
	Path	N/D	N/D	+	+	+	+	+	+	+	+	+	+
W. confusa (10^2)	Prob	N/D	N/D	-	-	-	-	-	+	-	-	+	+
	Path	N/D	N/D	-	+	-	+	+	+	+	+	+	+
W. confusa (10^4)	Prob	N/D	N/D	+	+	+	-	+	-	+	+	+	+
	Path	N/D	N/D	-	+	-	+	+	+	+	+	+	+
W. confusa (10^6)	Prob	N/D	N/D	+	+	+	+	+	+	+	+	+	+
	Path	N/D	N/D	-	+	+	+	+	+	+	+	+	+
W. cibaria (10^2)	Prob	N/D	N/D	-	-	-	-	-	-	-	-	+	-
	Path	N/D	N/D	-	+	+	+	+	+	+	+	+	+
W. cibaria (10^4)	Prob	N/D	N/D	-	-	+	-	+	-	+	-	+	+
	Path	N/D	N/D	+	+	+	+	+	+	+	+	+	+
W. cibaria (10^6)	Prob	N/D	N/D	+	-	+	-	+	+	+	-	+	+
	Path	N/D	N/D	+	+	+	+	+	+	+	+	+	+

Prob: Probiotic, Path: Pathogen, W: Culture water, L: Larvae, N/D: Not did, +: Yes, -: No. Blank squares means larvae were died due to pathogenic inoculations

inoculated with probionts and non inoculated control. *V. harveyi* was believed to be in culture system due to shed of adult female crab in hatching tanks. No probiotic bacteria was detected in non inoculated control either in water or with live larvae and dead larvae. It was observed that,

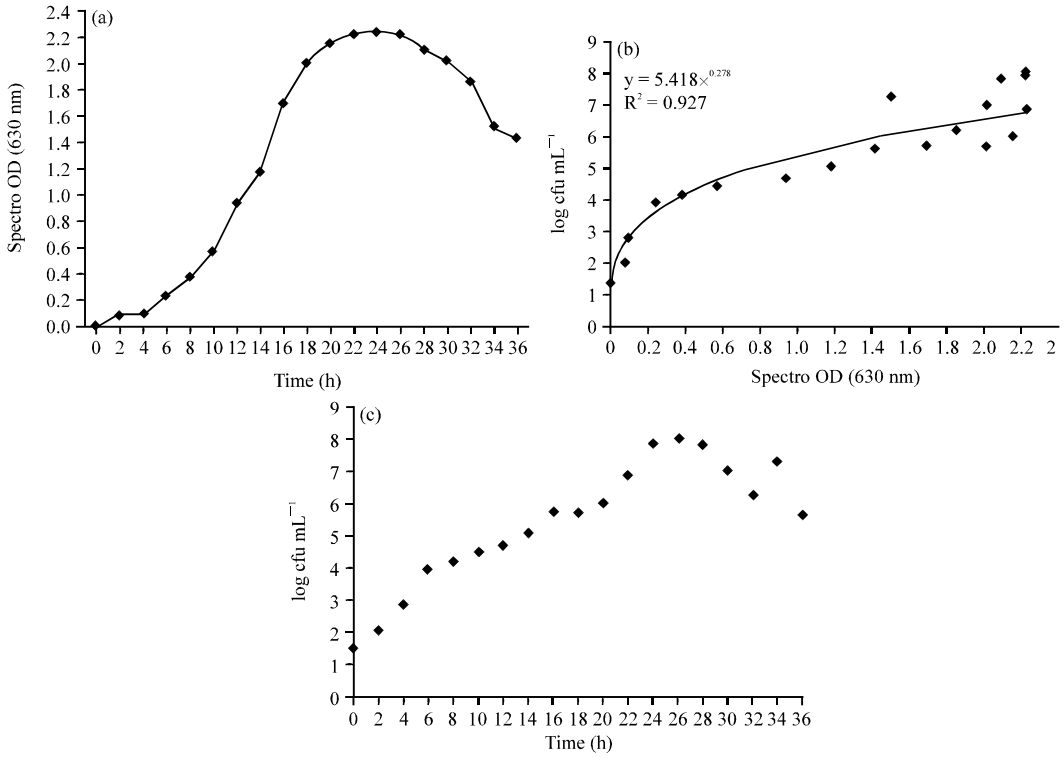

Fig. 5 (a-c): Growth curve of *L. plantarum*

even at low dose frequency of pathogen was inoculated, the incidence of pathogen was evident in culture water from day two to day six even larvae were died.

