

The Use of Indigenous Probiotic *Halomonas aquamarina* and *Shewanella algae* for White Shrimp (*Litopenaeus vannamei* Boone) Hatchery Productivity in Zero Water Discharge System

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Abstract

This research was aimed to improve the performance of white shrimp postlarvae culture through the application of indigenous probiotic bacteria, *Halomonas aquamarina* and *Shewanella algae* in zero water discharge system. The research was conducted by following two consecutive steps: (1) pathogenicity test of both probiotic in white shrimp culture, and (2) probiotic effect test to water quality and vibriosis syndrome control. From the first step, the use of probiotics had no pathogenicity effect to shrimp PL since survival rate of 84-98% was documented. From step two, the application of both probiotic bacteria was able to inhibit the population growth of *V. harveyi* in which the highest survival rate of 93.94% obtained from *H. aquamarina* addition, followed by *S. algae* addition (92.12%), *H. aquamarina*: *S. algae* addition (90.60%), *S. algae*: *V. harveyi* addition (89.39%), *H. aquamarina*: *S. algae*: *V. harveyi* addition (87.87%), *H. aquamarina*: *V. harveyi* addition (87.57%), no addition of bacteria (84.84%) and *V. harveyi* addition (82.42%). There was no significantly different ($p>0.05$) among the treatments on all water quality parameters which were still in tolerance range of white shrimp PL culture (salinity 26-30 ppt; temperature 26-28°C; pH 7.5-8.5; DO 5.7-6.4 mgL⁻¹; ammonia 0.1-0.5 mgL⁻¹; nitrite 0.02-0.25 mgL⁻¹; nitrate 5-40 mgL⁻¹). In term of other biological parameter, the use of these probiotics was significantly affecting the weight increase of shrimps. Bacterial identification showed that there was a major similarity of microbial diversity found both in water and *L. vannamei* intestine. It could be concluded that the use of *H. aquamarina* and *S. algae* as indigene probiotics contributed to the increase of shrimp survival rate. However this effect was not clearly described by the effect of water quality parameters improvement and it most probably due to the inhibition activity of these two probiotics on *V. harveyi*.

Keywords: *L. vannamei*; Postlarvae culture; Probiotic; *V. harveyi*; *H. aquamarina*; *S. algae*

Introduction

Fishery as main food source for human has been limited due to over exploitation, pollution, and disturbance of natural balance. One of way to meet demand of food from fishery is aquaculture, as a system to cultivate aquatic organism as a food source under human control, involving at least one step of aquatic organism life cycle to increase culture productivity [1]. Several aquatic organisms have been cultured in aquaculture, such as many kinds of fish and shrimp [2]. Aquaculture has important role either in national or global economical development, since it has the fastest growth of food industry (9.6% each year). As one of the biggest aquaculture producer, Indonesia has growth of 10.26% annually [3].

In 2008, total aquaculture production in the world reached the point of 52.5 million metric tons, which 40% of them was contributed by modern aquaculture. Crustacean production as one of the important commodity contributed up to around 62.5% of total crustacean production in the world [4].

One of the main aquaculture commodities of crustacean group in Indonesia is shrimp, especially white shrimp (*Litopenaeus vannamei* Boone.) as one of Penaeidae shrimp, which has high economical and nutritional value and contributes to 50% of total world production [5]. This commodity contributes significantly to the total of Indonesian aquaculture productions and by 2003 reached 115.441 ton or 47.61% of world production total [6]. However there are still many disadvantages attributed to the culture particularly low culture productivity during hatchery phase, due to the application of batch culture system which does not pay attention in water quality and disease management. Low water culture management triggers increasing of white shrimp disease infection [7,8].

One of the main disease infection of white shrimp culture is vibriosis syndrome, due to *Vibrio harveyi* infection when it population reach more than 10⁴ Colony Forming Units.mL⁻¹ (CFU.mL⁻¹) [9]. Several ways have been applied to solve this problem i.e., use of recirculation system, antibiotic, chlorine, and disinfection. However, limited of simplicity and reliability recirculation system make it difficult to be applied in traditional hatchery. In addition, use of chlorine, antibiotic and disinfection support the increase of resistant bacteria strain population and accumulation of residue in the water [10,11]. Some kinds of bacteria, called probiotic bacteria, have been found to solve ineffective methods above. However, until now the application of probiotic is still limited because unresolved action mechanism of each probiotic, and questionable of probiotic as indigene or local bacteria in white shrimp culture [7].

Based on the above explanation, the research about the use of local probiotic application in white shrimp static culture system to increase water quality and to solve vibriosis syndrome is needed. In this research

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two kinds of probiotic that had been isolated were used, *Halomonas aquamarina* and *Shewanella algae* [12]. This research was aimed to determine effect of these two probiotics application to white shrimp culture water quality, survival and growth of white shrimp.

