Evaluation of *Enterococcus hirae* LAB3 as potential probiont against *Vibrio harveyi* in *Artemia* nauplii and Asian seabass larvae (*Lates calcarifer*) cultures

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Abstract

Aim: To evaluate the ability of *Enterococcus hirae* strain LAB3 to inhibit the growth of *V. harveyi* in *in-vitro* co-culture assay and confer protection towards *Artemia* nauplii and seabass larvae against *V. harveyi* in *in-vivo* assay.

Methodology: *Enterococcus hirae* was co-cultured with *V. harveyi* for 96 hrs and the samples were taken every 6 hr interval to determine the growth of the pathogen in *in-vitro* assay. In the *in-vivo* assay, *E. hirae* at different concentrations were introduced to the *Artemia* nauplii and seabass larvae cultures. After 24-hr incubation period, *V. harveyi* at concentration 10^6 CFU ml⁻¹ were added into the respective experimental tanks. Mortality was observed and recorded daily, until 50% mortality was observed in negative control group which challenged with *V. harveyi* only.

Results: *E. hirae* was able to reduce the numbers of *V. harveyi* after 12 hr of co-culture incubation. In addition, *E. hirae* was administered into the *Artemia* nauplii and conferred protection against *V. harveyi* with the best survival rate at concentration of 10^6 CFU ml⁻¹ ($70 \pm 3.1\%$). The untreated group seabass larvae challenged with *V. harveyi* presented low survival of ($16.7 \pm 3.3\%$), while fish treated with probiotic *E. hirae* at 10^6 CFU ml⁻¹ showed significantly increased survival rates ($68.3 \pm 0.9\%$) after challenged. The survival of healthy unchallenged fish treated with probiotic was not significantly different with the control group. The pathogen loaded also reduced in groups treated with *E. hirae* respectively.

Interpretation: *E. hirae* strain LAB3, a potential probiont was able to reduce the number of Vibrios and conferred protection to *Artemia* and seabass larvae.

Key words: *Artemia* nauplii, *Enterococcus hirae*, Probiotic, Seabass larvae, *V. harveyi*

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Introduction

Intensification of fish farming industry has increased the incidence of diseases that has become major constraint to the system resulting in economic loss (Stentiford *et al.*, 2012; Joffre *et al.*, 2018). In euryhaline fish farming, the potential pathogenic bacteria belongs to *Vibrio* spp. *Vibrios* belong to *Harveyi* clade and are considered as important pathogens for a large number of marine animals in the aquaculture industry (Vanmaele *et al.*, 2015). *Vibrio harveyi* express the virulence factor known as gene product enabling them to infect and damage the host (Defoirdt *et al.*, 2014). Meanwhile, In Malaysia, outbreak of vibriosis reported in year 2008 in open cage in Sabah mainly caused by *V. harveyi* infection among the fish stocked lead to huge losses (Ransangan *et al.*, 2012). In addition, *V. harveyi* is known as pathogenic organism associated with luminous vibriosis that causes mortality in penaeid shrimp farm (Wang *et al.*, 2015) and is highly infectious at early stages of fish (Nandi and Ray, 2017). Application of antimicrobials as well as chemicals have been well documented to portray beneficial effects to infected animals. However, it might be hazardous to the environment due to microbial resistance developed by pathogens (Romero *et al.*, 2012). Hence, holistic approaches that include pathogen, host, and environment can be more effective for long term usage (Defoirdt *et al.*, 2011). It refers to the strategies directed toward pathogenic bacteria without using antibiotics as a preventive method. For past few years, using probiotics have been the most promising preventive method in controlling pathogens in fish culture (Kesarcodi-Watson *et al.*, 2008). Generally, probiotics known as beneficial microorganisms work as a live microbial feed supplement when administered in appropriate amount are beneficial to the host (Fuller, 1989; FAO, 2001; Lara Flores and Aguirre-Guzman, 2009). The diversity of probiotics in the host gut is correlated with their habitats and closely interacts with these microbes. For instance, probiotics of *Bacillus*, *Lactobacillus* and *Enterococcus* have the potential to influence the immune system of the host (Sayes *et al.*, 2018). However, administration of probiotic in live fish feed such as *Artemia* was reported to be an interesting but rarely studied. Due to the fact that live feed plays an important role in dietary systems, especially at larval stages of fish and shellfish (Haq *et al.*, 2011). Various live feed have been used commercially in aquaculture. *Artemia* is widely used in marine finfish and crustacean hatcheries due to high nutrient content and is used as vector for probiotic as an excellent preliminary model organism (Soltanian *et al.*, 2007). *Enterococcus hirae* strain LAB3 isolated from the intestine of seabass and have been undergoing several *in-vitro* assay to assess their potential as probiotics. In a study, LAB3 showed positive characteristics of probiotic as it was able to grow at pH2 to pH10 within 3hr incubation period as well as as able to secrete some extracellular enzymes and produce biofilm up to 30hr incubation period. Exhibit resistance to low pH is a crucial key to be appointed as probiotic (Noriega *et al.*, 2004; Fathiah Masduki, 2018). Moreover, probiotics that secrete and