It was interestingly noticed that during the small scale *in vivo* probiotic administration, pathogen was not inhibited either with larvae or in culture water until day six by any of probiont bacteria.

All LAB isolates were capable to grow on salt 2, 5 and 7%.

Growth of probiotics: Bacterial growth characteristics have also been used to screen probiotic bacteria and it has been proposed that probiotic bacterium with a short lag phase and a fast growth will be the most competitive *in vivo* (Vine *et al.*, 2004), Although probiotic bacteria with these growth characteristics might have better competitive abilities. Growth of *L. plantarum*, *L. salivarius*, *L. rhamnosus*, *W. confusa* and *W. cibaria* was determined and growth curves obtained are shown in Fig. 5-10, respectively. Data of OD and log cfu mL^{-1} were analysed using two tailed correlation and was found statistically significant ($p<0.05$).

Probiotic preservation and maintenance: LAB Isolates were preserved in deMan, Rogosa and Sharpe (MRS) broth medium which contained 30% (v/v) sterile glycerol as frozen stocks at -80°C for long term preservation and for short-term in -20°C. Working cultures were maintained on 20 mL MRS agar slants. After incubation at 37°C until bacterial growth was evident, the slants were maintained at temperature 10-12°C or in refrigerator at 4°C. All agar plates were tightly two fold parafilm in order to avoid dryness and contamination. From these, new slants were re-streaked

