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Evaluation of *Enterobacter* sp. Strain G87 as Potential Probiont against *Vibrio harveyi* Infection in *Artemia* Nauplii and Asian Seabass (*Lates calcarifer*) Larvae

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ABSTRACT

Probiotic has gained many interests as an alternative method in preventing and treating diseases in aquaculture. The benefits include improving feed value, inhibition of pathogenic microorganisms, anti-mutagenic and anti-carcinogenic activity, growth promoting factors, and increase host immune response. This research was carried out in order to evaluate the potential of probiont *Enterobacter* sp. G87 in conferring protection to *Artemia* and seabass larvae against *Vibrio harveyi* infection. In preliminary *in vivo* test, *Artemia* nauplii was treated with *Enterobacter* sp. G87 at three different concentrations 10^4, 10^6 and 10^8 CFU mL^{-1} and challenged with *V. harveyi* at 10^5 CFU mL^{-1}. After challenged, significant increased survival was found in *Artemia* (78±2%) treated with 10^6 CFU mL^{-1} of *Enterobacter* sp. G87 compared with challenged group with no probiont added (48±2%). From the results, two concentrations of probiont (10^6 and 10^8 CFU mL^{-1}) were selected to be used in seabass larvae *in vivo* challenge assay. After challenged with *V. harveyi* at 10^5 CFU mL^{-1} highest survival was found in seabass larvae treated with 10^6 CFU mL^{-1} of *Enterobacter* sp. G87 (95±3%). Additionally, *Enterobacter* sp. G87 was also able to reduce *Vibrio* counts both in *Artemia* and seabass larvae culture. This study showed that probiont *Enterobacter* sp. G87 was able to protect *Artemia* nauplii and seabass larvae from *Vibrio harveyi* infection and has a potential to be further studied in a larger scale.

Keywords: *Artemia*, *Enterobacter*, larvae, probiotics, seabass, *Vibrio harveyi*

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INTRODUCTION

World is predicted to undergo food crisis in 2050 if the alternative to captured fisheries could not be found (Béné et al., 2015). Fish landing have declined drastically for the past 10 years due to overfishing and environmental issue (Food and Agriculture Organization [FAO], 2015). Aquaculture is the best option to fulfill the protein requirement of the country which projected to grow more than 100% production within five years ahead (FAO, 2016).

However, major disease outbreaks have been reported within the aquaculture industry around the world due to the increased in stocking density, over-crowding and poor husbandry management along with the rapid growth of aquaculture (Tan et al., 2016). The annual economic losses associated with diseases worldwide are estimated to be in excess of US$9 billion per year (Ruwandeepika Hettipala Arachchige, 2010). One of the common disease outbreaks is bacterial infection known as vibriosis which is commonly caused by *Vibrio harveyi* (Talpur, 2014). *Vibrio harveyi* is one of the *Vibrio* sp. which is an important aquaculture pathogen that can infect large number of marine animals (Li et al., 2011).

In Malaysia, Asian Seabass is one of the top demand species from the locals; probably due to its unique taste and reasonable price. In 2017, production seabass in Malaysia was recorded nearly at 30,000 metric tons (Department of Fisheries [DOF], 2017). The production of seabass increased through the years due to high demand from consumers which make seabass culture to be a profitable industry. The annual production of worldwide for this type of fish increased from 93,422 metric tons in 2012 to 101,231 metric tons in 2013 (FAO, 2016). Production of seabass is greatly affected by the occurrence of vibriosis, which causes heavy mortality of more than 50% (Ransangan & Mustafa, 2009).

The use of antibiotic as preventive measures are limited in most country including Malaysia because of its negative effect to environment, human health and causing antimicrobial ressistance. Alternatively, the use of probiotic is one of the best option to control diseases in aquatic environment (Harikrishnan et al., 2011). Among the common microorganism used as probiotics are *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bacillus* sp., *Lactococcus* sp. and also yeast *Saccharomyces cerevisiae* (Salamoura et al., 2014).