synthesize different extracellular enzymes are able to improve feed utilization for better growth rate (Balzacar *et al.*, 2006). *In-vitro* evaluation prior to *in-vivo* testing is a good indicator for probiotic effectiveness in controlling disease in host (Balzacar *et al.*, 2006; Aly *et al.*, 2008). Thus, present study aimed to evaluate the protective effect of potential probiotic *E. hirae* strain LAB3 towards *Artemia* nauplii and Asian seabass larvae after challenged with *V. harveyi*.

Materials and Methods

Bacterial strains and culture condition: *E. hirae* strain LAB3 (Accession number: MK757970) isolated previously from the intestine of healthy Asian seabass was used in this study. Meanwhile, *V. harveyi* was isolated from diseased Asian seabass. Probiotic was grown in Man Rogosa and Sharp (MRS, Himedia, India) broth supplemented with 1.5% NaCl while pathogen was cultured in Trypticase Soy Broth (TSB, Difco, USA) supplemented with 1.5% NaCl at 30 °C for 24 hr.

Growth inhibition in co-culture assay: Candidate probiotic strain was tested for antagonistic effect against *Vibrio harveyi* using broth co-culture assays. In this assay, the initial density of *V. harveyi* was calculated to be approximately 10^5 CFU ml⁻¹, while that of probiotic was 10^4 , 10^6 and 10^8 CFU ml⁻¹ respectively. Each pathogen and probiotic inoculum were co-cultured in 10 ml TSB supplemented with 1.5% NaCl and incubated at 30 °C, respectively. Sample was taken at 0, 6, 12, 24, 48 and 96 hr of incubation. A 100 µl of culture from each inoculum was plated on Thiosulfate Citrate Bile Salt Sucrose (TCBS, Difco, USA) agar in order to quantify the number of cells. All plates were incubated overnight at 30 °C. The number of colonies for each inoculum was recorded as CFU ml⁻¹ and counted by the formula:

CFU ml⁻¹ = (No. of colonies x dilution factor) / Volume of culture plate (ml)

Challenge of *Artemia nauplii* enriched with probiotic against *V. harveyi* : Experiment was performed on *Artemia salina* cyst (Bio-Marine, USA). *Artemia* cyst were hatched for 24 hr, at 28-30 °C in 35 ppt of filtered sterile seawater with continuous aeration and light intensity. After 24 hr, 20 nauplii were divided into sterile 50 ml falcon tube filled with 30 ml of filtered sterile seawater (Table 1). *Artemia* were first pre-incubated for 24 hr with probiotic in three different concentrations (10^4 , 10^6 and 10^8 CFU ml⁻¹). After 24 hr incubation, *V. harveyi* was introduced at 10^5 CFU ml⁻¹. All the tubes were kept on orbital shaker for continuous aeration purpose. Observation was made until 50% mortality occurred in the group that contained *Artemia* and pathogen only. Survival of *Artemia* was recorded and the data were analyzed.