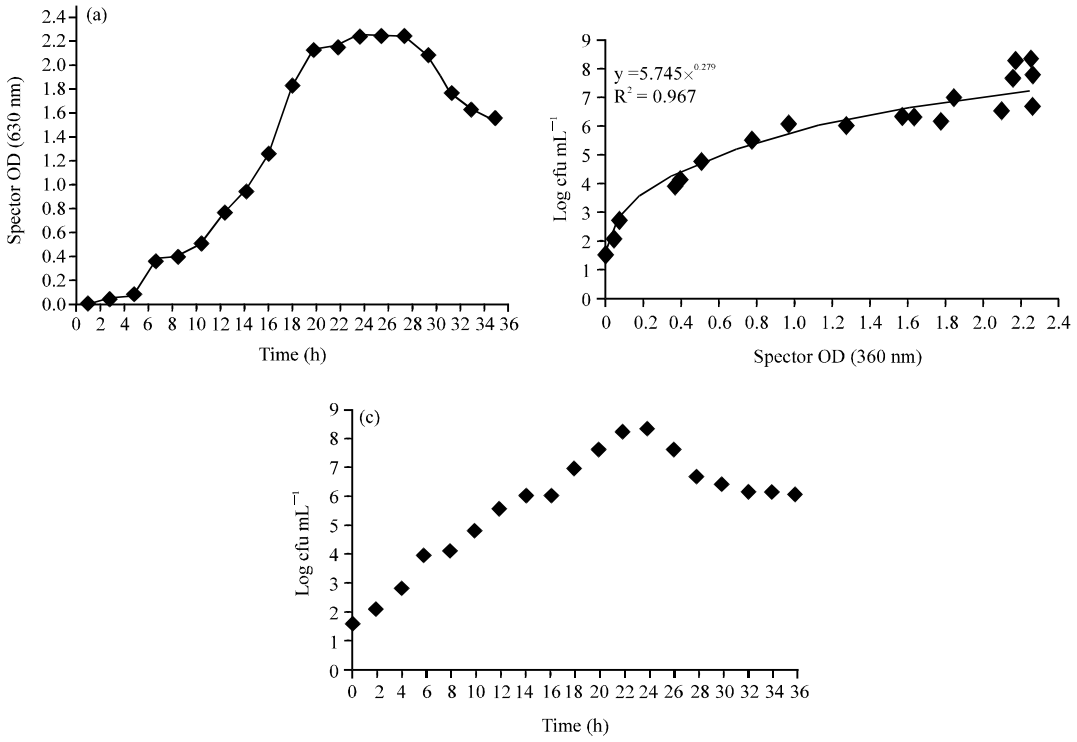

Fig. 6 (a-c): Growth curves of *L. salivarius*

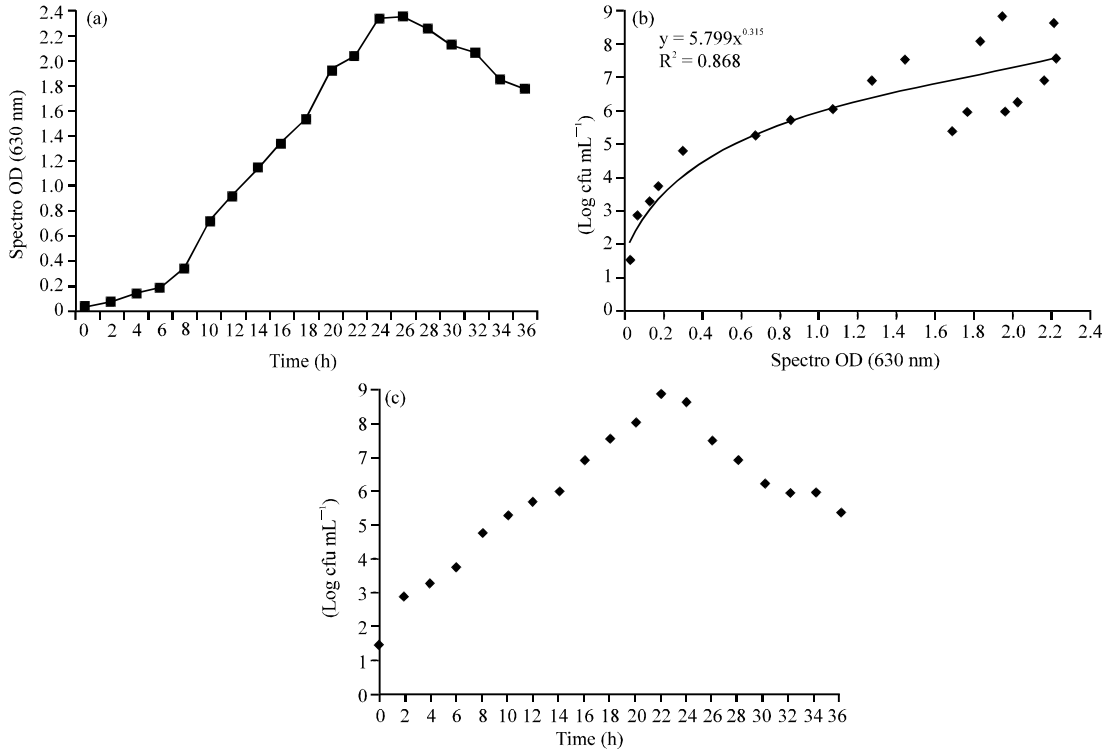

Fig. 7 (a-c): Growth curve of *L. rhamnosus*

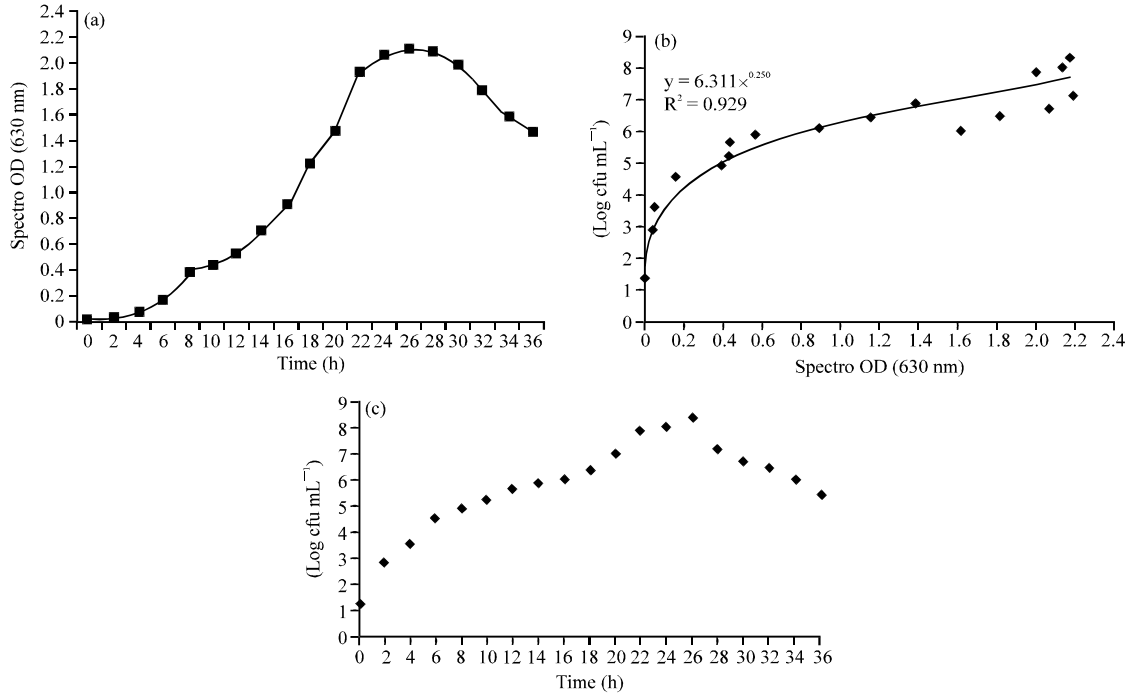

Fig. 8 (a-c): Growth curves of *W. confusa*

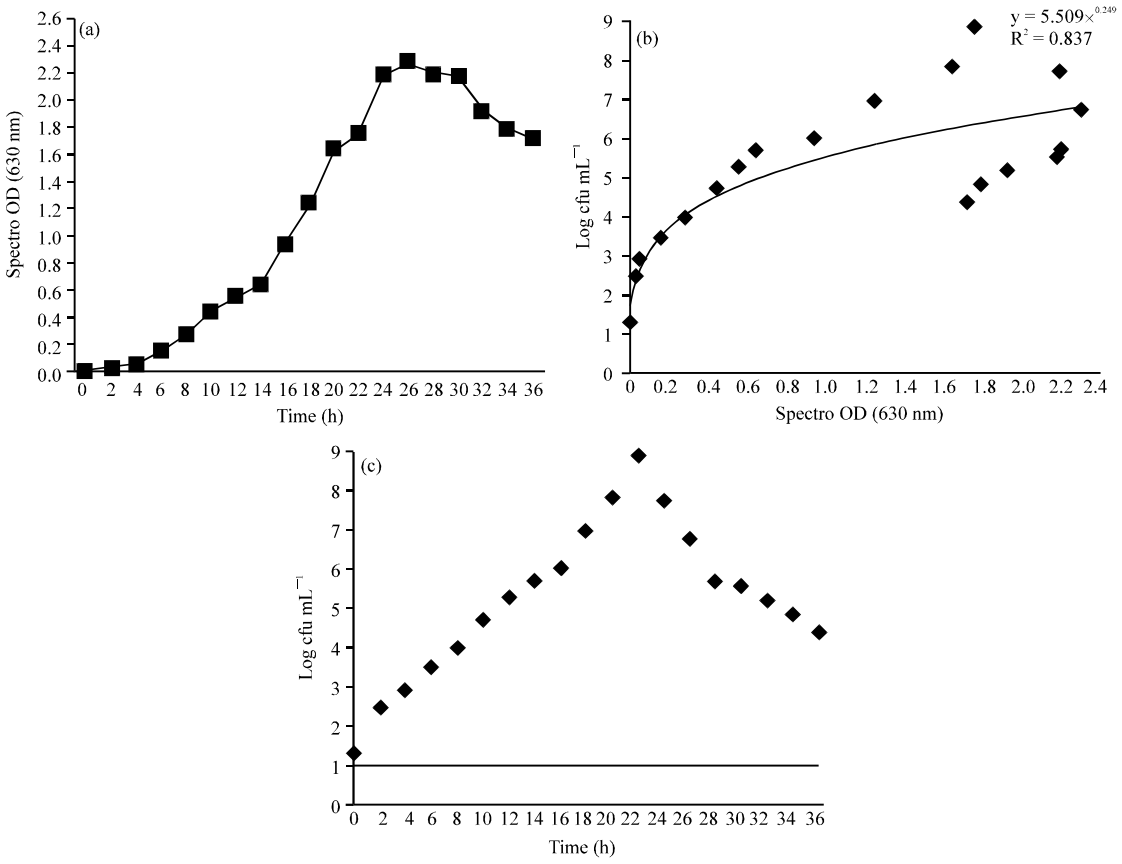

Fig. 9 (a-c): Growth curves of *W. cibaria*

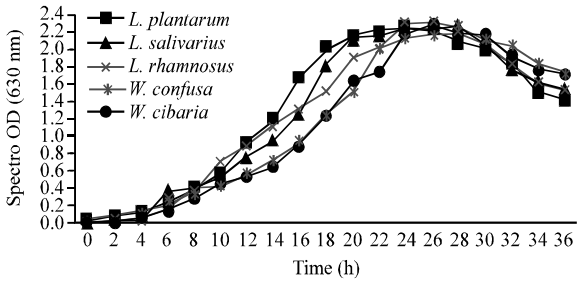

Fig. 10: Comparative growth pattern of five LAB isolates

every one to two weeks unless contamination occurred. When contamination of the slants occurred, new slants were prepared from the -80°C or -20°C stock cultures.

