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5 authors, including:

Ina Salwany Md Yasin
Universiti Putra Malaysia

Mohd Zamri Saad
Universiti Putra Malaysia

Nurhidayu Al-saari
International Islamic University Malaysia

Aslizah Mohd Aris
Universiti teknologi MARA, cawangan negeri sembilan, kampus kuala pilah

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Administration of live-attenuated vaccine of *Vibrio harveyi* to improve survival of gnotobiotic brine shrimp (*Artemia salina*) model against multiple *Vibrio* infection

Y.K. Chin[1], Nurhidayu Al-saari[1,5], Aslizah Mohd Aris[1,3], Mohd Zamri-Saad[1,4], Ina-M.Y. Salwany[1,2*]

[1]*Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Malaysia*

[2]*Department of Aquaculture sciences, Faculty of Agriculture sciences, Univeriti Putra Malaysia, 43400 Serdang, Malaysia*

[3]*Department of Biology, School of Biology, UniversitiTeknologi MARA, Kampus Kuala Pilah, 72000 Kuala Pilah, Negeri Sembilan, Malaysia*

[4]*Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, University Putra Malaysia, Serdang, Malaysia*

[5]*International Institute for Halal Research and Training (INHART), International Islamic University Malaysia, 50728 Kuala Lumpur, Malaysia*

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Abstract

Newly developed live-attenuated protease derivative from pathogenic *Vibrio harveyi*s train *Vh1* as a live vaccine to against Vibriosis of aquatic animals. In the current study, we used the gnotobiotic *A. salina* as model to evaluate the safety and efficacy of the live-attenuated. This study was conducted by bacterial safety experiment and bacterial efficacy experiment. During the bacterial safety, the wild type and live-attenuated of *V. harveyi* (MVh-vhs) were tested for 48 hours on the Artemia larvae (instar II). During the efficacy experiment, the *A. salina* larvae were incubated with different concentration of live-attenuated *V. harveyi* (MVh-vhs), then challenged with *V. harveyi, V. alginolyticus* and *V. parahaemolyticus*. The result of safety experiment showed that the high concentration of live-attenuated mutant *V. harveyi* (MVh-vhs) at concentration of 10^9 CFU/mL is safe and had improved the *A. salina* larvae survival compared to other groups. On the other hand, pathogenic wildtype *V. harveyi* caused lethal effect on *A. salina* larvae by decreasing their survival. The surprising result of efficacy experiment showed that 10^7 CFU/mL of live attenuated MVh-vhs with 6 hours post incubation with*A. salina* larvae contributed higher survival while 10^9 CFU/mL of live attenuated MVh-vhs with 24 hours incubated *A. salina* larvae contributed higher survival against multiple *Vibrio* challenge. In this study, we concluded that the incubation time had affect bacterial concentration uptake by *A. salina* larvae and affect the effectiveness of Artemia bioencapsulation for targeted hosts.

*** Corresponding Author:** M.Y Ina-Salwany ✉ salwany@upm.edu.my

Introduction

Artemia sp. is characterize as having a short life-span with good resilience and able to survive in high salinity environment which is suitable as good model organisms to study virulence of marine pathogenic bacteria (Lee *et al.*, 2014). In fact, *A. salina* is a small crustacean species which highly depending on the innate immune system that consists of cellular components and humoral components due to lacking of adaptive immune system (Soderhall and Lee, 2002; Hauton, 2012). Similar with other invertebrates, cellular and humoral mechanisms contribute defense reaction through preventing microbial invasions or assisting the elimination of the invading microbes in their bodies (Destoumieux-Garzon *et al.*, 2001). Moreover, during early stages of fish growth development including the developing embryos until further larval stages are all rely on innate immune system to regulate quick immune responses and protect the host against unfavorable condition (Vadstein *et al.*, 2013). Therefore, *A. salina* larvae can be a pathogen-disease study model for all live stages of marine crustacean and early developing life stages of marine fishes because they all have similar immune system. Live *Artemia* nauplii have been used as vectors for delivering compounds to larvae stages of aquatic animals, which known as bioencapsulation. Moreover, bacteria with various characteristics had been incorporated into *Artemia* nauplii and this route has been used to vaccinate fry.

Vibriosis is a disease caused by pathogenic *Vibrio* spp. that has negatively affected worldwide marine aquaculture by increase mortality of farmed fishes and potentially zoonotic to human (Haenen *et al.*, 2014; Aris *et al.*, 2018). *Vibrio* species including *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* are few of the major species that cause vibriosis in marine crustacean shrimp, *Penaeus vannamei* and *P. monodon* (Chatterjee and Haldar, 2012) and marine finfish such as large yellow croaker, *Pseudosciaenacrocea* (Liu *et al.*, 2016). On the other hand, the fact that seawater is a reservoir of Vibrios, water transmission is suggested as another primary route for *Vibrio* infection in aquatic organisms, collapsing the first physical and chemical defense barrier when the bacteria penetrates the wounded or exposed skin (Frans *et al.*, 2011).Previously, our laboratory has successfully constructed a live-attenuated serineprotease vaccine by genetically modified a local isolate of virulent *V. harveyi* (Aris *et al.*, 2018). The novel live vaccine candidate namely *V. harveyi* strain MVh-vhswas constructed by site directed mutagenesis, conjugation and allelic exchange (Aris *et al.*, 2018).