As for now, probiotic usage in Malaysia has been a popular option however, there are still lack of local probiotic products. This mainly because of limitation of knowledges, awareness, resources, research and development on local probiotics strain. Among the earliest study done on application of probiotics in aquaculture in Malaysia was by Al-Dohail et al. (2009) who reported the beneficial effects of *L. acidophilus* on growth performance and immune response of African catfish *Clarias gariepinus*. *Lactobacillus plantarum* was also proven to be able to reduce *Vibrio* loads in culture water of *Portunus pelagicus* larvae as well as improving the survival rate of the larvae (Talpur et al., 2013)

Live feeds are crucial at early stages of larvae. *Artemia* is one of the live feeds that are commomly used for marine larvae. One of the pathway to intorduce probiotics to the cultured hosts are by using *Artemia* as the medium or transporter. Enrichment of *Artemia* using probiotics able to increase the nutrients contents as well as provide protection towards any pathogens since feed could be a possible carrier for diseases as well (Hai, 2015).

Potential probiont *Enterobacter* sp. G87 was isolated from gut of healthy adult Asian seabass. In earlier studies, our lab had confirmed the antagonistic properties of this strain against *V. harveyi* strain NBRC 15634 in *in vitro* assay. Thus, this study was undertaken to determine the ability of potential probiont *Enterobacter* sp. G87 in protecting *Artemia* which is one of an important live feed in larviculture as well as the most preferable model for a preliminary *in vivo* test prior testing to the real host (Frans et al., 2013). The study also includes on the effect of *Enterobacter* sp. G87 towards seabass larvae after being challenged with *V. harveyi*.

MATERIALS AND METHODS

Artemia Nauplii and Seabass Larvae

The *Artemia* (Bio-Marine, USA) cyst was obtained from the laboratory of Fish Health Laboratory, Faculty of Agriculture, UPM. Meanwhile, larvae of seabass (*Lates calcarifer*) at size average of 1 inch were obtained from fish farm in Banting, Selangor. The fish were acclimatized for 24 hr in separate tanks prior use for experiments. Any presence of pathogenic vibrios were tested by taking few samples of fish and streak on Thiosulphate Citrate-Bile Salt (TCBS, Difco Company, USA) agar.

Bacterial Cultures and Growth Condition

The potential probiont *Enterobacter* sp. G87 and pathogenic *V. harveyi* strain NBRC 15634 were obtained from Fish Health Laboratory, Department of Aquaculture, UPM. *Enterobacter* sp. G87 was previously isolated from the gut of healthy adult seabass *Lates calcarifer*. Tryptic soy agar (Difco Company, USA) + 1.5% NaCl was used to culture *Enterobacter* sp. G87 and TCBS media was used for *V. harveyi*. Both isolates were incubated at 30ºC for 24 hr prior to use. Meanwhile for broth cultures, the isolates were inoculated in TSB (Difco Company, USA) + 1.5% NaCl and incubated 24 hr in the innova®42 incubator shaker (Eppendorf, Germany) series at 120 rpm, 30ºC prior used in challenged assay. Concentrations were adjusted accordingly using spectrophotometer and McFarland Standard.

Preliminary Challenged Assay using *Artemia*

Artemia cyst (Bio-Marine brand) was cultured for 24 hr using sterile seawater (SSW) at 28-30°C with continuous aeration and light intensity. After 24 hr of incubation, 20 hatched *Artemia* nauplii were divided into falcon tube containing 30 ml SSW. All treatments were run in triplicate. *Artemia* nauplii were immersed for 24 hr with *Enterobacter* sp. G87 at concentration of 10^8, 10^6 and 10^4 CFU mL^{-1} which were

selected based on *in vitro* results in previous studies. *Vibrio harveyi* at concentration of 10^5 CFU mL^{-1} was added into the respective falcon tubes after 24 hr. Control was run with no bacteria added. The tubes were placed on the orbital shaker, 50rpm at room temperature. *Artemia* was fed with yeast once daily. Experiment was stopped when the group that was challenged with *V. harveyi* only reached 50% mortality. The mortality and water quality parameters (salinity, pH and dissolved oxygen) were recorded everyday. Experiment on each of the group was run in triplicates and water quality was checked daily.

