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USE OF DIFFERENT PROBIOTICS FOR PREVENTION OF VIBRIOSIS DISEASE ON TIGER SHRIMP LARVAE REARED IN FIBERGLASS TANKS

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ABSTRACT

To counter disease problems caused by vibriosis in shrimp hatchery, this recent study used three different probiotics to be tested on tiger shrimp (*Penaeus monodon*) postlarvae. The study arranged four treatments as follows: A: a combination of three liquid-form probiotics *Brevibacillus laterosporus* BT951, *Bacillus subtilis* BM12, and *B. licheniformis* BM58; B: a combination of three powder-form probiotics *Brevibacillus laterosporus* BT951, *Bacillus subtilis* BM12, and *B. licheniformis* BM58; C: a commercial powder probiotic containing *Bacillus subtilis*; and D: control (without probiotic), each treatment with three replications. This study was set up in a completely randomized design experiment using twelve fiberglass tanks filled with 750 L sterile sea water and stocked with 30,000 nauplii in the Awarange shrimp hatchery of the Research Institute for Brackishwater Aquaculture and Fisheries Extension Installation in Barru. Variables observed in this study were the survival rate of the shrimp postlarvae at the end of the experiment, total vibrio count (TBV) and total plate count of common bacteria (TPC) in the culture water. The results showed that the survival rate of tiger shrimp applied either in liquid (A: 61.5 ± 4.7%) or powder form (B: 48.6 ± 6.8%), and control (without probiotic) (D: 51.2 ± 4.4%) were not significantly different ($P > 0.05$). However, survival rates in these three treatments differed ($P < 0.05$) with that of the commercial probiotic (C: 21.7 ± 9.9%). TBV/TPC ratio in the tank waters treated with the commercial probiotic (2.26-37.52%) was much higher than that of the liquid form probiotic (0.86-1.98%), powder form probiotic (1.25-8.37%), and control (1.93-2.84%). Ammonia-nitrogen in treatment C (1.462-2.989 mg/L) was relatively higher than that of in treatment A (1.595-2.435 mg/L), treatment B (1.644-2.115 mg/L), and treatment D (1.051-1.858 mg/L).

KEYWORDS: probiotic; survival rate; tiger shrimp postlarvae; vibriosis

INTRODUCTION

Tiger shrimp (*Penaeus monodon*) aquaculture has suffered various shrimp diseases in both hatchery and grow-out systems, worldwide. Shrimp diseases caused by *white spot syndrome virus*, *yellow head virus*, and vibriosis occurred not only in tiger shrimp culture, but also in whiteleg shrimp culture (Lightner, 2011; Atmomarsono & Susianingsih, 2013). The use of chemicals and antibiotics to cure shrimp diseases have been practiced in Thailand and Vietnam (Tran *et al*., 2017). Because of their negative impacts on human health, these chemical uses in aquaculture have now been banned in Indonesia. This has left fewer alternatives to combat or prevent these diseases occurrence. One way to solve this problem is using probiotic to prevent shrimp diseases (Poernomo, 2004; Waston *et al*., 2008). The main culprit of shrimp diseases is vibriosis caused by *Vibrio harveyi* which could wipe out 100% of the cultured shrimp (Flegel, 2012; Atmomarsono & Nurbaya, 2014). As an opportunistic pathogen, *Vibrio harveyi* will only become pathogenic bacteria causing shrimp diseases when the pond water quality is poor (Atmomarsono & Susianingsih, 2015). This problem could be minimized by practicing the best aquaculture practices and applying proper probiotics. Probiotic bacteria are needed in the culture of larval aquatic organisms including black tiger shrimp to prevent bacterial diseases (Atmomarsono *et al*., 2009; Uddin *et al*., 2013).

Probiotic bacteria are non-pathogenic bacteria usually used to protect the shrimp from pathogenic microorganisms, inhibit the growth of pathogenic bacteria, neutralize poor pond water quality such as

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organic matter, H_2S, NH_3-N, and NO_2-N, and function as food (Poernomo, 2004; Wang *et al*., 2008). Probiotic bacteria can be isolated from sea sediment, pond sediment, mangrove leaves, and macroalgae (Atmomarsono *et al*., 2009; Susianingsih *et al*., 2012; Tampangallo *et al*., 2013; Atmomarsono & Nurbaya, 2014). There are various types of domestically produced and imported probiotics available in the market. Some of them are useful in the shrimp culture, but many of them are in effective.

