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PERFORMANCE OF CULTURED WHITE-LEG SHRIMP IN RICA PROBIOTIC APPLICATION METHOD IN PONDS AERATED WITH SUPERCHARGE BLOWER

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ABSTRACT

Several ways have been done to encounter shrimp disease affecting cultured shrimp in Indonesian ponds in the last two decades. This research was aimed to find out the effect of different application of probiotic RICA4, RICA5, and RICA3 method on survival rate and production of white-leg shrimp (*Litopenaeus vannamei*) cultured in ponds aerated with supercharge blower. RICA probiotics are bacteria probiotics produced by the Research and Development Institute for Coastal Aquaculture, originally isolated from seaweed and sea sediment. This experiment was carried out in completely randomized design using nine 250-m^2 experimental ponds stocked with 15 shrimp fries/m^2. There were three treatments namely: A = alternate use of three probiotics RICA4, RICA5, and RICA3; B = combination use of three probiotics RICA4, RICA5, and RICA3; and C = control (without probiotic), each treatment with three replications and cultured with supercharge blower. Variables observed in this study were survival rate and production of the shrimp calculated at the end of experiment, total vibrio count (TBV) and total plate count of common bacteria (TPC) of the pond waters and sediments monitored every two weeks. The results showed that application of probiotic RICA4, RICA5, and RICA3 applied either in alteration or in combination significantly increased survival rate ($P < 0.05$) but not on production ($P > 0.05$) of the white-leg shrimp. TBV/TPC ratio in the control pond waters after 10-weeks culture (over than 10%) was relatively dangerous for the cultured white-leg shrimp. This shows that application of probiotic could prevent the growth of *Vibrio* spp in the cultured shrimp pond water.

KEYWORDS: **application method; RICA probiotic; TBV/TPC ratio; white-leg shrimp**

INTRODUCTION

Over than two decades tiger shrimp (*Penaeus monodon*) culture in Indonesia has been affected by pathogenic *Vibrio harveyi* and *White Spot Syndrome Virus* (WSS). Then, the white-leg shrimp (*Litopenaeus vannamei*) has been domesticated to replace it in some areas like in Lampung, East Java, and South Sulawesi. Initially, white-leg shrimp was known to be more resistant than tiger shrimp to pathogenic viruses (Subyakto *et al*., 2009). Factually now some pathogens like vibrios and viruses have been destroying the white-leg shrimp culture. Luminescent *Vibrio harveyi* as well and is considered to be opportunistic pathogenic bacteria causing shrimp disease when the pond water quality is worse (Atmomarsono & Susianingsih, 2015). To encounter this problem, best aquaculture practices should be done properly as well as using local probiotics.

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According to Poernomo (2004) probiotic bacteria is non pathogenic bacteria usually used for protecting the shrimp from pathogenic bacteria, neutralizing of poor pond water quality (demineralization of organic matter, H_2S, NH_3-N, and NO_2-N). Probiotic bacteria can be isolated from the sea sediment, pond sediment, mangrove leaves, and macroalgae (Haryanti *et al*., 2000; Muliani *et al*., 2003, 2004; Atmomarsono *et al*., 2009; Susianingsih *et al*., 2012; Tampangallo *et al*., 2013).

Until today *Balai Penelitian dan Pengembangan Budidaya Air Payau Maros* (Research and Development Institute for Coastal Aquaculture) has collected 4,226 bacteria isolates originated from the sea sediment, brackishwater pond, mangrove, and seaweed. Five isolates were finally chosen for probiotic called as RICA (Research Institute for Coastal Aquaculture) probiotics. RICA1 was useful for controlling organic matter and H_2S in the ponds, while RICA2 was important for the shrimp growth and controlling ammonia-nitrogen (Muliani *et al*., 2006; Atmomarsono *et al*., 2009). RICA3 had important role for controlling nitrite and pathogenic *Vibrio harveyi* (Muliani *et al*., 2003; Atmomarsono & Susianingsih, 2013). RICA4 was useful for controlling organic matter and *Vibrio harveyi* in the pond water. While RICA5 was useful for controlling ammonia and nitrite in the pond water (Tampangallo *et al*., 2013). If several different useful bacteria are available in the pond waters naturally or by application, then pond water quality will be better for the shrimp life.