DISCUSSION

All of the bacteria with antagonistic abilities that were isolated in this study were members of LAB. Out of seven female crabs, LAB were found in the gut of three females while rest did not responds the growth of LAB. This is may be due to either the LAB were not available or did not grow on MRS agar made with sea water which could not fit the environment for their growth. Based on preliminary *in vitro* test, LAB isolates were identified through 16S rRNA gene sequencing and necessary tests were performed. Erkkila and Petaja (2000) reported that the strains of LAB could be resistant to 3000 ppm bile salt. Pennacchia *et al.* (2004) reported that the bile salt tolerance of the *Lactobacillus* strains were able to grow in MRS agar supplemented with 3000 ppm bile salt. However, *L. rhamnosus* strains isolated from Parmigiano Reggiano cheese were able to survive at bile salt concentration of 10,000, 15,000 and 20,000 ppm after 48 h of incubation at 37°C (Succi *et al.*, 2005). Here in the present study we reported that all LAB were able to grow in bile. The present results are in accordance to the results of previous researchers that LAB has ability to grow in high bile salt and showed viability at various acid conditions of pH. *L. rhamnosus* was the most acid tolerant isolate. The results of present study are in agreement with Succi *et al.* (2005) who selected *L. rhamnosus* strain isolated from Parmigiano Reggiano cheese based on their survivals after 2 and 4 h of incubation at pH 3.0 which was similar to the study performed by Chou and Weimer (1999) and on *L. acidophilus* strain from the American Type Culture Collection. Some *Lactobacillus* strains retained their viability, at pH 1 for 1 h (Maragkoudakis *et al.*, 2006). However, in the present study no LAB showed viability against pH 1.0. Results of present study are in accordance to findings of Buntin *et al.* (2008) who investigated a large bunch of LAB isolates isolated from marine fish and shell fish.

Most studies searching for probiotic candidates focus on bacteria that show inhibitory activity in an *in vitro* assay against a target pathogen (Gram and Ringo, 2005). The rationale behind this strategy is that when these probiotic bacteria are established in the gut, they will produce substances that inhibit growth and thus colonization by pathogenic bacteria. *In vitro* inhibition activity via disc diffusion assay, clear zone around the discs is due to bacteriocin produced by LAB (Cadirci and Citak, 2005).

Lactobacillus is present in the gastrointestinal tract of various vertebrates, including freshwater fish, cold water fish and marine fish (Ringo *et al.*, 1995; Ringo and Gatesoupe, 1998; Gonzalez *et al.*, 2000; Balcazar *et al.*, 2008; Vijayabaskar and Somasundaram, 2008). In an other study Buntin *et al.* (2008), isolated variety of LAB from the gastrointestinal tracts of marine fish,

shellfish shrimp and crab. A wide range of 18 LAB species and strains were discovered by (Lee *et al*., 2010) from the marine Oyster (*Crassostrea gigas*) including *L. paracasei*, *L. johnsonii*, *L. plantarum*, *L. rhamnosus*, *L. parabuchneri*, *L. pentosus* and *L. paraplantarum*. (Mahious *et al*., 2006) isolated allochthonous LAB from the whole intestine of Siberian sturgeon (*Acipenser baeri*). However, the present study is the first to isolate LAB from the gut of the *P. pelagicus*. It is has been worked out that LAB is not dominant flora in fish intestines (Ringo and Gatesoupe, 1998; Ringo, 2004; Balcazar *et al*., 2008). But, it is possible to maintain a high population level of LAB artificially in the fish gut by regular intake of food containing LAB (Ringo, 2004). In case of *P. pelagicus*, particularly at larval stages when they were faint and easily susceptible to bacterial pathogens, LAB should be further considered as therapeutic agents to improve the survival and health of the larvae. The importance of a stable gut microbiota might have some application, as the gut is one of the major infection routes in fish (Birkbeck and Ringo, 2005; Ringo *et al*., 2007). During the study, total nine LAB bacteria were isolated and tested for antibacterial activity. Out of nine only five (56.56%) LAB produced antibacterial activity against indicator pathogens namely *V. harveyi*, *V. parahaemolyticus* and *P. piscicida* used for the study while rest showed mix up results and were eliminated from the study. *L. plantarum* showed the broadest range of inhibitory action. *L. plantarum* has high potential probiotics properties due wide range of antimicrobial activities against fatal pathogens (Gharaei-Fathabad and Eslamifar, 2011).