Challenge Assay on Seabass Larvae

The larvae were acclimatized for 24 hr prior to use. Next, 20 larvae were divided into 5L aquarium contained 2L SSW with continuous aeration. In this assay, two concentrations of probiont *Enterobacter* sp. G87 were used (10^8 and 10^6 CFU mL^{-1}) based on the findings from preliminary *in vivo* assay using *Artemia* and challenged with 10^8 CFU mL^{-1} of *V. harveyi*. Larvae were pre-incubated with probiont *Enterobacter* sp. G87 on the first day and challenged with *V. harveyi* on the next day (after 24 hr). No bacteria either probiont or pathogen were added in the control. Mortality and water quality were checked and recorded every day. Experiment was stopped until group challenged with *V. harveyi* with no probiont reached 50% mortality. Each of the treatment group was run in triplicate according to Table 1 and Table 2 and water quality was checked daily.

Table 1
Treatments for preliminary in vivo *challenged using* Artemia *nauplii*

Label	Treatment
C	Positive Control (with no addition of bacteria)
T1	*Vibrio harveyi* 10^5 CFU mL^{-1} (Negative Control)
T2	*Enterobacter* G87 10^8 CFU mL^{-1}
T3	*Enterobacter* G87 10^6 CFU mL^{-1}
T4	*Enterobacter* G87 10^4 CFU mL^{-1}
T5	*Enterobacter* G87 10^8 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}
T6	*Enterobacter* G87 10^6 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}
T7	*Enterobacter* G87 10^4 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}

Table 2
Treatments for seabass larvae in vivo *challenge assay*

Label	Treatment
C	Positive Control (with no addition of bacteria)
VH	*Vibrio harveyi* 10^8 CFU mL^{-1} (Negative Control)
CT1	*Enterobacter* G87 10^6 CFU mL^{-1}
CT2	*Enterobacter* G87 10^8 CFU mL^{-1}
T3	*Enterobacter* G87 10^6 CFU mL^{-1} + *Vibrio harveyi* 10^8 CFU mL^{-1}
T4	*Enterobacter* G87 10^8 CFU mL^{-1} + *Vibrio harveyi* 10^8 CFU mL^{-1}.

Vibrio Counts in *Artemia* and Seabass Larvae

Five *Artemia* nauplii from each tank were separated from culture water using sieve. Next, *Artemia* was suspended in 1 mL SSW and meshed using sterile mortar and pestle. Serial dilutions were made up to 10^8 CFU mL^{-}1. In order to determine *Vibrio* loads in larvae, 10 µl of each diluted sample was plated on TCBS agar. The plates were incubated at room temperature for 24 hr. The colonies of *Vibrio* formed were counted using ROCKER galaxy 230 colony counters and calculated as CFU mL^{-1} using this formula:

Concentration of bacteria =
(Number of CFU)/(Volume plated × Total dilution)

In order to determine the *Vibrio* counts in seabass larvae the same method as *Artemia* was applied.

Statistical Analysis

All the data collected were analyzed using One-way Analysis of Variance (ANOVA). Multiple comparison tests (Tukey test) were used (IBM SPSS Statistic 20 software) in order to determine the significance among groups. Results were expressed as the mean ± standard error and the differences were considered significant at $p<0.05$.