Shrimp diseases are usually occurred when accumulation of organic matter in the pond waters and sediments could not be controlled properly by natural bacteria. Since each RICA probiotic bacteria has different role, then three different RICA probiotics are selected to control organic wastes, ammonia-nitrogen, nitrite-nitrogen, and pathogenic *Vibrio harveyi*.

This research was aimed at knowing the effect of RICA probiotic application (RICA3, RICA4, and RICA5) on survival rate and production of white-leg shrimp in the ponds aerated with blower supercharge.

MATERIALS AND METHODS

This experiment was carried out in Marana Experimental Ponds of BPPBAP (Research and Development Institute for Coastal Aquaculture) Maros from March to July 2015 using nine 250-m^2 concrete-wall experimental ponds aerated with *blower supercharge*. Each pond was stocked with 3,750 pieces PL-12 of WSSV-negative fries white-leg shrimp (15 pcs/m^2). Completely Randomized Design (CRD) was applied in this research for three treatments and three replications each. The three treatments tested were: A) alternate use of three probiotics RICA4, RICA5, and RICA3; B) combination use of three probiotics RICA4, RICA5, and RICA3; and C) control (without probiotic). Each probiotic was cultured separately for three days before applied weekly in the shrimp ponds. The reared shrimp was fed with shrimp pellet for about 50% of total biomass per day at initial stocking, and decreased to 2% of total biomass per day up to harvest time (98 days).

For A treatment, RICA4 was applied on the first and second week, then replaced by RICA5 on the third and the fourth week, then followed by RICA3 on the fifth and the sixth week, then followed by RICA4 and RICA5 again. For B treatment, the three RICA probiotics were applied weekly in the ponds. Each probiotic was cultured for three days using rice bran (1,000 g), fishmeal (400 g), molasses (500 g), and yeast (100 g) for 20 L of water media. Rice bran and fishmeal should be cooked and boiled for about 10 minutes with the pond water. Molasses and yeast were added to the mixture after the stove was turned off. Certain RICA probiotic was added when the mixture was cooled (less than 40°C). The mixture was then aerated with AC/DC aerator for about three days. The RICA probiotic was applied once a week separately or in combination. All ponds were provided with supercharge blower.

RICA3 is *Pseudoalteromonas* sp Edeep-1, isolated from the sea sediment. This bacterium is useful for killing *Vibrio* spp. and considered to have role in changing nitrite-nitrogen to nitrate-nitrogen. RICA4 is *Bacillus subtilis*, isolated from sea macroalgae (*Kappaphycus alvarezii*). This bacterium is useful for organic matter demineralization and controlling the Vibrio growth of the pond water. RICA5 is *Bacillus licheniformis*, isolated from sea macroalgae (*Kappaphycus alvarezii*). This bacterium is mainly used for controlling ammonia-nitrogen in the pond water.

Survival rate and production of white-leg shrimp in each pond were calculated at the end of research (98 days) and analyzed quantitatively. Using modification of spreading method (Buller, 2004), total bacteria of *Vibrios* (TBV) were measured in *Thiosulphate Citrate Bile Sucrose Agar* (TCBSA) and total general bacteria (total plate count = TPC) were measured in *Tryptic Soy Agar* (TSA). Both TBV and TPC were monitored every other week from pond waters and sediments, and they were analyzed descriptively. TBV/TPC ratios of the pond waters were also calculated in percentage of the number of TBV and TPC in each sample. When TBV/TPC ratio is increase more than 10%, some pathogenic *Vibrio* spp. might be harmful to the shrimp (Atmomarsono & Nurbaya, 2014).