L. rhamnosus stand second in inhibitory activity followed by *L. salivarius*, *W. confusa* and *W. cibaria* were the fourth and fifth in the inhibitory activity. The virulence potential of pathogens can be reduced by inhibitory or antagonistic activity of LAB. This reveals potential applications of *Lactobacillus* strains as protective cultures for the improvement of the microbial safety (Osuntoki *et al*., 2008).

In the search for probiotic candidates, the following criteria should be considered (Gatesoupe, 1999; Verschuere *et al*., 2000): The microorganisms should (1) be non-pathogenic to the host (2) compete with or hinder the growth of undesirable microbes (3) adhere to, develop within the host and (4) be indigenous to the environment to which it will be used. Many studies have demonstrated that the indigenous microbiota of fish or rearing environment can inhibit pathogen growth (Fjellheim *et al*., 2007; Joborn *et al*., 1997; Robertson *et al*., 2000; Vijayan *et al*., 2006; Vine *et al*., 2004). Several probionts tested in fish aquaculture have been selected based on their effects in humans (Balcazar *et al*., 2007a; Nikoskelainen *et al*., 2001a; Panigrahi *et al*., 2004; Nikoskelainen *et al*., 2001b) and livestock (El-Haroun *et al*., 2006; Gatesoupe, 2002; Suzer *et al*., 2008). These solutions may prove safe for human consumption but their efficiency in marine environment; may be expected to be poor due to different requirements of non-marine, mesophilic microbes. Other researchers have focused their selection on autochthonous bacteria, i.e., those originating from the respective aquatic animals (Abd El-Rhman *et al*., 2009; Aly *et al*., 2008a; Bjornsdottir *et al*., 2010; Brunt *et al*., 2007; Caipang *et al*., 2010; Fjellheim *et al*., 2010; Jatoba *et al*., 2008).

In order to select the probiotic candidates, it is important to screen the isolates for beneficial properties that are important to probiotic bacteria. Screening for bacterial antagonism against a target pathogen is the most common method used (Gram and Ringo, 2005). However, screening for antagonism against dominant bacterial strains, to see if the normal bacterial flora is inhibited, is also relevant. It is widely considered that, probiotic bacteria may help its host by contributing to digestive processes in the gut (Rowland, 1992), as the intestinal bacterial flora in fish produces extracellular enzymes (Ramirez and Dixon, 2003; Bairagi *et al*., 2002). Probiotic bacteria should

also be resistant to bile to persist in the digestive tract (Nikoskelainen *et al.*, 2001b) and they should tolerate low pH to pass through the acidic environment in the stomach. However, resistance to acid is not required for probiotic candidates aimed at marine larvae, as the digestive system is alkaline during first feeding (Hoehne-Reitan *et al.*, 2001).

The *in vitro* experiments can and should only be used as an indication of possible successes *in vivo*. Some assumptions regarding the *in vivo* mode of action based upon *in vitro* experiments may not hold. Therefore, the purpose of the *in vitro* experiments is to gain a better understanding of the potential of specific candidate probiotics but it is necessary before subjecting them to costly and time-consuming in large scale *in vivo* trials, should be validated in small scale *in vivo* protocol as probiotics. Bacteria that contribute to the adult fish's normal microflora may provide the larvae with protection against opportunistic or pathogenic bacteria (Olson *et al.*, 1992; Gatescope, 1994; Ottesen and Olafsen, 2000). It therefore seems reasonable to screen for candidate probiotics isolated from adults of the same species into which they are to be introduced.