Challenge of fish larvae enriched with probiotic against *V. harveyi* : Twenty three-day-old asian seabass larvae (approximately weighting 0.34 g, 2.5-2.9 cm long), was procured

Table 1: Treatments in *Artemia* challenge assay

Treatments	Descriptions
T1	<i>Artemia</i> only
T2	<i>Enterococcus</i> LAB3 10^4 CFU mL ⁻¹
T3	<i>Enterococcus</i> LAB3 10^6 CFU mL ⁻¹
T4	<i>Enterococcus</i> LAB3 10^8 CFU mL ⁻¹
T5	<i>Vibrio harveyi</i> 10^5 CFU mL ⁻¹
T6	<i>Enterococcus</i> LAB3 10^4 CFU mL ⁻¹ + <i>Vibrio harveyi</i> 10^5 CFU mL ⁻¹
T7	<i>Enterococcus</i> LAB3 10^6 CFU mL ⁻¹ + <i>Vibrio harveyi</i> 10^5 CFU mL ⁻¹
T8	<i>Enterococcus</i> LAB3 10^8 CFU mL ⁻¹ + <i>Vibrio harveyi</i> 10^5 CFU mL ⁻¹

Table 2 : Treatments of seabass larvae challenge test

Treatments	Descriptions
T1	Control
T2	<i>Vibrio harveyi</i> 10^5 CFU mL ⁻¹
T3	<i>Enterococcus hirae</i> LAB3 10^4 CFU mL ⁻¹
T4	<i>Enterococcus hirae</i> LAB3 10^6 CFU mL ⁻¹
T5	<i>Enterococcus hirae</i> LAB3 10^8 CFU mL ⁻¹
T6	<i>Enterococcus hirae</i> LAB3 10^4 + <i>Vibrio harveyi</i> 10^5 CFU mL ⁻¹
T7	<i>Enterococcus hirae</i> LAB3 10^6 + <i>Vibrio harveyi</i> 10^5 CFU mL ⁻¹
T8	<i>Enterococcus hirae</i> LAB3 10^8 + <i>Vibrio harveyi</i> 10^5 CFU mL ⁻¹

from by FRI Gelang Patah, Johor, Malaysia and acclimatized for 2 days prior to use. All experiments were conducted in triplicates with 15 larvae per experimental tank (Table 2). A negative control group consisted larvae that were neither treated with probiotics nor challenged with pathogens. The positive mortality control was the group that was challenged with pathogens but was not treated with probiont. *E. hirae* was introduced on the first day using immersion method into the culture water at 10^4 and 10^6 CFU mL⁻¹, respectively. After 24-hrs, the larvae were challenged with *V. harveyi* at a concentration of 10^5 CFU mL⁻¹. Observations were made until 50% mortality was observed in positive mortality control group. Survival and mortality were observed and recorded daily.

Reduction on pathogen loads: Once the experiment ended, the number of pathogen loads were determined in the host and culture water. As for *Artemia*, a sterile 100µm mesh was used. Host (*Artemia* and seabass larvae) were mashed and homogenized using sterilized stick for *Artemia* and also mortar and pestle for seabass larvae. Serial dilution was made up to 10^8 to quantify the number of Vibrios. To determine the pathogen loads in host and culture water, 100µl of each sample was plated on TCBS agar with triplicate each. The plates were incubated at 30 °C for 24 hr and the number of colonies were calculated as CFU mL⁻¹.

Statistical analyses : Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey test in order to test for significant differences between the groups. Statistical significance was tested at probability level of 0.05.