Measurement of total haemocyte (THC) from the shrimp haemolymph to see immune response of the white-leg shrimp due to RICA probiotic application was carried out before and after probiotic application for three times (first month, second month, and third month of experiment). Total haemocyte (THC) of the shrimp hemolymph was measured through modified procedures used by Blakxhall & Daishley (Braak, 2002). About 0.1 mL of the white-leg shrimp haemolymph was taken from the second abdomen segmen using 1.0-mL 26-G syringe already filled with 0.3 mL anticoagulant of Na-Citrate 3.8%. The mixture was then homogenized by eight-formed hand shaking. The first drop was discharge, then the second drop was put into haemocytometer (*Improved Neubauer type*) to count haemocyte under light binocular microscope using 10 x 10 magnification. Total haemocyte was then calculated using below formula:

$$N = \left(\frac{n1 + n2 + n3 + n4 + n5}{5} \right) x\ 25\ x\ 10^4$$

where:
N = total haemocyte (cell/mL)
n1, n2, n3, n4, n5 = haemocyte number in each view of haemocytometer (cell)

Water quality parameters like total organic matter (TOM), ammonia-nitrogen (NH_3-N), nitrite-nitrogen (NO_2-N), and nitrate-nitrogen (NO_3-N) were monitored every other week using spectrophotometer in water quality laboratory of the Research and Development Institute for Coastal Aquaculture.

Analysis of varian was applied for quantitative data like survival rate and the shrimp production in each treatment. TBV and TPC of the pond waters and sediment, and total haemocyte were descriptively reported using graph and figures.

RESULTS AND DISCUSSIONS

Survival Rate and Production

Survival rate and production of white-leg shrimp (*Litopenaeus vannamei*) by the end of experiment in each treatment are shown in Figure 1. It is shown in this figure that the use of three combination of RICA probiotics (B treatment) had relatively better result ($P>0.05$) in survival rate (81.4 ± 3.9%) and production 42.1 ± 4.2 kg/250 m^2 (1,685 ± 168 kg/ha) than that of alternate use of the three RICA probiotics (A treatment) having (79.1 ± 2.3%) in survival rate and 38.4 ± 4.1 kg/250 m^2 (1,537 ± 164 kg/ha) in shrimp production. Control ponds (C treatment) had the lowest survival rate (70.9 ± 2.7%) ($P<0.05$) and shrimp production 36.4 ± 2.6 kg/250 m^2 (1,455 ± 104 kg/ha) ($P>0.05$).

Survival rate of the shrimp in treatment A was not significantly lower ($P>0.05$) than that of in treatment B. There were no significantly different ($P>0.05$) in production of white-leg shrimp in this experiment, even though application of the RICA probiotics tended to have relatively better production than control. This result indicated that application of RICA4, RICA5, and RICA3 either applied in alternately or in combination caused less shrimp mortality than that of control. Similar result found by Widanarni *et al*. (2014), that the highest survival rate of vanname shrimp (*Litopenaeus vannamei*) challenged with *Vibrio harveyi* and infectious myonecrosis virus was achieved by probiotic application (88.33%), followed by sinbiotic (85%), and control (81.67%), while the lowest survival rate was achieved by prebiotic application (76.67%). Amin & Hendrajat (2008) reported that application of commercial probiotic at concentration of 0.5–1.5 mg/L/week in white-leg shrimp pond could enhance better survival rates (92.33%–94.33%) than that of in control shrimp pond (86.33%). Verschuera *et al*. (2000) also mentioned that adding probiotic bacteria in the shrimp culture media could function as food complement or contribute to food digesting system and prevent the cultured shrimp from pathogenic microorganisms.