Moreover, non-selective filter feeding of *A. salina* nauplii (Instar II) was used for bioencapsulation and become vector of *Vibrio*sp (Interaminense *et al.*, 2014). Therefore, the current experiments to evaluate the bioencapsulation and safety of live attenuated *V. harveyi* strain MVh-vhs on gnotobiotic *A. salina* larvae (Instar II of nauplii) by immersion method.

Materials and Methods

Gnotobiotic Artemia sp. culture

Axenic *A. salina* nauplii were obtained by decapsulation and hatching procedures. 0.2 g of commercial Artemia cysts (O.S.I. PRO 80TM Brine shrimp cysts) were hydrated with 18 L of tap water for an hour of strong aeration in sterile falcon tube. The following steps were then undertaken in sterile condition at which 10 ml of 50% cold NaOCl and 0.66 ml of 32% NaOH were added to the hydrated cysts suspension for decapsulation and sterilization. After that, the sterilized decapsulated cyst suspension was then transferred to new sterile falcon tube equipped with a 0.22 μm-filtered aeration. Later, 14 ml of sodium thiosulphate was added to remove chlorine residue and the cysts were sieved with 100 μm mesh size and washed over by using sterile autoclaved seawater. The cysts were then transferred into new sterile falcon tube with 30 ml of sterile autoclaved seawater. The strong filtered aeration was supplied and waited for 24 hours to hatch. The newly hatched *Artemia*were then continue growing for another 6 to 8 hour to let the nauplii grow into instar ii of nauplii in sterile condition (referred as gnotobiotic larvae). Axenic confirmation was performed by plating *Artemia* naupliion TSA (Tryptic Soy Agar). No bacteria growth on the agar indicates the success of gnotobiotic Artemia culture.

Identification of Vibrio spp. strain and culture conditions

Vibrio spp. were retrieved from glycerol stock stored at -80 ˚C or slant culture, followed by streaked plate on the TSA agar supplemented with 1.5% NaCl. The plates were incubated at 28 ˚C for 16 hours.For identifying the wildtype strain of *V. alginolyticus* and *V. parahaemolyticus*, DNA gyrase subunit B (gyrB) was targeted during PCR amplification (Table 1). For the characterization of wildtype *V. harveyi* (VH1) strain and live-attenuated *V. harveyi* (MVh-vhs) strain, serine protease gene (VHS) was targeted during PCR amplification (Table 2) and the identity of the gene sequence was re-confirmed using nucleotide BLAST in NCBI database. The three bases deletion (D153, H123 and S228) on VHS of MVh-vhs was checked by using gene sequence alignment software, BioEditTM.

The *Vibrio*s were cultured on either tryptic soy agar (TSA) supplemented with 1.5% NaCl or thiosulfate citrate bile salts sucrose (TCBS) agar and the plates were incubated at 28 ˚C for 24h. A colony of the vibrio was re-inoculated into tryptic soy broth (TSB) supplemented with 1.5% NaCl and incubated with at 28 ˚C for 24 hours. After that, the broth containing the bacteria was centrifuged at 12000 rpm for 15 min at 28˚C. The concentration of bacteria solution was determined by spectrophotometrically at 600 nm (OD600) with McFarland standard calculation method. List of the *Vibrio* spp. used for experiment is shown in Table 3.

Brine shrimp larvae survival studies

Two separate experiments were performed to assess the safety and efficacy of the live attenuated vaccine in cultured *A. salina* larvae (Instar II).

In the first experiment was aimed to study the dose-response relationship of wildtype strain *V. harveyi*(VH1) and live-attenuated*V. harveyi* (MVh-vhs) in*A. salina* larvae survival. For each vibrio, 20 of *A. salina* larvae (Instar II) were initially transferred into sterile falcon tubes containing 25 ppt of sterile autoclaved seawater. The *A. salina* larvae were incubated with three different concentrations (10^5 CFU/mL, 10^7 CFU/mL and 10^9 CFU/mL) of wildtype *V. harveyi* (VH1) strain and live-attenuated *V. harveyi* (MVh-vhs) strain into 30 mL of final volume. For the control treatment, no bacteria were added in the *A. salina* culture. All falcon tubes were placed horizontally on a rotor at 50 rpm at 24˚C. Each treatment was performed in triplicate. The survival of *A. salina* larvae was observed at 12th hour, 24th hour, 36th hour and 48th hour of incubation time. During observation, the suspension was poured into sterile petri dish, and was poured back into their respectively Falcon tube after observation under laminar flow hood. For estimating the survival, the number of swimming larvae were counted followed by calculating the survival percentage.

The second experiment was studied to verify the protective effect of Bioencapsulated live attenuated *V. harveyi* (MVh-vhs) into *A. salina* larvae with two incubation time which is 6 hours and 24 hours on different concentrations (10^5 CFU/mL, 10^7 CFU/mL and 10^9 CFU/mL). Each set was conducted by 300 *A. salina* larvae were transferred into sterile falcon tubes consisting sterile autoclaved seawater. Then, the larvae were incubated initially with three different concentrations (10^5 CFU/mL, 10^7 CFU/mL and 10^9 CFU/mL) of live-attenuated strain *V. harveyi* (MVh-vhs) into final volume of 30 mL for the pre-determined duration of incubation (6 hours or 24 hours). After incubation, the 20 of swimming larvae were collected and transferred into sterile falcon tubes that contain sterile autoclaved seawater.