Materials and Methods

Research preparation

Litopenaeus vannamei postlarvae-4 (PL-4) was obtained from Suritani Pemuka hatchery, Anyer, Indonesia. It was acclimated in the tank for 24 hours before the experimentation time in Aquatic Ecology Laboratory, Institut Teknologi Bandung (ITB). *V. harveyi* culture was obtained from Institut Pertanian Bogor (IPB) collection, whereas the two probiotic were isolated and selected from Situbondo hatchery, East Java. Optimum cell number of *H. aquamarina* is 10^6 CFU.mL⁻¹ and 10^5 CFU.mL⁻¹ for *S. algae* [12]. Optimum cell number of *V. harveyi* to cause shrimp infection is 10^4 CFU.mL⁻¹ similar to [9]. Bacteria were used at 24 hours inoculum age by inoculum number as 0.1% v.v⁻¹.

Pathogenicity test of probiotic to PL-4 white shrimp: *H. aquamarina* and *S. algae* were tested for their Pathogenicity potential to PL-4 white shrimp culture. Pathogenicity test used four chemical glasses (2L), each of them was contained 1.5 liter sea water. As 0.1% v.v⁻¹ of each bacteria was used [(K) no addition of bacteria; (P8) addition of *H. aquamarina*; (P9) addition of *S. algae*; and (V) addition of *V. harveyi*] in cell optical density 10^6 CFU.mL⁻¹, 10^5 CFU.mL⁻¹ and 10^4 CFU.mL⁻¹ for *H. aquamarina*, *S. algae* and *V. harveyi* respectively. Age of these three inoculums bacteria were 24 hours. In this test, 50 PL-4 white shrimp were added to each test group. Pathogenicity test was done for 12 days. Remain number of white shrimp in each test group was calculated at the end of culture period to know the survival rate.

Application of probiotic bacteria in PL-4 white shrimp culture

Experimental set up: 10L sterilized sea water with 30 ppt salinity was stocked per tank which the bottom has been covered by sterilized bicarbonate stone for buffering. Aeration line was settled then connected to aeration pump (Figure 1) and each tank with aeration rate at 200 mL.min⁻¹.

Probiotic bacteria was activated for 24 hours, and added to the culture based on treatment group (Table 1) as 0.1% v.v⁻¹ for concentration and optical density 10^6 CFU.mL⁻¹ for *H. aquamarina*, 10^5 CFU.mL⁻¹ for *S. algae*. Bacteri *V. harveyi* (0.1% v.v⁻¹) was added to the culture based on treatment after 24 hours from addition of probiotic. Then, PL-4 was added to the culture by stocking density 330 individuals/tank. Experiment was conducted for 12 days in $26 \pm 2^\circ\text{C}$ temperature, by

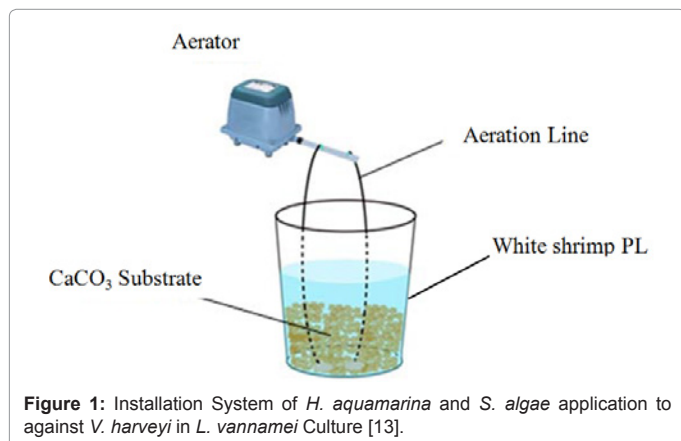


Figure 1: Installation System of *H. aquamarina* and *S. algae* application to against *V. harveyi* in *L. vannamei* Culture [13].

Group	Bacteria Inoculum Addition
K	No bacteria added
KP8	Added 0.1% v.v ⁻¹ <i>H. Aquamarina</i>
KP9	Added 0.1% v.v ⁻¹ <i>S. algae</i>
KV	Added 0.1% v.v ⁻¹ <i>V. harveyi</i>
KC	Added 0.1% v.v ⁻¹ <i>H. aquamarina</i> and <i>S. Algae</i>
P8	Added 0.1% v.v ⁻¹ <i>H. aquamarina</i> and <i>V. harveyi</i>
P9	Added 0.1% v.v ⁻¹ <i>S. algae</i> and <i>V. harveyi</i>
PC	Added 0.1% v.v ⁻¹ <i>H. aquamarina</i> , <i>S. algae</i> and <i>V. harveyi</i>

Table 1: Treatments of pathogenesis probiotics bacteria test againts *Vibrio harveyi* in white shrimp culture.

three repetitions for each treatment. Zero water discharged method was used in this experiment by adding CaCO₃ stone on each bottom tank as culture substrate [13]. The culture was fed with microalgae (*Chaetoceros* sp.) and sterilized *Artemia* sp. larvae. The amount of feed was adapted to the number of white shrimp PL stocking density. Microalgae were delivered manually two times a day at 09.00 am and 03.00 pm, and *Artemia* sp. was delivered three times a day at 08.00 am, 12.00 pm, and 08.00 pm.

Observation was consist of white shrimp survival in the end of culture period, length and weight of white shrimp at the beginning and the end of the culture, water quality parameter measurements (temperature, salinity, DO, pH, ammonia, nitrite, and nitrate), bacterial community counting, bacterial identification and bacteria population dynamic during culture.