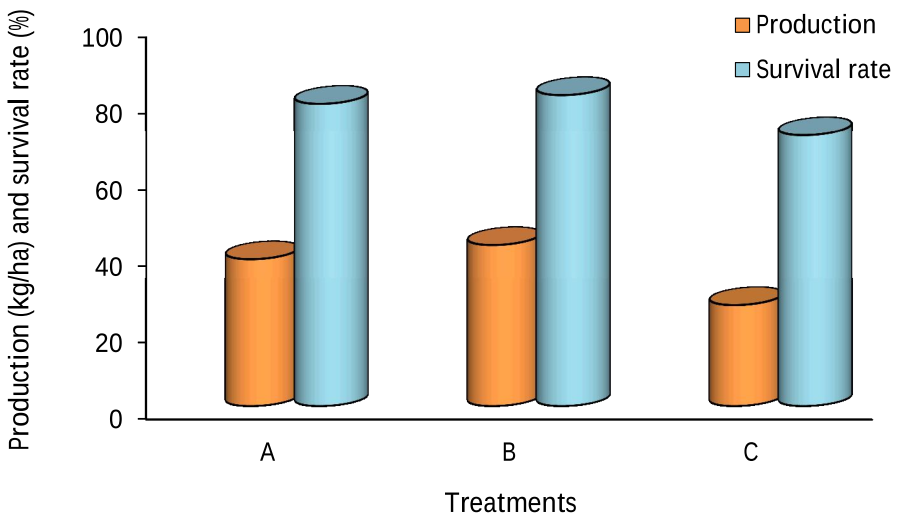

Figure 1. Production and survival rate of white-leg shrimp (*L. vannamei*) after 98-d treatment with different method of the RICA probiotic application (Remarks: A= alternate use of three probiotics RICA4, RICA5, and RICA3; B= combine use of three probiotics RICA4, RICA5, and RICA3; C= control (without probiotics) (It should be shown in two-axes graph).

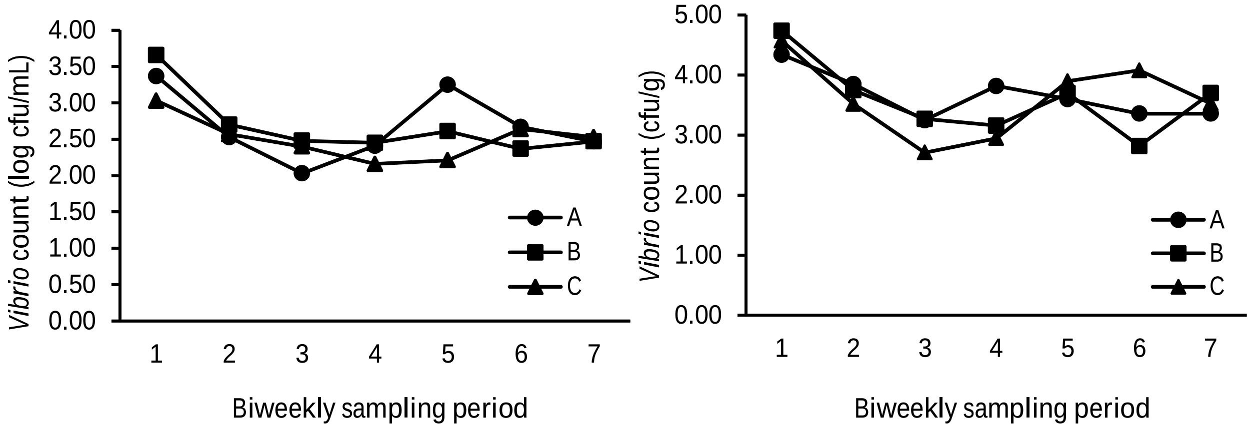

Figure 2. Total bacteria of vibrios (TBV) in white-leg shrimp pond waters (a) and pond sediments (b) during 98-d experiment. A= alternate use of three probiotics RICA4, RICA5, and RICA3; B= combine use of three probiotics RICA4, RICA5, and RICA3; C= control (without probiotic).

Total Bacteria of Vibrios (TBV) and Total Common Bacteria (TPC)

During 98-d experiment, ranges of TBV in white-leg shrimp pond waters were 10^2-10^3 cfu/mL, while in the pond sediments were 10^2-10^4 cfu/g (Figure 2).