The encapsulated *A. salina* larvae were challenged with wildtype *V. harveyi* (VH1) strain at concentration of 10^9 CFU/mL for 48 hours. The amount of spent medium transferred into each treatment was balanced by adding a complementary autoclaved seawater to make up the final volume of 30mL and the falcon tubes were added horizontally on a rotor at 50 rpm at 24 ˚C. Triplicate for each treatment was performed.The survival of larvaewere observed at 12th hour, 24th hour, 36th hour and 48th hour after challenge test at which the suspension was poured into sterile petri dish and later was poured back into their respective falcon tube after the observation under laminar flow hood. After that, the survival percentage was determined.

The experiment were repeated for *V. alginolyticus* (VA2) and *V. parahaemolyticus* (FORC) challenge for cross-protective effect test. Non-encapsulated gnotobiotic *A. salina* larvae were used for the *Vibrio* spp. challenge for negative control. On the other hand, the encapsulated *A. salina* larvae without *Vibrio* sp. challenge for positive control.

Data analysis

The differences in survival were analyzed by one-way analysis of variance (ANOVA) with post-hoc Turkey test in IBM SPSS® software.

The data was transformed into Arc-Sin and express as average ± stdev. The values were considered significantly different if $p < 0.05$

Results

Effect of live-attenuated V. harveyi (MVH-VHS) and wild type V. harveyi (VH1) on survival of A. salina larvae

Fig. 1 shows the survival of *Artemia* larvae incubated with different concentration of liveattenuated *V. harveyi* (MVh-vhs) and wildtype *V. harveyi* (VH1) compared to the untreated group. According to Fig. 1 (A), no significant differences ($p<0.05$) were observed on the survival of *A. salina* larvae incubated with 10^5 CFU/mL bacteria and those in the control group. Insignificant larvae survival between treatments was maintained even at prolong incubation with 48 to 62% larvae were survived at 48th hour incubation.

In Fig. 1 (B), *A. salina* larvae treated with live attenuated *V. harveyi*(MVh-vhs)at 10^7 CFU/mL has significantly ($p>0.05$) improved the survival of *A. salina* larvae compared to the larvae that were immersed with wildtype *V. harveyi* (VH1) and control at 36th hour to 48th hour incubation. However, in Figure 1 (C) shown Immersion of Live attenuated *V. harveyi* (MVh-vhs) improved high survival performance of *A. salina* larvae.

Table 1. Primers used for the PCR amplification.

Primer	Primer Sequence (5' to 3')	Expected sizes (bp)	Reference
Serine protease (VHS)			
F_vhs	GGTACCATGAAAAAACCATTGCTTGCG	1368	Aris *et al.*, 2018
R_vhs	GAGCTCTTAGCGGATAACGAGGTAAAC		
DNA gyrase subunit B (gyrB)			
F_gyrB	GAGAACCCGACAGAAGCGAAG	332	Chatterjee and Haldar, 2012
R_gyrB	CCTAGTGCGGTGATCAGTGTTG		

This proved by the performance of *A. salina* larvae were tested with live attenuated *V. harveyi* (MVh-vhs) significantly higher ($p>0.05$) than both tested by Wildtype *V. harveyi* and control from 36th hour to 48th hour.

Efficacy of live-attenuated V. harveyi (MVh-vhs) on A. salina survival against V. harveyi, V. alginolyticus and V. parahaemolyticus

In the second experiment, protective ability of live-attenuated *V. harveyi* was investigated against different wild type of *Vibrio*sfor *A. salina* larvae challenge. There are two trials with different live-attenuated *V. harveyi* (MVH-vhs) incubation time which are 6 hours and 24 hours that indicated by Fig. 2 and Fig. 3 respectively.

Fig. 2 demonstrated the result for the survival of *A. salina* larvae after 6 hours pre-treated with different concentration of live-attenuated *V. harveyi* (MVh_vhs) and challenge with *V. harveyi* (VH1), *V. alginolyticus* (VA2) and *V. parahaemolyticus* (FORC-

_008). After 6 hours encapsulation, administration of concentration 10^5 and 10^7 CFU/mL of live attenuated *V. harveyi* (MVh_vhs) gave poor significant different ($p<0.05$) in the challenged *A. salina* survival at 48th hour. However, In Fig. 3. After 24 hours encapsulation, administration of concentration 10^5 and 10^7 CFU/mL of live attenuated *V. harveyi* (MVh_vhs) gave no significant different ($p<0.05$) but 10^9 CFU/mL contributed significantly ($p<0.05$) high survival in the challenged *A. salina* survival at 48th hour.

Table 2. The deletion bases in catalytic sites of serine protease gene (VHS) sequence of MVh-vhs strain. (Source: Aris, unpublished).

Target Bases for deletion	Gene sequence (5' to 3')
Aspartate (D_{153})	GAGACGAGATGTCAgacATTGCCTTGCTTAAG
Histidine (H_{123})	ATCGTAACGAACTATcacGTTATCAAAGGCGC
Serine (S_{228})	CAATTAACAGTGGTAACtccGGTGGCGCTT

Note: the small capital with underline indicate a target deletion in specific catalytic gene.

Table 3. Bacteria strains that used for the experiment.