Taking into consideration of previous researches done, the probiotic candidate isolates were selected based on bile tolerance, salt resistant can survive under low pH and sea water. Probionts on strategies of the antagonistic activity *in vitro* against indicator pathogens were selected for small scale *in vivo* validation as a putative probiotics for larviculture of *P. pelagicus*.. Two types of experiments were accorded (I) only one time inoculation (ii) inoculation made daily for five days. Three different concentrations (10^2 cful mL^{-1}, 10^4 cful $mL^{-1}$and 10^6 cful mL^{-1}) of candidate probiotics and same concentration of *V. harveyi* were inoculated to +control while -ve control was employed with no inoculation.

All the Gram negative indicators *in vitro* test were inhibited by the culture supernatants. That all LAB showed promising inhibition against indicator pathogens but it was observed that all five LAB showed less zone inhibition against *V. parahaemolyticus*, this was noticed as common fascination.

Antibacterial activity is common among bacteria from the marine environment (Long and Azam, 2001; Grossart *et al.*, 2004). Bacteria from different genera (Sugita *et al.*, 1996; Ringo and Gatesoupe, 1998; Long and Azam, 2001; Hjelm *et al.*, 2004; Makridis *et al.*, 2005; Aslim *et al.*, 2005) produce chemical substances that cause antibacterial activity, including antibiotics, organic acids, hydrogen peroxide, siderophores and bacteriocins. These compounds could be stable in low or high temperature. Antimicrobial compound of LAB was found stable 121°C for 20 min and 4°C which is possible potential for probiotics, could be used at high or low temperature and in the therapy of infectious diseases (Amin *et al.*, 2009). A few percent of the cultivable bacterial flora from fish is usually antagonistic to pathogenic bacteria *in vitro* (Sugita *et al.*, 1996; Hjelm *et al.*, 2004), although in some cases, higher percentages of antagonistic bacteria have been reported (Westerdahl *et al.*, 1991; Makridis *et al.*, 2005). Under *in vitro* conditions bacterial antagonism is medium-, growth phase- and temperature- dependent (Bizani and Brandelli, 2004; Hjelm *et al.*, 2004; Monteiro *et al.*, 2005). Thus, it can be questioned whether conditions suitable for production of inhibitory compounds *in vitro* will occur in the *in vivo*. To overcome the question after *in vitro* testing of probionts, to validate them further, a small scale *in vivo* was conducted for selection of putative probiotics. Here ready to report that two LAB bacteria *W. confusa* and *W. cibaria* that produced well antagonistic activity during antimicrobial assay against indicator pathogens (*V. harveyi, V. parahaemolyticus* and *P. piscicida*) on one hand. On other hand when both LAB were tested in small scale *in vivo* validation against larvae of *P. pelagicus* they did not perform role of probiotics due to unknown reasons. Whereas three LAB namely *L. plantarum, L. salivarius* and *L. rhamnosus* showed the characteristics of probiotics and increased the larval survival.

The present study and previous studies donor by various researchers such as (Long and Azam, 2001; Sugita *et al.*, 2002); Grossart *et al.* (2004) have proven that a range of marine bacterial genera produce antimicrobial compounds in an *in vitro* tests. *In vitro* test conditions are standardized with regards to growth media and temperature and selected bacterial isolates tested against pathogens. Actual rectification of any probiotic bacteria can be achieved through *in vivo* application where it should show probiotics characteristics. During the present research, small scale *in vivo* bioassay for screening of probiotics was conducted in 1 L transparent aquaria. 1 litre transparent aquaria offered a useful avenue in developing a larval *in vivo* bioassay for the purpose of screening for probiotic bacteria. Benefits of small aquaria included: easy to handle; allowance of up to many replicates in small space; small and stackable to facilitate a large number of tests with ease; visualization of a whole population throughout an experiment and ease in maintaining a constant experimental environment. This technique of small scale *in vivo* was first time initiated for the probiotic recognition. Previously Tissue Culture Dishes (TCDs) were used in bioassays for muscles (Kesarcodi-Watson *et al.*, 2010) because they either require no or low oxygen demand and is infeasible for *P. pelagicus* larvae which require high demand of oxygen.