Relatively similar fluctuation of *Vibrio* bacteria number in the pond waters of the three treatments tested were shown here regardless in probiotic-treated ponds or in control ponds (Figure 2). According to Taslihan *et al*. (2004), the maximum number of *Vibrio* spp. in the pond waters should be less than 10^4 cfu/mL. If the Vibrio number is more than this level, the mass mortality of the cultured shrimp might occur in the pond. Based on this reference, the *Vibrio* numbers in the cultured shrimp pond water during experiment (about 10^3 cfu/mL) were still safely for the life and the growth of cultured white-leg shrimp.

Total bacteria of *Vibrios* (TBV) in the shrimp pond sediments in C treatment (control) increased over than 10^4 cfu/g at the sixth sampling period (after 10-wk culture), while the vibrio numbers in A and B treatments were about 10^3 cfu/g (Figure 2b). These results indicated that accumulation of uneaten feed and shrimp feces in the pond sediment might enhance the growth of *Vibrio* spp. in C treatment (control), while the use of three probiotics RICA4, RICA5, and RICA3 could prevent the growth of *Vibrio* spp. in the pond sediment of A and B treatments. Muliani *et al*. (2006) and Atmomomarsono *et al*. (2009) reported that probiotic RICA3 (BL542, *Pseudoalteromonas* sp. Edeep-1) applied in this experiment could prevent the growth of *Vibrio* spp. Yudiati *et al*. (2010) also reported that population of *Vibrio* spp. were much more in control water than in probiotic-treated water. Preventing the growth of pathogenic *Vibrios* by probiotic bacteria is mainly caused by probiotic producing anti bacterial substances like bacteriosin, lysozime, protease, siderophore, hydrogen peroxide, and organic acids (Verschuera *et al*., 2000).

During 98-d experiment, ranges of total common bacteria (TPC) in the shrimp pond waters were 10^4–10^5 cfu/mL (Figure 3a), while in the pond sediments were 10^5-10^6 cfu/g (Figure 3b). According to Taslihan (2004) these numbers of TPC in the pond water were still safely for the shrimp life that is less than 10^6 cfu/mL.

According to Widiyanto (2006), population of bacteria in the pond sediment is generally much more than that of in the pond water because of more organic waste in the pond sediment caused by accumulation of remnant feed and fish/shrimp feces. Beside that the pond water is more frequently changed to get better water quality through periodic water exchange.

Total common bacteria (TPC) in the pond waters and sediments for the whole ponds were relatively similar fluctuation (Figure 3). This means that application of probiotic bacteria could not change the population of common bacteria in the pond waters and sediments. Sukenda *et al*. (2006) reported that adding some probiotics in the culture system could increase the bacterial biomass in the waters caused by probiotic bacterial biomass itself and relationship between probiotic bacteria and the natural bacteria in the culture media producing their proliferation.

The ratio of *Vibrio* spp. numbers (TBV) and common bacteria (TPC) seemed to have relationship with the survival rate of cultured white-leg shrimp. As previously mentioned that survival rate of cultured white-leg shrimp in C treatment (70.9%) was significantly