Bacteria	Relevant characteristic	Source or Reference
Vibrio harveyi Strain VH1 Strain MVh-vhs	Complete serine endoprotease gene (VHS) 3 base deletion of DNA sequence on deficit serine endoprotease gene (VHS)	GenBank:KT266880.1, Aris *et al.* 2016. Isolation from Brown marbled grouper, *Epinephelusfuscoguttatus* Aris *et al.*, 2018 (Unpublished) Lab collection
Vibrio alginolyticus Strain VA2	A strain of *V. alginolyticus*	GenBank:KU141337.1 Nehlah, Ina-Salwany&Zulperi, 2016. Isolation from Brown marbled grouper, *Epinephelusfuscoguttatus*
Vibrio parahaemolyticus Strain FORC_008	A strain of *V. parahaemolyticus*	GenBank:CP013826.1 Isolation from Brown marbled grouper, *Epinephelusfuscoguttatus*

In this experiment, the strain significantly increased the survival of *A. salina* larvae that encapsulated 10^9 CFU/ML of live attenuated *V. harveyi* (MVh_vhs) strain for 24 hours encapsulation after challenged withthree *Vibrio*sp.

Therefore, administration of Live-attenuated *V. harveyi* (MVh_vhs) at the concentration 109 CFU/ML for 24 hours encapsulation in *A. salina* larvae provided the best protection from vibriosis.

Discussion

The results showed that the live-attenuated *V. harveyi* (MVh-vhs) is not only safe and harmless for *A. salina* larvae but also can recover their survival at prolong incubation of 36 h to 48 h. Previous studies showed that the same live-attenuated *V. harveyi* (MVh-vhs) at three different concentrations 10^5 CFU/mL, 10^7 CFU/mL and 10^9 CFU/mL as vaccine candidates was harmless for tiger grouper juvenile, *Epinephelusfuscoguttatus*(Aris, 2018 Unpublished). This is possible due to loss of pathogenic and virulence factor of serine endoprotease gene which was attenuated in the *Vibrio* strain. Serine protease gene (VHS) for pathogenic *V. harveyi* has contributed as chaperone or provide thermal resistant properties for activity of proteolytic enzymes (Aris *et al.*, 2016). Therefore, the live-attenuated *V. harveyi*MVh-vhsis harmless for *A. salina* larvae. Live-attenuated *V. harveyi* (MVh-vhs) was developed previously based on deletion of three catalytic amino acids sites including Aspartate (D153), Histidine (H123) and Serine (S228) of the bacterial serine protease gene (VHS) (Aris *et al.*, 2016).