Water quality parameter: Several water quality parameters, i.e., temperature, salinity, pH level, and dissolved oxygen (DO) were measured every day, while ammonia, nitrite, and nitrate concentrations were measured every two days. Temperature was measured using thermometer, DO was measured using calibrated DO meter YSI Type 51B, pH level was measured using pH meter OAKTON waterproof Type PC10, while salinity was measured using refractometer. The ammonia, nitrate and nitrite concentration was measured using measurement kit and HACH Spectrophotometer by following the Nessler-, Diazotiation-, and Nitrate HCl method at the wavelengths of 425, 371, 275 and 220 nm respectively.

Zootechnical parameters of white shrimp: Survival rate of *L. vannamei* was calculated at the end of culture period by counting the number of remaining shrimp as compared to the initial stock number. The survival rate of the PL was conducted by using the following equation [13]:

$$SR = \frac{N_t}{N_o} \times 100\%$$

Where:

SR=Survival Rate

No=Initial shrimp number

Nt=Final shrimp number

Mean individual body weight and length of shrimp postlarvae were recorded for 20% of the population in each treatment at the end of culture period.

Bacterial sampling: Microbiological analyzes performed by sampling 10 mL water from each rearing tank and sampling shrimp for bacterial intestine analysis. Serial dilution (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} etc.) were prepared in sterile saline solution NaCl (1.5%) from the homogenized samples and 0.1 mL was plated in duplicate on NA (nutrient agar) and TCBS (Thio agar. The plates were incubated at 25°C and bacterial

counts performed after 24-48 hours. Number of bacteria was resulted of number colony counted on Petri dish multiply to dilution factor [14].

Bacterial identification: Bacterial identification was done by checking colony morphology of bacteria, Gram test, and 17 biochemical activity tests (carbohydrate fermentation, lipid hydrolysis, starch hydrolysis, casein hydrolysis, urease, TSI, IMViC, MRVP, gelatine, catalase, nitrate reduction, and H₂S) to figure out bacteria diversity in the water during culture period [14]. The result of determination bacterial properties was checked using “Bergey’s Manual of Determinative Bacteriology”.

Statistical analyses: All data were statistically treated using one-way ANOVA. Significant differences among means ($p < 0.05$) were tested by Duncan’s multiple range test.

Results and Discussion

Pathogenicity test of probiotic bacteria to white shrimp postlarvae

Pathogenicity test was done to check whether *H. aquamarina* and *S. algae* give negative effect to white shrimp PL culture or not. The result of pathogenicity is represented in white shrimp survival rate (Figure 2). The result shows that the probiotic bacteria were harmless to shrimp postlarvae and the highest survival rate per centation was in the group addition of *H. aquamarina* probiotic (98%), and followed by control (96%), addition of *S. algae* (84%) and the lowest was result of addition *V. harveyi* (54%). Addition *H. aquamarina* gave better survival rate than addition of *S. algae*. This implied that *H. aquamarina* gave better effect as probiotic than *S. algae*, which was also proven by inhibition zone diameter produced by each probiotic from Kirby Baueur method [12]. Result of addition *V. harveyi* shows that this bacteria give pathogenicity effect to the white shrimp postlarvae, similar to result of former research [11].

Application of probiotic bacteria in PL-4 white shrimp culture

Water quality parameter: Water quality is important factor in white shrimp culture, since water is its life habitat so they can effects each other. Water quality needs to be observed because low water quality will facilitates pathogenic bacteria activity to infect shrimp in the culture and resulted in lower growth and survival rate [15]. Result of water quality parameter measurement including temperature, salinity, DO, pH, and level of ammonia, nitrite, nitrate is presented in Table 2.

Temperature: Water temperature is one factor that affects shrimp metabolism rate, food and oxygen consumption rate, ammonium excretion rate, and growth rate. The increase of temperature trigger the

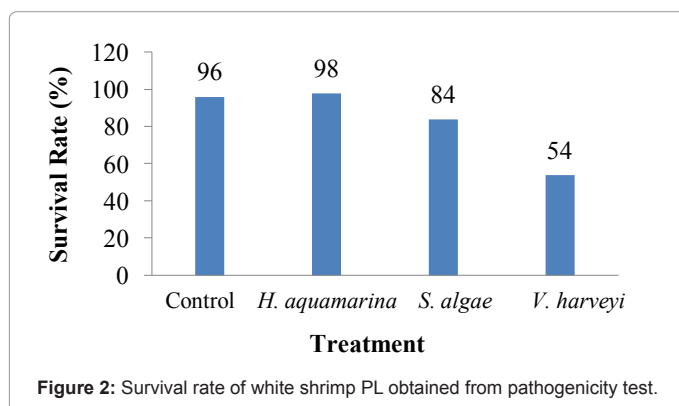


Figure 2: Survival rate of white shrimp PL obtained from pathogenicity test.