RESULTS

Preliminary Challenged Assay using *Artemia*

After four days of observation, the survival rate of *Artemia* treated with *Enterobacter* sp. G87 (T2, T3 and T4) were between 77-82% for all concentrations. The results demonstrated that *Enterobacter* sp. G87 was not harmful to the *Artemia* (Figure 1). The highest survival was shown at concentration of 10^8 CFU mL^{-1} (T2, 82±2%). In challenged group, after *V. harveyi* was added to the

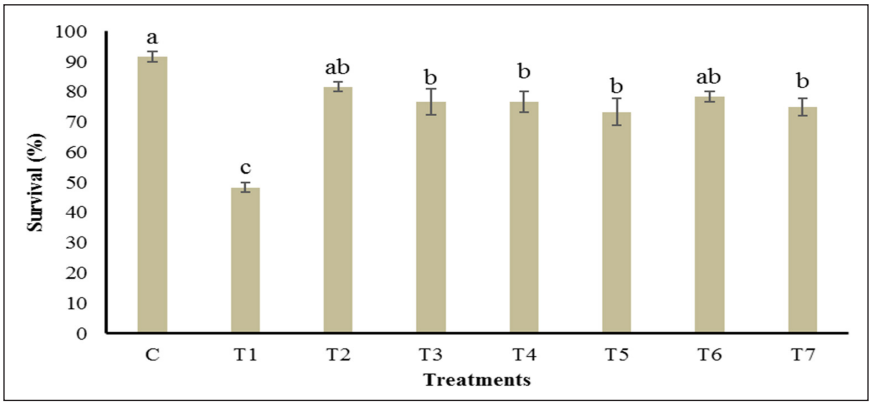

Figure 1. The survival rate of *Artemia* nauplii after pre-incubated with different concentrations of *Enterobacter* sp. G87 (10^8, 10^6, 10^4 CFU mL^{-1}) and challenged with 10^5 CFU mL^{-1} of *Vibrio harveyi*. Error bars indicate standard error. Mean with different alphabet letters indicate significant difference ($p<0.05$). *Note*. C: Control; T1: *Vibrio harveyi* 10^5 CFU mL^{-1}; T2: *Enterobacter* sp. G87 10^8 CFU mL^{-1}; T3: *Enterobacter* sp. G87 10^6 CFU mL^{-1}; T4: *Enterobacter* sp. G87 10^4 CFU mL^{-1}; T5: *Enterobacter* sp. G87 10^8 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}; T6: *Enterobacter* sp. G87 10^6 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}; T7: *Enterobacter* sp. G87 10^4 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}

respective treatments, the survival rate for group T5, T6, T7 was between 73-78% which was significantly higher compared to group with *V. harveyi* only (T1, 48±2 %). *Artemia* treated with 10^6 CFU mL^{-1} (T6) showed the highest survival (78±2%) after being challenged with *V. harveyi*. The results showed that *Enterobacter* sp. G87 was capable to confer protection to *Artemia* against *V. harveyi* infection (Figure 1).

Challenged Assay on Seabass Larvae

After four days of experiment, results demonstrated the survival of seabass larvae treated with potential probiont *Enterobacter* sp. G87 at concentration of 10^6 CFU mL^{-1} (CT1, 93±2 %) showed no significant different with the control (98±2%) indicated this concentration was not harmful to the larvae. Moreover, after challenged with *V. harveyi*, this concentration provided full protection (T3, 95±3%) to the larvae with significant difference compared to the control group with *V. harveyi* only (VH, 45±3%). However, pre-incubation of seabass larvae at concentration of 10^8 CFU mL^{-1} of *Enterobacter* sp. G87 (CT2) reduced the survival to 55±5% which was significantly different compared to control group and no protection was observed after challenged. Results suggest this concentration was too high and not suitable

