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RESEARCH ARTICLE

Control of *Vibrio harveyi* Infection in Blue Swimming Crab, *Portunus pelagicus* Larvae by the Gut Isolated Lactic Acid Bacteria under Challenge Bioassay

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ABSTRACT

Five isolates of lactic acid bacteria (LAB) isolated from the gut of female *Portunus pelagicus* with inhibitory activity against shellfish pathogens and validation as probiotics via small scale *in vivo* model were tested for hatchery trials pathogen probiotic assay. *Vibrio harveyi* previously isolated from the gut of *P. pelagicus*, was added at 10^4 cfu mL^{-1} to test larvae for 10h. Test (LAB) isolates were inoculated at a concentration of 10^6 cfu mL^{-1} to pathogen addition aquaria once and until day four during the experiment. 20 larvae/liter were stocked and larval survival was determined over five days. *Lactobacillus plantarum* did produce highest survival 28.33% to 48.33% in one day and daily inoculations respectively and in probiotic control it produced 58.33% survival followed by *L. rhamnosus* 55% and *L. salivarius* 53.33% respectively over non inoculated control 43.33% and 0% survival in *V. harveyi* inoculated control. However, *Weissella confusa* and *W. cibaria* did show less probiotic activity compared to rests of three LAB isolates. In the present study, it was determined that three LAB probiotics were effective in hatchery trials challenge assays which may significantly control the infection and increased the survival of larvae.

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INTRODUCTION

Blue swimming crab, *P. pelagicus* is a popular recipe of the table in south East Asia and others parts of the world. The aquaculture of this species has not been boosted yet owing to non availability of commercial seed production. Mortalities are vast at early stages of larvae due to microbial infections particularly *Vibrio harveyi* transmitted from mother crab to larviculture system (Talpur *et al*., 2011a). *V. harveyi* isolated from the gut of female *P. pelagicus* was previously demonstrated using a pathogenicity bioassay using 1L aquaria resulted fatal effect on larval survival of *P. pelagicus* crab (Talpur *et al*., 2011b). However, in the hatchery production environment of *P. pelagicus* larvae, conditions are conspicuously different from those of 1L bioassay. In order to understand the effects of pathogenic *V. harveyi* and to facilitate effective management of microbial infections happening at a hatchery facility, in this disquiet, it is necessary to have the knowledge of the circumstances that initiate infection and to develop remedy plan through environment friendly microbes.

Literature citation reveals that numerous researchers have work out to control the infections caused by the fatal pathogens and results of their studies have revealed significant survival of animals challenged by bacterial pathogens as a result of probiotic use (Gibson *et al*., 1998; Ruiz-Ponte *et al*., 1999; Kumar *et al*., 2006; Li *et al*., 2006; Taoka *et al*., 2006).

It has believed that propagation of opportunistic and pathogenic microorganisms in intensive rearing systems is believed to cause poor larval growth and high mortality rates (Munro *et al*., 1995). Therefore, alternative therapeutic approaches other than antibiotics are necessary to achieve environment friendly fish hatchery operations for sustainable larval productions. In this regard effective probiotics should be investigated against fatal pathogens under hatchery conditions. Relating to probiotics pathogens challenge for *P. pelagicus* larviculture, no research has been investigated. To

date, there is no published literature on probiotics pathogens bioassay for *P. pelagicus* larvae and there is a lack of literature on pathogen probiotic challenge models during hatchery conditions at present.

The main objective of this study was to use hatchery trial bioassay to screen and validate, naturally existing non-pathogenic bacterial isolates from the gut of *P. pelagicus* as potential probiotics for control of infections in hatchery environment for larval production of *P. pelagicus*.

MATERIALS AND METHODS

Study site and Sampling Site: The present study was conducted in the marine hatchery and the laboratory of the Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, Kuala Terengganu, Terengganu Malaysia.

Seawater for experiment: UV treated seawater was filtered through a 10 µm net and stored in 1000L tank and treated with chlorine for 24h and supplemented with EDTA (after 24h of chlorine treatment) to settle down the heavy metals and then de-chlorinated with sodium thiosulphate for larval and brood stock use.