Parameters	Optimum Range	Culture Range
Temperature (°C)	28-32	26-28
Salinity (ppt)	0.5-35	26-30
DO (mg.L ⁻¹)	5.0-9.0	5.7-6.4
pH	7.0-8.3	7.5-8.5
Ammonia (mg.L ⁻¹)	<0.03	0.1-0.5
Nitrite (mg.L ⁻¹)	<1.0	0.02-0.25
Nitrate (mg.L ⁻¹)	<60	5-40

Table 2: Water quality parameters of white shrimp PL culture in different treatment during 12 days culture period.

metabolism rising to the maximum rate [16]. Based on the result, there was no fluctuative changes in water temperature. Water temperature during culture period always within range 26-28°C. These were still in the tolerance range of temperature requirement of shrimp to survive (24-32°C). Statistical analysis shows that there was no temperature significant difference of all of culture treatment during culture period ($p > 0.05$). *L. vannamei* is included to poikilothermic organism, therefore the body temperature is influenced by ambient temperature. White shrimp has wide tolerance range of temperature, with maximum temperature achieved within range 28-32°C. Water temperature less than 15°C and more than 35°C (long term) or 45°C (short term), may trigger shrimp mortality [17].

Salinity: Result of this research shows that salinity during culture period were not extremely change. Range of culture salinity is around 26-30 ppt, and it is still in salinity requirement tolerance range for white shrimp [16]. Statistical analysis shows that there was no salinity significant difference of all of culture treatment during culture period ($p > 0.05$).

Based on tolerance range for water salinity, aquatic organism classified into two groups, stenohaline and eurihaline. Stenohaline is organisme which is adaptable to narrow tolerance range environment salinity, and eurihaline is organisme which is adaptable to dynamically changing salinity (wide tolerance range). White shrimp is classified as eurihaline organisme that adaptable to fluctuative salinity [16]. Penaeid shrimp has different salinity range during life time cycle. Larvae and juvenile can adapt to survive even in the high salinity environment, range 1-40 ppt. Adult shrimp needs salinity 28-35 ppt to active reproduce offspring. Shrimp has good osmoregulation capability even life in low salinity. This capability developed gradually since postlarvae [17].

Dissolved oxygen (DO): Oxygen is physiologically needed in the cell for carbohydrate oxidation. In the aquatic environment, DO level is important factor not only for aquatic organism respiration, but also important to maintain chemical environment and water hygiene for organism. Two kind of biologic factors that effect DO level are respiration and photosynthesis [18].

Table 2 shows that DO level of this research were between 5.7-6.4 mg.L⁻¹. As of DO level of this culture still in the tolerance range of shrimp, 5-9 mg.L⁻¹ [16]. DO level may affects food metabolism rate, growth rate, and food conversion rate. Decreasing of DO will lead to decreasing of food metabolism rate, growth rate, and food conversion rate. DO less than 1.5 mg.L⁻¹ for long time can cause shrimp mortality [17]. Statistical analysis shows that there was no DO level significant difference of all of culture treatment during culture period ($p > 0.05$).

Acidity level (pH): pH level is a negative log of hydrogen ion concentration in the water. pH is affected by respiration process, which can make the pH level decrease. CO₂ in the water dissolved to

form H_2CO_3 , pH level less than 6.5 or more than 10 will be dangerous for shrimp gills and affect in decreasing shrimp growth rate. pH range of water culture was 7.5-8.5 and still in the tolerance range pH requirement, 7-9. Statistical analysis shows that there was no salinity significant difference of all of culture treatment during culture period ($p>0.05$). pH level is affected by respiration which can makes pH decreases, and photosynthesis which can makes pH increases. If pH level is too high of low, cultured white shrimp will be influenced. pH <6.5 or >10 is harmful for gill and will reduces shrimp growth rate [16].

One of the main problem source related to disease in shrimp culture is deterioration of water quality. Zero water discharge applied in this research contributes in maintaining water quality parameter within tolerance level of white shrimp PL culture. Addition of $CaCO_3$ stone on the culture tank bottom worked as culture substrate which could help in buffering the pH stability [19].

Ammonia: Ammonia is one of nitrogen compound in the water culture. One of ammonia source is shrimp feed. Feed protein which have been consumed are assimilated and excreted through feces. Twenty percent of food protein enter the culture system and converted to ammonia. Ammonia excreted through gills and dissolved in water. Beside feed, ammonia is also produced from organic matter decomposition by heterotroph bacteria [17].

Ammonia concentration during culture period ranged from 0.1 to 0.5 $mg.L^{-1}$ (Table 2). It shows that ammonia concentration in culture exceeded maximum concentration of shrimp water culture, 0.1 $mg.L^{-1}$. Particularly this high ammonia concentration did not affect white shrimp life significantly. It was caused by the water pH still optimum. Statistical analysis shows that there was no significantly difference of ammonia concentration for every treatment.

In the water system, there are two kinds of ammonium compound. Unionized form as ammonia and ionized form as ammonium. Total nitrogen ammonium of water system is a combination of both ammonium form. Ammonia concentration in the water is affected by pH level and water temperature. At pH level of 7, most ammonium total are in the unionized form. The increase of pH level and temperature trigger the raising of unionized ammonium level. pH level 9 show that 50% of total ammonium are in the unionized form [17]. Unionized ammonium form is toxic compound for shrimp, but ionized form is intoxic. LC_{50} level of unionized ammonium form for juvenile shrimp is 1.26 $mg NH_3-N.L^{-1}$. Unionized ammonium more than 0.1 $mg.L^{-1}$ is still safe and does not trigger shrimp mortality. Unionized ammonium more than 0.3 $mg.L^{-1}$ causing non-lethal effect such as low growth rate, increase of food conversion, decrease of low DO tolerance and disease resistancy [16].