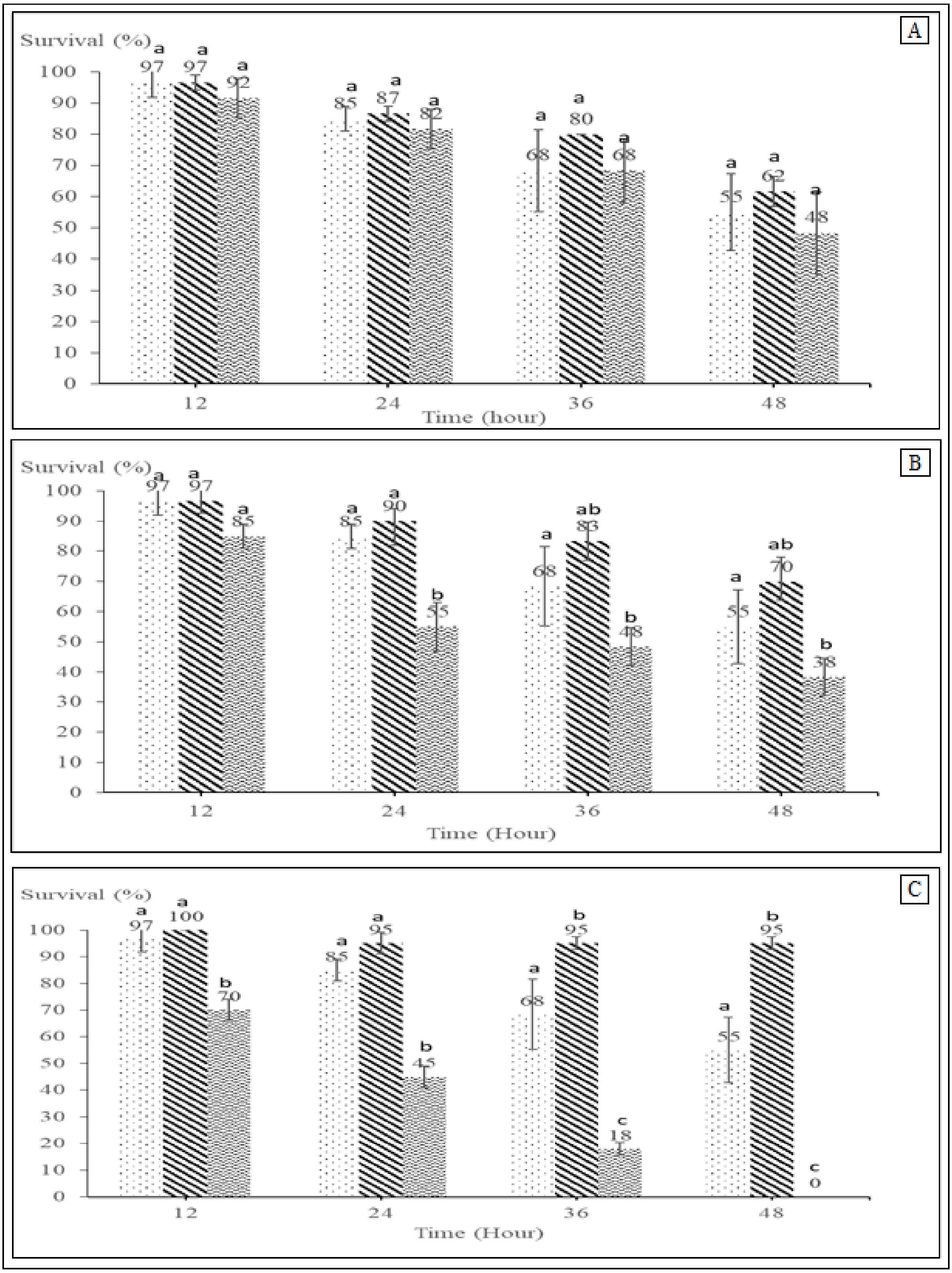

Fig. 1. Bacterial safety assay of *Vibrio harveyi* at different concentrations against *Artemia salina*. (A): *A. salina* larvae survival at 10^5cfu/mL of *Vibrio* spp. (B): *A. salina* larvae survival at 10^7cfu/mL of *Vibrio* spp. (C):*A. salina* larvae at 10^9cfu/mL of both *Vibrio* spp. , live-attenuated *V. harveyi* (MVh-vhs); , wildtype *V. harveyi (VH1)*; and , control. Different superscript letter above the bar graph indicated significant differences (p<0.05).

According to Fig. 1, 10^7 CFU/mL and 10^9 CFU/mL bacterial concentration of live-attenuated *V. harveyi* (MVh-vhs) treatments contributed to higher survival of *A. salina* larvae compared to the wildtype *V. harveyi* (VH1) treatment and control treatment throughout the experiment period. Survival of *A. salina* larvae had improved possibly due to *A. salina* larvae consume the bacteria as their food because certain bacteria contribute nutritional value and positive effect of the growth rate as well as survival for *Artemia* spp. (Tkavc *et al.*, 2010) Obviously, high concentrations of live-attenuated *V. harveyi*MVh-vhs (at 10^7 and 10^9cfu/mL) were more effective in improving the survival of *A. salina*larvae.

The concentration of bacterial suspension has shown to have effect on the accumulated bacteria quantity in *Artemia* spp. (Makridis *et al.*, 2000). Furthermore, the effectiveness of *Artemia* nauplii bioencapsulation depends on targeted bacteria type, exposure time and status of *Artemia* nauplii (Gomez-Gill *et al.*, 1998).

In this study, the harmless live-attenuated *V. harveyi* (MVh-vhs) has high potential for use as bio-encapsulated vaccine by encapsulate the *A. salina*.

Based on experimental result of bacterial protective efficacy assay with *A. salina* larvae (Fig. 2 and 3), the live-attenuated *V. harveyi* (MVh-vhs) was encapsulated within the *A. salina* to test its protectivity against wildtype *V. harveyi*(VH1). Besides, *V. alginolyticus* (VA2) and *V. parahaemolyticus* (FORC) were used also to test on cross-protectivity of the live-attenuated *V. harveyi* (MVh-vhs) for *A. salina* larvae. Similar to Fig. 1, the high survival of *A. salina* larvae incubated with MVh-vhs without the *Vibro* challenge in positive controls was recorded in this experiment throughout the experiment period. However, there is no surprise that low survival of *A. salina* larvae for negative control which was challenged by wildtype of *Vibrio* sp. We investigated the optimum incubation time of *A. salina* larvae with live-attenuated *V. harveyi* (MVh-vhs) to win over the infection by *Vibrio* spp. effectively. However, there are different time required for encapsulation or enrichment of gnotobiotic *Artemia* sp. larvae were used by researchers to test their different probiotic or immune-stimulant substance on their experiments. For example, active or autoclaved *Bacillus* sp. LT3 was incubated 6 hours with gnotobiotic *Artemia* sp. larvae for *V. campbellii* challenge test (Niu *et al.*, 2014).

The probionts, *Lactobacillus acidophilus, L. sporogenes* and yeast, *Saccharomyces cerevisiae* were incubated 24 hours with *Artemia* sp. larvae respectively for *V. parahaemolyticus* and *V. cholerae* challenge tests (Immanuel, 2016).

According to Fig. 2, the result showed good performance for larvae survival when 10^7 CFU/mL of 6h pre-incubated MVh-vhs at which it has improved *A. salina* larvae survival after challenge with 10^9cfu/mL wildtype *V. alginolyticus* (VA2) and *V. parahaemolyticus* (FORC) respectively.