The Research Institute for Brackishwater Aquaculture and Fisheries Extension develops and produces RICA (Research Institute for Coastal Aquaculture) probiotics originally isolated from the sea sediment, brackishwater pond, mangrove, and seaweed. RICA-1 (*Brevibacillus laterosporus* BT951) is useful in controlling organic matter and H_2S in the ponds, while RICA-2 (*Serratia marcescens* MY1112) is beneficial for the shrimp growth and controlling ammonia-nitrogen (Atmomarsono *et al*., 2009). RICA-3 (*Pseudoalteromonas* sp. Edeep-1 BL542) has an important role in controlling nitrite and pathogenic *Vibrio harveyi* (Atmomarsono & Susianingsih, 2013). RICA-4 (*Bacillus subtilis* BM12) is useful in controlling organic matter and *Vibrio harveyi* in pond water. RICA-5 (*Bacillus licheniformis* BM58) is useful in controlling ammonia and nitrite in pond water (Tampangallo *et al*., 2013). A shrimp disease usually occurs when the accumulation of organic matter in the pond waters and sediments can not be controlled properly by the available natural bacteria. Since each RICA probiotic bacteria has a different role, the combination use of three different RICA probiotics might offer a full potential solution package to control organic wastes, ammonia-nitrogen, nitrite-nitrogen, and pathogenic *Vibrio harveyi* in shrimp ponds.

The aim of this study was to determine the best combination of probiotics in hatchery larval rearing of tiger shrimp using fiberglass tanks.

MATERIALS AND METHODS

This research was carried out in the shrimp hatchery of the Research Institute for Brackishwater Aqua-culture and Fisheries Extension Installation, Awarange, Barru Regency, South Sulawesi. A Completely Randomized Design (CRD) experiment with four treatments and three replications was used in this research. The four treatments applied were: A: a combination of three liquid-form probiotics RICA-1 (*Brevibacillus laterosporus* BT951), RICA-4 (*Bacillus subtilis* BM12), and RICA-5 (*Bacillus licheniformis* BM58); B: a combination of three powder-form probiotics RICA-1 (*Brevibacillus laterosporus* BT951), RICA-4 (*Bacillus subtilis* BM12), and RICA-5 (*Bacillus licheniformis* BM58); C: a commercial powder probiotic containing *Bacillus subtilis*; and D: control (without probiotic).

This research used twelve fiberglass tanks of one m^3 in volume filled with 750 L of chlorinated-sterile sea water of about 30 ppt and continuously aerated. Each tank was stocked with 30,000 nauplii (40 pcs/L), fed with *Skeletonema* sp. 15,000-75,000 cell/mL (started from the second day, just before Zoea-1 stage), commercial feed (started from Zoea-1 stage), and brineshrimp 10-20 nauplii *Artemia*/shrimp larvae (started from Mysis-3 stage or about day-9). Water exchange for about 5%-20% was carried out daily after Mysis-3 stage. The probiotic applications for treatment A and B were as follows RICA-1 probiotic (*Brevibacillus laterosporus* BT951) was applied on day-3 (D-3), RICA-4 (*Bacillus subtilis* BM12) was applied on D-6, RICA-5 (*Bacillus licheniformis* BM58) on D-9, and followed by RICA-1 applied daily from day-10 (PL-1) to day-21 (PL-12). For treatment C, a commercial powder probiotic was applied on the same day and the same amount with treatment B (1 g/m^3). All of the applied probiotics in the shrimp culture water were estimated to reach about 10^1 cfu/mL in population. To make this density, 100 mL probiotic in nutrient broth (liquid-form probiotic) was added to 900 mL sterile sea water and stirred for about 24-hours before used for the shrimp culture water in treatment A. To make powder-form probiotic (treatment B), 200 mL liquid-form probiotic was added to 400 g sterile cassava meal, then dried at 40°C in an oven for about 48-hours before used. The density of the applied bacteria in each treatment was checked before application.