Experimental Larvae: Berried females were caught from Strait of Tebrau (1° 22' N and 103° 38' E), Johor, W. Malaysia. Berried females were disinfected and breed in marine hatchery of the Institute of Tropical Aquaculture, Universiti Malaysia Terengganu according to Talpur *et al*. (2011a). Day one hatches Zoea (1DAH) were used as experimental larvae. Prior to exposing challenge test or for control, energetic larvae were conditioned with sterilize seawater (SW) with similar parameters as in treated aquaria in order to minimise the bacterial load with larvae adhering from hatching tank water.

LAB bacteria: Five lactic acid bacteria previously isolated from the gut of female *P. pelagicus* includes *Lactobacillus plantarum*, *L. salivarius, L. rhamnosus, Weissella confusa* and *W. cibaria*, which showed *in vitro* inhibition against indicator pathogens were used in the bioassay study.

Bioassay challenge experimental design: A number of 20 larvae/L were added to each aquaria having 10L capacity where each aquaria contained 10L of disinfected seawater. Each treatment was conducted in triplicate. The larvae were fed daily with a mixture of live prey composed of 30-40 rotifers mL^{-1} (*Brachionus* sp.) and *Nannochloropsis* sp ($8x10^5$ cells mL^{-1}). The pathogen, *V. harveyi* previously isolated from the gut of *P. pelagicus* was added at 10^4 cfu mL^{-1} to test larvae for 10 hours. Test probiotics (LAB) isolates were inoculated at a concentration of 10^6 cfu mL^{-1} to aquaria added with pathogen after 10 h one time and daily until day 4 of the experiment. Larval survival was measured over five days. Aquaria containing non-inoculated larvae served as survival controls, while those inoculated with the pathogen alone served as mortality controls. Exclusively aquaria treated with LAB isolates were served as probiotic control. Larvae were maintained at 28°C and 28ppt throughout the experiment. All aquaria were provided aeration continuously.

Bacterial culture: Prior to use in experiments, pathogenic bacteria were grown in marine broth (28 ppt) for 24 hours at 37°C on shaker with 80 rpm prior to inoculation to larvae. Probiotic bacteria were grown in MRS broth prepared with seawater (28ppt) for 24 hour at 37°C on shaker with 1500 rpm. Bacteria were then centrifuged 12000x g for 10 min and washed two times with sterilise seawater (28ppt) and suspended in sterilize seawater (28ppt) to a concentration of 10^4 cfu mL^{-1} for *V. harveyi* and 10^6 cfu mL^{-1} for LAB probiotics sing OD_{630nm} according standards previously established.

Data analysis: Treatment differences were analysed using ANOVA (P=0.05). Post Hoc comparisons between survivals were compared using Tukey's test according to Talpur *et al*. (2012c).

RESULTS

Based on larval survival, the results of screening trials showed that three putative probiotics includes *L. plantarum*, *L. salivarius* and *L. rhamnosus* did show better effectiveness against the infection. In total, five isolates were screened those three providing significant protective effect. In one day inoculation test all five LAB isolates were comparatively showing same degree protective effect (Table 1) but in daily inoculation only three of the putative probiotics (*L. plantarum*, *L. salivarius* and *L. rhamnosus*) provided a better protective effect against infection, while two isolates *W. confusa* and *W. cibaria* isolates respectively, provided less promising benefit to larvae challenged with pathogen *V. harveyi* over three other LAB probiotics (Table 1). Results shows across all experiments, effectiveness from LAB isolates that were determined significant ranged from 25.00 to 28.33% improved larval survivals when inoculated pathogen allowance and probiotic ration once. However, survival ranged from 31.67 to 48.33% when inoculated pathogen allowance once and probiotic allowance daily. Furthermore, *L. plantarum*, *L. salivarius* and *L. rhamnosus* isolates contribution momentous protection provided better larval survival >50% in exclusive probiotic control compared with those non inoculated control (Table 1) and larvae infected with pathogen controls and inoculated probiotic once and daily (Table 1). No survival was obtained in pathogen inoculated control. All pathogen-probiotic treated groups were statistically significant (P<0.05).