Nitrite: Result of nitrite measurement during 12 days culture ranged from 0.02-0.25 $mg.L^{-1}$ (Table 2). This nitrite concentration is still under the maximum concentration which allowed in the shrimp water culture. Maximum nitrite allowed is 1 $mg.L^{-1}$ [2]. Result of statistical analysis shows that nitrite concentration of each group treatment was not significantly different.

Nitrite is also one of toxic compound for shrimp at the certain level. LC_{50} of nitrite concentration for shrimp is 13.6 $mg.L^{-1}$ in the 96 hours, with safe level recommendation at 1.36 $mg.L^{-1}$ [17]. High nitrite concentration affects oxygen uptake in the red blood cell haemoglobine, and cause asphyxiation. Nitrite is a product from nitrification process by *Nitrosomonas* bacteria. Nitrite can be accumulated in the water when nitrification rate is low. Nitrite toxicity for shrimp is affected by shrimp life cycle and water salinity [17].

Nitrate: Actually nitrate compound is not toxic in the shrimp culture. Based on that result, nitrate concentration was ranged 5-40 $mg.L^{-1}$. These concentration is still below maximum nitrate concentration for shrimp culture, that is 60 $mg.L^{-1}$ [19]. Based on statistical analysis, there was no significantly difference between nitrate concentration of each groups treatment ($p>0.05$). Nitrate concentration from the beginning to the end culture period increase for each of groups treatment. It may be indicates that nitrite in the water system has been converted to nitrate form in the nitrification process. Nitrite is unstable compound with the oxygene level. Because this unstability, nitrite is easy to converted to form nitrate by bacteria [17]. It seems that nitrite concentration in the water system particularly at low level, but nitrate concentration is at high level.

White shrimp PL survival rate and growth

Survival rate of white shrimp larvae was also determined to know the effect of addition *H. aquamarina* and *S. algae* to white shrimp culture infected by *V. harveyi*. Figure 3 shows survival rate of white shrimp PL in the antagonist effect of probiotic bacteria to the *V. harveyi*. The highest survival rate was in the addition of *H. aquamarina* to the culture (KP8) followed by addition of *S. algae* (KP9), *H. aquamarina-S. algae* (KC), *S. algae-V. harveyi* (P9), *H. aquamarina-S. algae-V. harveyi* (PC), *H. aquamarina-V. harveyi* (P8), and control (K). The lowest survival rate obtained from addition of *V. harveyi* (KV).

Statistical analysis shows that there was significant difference for the result of survival rate on addition probiotic treatment with no probiotic. Survival rate of K was different significantly with others treatment, where the survival rate was higher than KV and lower than other six treatment groups. KV also has significant difference with other groups treatment, it shows that addition of pathogenic bacteria, *V. harveyi*, in the culture give lower white shrimp survival rate significantly ($p<0.05$). On the other hand, addition probiotics to the culture help decrease infection of *V. harveyi*.

Addition probiotic to white shrimp PL culture as a single inoculum was not significantly different with addition of mix culture of both probiotics. However, addition of *S. algae* as single probiotic (P9) gave higher survival rate and different significantly with *H. aquamarina* treatment (P8). There are some factors that affect lower survival rate

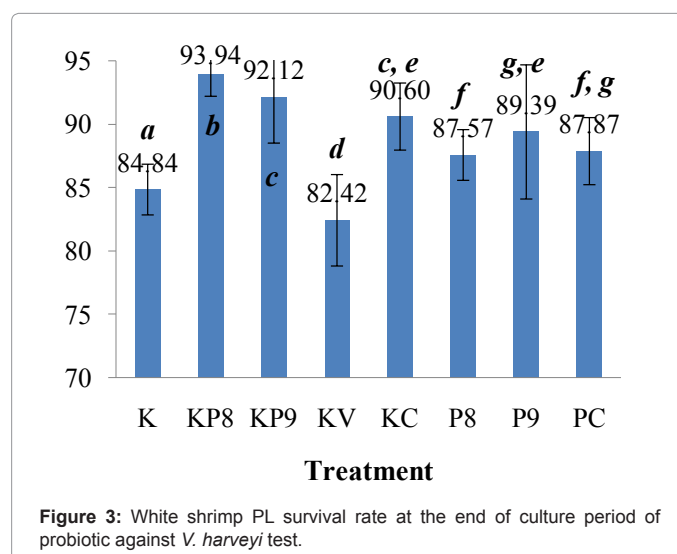


Figure 3: White shrimp PL survival rate at the end of culture period of probiotic against *V. harveyi* test.

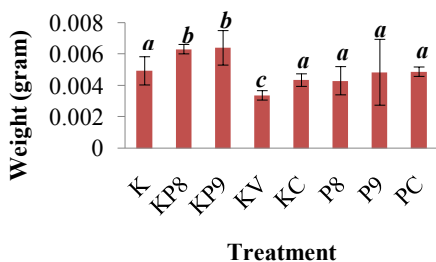


Figure 4: Increase of white shrimp PL weight at the end of culture period.