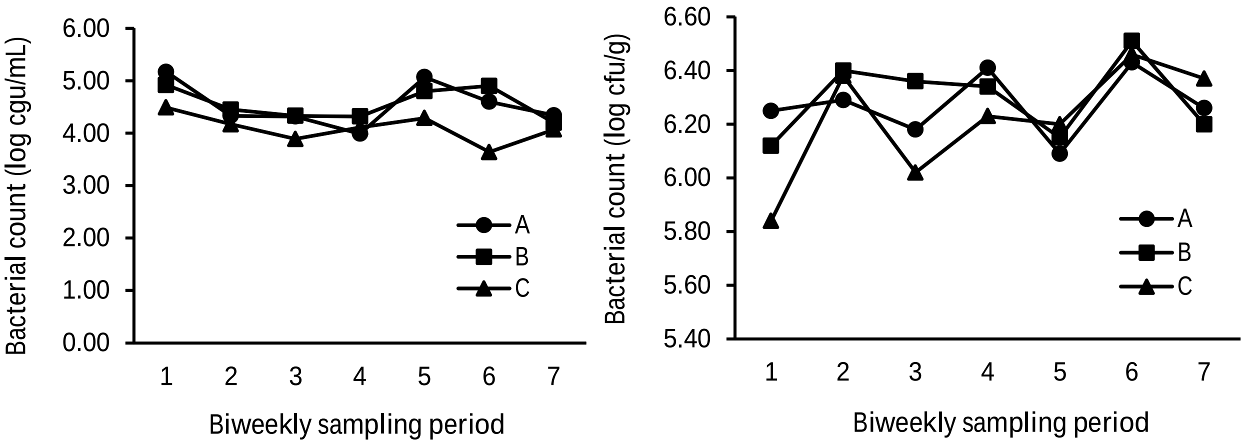

Figure 3. Total common bacteria (TPC) in white-leg shrimp pond waters (a) and pond sediments (b) during 98-d experiment; A= alternate use of three probiotics RICA4, RICA5, and RICA3; B= combine use of three probiotics RICA4, RICA5, and RICA3; C= control (without probiotic).

(P<0.05) lower than those of in treatment A (79.1%) and B (81.4%). Some mortality of the cultured white-leg shrimp in control (C) ponds may have been caused by the increase number of the *Vibrio* ratio in the reared white-leg shrimp pond water that increased over 10% of total common bacteria (Table 1). Actually TBV in the control shrimp pond water did not increase much, but TBV/TPC ratio increased to 17.48% since TPC in the control shrimp pond water decreased. Atmomarsono & Nurbaya (2014) also reported that percentage of TBV/TPC ratio in the shrimp pond water over than 10% was dangerous for the cultured shrimp.

Related to this finding, Sukenda *et al*. (2006) also reported that *Vibrio* number (TBV) in the culture media treated with probiotic was less than those of in control culture media. This might be caused by enzyme or other chemical substances produced by probiotic that prevent the growth of *Vibrio* spp. Verschuera *et al*. (2000) reported that the growth of *Vibrio* might be prevented specifically by other bacteria producing inhibitor substances, developing their ability to use iron and other chemical substances for their energy. When the *Vibrio* mortality increased, the number of total common bacteria (TPC) would increase and consequently the TBV/TPC ratio would decrease (Moriarty, 1999).

Immune Response

Total haemocyte of the shrimp haemolymph at three sampling periods (first month, second month, and third month of experiment) is presented in Figure 4.

Based on the three sampling periods shown in Figure 4, total haemocyte in the shrimp haemolymph (THC) in each treatment varied. Increasing of THC after treatment was only found in treatment A, that was from 23.47 x 10^6 cells/mL to 27.33 x 10^6 cells/mL at early culture period, and from 5.9 x 10^6 cells/mL to 6.63 x 10^6 cells/mL at the end of experiment. Application of three RICA probiotics RICA4, RICA5, and RICA3 alternately increased THC of the shrimp haemolymph,

Table 1. The ratio of *Vibrio* spp. number compared to total bacteria (%) in the white-leg shrimp culture pond water; A= alternate use of three probiotic bacteria RICA4, RICA5, and RICA3; B= combine use of three probiotic bacteria RICA4, RICA5, and RICA3; C= control (without probiotic bacteria)

Treatments	TBV/TPC ratio (%) at different shrimp culture period (weeks)						
	0	2	4	6	8	10	12
A	3.14	2.56	0.55	3.05	1.37	1.14	1.35
B	6.33	2.11	4.23	4.61	2.52	0.63	1.73
C	3.47	3.19	**14.27**	1.72	0.89	**17.48**	**34.51**