The mechanism of live-attenuated *V. harveyi* (MVh-vhs) for improving survival of *A. salina* larvae is still unknown against *Vibrio* challenge. However, there are possible reasons which can explain the ability of MVh-vhs to confer protection and cross-protection to the *Artemia* larvae as demonstrated in the current experiment. Firstly, the live attenuated *V. harveyi* (MVh-vhs) enhanced the *A. salina* immune response possibly through prevention of the rapid reproduction of pathogens by seizing the available resources including nutrients, space, adhesion sites on the *A. salina* larvae' guts or surface etc. or secondly, through production of toxic or inhibitory substances to against pathogens (Marques *et al.*, 2005). *Artemia* spp. are lack of adaptive immune system and fully depend on innate immune system of which recognized the pathogen associated molecules to activate cellular or humoral effector mechanisms to eliminate invasive pathogens (Vazquez *et al.*, 2009).

The other possible reason to such results is the *Artemia* spp. larvae' tolerance to infection is enhanced due to stimulation of their non-specific or innate immune response (Sung *et al.*, 2009).

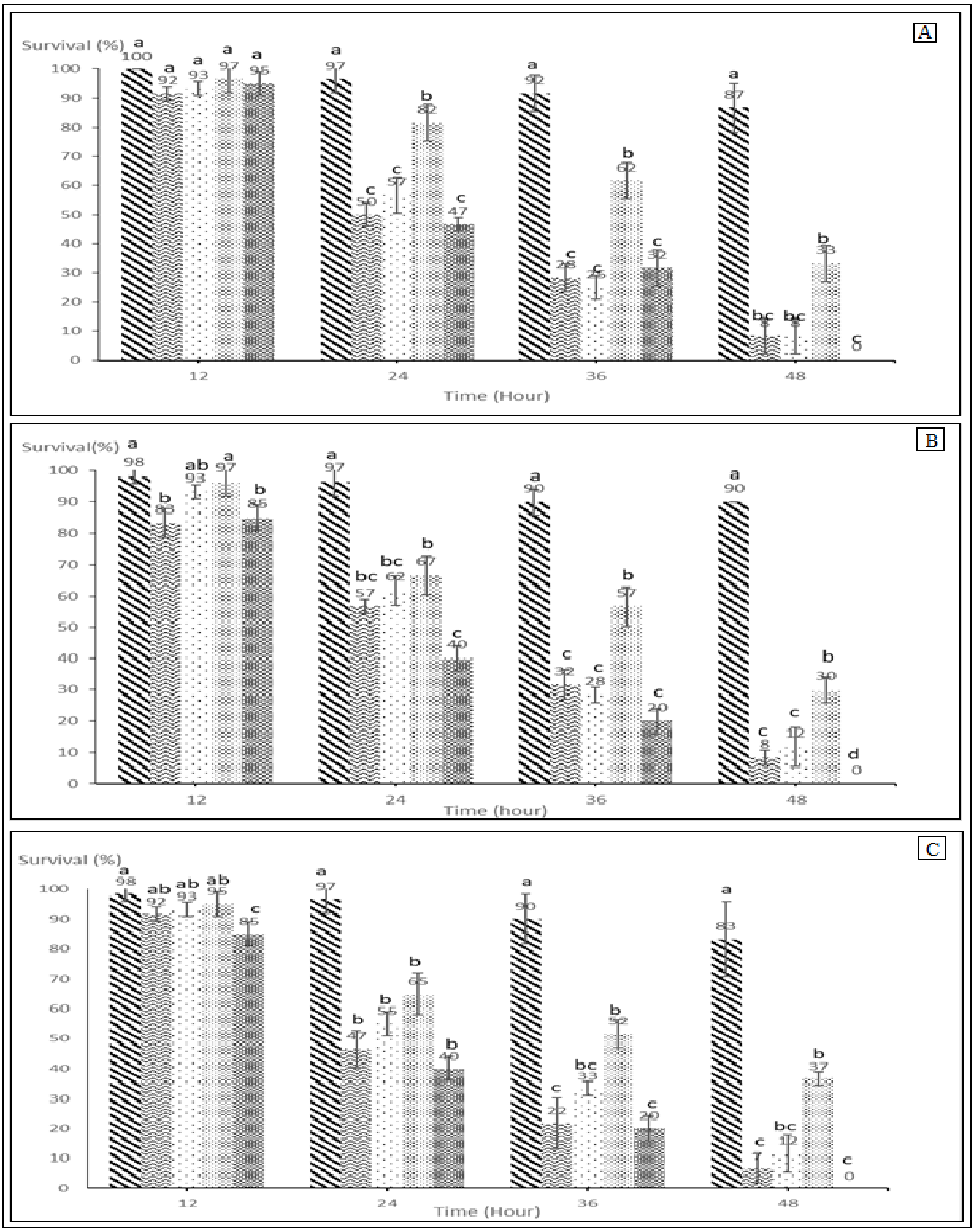

Fig. 2. Survival upon 10^9 CFU/mL of *Vibrio*spp challenge for 10^5 CFU/mL, 10^7 CFU/mL and 10^9 CFU/mL of Live-attenuated *V. harveyi* (MVh-vhs) with 6 hours encapsulated *A. salina* larvae respectively. A: *V. harveyi* (VH1) challenge test. B: *V. alginolyticus* (VA2) challenge test. C: *V. parahaemolyticus* (FORC_008) challenge test. , Positive control; , Negative control; , 10^5 CFU/mL of live-attenuated *V. harveyi* (MVh-vhs) incubation; , 10^7 CFU/mL of *live-attenuated V. harveyi (MVh-vhs)*; and , 10^9 CFU/mL of live-attenuated *V. harveyi* (MVh-vhs) incubation respectively. Different superscript letter above the bar graph indicated significant differences ($p<0.05$).