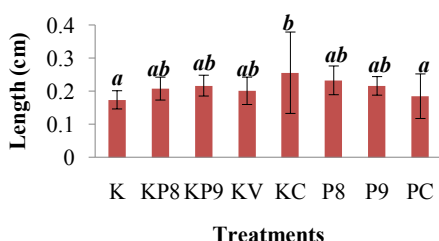


Figure 5: Increase of white shrimp length at the end of culture period.

Species	Name
Species 1	<i>H. aquamarina</i>
Species 2	<i>S. algae</i>
Species 3	<i>V. harveyi</i>
Species 4	<i>Spingobacterium</i> sp.
Species 5	<i>Bacillus</i> sp.
Species 6	<i>Micrococcus</i> sp.
Species 7	<i>Pseudomonas</i> sp.
Species 8	<i>Alcaligenes</i> sp.

Table 3: Species of identified bacteria during white shrimp culture period.

due to application of mix probiotic culture: different of probiotic growth characteristic, uptake nutrient ability, doubling time, space competition and communication [20].

Growth of white shrimp postlarvae was observed from individual average of body weight and length that measured at the beginning and the end of the culture period. In general, the average of body weight and length of individual postlarvae had increased. From the collected data (Figure 4), the highest body weight acquired by KP9, KP8, K, PC, P9, KC, P8, and the last is KV. The result of statistical analyses indicating that growth of body weight is significantly different for group KV, it indicated that addition of *V. harveyi* to the culture provided negative effects shown by the lowest final body weight. Over all, there was no significantly differences of final body weight for each group. The addition of probiotic to challenge *V. harveyi* did not give a different higher final body weight significantly.

Figure 5 shows data of white shrimp PL body length. The highest body length increase acquired by KC, followed by P8, KP9, P9, KP8, KV, PC, and the last is K. Based on statistical analyses, there is no any significantly differences of body length for all of the group ($p>0.05$). It indicates that addition of probiotic did not give any effect to body length. However, there were some significant differences of body weight increase for some treatment group. KV was different significantly with

other groups, by the lowest body weight. And addition of probiotic (KP8 and KP9) were different significantly with other groups.

Bacteria identification: Bacteria which were found both in water and shrimp's intestine sample were identified through biochemistry test method (Table 3). Generally, *H. aquamarina* and *S. algae* were found in all of culture treatment group. It shows that these two probiotics are indigenous bacteria in white shrimp culture.

Population of *H. aquamarina*, *S. algae* and *V. harveyi* during culture

Bacteria population in culture water: Bacteria population checked from 1 mL sample of *L. vannamei* culture water shows that there were various bacterial species found in each treatment as shown in Figure 6. Probiotic *H. aquamarina* and *S. algae*, as well as the pathogen, *V. harveyi* could be found in all treatment with different level of cell number. This condition means all bacteria were actually also indigenous bacteria that originally inhabit seawater or sea culture.

Treatment K with no isolate was added, still shown presence of both probiotics and *V. harveyi*. It shows that these three bacteria are indigenous bacteria in the white shrimp culture. *H. aquamarina*, *S. algae* and *V. harveyi* were found since the beginning of culture period. *V. harveyi* could grow in water culture even there was inhibition by the two probiotics. Number of *V. harveyi* was reached 10^4 CFU.mL⁻¹ since day 2. In the middle of culture period, *V. harveyi* was increased and then followed by decrease that resulted of *H. aquamarina* and *S. algae* activity interaction.