Results and Discussion

The antagonistic effects of potential probiotic strain was tested for the growth of *V. harveyi* in broth co-culture assays. The results demonstrated that *E. hirae* managed to reduce the growth of *V. harveyi* 12 to 48 hrs. 10^4 and 10^6 CFU mL⁻¹ probiont inhibited the growth of *V. harveyi* significantly. Whereas in control groups, the growth of pathogens increased gradually with time until 48 hr incubation. All the probionts treatment significantly decreased ($p < 0.05$) the number of pathogens.

In order to evaluate any possible protection offered to *Artemia* by the probionts, the nauplii were treated with probiont (Fig. 2). The results demonstrated that the survival rate of *Artemia* inoculated with 10^6 CFU mL⁻¹ probiont was higher (70±3%) compared to *Artemia* treated with pathogen alone (13.3±2%). Challenged groups of *Artemia* with 10^4 and 10^6 CFU mL⁻¹ probiont showed increase in the survival rate (50-70%) compared to *Artemia* treated with *V. harveyi* only.

Fig. 3 indicated that, larvae treated with 10^6 CFU mL⁻¹ *E. hirae* LAB3 (T3) showed the highest survival rate (66.7%) with no significant difference ($p > 0.05$) with the control group (70%). Larvae treated with *E. hirae* LAB3 at 10^4 CFU mL⁻¹ and 10^8 CFU mL⁻¹ showed survival rate of 46.7% and 18.3% respectively, which significantly differed ($p < 0.05$) with the control group. Among the treatments, 10^8 CFU mL⁻¹ concentration was found harmful to the host. In the challenge assay (Fig. 3), survival rate of negative control group was 16.7%. Meanwhile, survival of seabass larvae treated with probiont and challenged with *V. harveyi* was 36.7% in

Fig. 1 : Growth pattern of *Vibrio harveyi* at 10^5 CFUml⁻¹ incubated with different concentrations of *Enterococcus hirae* against time. T1 (*Vibrio harveyi* 10^5 CFUml⁻¹), T2 (*Vibrio harveyi* 10^5 CFUml⁻¹ + *Enterococcus hirae* 10^4 CFUml⁻¹), T3 (*Vibrio harveyi* 10^5 CFUml⁻¹ + *Enterococcus hirae* 10^6 CFUml⁻¹) and T4 (*Vibrio harveyi* 10^5 CFUml⁻¹ + *Enterococcus hirae* 10^8 CFUml⁻¹). Different alphabet indicates significant differences among treatments ($p < 0.05$)

group T6 (10^4 CFU ml⁻¹) and 68.3% in T7 (10^6 CFU ml⁻¹), respectively.

Enterococcus hirae LAB3 was able to reduce the pathogen load in both culture water and *Artemia*. The number of *Vibrios* in *Artemia* reduced significantly after treatment with the probionts as compared with the control group (T2). The highest reduction was observed at 10^6 CFU ml⁻¹ (5.82 CFU ml⁻¹). In culture water, group T7 treated with 10^6 CFU ml⁻¹ *E. hirae* also showed the highest reduction (1.69 ± 0.86 CFU ml⁻¹) as compared with other treatments (Table 3).

Fig. 2 : Survival of *Artemia* after pre-incubated with probiont *Enterococcus hirae*. Error bars indicate standard error. Different alphabet indicates significant difference among treatments ($p < 0.05$). T1 (Control), T2 (*Vibrio harveyi* 10^5 CFUml⁻¹), T3 (*Enterococcus hirae* LAB3 10^4 CFUml⁻¹ + *Vibrio harveyi* 10^5 CFUml⁻¹), T4 (*Enterococcus hirae* LAB3 10^6 CFUml⁻¹ + *Vibrio harveyi* 10^5 CFUml⁻¹), T5 (*Enterococcus hirae* LAB3 10^8 CFUml⁻¹ + *Vibrio harveyi* 10^5 CFUml⁻¹).

Similar results were obtained for seabass larvae where significant reduction of vibrios were observed in culture water as well as in larvae after treated with *E. hirae* at 10^6 CFU ml⁻¹ compared to the untreated larvae (Table 4).