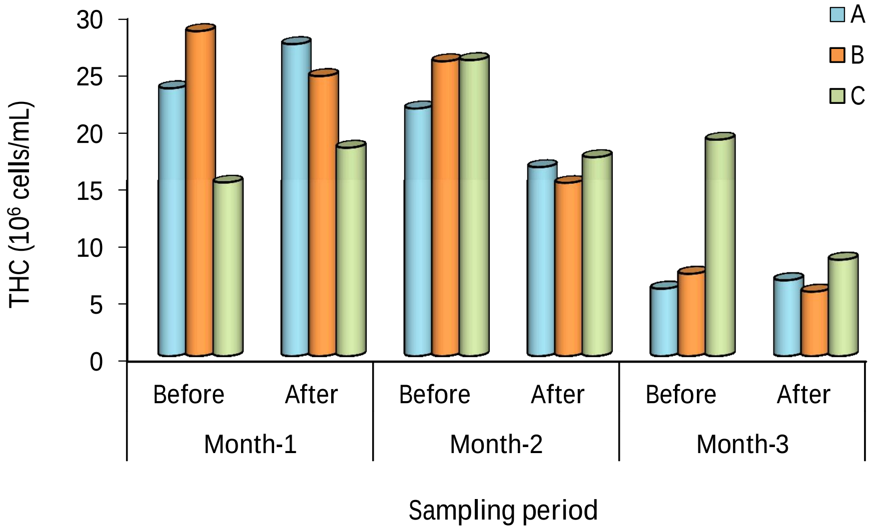

Figure 4. Total haemocyte (THC) of the cultured white-leg shrimp haemolymph measured three times before and after treated with probiotics (Remarks: A= alternate use of three probiotics RICA4, RICA5, and RICA3; B= combine use of three probiotics RICA4, RICA5, and RICA3; C= control (without probiotic)).

even it was not so high. This meant that probiotic application did not have effect on the immune response of the white-leg shrimp, since there were no special pathogens might be harmful to the shrimp during that time. Generally, the vibrio numbers (TBV) in the shrimp pond water during 98-d experiment were mostly safe for the cultured shrimp (less than 10^4 cfu/mL). The vibrio numbers (TBV) in the shrimp pond water at beginning were quite high (close to 10^4 cfu/mL) (Figure 2). That is why the shrimp THC was relatively high at beginning as its response.

As we know that haemocyte has very important role on the shrimp immune response in protecting the shrimp from pathogenic organisms. Haemocyte actively eliminates all foreign substances in haemocoele through phagocyte, encapsulation, and nodular aggregation. Phagocyte is a very first common reaction in the shrimp cellular to protect the cells from pathogenic organisms. Phagocyte mechanism starts from particle attachment and particle swallowing into phagocyte cell. Then this phagocyte cell forms phagosome and along with lysosome will form phagolysosome that will destroy pathogenic microorganisms and eliminate them from the cells through digestion process (Rodriguez & Lee Moullac, 2000).

Fluctuation of total haemocyte (THC) of the white-leg shrimp haemolymph was mainly caused by no stressor factors in the shrimp pond that might enhance the immune response of the shrimp showed by prophenol-oxidase activity. Usually this reaction will happen if any stressor factor in the pond water like the increase number of TBV (over 10^4 cfu/mL). However this number could be minimized to less than 10^3 cfu/mL by applying certain appropriate RICA probiotics especially RICA1 (*Brevibacillus laterosporus*) or RICA3 (*Pseudoalteromonas* sp. Edeep-1) (Atmomarsono & Nurbaya, 2014). This RICA3 probiotic could protect the growth of *Vibrio* spp. through controlling the organic matter first.

Pond Water Quality

Generally, white-leg shrimp pond water quality parameters like total ammonia-nitrogen (NH_3-N), nitrite-nitrogen (NO_2-N), and nitrate-nitrogen (NO_3-N) during experiment were still in good ranges for white-leg shrimp life.