The results of this study demonstrated that small scale *in vivo* screening of probiotics using one liter small aquaria for bioassays is feasible with *P. pelagicus* larvae. Larval survival was good, water quality degradation not evident but use of probionts improved the water by change in pH and a large number of treatments could be conducted over six days in the controlled environment of an incubator like experiment. Potentially, this bioassay design could be used with larvae of other aquatic species for screening purposes. Studies of baseline effects would need to be performed for individual species, as shown in this study. However, given insignificant detriment to test animals over a timeframe long enough to perform screening tests, the present designed bioassay offers an efficient and effective alternative method to validate probionts in large scale, laborious, costly and time consuming *in vivo* screening and application. Under the provided experiment, it was easy to visualise all effects of tested probionts reflected on larvae. This type of system can be used in pathogenicity test against indicator pathogens in larviculture to determine the pathogenic stress of microbes. Same technique was also used in the present study employing pathogens in+control of indicator pathogens against *P. pelagicus* larvae. Small scale *in vivo* test for screening of isolated LAB through this type of bioassay was the first attempt in the research history towards the *P. pelagicus* larvae. Mostly researchers used *in vitro* only antimicrobial test of probionts against indicator pathogens either through disc diffusion assay, overlay assay or well agar assay. However, here in small scale *in vivo* bioassay for screening of LAB probionts isolated from the gut of female crab, *P. pelagicus* led to increase in survival of larvae of *P. pelagicus*. Lactic Acid Bacteria (LAB) strains applied to different fish species led to increased survival compared with control groups when challenged with fish pathogens, as in trout (Irianto and Austin, 2002; Nikoskelainen *et al.*, 2001a; Vendrell *et al.*, 2008) and tilapia (Aly *et al.*, 2008b). Our results were in agreement with said studies, we got better survival of larvae with three probionts, *L. plantarum*, *L. salivarius* and *L. rhamnosus* in daily inoculation as well as one time inoculation over a control.

Pathogenic *V. harveyi* was frequently observed both in water and larvae in culture water inoculated with probionts and non inoculated control. No probiotic bacteria were detected in non inoculated control either in water or with live and dead larvae. It was observed that even at low dose frequency of inoculated pathogen, the incidence of pathogen was evident in culture water from day two to day six even larvae were died.

In all small scale *in vivo* validation of probiotics, it was evident that all inoculated LAB, were successfully recovered from culture water and larvae. Recovery of LAB from the larvae is the capability of adhesion which is positive characteristic for any probiont.

Another interest thing was noticed during the small scale *in vivo* probiotic administration, no inhibition of pathogen was evident on one hand and on other enhanced survival of larvae with LAB inoculated treatments was observed. This may be due to enhancement in immunity of the larvae against pathogen. Pathogenic *Vibrio* in larviculture was transmitted from the adult female crab during hatching in breeding tanks.

Interest in lactic acid bacteria is growing. In addition, bacteriocins produced by lactic acid bacteria are great interest to aquaculture industry because they are able to inhibit the growth of causative pathogenic bacteria and are friendly to the environment. Therefore, an investigation of antibacteriocin in lactic acid bacteria may offer potential applicability in aquaculture industry as environment friendly probiotic. Antimicrobial activity of *L. plantarum, L. salivarius* and *L. rhamnosus* have broad spectrum to pathogenic bacteria and their high potency in antibacterial activity is highly recommended as putative probiotic in larviculture.

An other interest thing during the present study was that pH of culture medium kept at 7.6. All isolates were optimised through sub culture in MRS agar made with sea water (SW) having 28 ppt, because this was set as the ambient salinity for larviculture. This technique was done in accordance to make the LAB capable to survive in culture sea water with salinity 28 ppt.

The selected probionts were *L. plantarum, L. salivarius* and *L. rhamnosus*. *L. plantarum* and *L. rhamnosus* are used in aquaculture but to date our knowledge *L. salivarius* have not been tested so far in aquaculture or literature is not cited. Another observation that makes selected LAB were suitable as a probiotic is that after being stored in refrigeration.

Pathogens are usually fast-growing (Andrews and Harris, 1986), therefore, a high starting concentration of probiotics may make them more competitive. This would favour the use of all LAB which showed relatively high inhibition potential. It is necessary to know the growth pattern, the bacteria could be cultured in large numbers before addition to the aquaculture system. Therefore, the maximum growth of all LAB was observed 20-30 h after culture. Decline in growth was determined after 30 h.

CONCLUSION

A new model of small scale *in vivo* was first time used to select the probiotics for larviculture of *P. pelagicus*. Based on results of small scale *in vivo* experiments three LAB species namely *L. plantarum, L. salivarius* and *L. rhamnosus* were selected for further pilot scale *in vivo* larviculture of *P. pelagicus*. Potentially, this bioassay design could be used with other aquatic species for screening purposes. Therefore, small scale *in vivo* test for validation of probiotics is the base line for large scale or pilot scale application of new probiotics.

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