Overuse of antibiotics in treating fish infections in aquaculture has led to search a novel alternative method such as usage of probiotics (Gomez-Gil et al., 2002). Use of probiotics as growth promoter and immune modulator system in fish has been investigated (Mohapatra et al., 2012). In recent years, manipulation of bacterial load in the live feed may contribute as a valuable mechanism for increasing the larval growth and survival rates (Villamil et al., 2003). Since microbiological pollution in the environment due to the administration of probiotics in culture water, cooperation between live feed and probiotics can possibly become an alternative approach in aquaculture. Hence, the present study demonstrates that *E. hirae* LAB3 can serve as a probiont to inhibit the growth of *V. harveyi* in *in-vitro* co culture assay and confer protection to the *Artemia* and seabass larvae after challenged with *V. harveyi* *in-vivo* studies. Co-culture assay was done to demonstrate the growth pattern of *V. harveyi* after incubating with the probiont *E. hirae* along with inoculation period intervals. The results showed that, probiont *E. hirae* LAB3 managed to reduce the growth of *V. harveyi* from 24 to 96 hr respectively. All the concentrations showed significant results compared to the control group. Probiont *E. hirae* was able to inhibit the growth of *V. harveyi* significantly within 48 hr of incubation.

Probiotics can be delivered into cultured organism in two ways. It can be blended with feed and administrated orally or through proliferation in water medium and exclude pathogenic

Fig. 3 : Survival of seabass larvae after pre-treated with probiont *Enterococcus hirae* LAB3 and challenged with *Vibrio harveyi*. Error bars indicate standard error. Different alphabet indicates significant differences among treatments ($p < 0.05$). T1 (Control), T2 (*Vibrio harveyi* 10^5 CFUml⁻¹), T6 (*Enterococcus hirae* LAB3 10^4 CFUml⁻¹ + *Vibrio harveyi* 10^5 CFUml⁻¹), T7 (*Enterococcus hirae* LAB3 10^6 CFUml⁻¹ + *Vibrio harveyi* 10^5 CFUml⁻¹).

Table 3 : Vibrios count in culture water and *Artemia* after pre-incubated at different concentrations of *Enterococcus hirae* LAB3 (10^4 , 10^5 and 10^6 CFUml⁻¹) and challenged with 10^5 CFUml⁻¹ of *Vibrio harveyi*. Different alphabet indicates significant differences among treatments ($p < 0.05$)

Treatments	Descriptions	Log 10 CFUml ⁻¹	
		Culture water	Artemia
T2	<i>Vibrio harveyi</i> 10^5 CFUml ⁻¹	5.65±0.64 ^a	6.62±0.11 ^b
T6	<i>Enterococcus hirae</i> LAB3 10^4 + <i>Vibrio harveyi</i> 10^5 CFUml ⁻¹	3.67±0.12 ^b	6.05±0.06 ^a
T7	<i>Enterococcus hirae</i> LAB3 10^6 + <i>Vibrio harveyi</i> 10^5 CFUml ⁻¹	1.69±0.86 ^c	5.82±0.05 ^a
T8	<i>Enterococcus hirae</i> LAB3 10^8 + <i>Vibrio harveyi</i> 10^5 CFUml ⁻¹	3.80±0.11 ^b	6.11±0.14 ^a

Table 4 : Vibrios count in culture water and larvae after pre-incubated at different concentrations of *Enterococcus hirae* LAB3 (10^4 , 10^5 CFUml⁻¹) and challenged with 10^5 CFUml⁻¹ of *Vibrio harveyi*. Different alphabet indicates significant differences among treatments ($p < 0.05$)