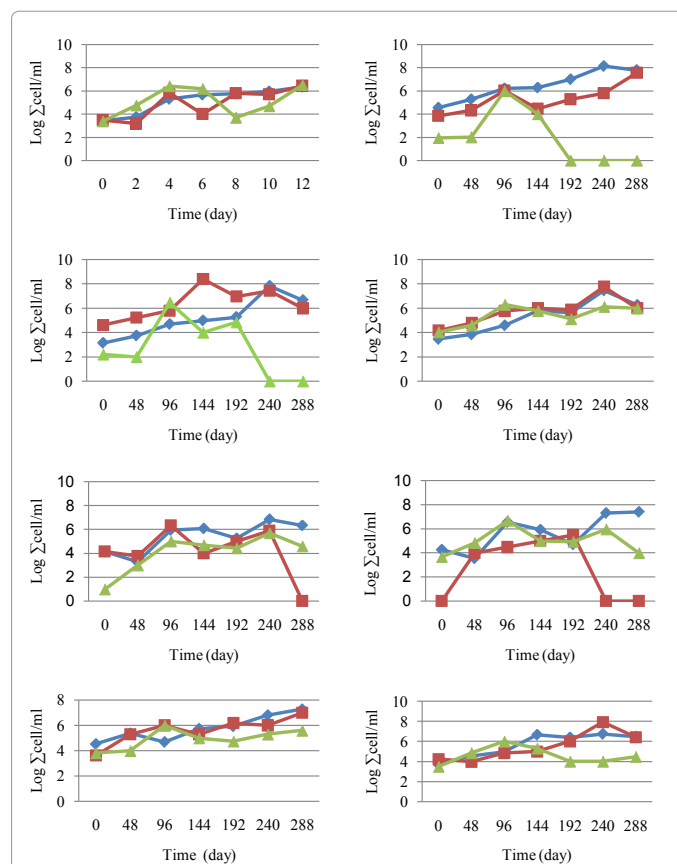
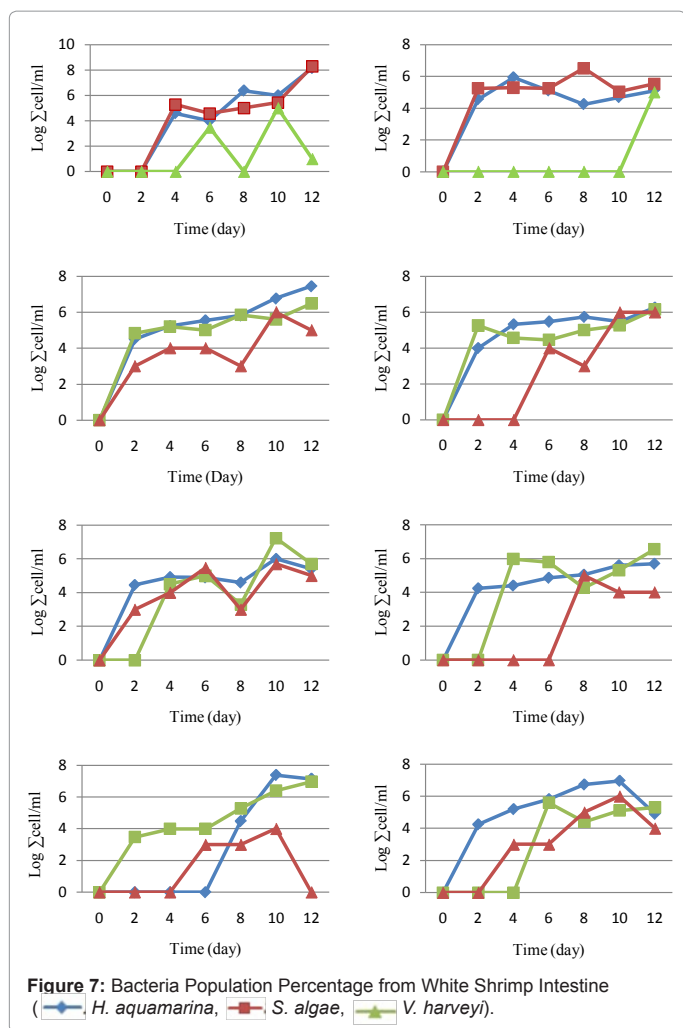


Figure 6: Bacteria Population Percentage from Water Sample of White Shrimp Culture (—◆— *H. aquamarina*, —■— *S. algae*, —▲— *V. harveyi*).



Treatment KP8 with addition of *H. aquamarina*, makes this bacteria able to dominate during white shrimp culture period. Growth of *H. aquamarina* tend to increase since the beginning until the end of culture period. *S. algae* were not added to the culture, still shown its existence and growth, which further indicates that this bacteria is indigene bacteria in white shrimp culture. *V. harveyi* was found in the culture since the beginning, but then was not found any more since the middle to the end of culture period. This possibly was a result of *H. aquamarina* and *S. algae* activity that existed and continued to dominated bacteria population in the culture. These results were also consistent with the result of survival rate, which was the highest, due to antagonistic activity of probiotics against *V. harveyi*.

In KP9 treatment group, *S. algae* were added to white shrimp culture. Based on the result, the addition of *S. algae* to the culture made these bacteria dominated at the beginning of culture period and then continues to grow. In this group, *H. aquamarina* and *V. harveyi* were also found. Similar with *S. algae*, *H. aquamarina* was grown during culture period. The growth of these two probiotics made growth of *V. harveyi* became decreased and then not found in the culture. The result of both probiotics activity in inhibiting *V. harveyi* was consistent with result of survival rate.

In KV treatment group, *V. harveyi* was added to white shrimp culture. Based on the result, addition of *V. harveyi* to the culture made

this bacteria population remain high during culture period, although probiotic *H. aquamarina* and *S. algae* also existed. The existence of these two probiotics did not give significant inhibition to *V. harveyi* growth in this group treatment. *V. harveyi* has achieved a high number since the beginning of culture period, which may support it to become pathogenic bacteria. *V. harveyi* infected white shrimp, so its survival rate became the lowest.

In KC treatment group, *H. aquamarina* and *S. algae* were added to white shrimp culture. Based on the result, addition of both probiotics as mix culture made their number become stable at the beginning of culture period, whereas *V. harveyi* number was low. During culture period, growth of *V. harveyi* was increased until the end of period. It shows that addition of probiotic as mix culture decreased both probiotics activity in against *V. harveyi*. In other word, growth type of both probiotics was neither symbiotic nor synergic. It was consistent with white shrimp survival rate, which shown that survival rate was lower than application of probiotic as single culture (KP8 and KP9).

In P8 treatment group, *H. aquamarina* and *V. harveyi* were added to the culture. *H. aquamarina* dominated bacteria population of this treatment group during culture period. Compared with the control (K) and addition of *H. aquamarina* (KP8), the growth of *H. aquamarina* in P8 was not as optimal as KP8. This was the result of competition between *H. aquamarina* and *V. harveyi*, in term of nutrient and space. This competition also affected inhibitory activity of *H. aquamarina* against *V. harveyi*, shown by lower survival rate obtained.