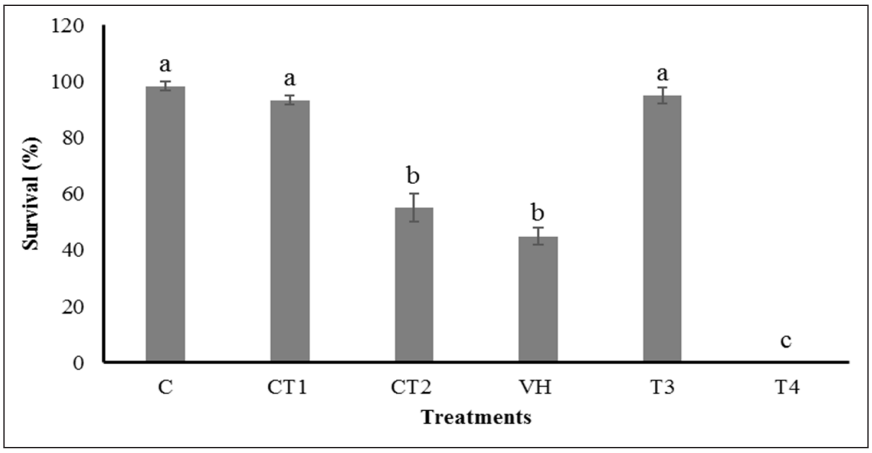

Figure 2. The survival rate of seabass larvae after pre-incubated with different concentrations of *Enterobacter* sp. G87 (10^8 and 10^6 CFU mL^{-1}) and challenged with 10^5 CFU mL^{-1} of *Vibrio harveyi.* Error bars indicate standard error. Mean with different alphabet letters indicate significant difference (p<0.05). *Note*. C: Control; CT1: *Enterobacter* sp. G87 10^6 CFU mL^{-1}; CT2: *Enterobacter* sp. G87 10^8 CFU mL^{-1}; VH: *Vibrio harveyi* 10^8 CFU mL^{-1}; T3: *Enterobacter* sp. G87 10^6 CFU mL^{-1} + *Vibrio harveyi* 10^8 CFU mL^{-1}; T4: *Enterobacter* sp. G87 10^8 CFU mL^{-1} + *Vibrio harveyi* 10^8 CFU mL^{-1}

for the larvae (Figure 2).

***Vibrio* Counts**

All concentrations of probiont *Enterobacter* sp. G87 (T5, T6, T7) were able to reduce the numbers of *Vibrios* in *Artemia* significantly at the end of the assay compared with control group of *V. harveyi* only (T1) (Table 3).

Meanwhile in seabass challenge assay, probiont *Enterobacter* sp. G87 at

concentration of 10^6 CFU mL^{-1} (T3) was able to reduce the numbers of *Vibrios* significantly compared to T1. However, at concentration of 10^8 CFU mL^{-1} (T4) no reduction in numbers of *Vibrios* was observed (Table 4).

Table 3
Vibrio *count in* Artemia *after pre-incubated with* Enterobacter *sp. G87 and challenged with* 10^5 *CFU* mL^{-1} *of* Vibrio harveyi

Treatments	Description	Log10(CFU mL^{1})
T1	*Vibrio harveyi* 10^5 CFU mL^{-1}	9.4 ± 0.0^a
T5	*Enterobacter* sp. G87 10^8 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}	5.3 ± 0.1^b
T6	*Enterobacter* sp. G87 10^6 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}	6.4 ± 0.2^b
T7	*Enterobacter* sp. G87 10^4 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}	7.2 ± 0.1^b

Note. Mean with different alphabet letters indicate significant difference ($p<0.05$)

Table 4
Vibrio *counts in seabass larvae after pre-incubated at different concentrations of* Enterobacter *sp. G87 and challenged with* 10^8 *CFU* mL^{-1} *of* Vibrio harveyi.