Treatments	Descriptions	Log 10 CFUml ⁻¹	
		Culture water	Larvae
T2	<i>Vibrio harveyi</i> 10^5 CFUml ⁻¹	5.65±0.16 ^a	6.98±0.16 ^a
T6	<i>Enterococcus hirae</i> LAB3 10^4 + <i>Vibrio harveyi</i> 10^5 CFUml ⁻¹	3.67±0.21 ^b	5.80±0.21 ^b
T7	<i>Enterococcus hirae</i> LAB3 10^6 + <i>Vibrio harveyi</i> 10^5 CFUml ⁻¹	1.69±0.16 ^c	4.47±0.16 ^c

bacteria by consuming all available nutrients (Azrin et al., 2017). *Artemia* was used in preliminary *in-vivo* challenged test in this study. Additionally, *Artemia* is considered a perfect candidate for preliminary interaction study between host and microbes (Lamari et al., 2014). Despite being a model host for challenge assay, it is commonly used as a medium for introducing probiotics to the cultured species (Seenivasan et al., 2012). *Macrobrachium rosenbergii* postlarvae fed with *Artemia franciscana* enriched with probiotic *Enterococcus durans* proves to have fewer pathogen load in their gut microflora compared to postlarvae fed with un-enriched *Artemia* (Bhaheerathan et al., 2020).

The findings of this study demonstrate that incubation of probiont *E. hirae* showed the ability to increase the survival of *Artemia* challenging with *V. harveyi*. These findings also corroborates with the finding of Lamari et al. (2014) where *Artemia* treated with probiotic *Lactobacillus casei* showed higher survival and faster growth rate when challenged with *V. alginolyticus*. Moreover, the administration of probiotics by live food was able to decrease and manipulate pathogen activity in some aquatic species (Suzer et al., 2008).

In this study, seabass larvae were used as the final host. There only a few reports emphasizing the use of *E. hirae* as probiotics in aquaculture and the present study proved that *E. hirae* administered at 10^6 CFU ml⁻¹ offered the best protection to larvae after treating with *V. harveyi*. The concentration of probiont used can influence the growth performance as well as the number of pathogen load in the host. Based on the results, 10^6 CFU ml⁻¹ appeared to be the optimal concentration that offered protection to both *Artemia* and seabass larvae against pathogenic *V. harveyi* without showing any toxicity effects to the host.

Artemia and seabass larvae incubated with probiont *E. hirae* LAB3 had lower pathogen loads after *V. harveyi* treatment. The results obtained showed significant decrease in the member of *Vibrios* in the experimental groups as compared to the control. The reduction could be an antagonism mechanism offered by the probiont for colonization activity and competition between probiotic and pathogen (Verschuere et al., 2000). Salinas et al. (2005, 2006) stated that administration of probiotic in culture water dramatically decreased the pathogenic bacteria activity in some teleost such as *Sparus aurata*.

Research done by Tachibana et al. (2020) also report on the use of probiotic *Enterococcus faecium* on Nile Tilapia which was able to give protection against *Aeromonas hydrophila* infection. Similar findings were observed in mud crab (*Scylla paramamosain*) treated with *Enterococcus faecalis* which exhibited higher survival after challenged with *Vibrio parahaemolyticus* (Yang et al., 2019).

Our findings revealed that the probiont concentration should be higher than or equal to that of the pathogen for the probiont to be effective. It is postulated that the mechanism of competitive exclusion occurred between the potential isolates and *Vibrio* spp.. They might be competing for the nutrients, spaces, and release water-soluble substances in order to inhibit the growth of other bacteria (Balcazar et al., 2006). Beneficial bacteria are widely used as an alternative method to control pathogens in aquaculture. However, more information is needed to understand the mechanism of action, which leads to good selection of probiotics.

In conclusion, *E. hirae* strain LAB3 demonstrated the ability to act as a probiotic which can inhibit the growth of

pathogenic *V. harveyi* in *in-vitro* assay. The potential probionts successfully reduced the pathogens counts and enhanced the survival of *Artemia* and seabass larvae in *in-vivo* assay. This study revealed positive correlation between *in-vitro* and *in-vivo* assay. Therefore, this strain is a potential probiotic which can be applied in seabass cultures. In order to confirm its potential, further studies should be carried out to strengthen the results of this research.

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