Note that the live attenuated strain of bacteria as vaccine deliver foreign antigen to stimulate both innate immune system and activate adaptive immune system against infectious diseases (Shahabi *et al.*, 2010).

In this experiment, the live-attenuated *V. harveyi* (MVh-vhs) also showed cross-protective potential for *A. salina* to against *V. alginolyticus*(VA2) and *V. parahaemolyticus* (FORC). Although the cross-protective effect was not understood, the possible reason is *V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus* are closely related that recognized as members of Harveyi clade which are subset of *Vibrio*s core group (Lin *et al.*, 2010). This might also related to the successful previous study which showed that formalin killed *V. anguillarium* can be cross-protected for Banana shrimp, *Fenneropenausmerguiensis* via oral vaccination to against *V. harveyi* challenge (Patil *et al.*, 2013).

Nevertheless, the *A. salina* survival performance for 10^7 CFU/ml of the live-attenuated *V. harveyi* (MVh-vhs) with 24 hours pre-incubation seems not enough to confer protection to the larvae after multiple *Vibrio* challenge as shown in Figure 3. Previous study proved that non-pathogenic *V. alginolyticus* CW8T2 contributed to a relatively lower biomass production that indicated nutrient value, body length and survival compare with other non-pathogenic bacteria strains for axenic *Artemia* juveniles (Verschuere *et al.*, 1999).

Therefore, we postulated that the nutrient value of MVh-vhs is similar with the other *Vibrio* sp. and are considered low for *A. salina* metabolism and the concentration of $\leq 10^7$ CFU/mL might be insufficient for larvae survival which affected indirectly on their overall stimulated immune response. The pre-incubation time are some significant factors for bioencapsulation of live bacteria supporting the statement made by Gomez-Gill *et al.* (1998).

In support to that, other authors claimed that the higher concentration of particles, the higher the percentage of particles filled in the *Artemia* digestive tract (Gelabert, 2003). In this experiment, *A. salina* larvae are proved as Bioencapsulation vector on live-attenuated *V. harveyi*(MVh-vhs) for other targeted organisms due to harmless if the bacteria.

In fact, *Artemia*sp were commonly exploited in vaccine development due to their characteristic of bacterial consumption and encapsulation (Mutoloki *et al.* 2015). Besides, although the live-attenuated *V. harveyi* (MVh-vhs) that more likely to categorise as a type of vaccine candidate, but we believe will have similar characteristic and functions as probiotic since they are genetic modified bacteria. Actually, microorganisms included pathogens that used for undergoing genetic modification into harmless strain can be fully new probiotics (Steidler 2003). Moreover, *Artemia* bioencapsulation effectiveness on live-attenuated *V. harveyi* (MVh-vhs) can be indicated by the survival of *A. salina* larvae after multiple *Vibrio*sp challenge.

This is because improvement of *Artemia* immune system due to probiotics retention during encapsulation can convey the probiotic for the main targeted host organism (Hai, Buller and Fotedar 2010). Previous studies showed that *Artemia* bioencapsulation with formalin killed *V. anguillarum* vaccine protected antigens during digestion to trigger immune response of juvenile carp, *Cyprinus carpio* and gilthead seabream, *Sparus aurata* via oral administration (Joosten *et al.*, 1995).

In contrast, the Fig. 3 showed that the 24h pre-incubation with high concentration (10^9 CFU/mL) of MVH-vhs contributed to significant high larvae survival than other treatment after the challenge test with high concentration 10^9 CFU/ml of multiple *Vibrio*. The survival showed the effect of pre-incubation time needed for live attenuated *V. harveyi* (MVh-vhs) encapsulation that might contributed to the effectiveness of vaccination. Bioencapsulation is said to depends on targeted bacteria type, exposure time and status of *Artemia* sp. nauplii (Gomez-Gill *et al.*, 1998).