Table 1: Survival (%) of *Portunus pelagicus* larvae at day five during pathogen-probiotics challenge test

Treatment	Survival %
No inoculated control	43.33±7.64
Pathogen inoculated control (10^4 cfu mL^{-1})	0.00±0.00
Pathogen infection once + probiotic added once	
L. plantarum	28.33±2.89
L. salivarius	25.00±5.00
L. rhamnosus	26.67±5.77
W. confuse	26.67±2.89
W. cibaria	25.00±5.00
Pathogen infection once + probiotic added daily	
L. plantarum	48.33±2.89
L. salivarius	41.67±5.77
L. rhamnosus	46.67±2.89
W. confuse	31.67±2.89
W. cibaria	33.33±2.89
Probiotic added control	
L. plantarum	58.33±7.64
L. salivarius	53.33±7.64
L. rhamnosus	55.00±5.00
W. confuse	45.00±5.00
W. cibaria	46.67±5.77

All treated groups were statistically significant (P<0.05). Pathogen dose=10^4 cfu mL^{-1}; Probiotic dose= 10^6 cfu mL^{-1}.

DISCUSSION

Screening process is an appropriate tool to validate and confirm the potential probiotics using certain models. Application technique is more worth to screen the potentiality of any probiotic when challenged against any infection therapy. During the present study, therapeutic response of LAB isolates against *V. harveyi* was tested. Five LAB isolates were tested *in vitro* against three indicator pathogens previously isolated from the gut of female crab, *P. pelagicus* including *V. harveyi*, *V. parahaemolyticus* and *P. piscicida*. All five showed inhibition against three indicator pathogens (Talpur *et al*., 2012c). Based on *in vitro* testing LAB isolates were validated through small scale *in vivo* model for *P. pelagicus* larviculture. Out of five isolates only *L. plantarum*, *L. salivarius* and *L. rhamnosus* showed potential probiotic characteristics while two, *Weissella confusa* and *W. cibaria* did not show promising response towards survival of larvae during small scale *in vivo* model for validation of probiotics (Talpur *et al*., 2012c). To further validate the probiotics, infection assay was imitated to determine the potential properties of LAB probiotics against indicator pathogen. The present screening bioassay showed that three isolates *L. plantarum*, *L. salivarius* and *L. rhamnosus* out of five tested isolates produced an effective protection upshot in pathogen challenged assay toward *P. pelagicus* larvae. The output of survival rate demonstrated by this type of screening approach has the worthiness. The result of present study is in agreement with previously described results in small scale *in vivo* model (Talpur *et al*., 2012c) where only three LAB were more effective while *Weissela* spp. were less promising. It can be assumed that the protection showed by the LAB isolates was due to a result of their ability to suppress the pathogen by enhancing the nutritional strength or immunity of larvae during the hatchery trials 10L bioassay, when compared with control larvae, which did not receive test bacteria. The observation is based on the high virulence of the *V. harveyi* pathogen, which was previously demonstrated with *P. pelagicus* larvae during pathogenicity challenge assay (Talpur *et al*., 2011b) where elevated concentrations caused severe mortalities . Therefore, further it could be explained that the survivals observed against pathogen challenges in the present study were owing to an inhibitory activity of LAB isolates that have suppressed the *V. harveyi* and healed up the infection. It is to explain that the screening were performed as an assessment of test isolates and all isolates which showed inhibition against indicator pathogens *in vitro* but *Weissella* spp. did not show promising response as potential probiotic during the hatchery trial pathogen probiotic challenge bioassays even though they showed some cure against infection. It is worth to mention that both *Weissella* spp showed the same degree of protection in one day inoculation parallel to other LAB but in rest experiments its result was inconclusive. The effectiveness of three LAB isolates against infection confirmed the recognition of results achieved from hatchery trial probiotic pathogen bioassay experiments validated them as putative probiotic. As a single isolate *L. plantarum* did appear as putative probiotic candidate following by *L. rhamnosus* during the hatchery trials bioassay. Both isolates did show significant protect action against pathogens and significantly increased survival in all trials tested. Moreover, *L. salivarius* did show better probiotic effectiveness compared to *Weissela* spp. From the literature cited, not many researchers have used different approaches in determining pathogens assays of larval fish such as Lovko *et al*. (2003), they tried to assess the pathogenicity of *Pfiesteria piscicida* and *Pfiesteria shumwayae* on tilapia ((*Oreochromis niloticus*, 2.5–5 cm length) where *P. shumwayae* cultures exhibited 80%–100% cumulative mortality in less than 96 h at initial zoospore densities of approximately 1000 cells·mL^{-1} in tissue culture dishes. In other study on molluscs *Crassostrea gigas* larvae by Estes *et al*. (2004) was designated to pathogenicity of *Vibri*o spp. in tissue culture plates rather than pathogen probiotic assay. In a study on eel fertilization (Unuma *et al*., 2004), bacterial monoculture effects upon fish larvae and *Artemia* sp. (Verschuere *et al*., 1999; Makridis *et al*., 2005) and turbot larvae (Hjelm *et al*., 2004). All mentioned studies were mostly performed for pathogenicity of microbes in tissue culture dishes or in small jars. In other development Kesarcodi-Watson *et al*., (2009) challenged pathogens against larvae of green shell muscle, *Perna canaliculus* to screen the putative probiotics. Like previous studies he also used tissue culture dishes for bioassay where achieved a 58% success rate. Furthermore, the commercial probiotic Sanolife ®MIC, INVE improved survival of *P. monodon* postlarvae after infection with *V. harveyi* added in the water (Das *et al*., 2010). Recently Rahman *et al*. (2011) in a co-culture experiment bioassay determined that the isolated probiotic bacteria were shown to outcompete the growth of pathogenic *V. harveyi* due to their antagonistic properties. However, the model developed for screening of probiotic during present study was unique in its design for *in vivo* trials assay. During the study it was observed that bioassay achieved > 48% success rate of larvae with continuous inoculation of probiotic against infected larvae and in probiotic inoculated control >58%.