In P9 treatment group, *S. algae* and *V. harveyi* were added to the culture. In this treatment group, each of *H. aquamarina*, *S. algae* and *V. harveyi* has balanced growth during culture period. Even *H. aquamarina* was not added to the culture, it was still existed during culture. The addition of *V. harveyi* increased its ability to infect white shrimp, eventhough both probiotics existed in the culture. It is consistent with result of survival rate, when *V. harveyi* was added to the culture, survival rate became lower.

In PC treatment group, *H. aquamarina*, *S. algae* and *V. harveyi* were added to the culture. In the graph, shown that each bacteria was fluctuated during culture period. The number of *V. harveyi* decreased at the end of culture, as a result of probiotics inhibition activity.

Bacteria population in *L. vannamei* intestine: Bacteria found in *L. vannamei* intestine samples are mostly same with those found in culture water (Figure 7), except one different species assumed to be normal microflora of *L. vannamei*, *Micrococcus* sp. Sample of crushed intestine was diluted in 0.85% NaCl solution is checked, it gave varies result among treatments. Result of treatment K demonstrates that both probiotics, *H. aquamarina* and *S. algae*, and *V. harveyi* were consumed by shrimp culture. *Micrococcus* sp. is found in this culture. Dynamic growth of each culture was shown, *H. aquamarina* was still increasing by days.

In treatment KP8, both probiotics could be found since day 2, each species fluctuated and tend to be decreasing. *V. harveyi* found only in the last day, dominating bacteria in this culture was *Bacillus* sp, which also classified as normal microflora of shrimp culture. Whereas in treatment KP9 *V. harveyi* has already found since the first sampling, it could not be reduced completely that it was still found in day 12. It shows that adding only *S. algae* to the culture is not an effective preventive method against pathogen.

Addition of pathogen isolate to the culture affects not only microbial diversity in water, but also in the shrimp's intestine. In treatment KV,

colonies of this pathogen found in a big number since day 6, it reaches the highest level in the last sampling day. Comparing to microbial diversity chart in culture water, it figures that in the middle of culture period where *V. harveyi* seemed to be highly reduced, it was actually consumed and penetrate *L. vannamei* intestine. Shrimp cultures have been attacked by *V. harveyi* colonization resulting a low percentage of survival rate.

Growth rate of both *H. aquamarina* and *S. algae* in treatment KC shows an antagonistic activity of both culture. *V. harveyi* was found since day 0 to day 12, means that if there was any inhibition action in shrimp's intestine, it could not work properly to inhibit this pathogen to grow continuously. *Micrococcus* sp could also grow well in the culture. It is the same as treatment 8, cell number of *H. aquamarina* was becoming more dominant.

Treatment P8 and P9 pictures the ability of *V. harveyi* to habitant *L. vannamei* intestine in the middle of culture period, around day 6 and 8. This condition shows that inhibition mechanism performed by *H. aquamarina* in secreting bacteriosin, which considered more effective than *S. algae*, works better in the water rather than inside the shrimp intestine.

Based on the whole result of bacterial activity and survival rate of white shrimp PL, *H. aquamarina* and *S. algae* have the ability to inhibit or reduce pathogenicity effect of *V. harveyi* in white shrimp culture. Although this research was not able to analyse the exact mode of action of each probiotic, there are some previous research which show several mechanisms of bacteria from Genus *Halomonas* and *Shewanella* as probiotic. As it stated by Bitzer et al. [21] that halophilic bacteria from Genus *Halomonas* are able to produce antibiotic aminophenoxazinones which has been proven in inhibiting growth of gram negative bacteria, *E. coli*. Based on these ability, it is likely that *Halomonas* also able to inhibit other Gram negative bacteria such as *Vibrio harveyi*. Even so, further research is needed to prove the hypothesis. Other research stated that *H. aquamarina* has the ability to produce siderophore molecule, aquachelins, which plays a role to bind, as a solvent, and transporter of Fe³⁺ complex from aquatic environment in to cell. This ability contribute in helping white shrimp to maintain important nutrition in the body [22]. In addition, *H. aquamarina* plays a role as non-pathogen bacteria which help reduce pathogen bacteria infection in shrimp intestine by producing exoenzyme and organic compound (compatible solutes) to control defense of intestinal mucus [23,24].

On the other hand, *S. algae* as probiotic produces biofilm which help attachment and colonization on white shrimp intestine, produce bacteriocin, and also produces pigmen compound (pyomelanin) which protect this bacteria from hydrolytic enzyme in aquatic environment. Pyomelanin also known has important role in biogeochemical cycle of Fe in aquatic environment [25-27].

Conclusion

During culture period, water quality parameters consisting of temperature, salinity, pH level, and dissolved oxygen (DO), ammonia, nitrite, and nitrate concentrations mostly tended to be stable and there were no significant difference by days. The lowest survival rate given by treatment 4 with addition of only *V. harveyi* isolate. Although both probiotics perform an inhibitory activity to challenge *V. harveyi*, *H. aquamarina* was dominating and found to be more effective than *S. algae*. Thus, single culture of *H. aquamarina* could perform better result to maintain *L. vannamei* postlarvae culture rather than mixed culture, since the growth rate of both probiotics did not show neither symbiotic or synergic activity.

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