Treatments	Descriptions	Log10 (CFU mL^{-1})
T1	*Vibrio harveyi* 10^8 CFU mL^{-1}	5.2 ± 0.0^a
T3	*Enterobacter* sp. G87 10^6 CFU mL^{-1} + *Vibrio harveyi* 10^8 CFU mL^{-1}	0.0 ± 0.0^b
T4	*Enterobacter* sp. G87 10^8 CFU mL^{-1} + *Vibrio harveyi* 10^8 CFU mL^{-1}	5.1 ± 0.1^a

Note. Mean with different alphabet letters indicate significant difference ($p<0.05$)

DISCUSSION

In this study, probiotic *Enterobacter* sp. G87 was tested to discover its potential to confer protection in *Artemia* and seabass larvae culture against *V. harveyi* infection. The results demonstrated the ability of *Enterobacter* sp. G87 to increase the survival of seabass larvae and *Artemia* nauplii when challenged with *V. harveyi*. The probiont also able to reduce the numbers of *Vibrio* in host after challenged.

Enterobacter is a Gram negative, rod shaped and facultative bacteria which is widely distributed in soil, water, intestinal tract of animals as well as sewage (Rogers, 2017). There are few reports on *Enterobacter* sp. which highlighted its potential as probiotics in *in vitro* assay. Wendy et al. (2014) reported on *Enterobacter ludwigii* inhibitory activity against two pathogenic strains; *Vibrio parahaemolyticus* and *Aeromonas hydrophila* in *in vitro* assay. *Enterobacter hormaechei* which was isolated from grey mullet (*Mugil cephalus*) was also reported to have antagonistic properties against *Vibrio cholera* in well diffusion assay (Ghosh et al., 2011). However, to our knowledge, there is less information on the application of *Enterobacter* sp. in *in vivo* study.

In this study, *Artemia* was used as host in the preliminary *in vivo* assay. *Artemia* is an important live feed for a variety of

finfish and shellfish and are given to over 85% of aquaculture species around the world. It is therefore necessary to control the bacterial population of *Artemia* to minimize the danger of bacterial infection before their use in culture systems (Lamari et al., 2014). With the presence of probiotic, it could provide protection for *Artemia* in terms of immunity and disease (Balcázar et al., 2007). Furthermore, it could act as a carrier to introduce probiotics to the targeted host (Seenivasan et al., 2012). Enrichment and bioencapsulation of *Artemia* have been widely applied in marine fish and crustacean culture around the world as it can enhance the nutritional value of *Artemia* (Immanuel, 2016). For an example, study done by Jamali et al. (2014) reported rainbow trout larvae fed with *Artemia* enriched with *Bacillus* sp. had a higher growth and survival rate.

The survival of *Artemia* was high after enrichment with probiont *Enterobacter* sp. G87. All concentrations tested were able to provide protection to the *Artemia* after challenged with *V. harveyi*. The optimum concentration was at 10^6 CFU mL^{-1}, where full protection in *Artemia* was observed. Similar finding was observed in *Artemia* when challenged with *Vibrio parahemolyticus* and *V. cholera* after being enriched with two probiotics, *L. acidophilus* and *Lactobacillus sporogenes* which showed higher survival (72%) compared to the normal *Artemia* (Immanuel, 2016). In the previous study, concentration of 10^6 CFU mL^{-1} had been identified as optimal concentration of *L. sporogenes* to attain good survival and growth in *Artemia* (Jacobsen et al., 1999; Seenivasan et al., 2012). *Bacillus* spp. JAQ04 and *Micrococcus* spp. JAQ07 at concentration 10^6 CFU mL^{-1} also resulted in better survival of *Artemia* (70%) when challenged with *Vibrio alginolyticus* compared to control group (20%) (Shazwani et al., 2015).

After being challenged, the numbers of *Vibrio* in *Artemia* was determined to observe the vibrios reduction if any. Results demonstrated the ability of *Enterobacter* sp. G87 in reducing the numbers of *Vibrio* in *Artemia*. This suggests the protection maybe due to antibacterial activity or the colonization factor provided by *Enterobacter* sp. G87. Mohan et al. (2014) proved this theory when two probiotics *Alteromonas* sp. and *Actobacterium* sp. were able to colonize better, grow faster resulting in high counts compared to pathogens (*V. harveyi* and *Aeromonas* sp.) when introduced together to *Artemia*.