However, the main purpose of the hatchery trial bioassay was to indicate whether potential probiotics identified using small scale *in vivo* bioassays (Talpur *et al*., 2012c) were effective under normal rearing conditions. The results were amazing, thus confirming the usefulness of the bioassay. Furthermore, potential isolates were recommended for further hatchery trials *in vivo* full-scale, replicated experiments.

Review of the literature suggests that this study is the first report to screen the probiotics for *P. pelagicus* larvae using probiotic pathogen bioassay under hatchery conditions and the results has demonstrated the benefits of tested probiotics including the survival of test animals in the screening stages.

The hatchery trial probiotic pathogen assay model exercised during the present study evidently defines highly pathogenic *V. harveyi* bacteria infection can be controlled via therapeutic applications of lactic acid bacteria probiotics. However, such assay was reliable for screening of effectiveness of probiotic strains/isolates against infections of pathogens prior to large scale *in vivo* trials of probiotics subjected to larviculture.

Therefore, it was important to mention that during the pathogenic probiotic assay the larvae were no longer swimming actively first 24 hour. This owed to stress of pathogen. Larvae were observed more actively swimming in exclusive probiotic control compared to other treated groups which was due to benefit effect of probiotics.

For putative probiotics validation, it important to explicate that the screening trials mainly hatchery trials bioassay are liable to show great promise in identifying potential probiotics for aquaculture animals. In particular it should also be considered that, when screening for effective probiotics, it is preeminent to isolate and use bacteria associated with the same host, because the isolates naturally from the same environment and able to require less manipulation to ensure that their probiotic activity. In the present study, this was revealed through the innovation of probiotics, which were effective in hatchery trials challenge assays significantly increased the survival of larvae by suppressing of pathogen infection. This type of bioassays further confirm the potential capability of probiotics under undesirable conditions and prove their ability to combat with the pathogen on one hand and enhance the survival of animal on other hand. This is the first report where an applied hatchery technique was used to recognise the conformity of probiotics against fatal pathogen.

Conclusions: The present study concludes that the probiotics can allow larval survival during the unfavourable conditions of an induced pathogen challenge; the potential benefits of LAB to larval rearing are motivating. This is the first report, probiotic bacteria effective against pathogens and shows the benefits of a bioassay procedure that includes *P. pelagicus* larvae in the first stage of probiotic screening. Moreover, the assay used under the present study can also be used as model for screening of probiotics for other fish larvae as well.

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