Concentrations of total organic matter (TOM) in the shrimp pond waters were 47.86-60.68 mg/L in ranges. These concentrations in the shrimp pond waters were relatively high. Madeali *et al.* (2009) reported that the increase of total organic matter (TOM) concentration in the shrimp pond water up to 30 mg/L could increase the virulence of *Vibrio* spp. The increase of TOM concentration in the pond water is considered to be one of the stressors for the reared shrimp. Application of certain probiotic bacteria like in A and B treatments could alleviate the problems through organic control in the pond water. Probiotic RICA3 (*Pseudoalteromonas* sp. Edeep-1) could destroy organic wastes in the ponds and also protect the growth of pathogenic *Vibrio harveyi* in the pond waters.

Total ammonia-nitrogen (TAN) concentrations in the shrimp pond waters were also fluctuated during culture period, there were 0.0316–0.1535 mg/L in A treatment, 0.0252-0.1542 mg/L in B treatment, and 0.0707–0.1221 mg/L in C treatment. Poernomo (2004) reported that the shrimp growth was decreasing about 50% when total ammonia-nitrogen concentration in the pond water reaches 0.45 mg/L, and some shrimp mortality occurred when TAN concentration increases up to 1.29 mg/L. Increasing of TAN concentration in the shrimp pond waters is mainly caused by the accumulation of organic wastes (uneaten feed and shrimp feces) related to the culture period. Besides that, the increase of pond water salinity also causes the increase of shrimp metabolic waste, since they eat more for their osmoregulation.

Ranges of nitrite-nitrogen (NO_2-N) in the shrimp pond waters were 0.0051–0.0758 mg/L in A treatment, 0.0049–0.1028 mg/L in B treatment, and 0.0105–0.0525 mg/L in C treatment. Nitrite-nitrogen is easily changed to nitrate-nitrogen that is less toxic, but this is only happened when the nitrification bacteria is available enough in the pond water media. Bacteria probiotic of RICA3 is *Pseudoalteromonas* sp. Edeep-1 originated from the sea sediment that function as nitrification bacteria in the pond water (Atmomarsono *et al*., 2009; Atmomarsono & Susianingsih, 2015).

Nitrogen is needed by bacteria in synthesis of amino acids and nucleotide. Nitrogen could be originated from organic and inorganic substances. Usually in the cultured shrimp pond, bacteria use organic wastes from uneaten feed and shrimp metabolic (including feces). Protein from the remnant feed is decomposed by bacteria to amino acids using protease produced by them and finally these amino acids will be used by bacteria cells (Sukenda *et al*., 2006).

Concentration of nitrate-nitrogen in the shrimp pond waters were fluctuated during culture period. Ranges of NO_3-N concentrations in the shrimp pond waters were 0.1132–0.565 mg/L in A treatment, 0.1253–0.7274 mg/L in B treatment, and 0.1251–1.4112 mg/L in control ponds. Similar to nitrite-nitrogen concentration, the nitrate-nitrogen concentrations increased sharply after 6-week culture (the fourth sampling period). Unlike nitrite-nitrogen and ammonia-nitrogen, nitrate-nitrogen is less toxic to the cultured shrimp, because nitrate-nitrogen could be used directly by primary producers in the rearing pond. Even the increase of nitrate-nitrogen concentration in the shrimp pond waters is not dangerous, but to prevent from any phytoplankton blooms, nitrate-nitrogen concentration should be less than 1.0 mg/L (Wedemeyer, 1996).

CONCLUSION

Compared to untreated (control) shrimp, application of the three RICA probiotics RICA4, RICA5, and RICA3 either used alternately (A) or in combination (B) increased ($P<0.05$) survival rate of white-leg shrimp but not their production ($P>0.05$). Generally total vibrio count (TBV) and total general bacteria (TPC) of the cultured white-leg shrimp pond waters were still in good ranges for the shrimp life. However, the increase of TBV/TPC ratio in the pond water over than 10% could be dangerous for the cultured shrimp in control pond.

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