Seabass larvae was chosen as species of interest because of its high economic importance for larvae culture and aquaculture (Frans et al., 2013). The role of beneficial probiotic to limit and to control environmental pathogens which become particularly important in the future of aquaculture, especially with regard to increasing number of antibiotic resistant strains of bacteria (Haq et al., 2012).

In seabass larvae challenge assay, *Enterobacter* sp. G87 at concentration of 10^6 CFU mL^{-1} showed higher survival after challenged with *V. harveyi* compared to 10^8 CFU mL^{-1}. The results suggest that higher concentration of probionts may not be

suitable in conferring protection for seabass larvae due to high mortality observed after challenged.

High amount of probiont sometimes may not be suitable in protecting the host against pathogenic infection. It might harm the host instead which leads to bad effect on the host's health (Martínez Cruz et al., 2012; Tuan et al., 2013). High concentration of probiotics may deplete the oxygen content in the water due to a very fast rate of bacterial colonization which can disturbed the oxygen level in water (Yaminudin, 2017). Suzer et al. (2008) also reported that high concentration of probiotics was not good for husbandry parameter of the culture water. Hence, it is crucial to use probiotics in a correct concentration in order to exert optimum beneficial effects on the growth and survival of hosts (Bagheri et al., 2008).

The ability of *Enterobacter* sp. G87 in conferring protection of *Artemia* and seabass larvae against *V. harveyi* was in line with study done by Capkin and Altinok (2009) that reported the survival of rainbow trout *Oncorhynchus mykiss* challenged with bacteria *Yersinia ruckeri* which caused Yersioniosis diseases was increased when fed with feed supplemented with *Enterobacter cloacoe* for 60 days. LaPatra et al. (2014) also demonstrated the use of *Enterobacter* strain C6-6 in rainbow trout which showed higher survival after challenged with *Flavobacterium psychrophilum* in both *in vitro* and *in vivo* assay.

At the end of challenge assay, the pathogen count was done to investigate the reduction numbers of *Vibrio* in *Artemia* and seabass larvae. Results revealed the potential of *Enterobacter* sp. G87 in reducing the numbers of *Vibrio* after being challenged. *Enterobacter* sp. G87 at 10^6 CFU mL^{-1} was able to reduce the numbers of *Vibrio* count completely in seabass larvae. This suggest the protection provided by *Enterobacter* sp. G87 may be due to its ability to produce antimicrobial compound or compete for the colonization sites with the pathogens. Water quality parameters were within optimal range during the experiment indicated it did not contribute to the mortality of *Artemia* and seabass larvae.

The potential probiotic *Enterobacter* sp. G87 showed capability to enhance the survival of *Artemia* and seabass larvae and able to provide protection against *V. harveyi* infection at concentration of 10^6 CFU mL^{-1}. The results were relevant with the definition of probiotic which an adequate amount of live microbial which has a beneficial effect on the host by modifying the host associated or ambient microbial community, ensuring improvement by use of the feed or enhancing its nutritional value, enhancing the host response towards disease, or by improving the quality of its environment (Verschuere et al., 2000). Other than that, when the culture system was provided with potential probiont, it will be ingested naturally by the host (Mahdhi et al., 2011).

CONCLUSION

This research was done to observe the ability of potential probiont *Enterobacter* sp. G87 to protect *Artemia* nauplii and seabass larvae against *Vibrio harveyi*. The results showed

that *Enterobacter* sp. G87 at concentration of 10^6 CFU mL^{-1} was the most effective to protect *Artemia* and seabass larvae against *V. harveyi* infection and reduced the numbers of *Vibrio*. Thus, *Enterobacter* sp. G87 was proven to have potential as a good probiotic for seabass larval culture and as enrichment of *Artemia*. The used of potential probiotic can be advantageous for the aquaculture production.

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