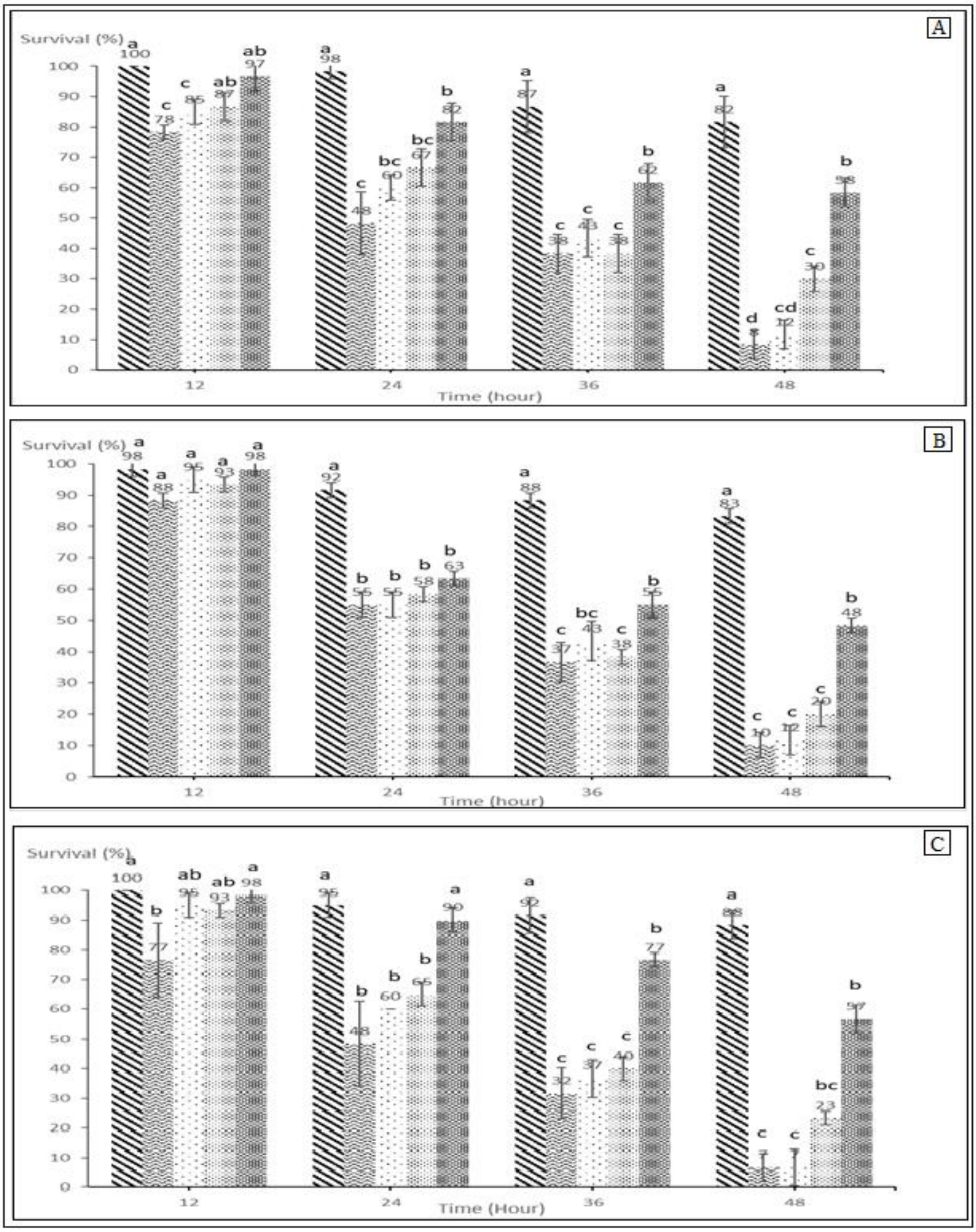

Fig. 3. Survival upon 109 CFU/mL of Vibrio spp challenge for 10^5 CFU/mL, 10^7 CFU/mL and 10^9 CFU/mL of Live-attenuated V. harveyi (MVh-vhs) with 24 hours encapsulated *A. salina* larvae respectively. A: *V. harveyi* (VH1) challenge test. B: *V. alginolyticus* (VA2) challenge test. C: *V. parahaemolyticus* (FORC_008) challenge test. , Positive control; , Negative control; , 10^5 CFU/mL of live-attenuated *V. harveyi* (MVh-vhs) incubation; , 10^7 CFU/mL of *V. harveyi*(MVh-vhs); and , 10^9 CFU/mL of live-attenuated *V. harveyi* (MVh-vhs) incubation respectively. Different superscript letter above the bar graph indicated significant differences (p<0.05).

Previously similar study showed that the survival of *Artemia* sp. larvae that were exposed for shorter time (less than 8 hours) to mnn9 yeast cells had decreased gradually after *V. campbellii* challenge (Soltanian *et al.*, 2007). Fig. 2 showed that, high concentration 10^9 CFU/mL of the live-attenuated *V. harveyi* (MVh-vhs) incubated *A. salina* larvae with 6 hours contributed to significant lower survival than negative control after challenge with 10^9 cfu/mL wildtype *V. harveyi* (VH1) and no significant different with negative control after challenge with 10^9cfu/mL wildtype *V. alginolyticus* (VA2) and *V. parahaemolyticus* (FORC) respectively. In this experiment, we figured that 24 hours is the most suitable and recommended pre-incubation time compared to 6 hours. Therefore, *A. salina* might could not fully activate the immune defence mechanism in a short period to withstand the accumulation of high concentration of bacteria followed by *Vibrio* spp. challenge. There is similar previous research shows that lowest survival of *Artemia* spp. larvae after incubated with 10^{10} CFU/mL of harmless probiotic yeast, *S. cerevisiae* suspension for 6 hours (Fazeli and Azari-Takami, 2006).

Conclusion

In a nutshell, concentration of live-attenuated *V. harveyi* (MVH) and incubation time are very crucial key factors for enhancing immune system of Artemia larvae as model during multiple *Vibrio* challenge. We believe that have immune-enhance potential as vaccine candidate for short term protection on other marine crustacean and early stages of marine fishes which shared similar innate immunity in the future. Therefore, further biomolecular studies are suggested will improve understanding of the actual immune mechanisms behind. Based on the experiment, we concluded that 6 hours incubation time is most suitable for 10^7 CFU/mL of *A. salina* larvae with live-attenuated *V. harveyi* (MVh-vhs) because sustainable cost-effective and time-effective for Artemia bioencapsulation as oral vaccination or its administration on the targeted animals directly to against effectively on high concentration of multiple *Vibrios* challenge.

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