Water salinity in the tank was maintained about 30 ppt, while water pH and temperature were monitored daily. The concentrations of total organic matter (TOM), ammonia-nitrogen (NH_3-N), and nitrite-nitrogen (NO_2-N) in the tank waters were monitored four times, namely at the stages of Zoea-1 (D-2), PL-1 (D-10), PL-4 (D-13), and PL-10 (D-19). Using a modified spreading method (Buller, 2004), the total number of common bacteria (Total Plate Count = TPC) in the tank water was measured in *Tryptic Soy Agar* (TSA), while the total number of *Vibrio* spp. (Total Bacteria of Vibrios = TBV) in the tank water was measured in *Thiosulphate Citrate Bile Sucrose Agar* (TCBSA). Both TBV and TPC were monitored in the same days with the water quality monitoring. The data on TBV and TPC were then analyzed descriptively. TBV/TPC ratios of the tank waters were also calculated based on the percentage of the number of TBV and TPC in each sample. When TBV/TPC ratio had increased more than

10%, some pathogenic *Vibrio* spp. might be harmful to the shrimp (Atmomarsono & Nurbaya, 2014). The survival rate and weight of tiger shrimp postlarvae in each tank were calculated after the shrimp reached postlarvae 12 (PL-12) and analyzed statistically to see if any differences among the treatments tested (Steel & Torrie, 1981).

RESULTS AND DISCUSSION

Table 1 shows that the average survival rate of tiger shrimp postlarvae (PL-12) in the tank water treated either liquid-form or powder-form RICA probiotics were significantly ($P<0.05$) higher (61.5 ± 4.7% and 48.6 ± 6.8%) than that of the commercial probiotic powder (21.7 ± 9.9%). However, there were no significant differences ($P>0.05$) between the average survival rates of tiger shrimp postlarvae in the control (51.2 ± 4.4%) and those of in treatment A and B. These results showed that RICA probiotics either in liquid-form or in powder-form had better results than the commercial probiotic powder, but not better than the control (without probiotic). The shrimp mortality might be caused by the increasing number of vibrios and the increased level of ammonia-nitrogen concentration in the tank water.

RICA probiotics (treatment A and B) contained three different bacteria, e.g.: *Brevibacillus laterosporus* BT951, *Bacillus subtilis* BM12, and *Bacillus licheniformis* BM58, functioning together to control *Vibrio* spp., total organic matter, ammonia-nitrogen, and nitrite-nitrogen. Treatment C (commercial probiotic) contained only *Bacillus subtilis* functioning in controlling *Vibrio* spp. and total organic matter. This was the reason why the concentration of ammonia-nitrogen (1.462-2.989 mg/L) and TBV/TPC ratio in treatment C (2.26%-37.52%) were relatively higher than those in the liquid form probiotic (1.595-2.435 mg/L and 0.86%-1.98% respectively) and powder form probiotic (1.644-2.115 mg/L and 1.25%-8.37% respectively). However, RICA probiotics (treatment A and B) and control (without probiotic) had not different significantly on the survival rates of the tiger shrimp postlarvae (Table 1). This might be caused by a low concentration of probiotic bacteria in the tank water (about 2.3×10^1 cfu/mL) at the initial stocking. This research advises that the probiotic bacteria should be applied at least ten times of the current application for future experiment or commercial applications.

The survival rates of tiger shrimp postlarvae resulted in this research were similar to those of Uddin *et al*. (2013) results, which were 52% for probiotic-treated postlarvae and 35% for untreated postlarvae. However, based on the individual survived postlarvae, the study findings (8-25 pcs/L) were relatively lower than that of Uddin *et al*. (2013) results (35-52 pcs/L). This difference might be caused by a higher ammonia-nitrogen concentration in the shrimp culture tank water in this research after PL-1 stage (2.30-5.08 mg/L) (Table 2) compared to Uddin *et al*. (2013) results (0.9-2.5 mg/L).

It was previously reported by Atmomarsono *et al*. (2009) that RICA-1 probiotic consisting of *Brevibacillus laterosporus* BT951 could be used as an anti vibriosis, and was useful in demineralization of total organic matter. Tampangallo *et al*. (2013) also reported that RICA-4 (*Bacillus subtilis* BM12) functioned as organic matter demineralizer, and RICA-5 (*Bacillus licheniformis* BM58) functioned as ammonia-nitrogen and nitrite-nitrogen controller in the culture media. Treatment C consisting of *Bacillus subtilis* could demineralize total organic matter, but not ammonia-nitrogen. This was the reason ammonia-nitrogen in treatment C was relatively higher than those of the other treatments. This ammonia-nitrogen could be toxic for tiger shrimp postlarvae.

According to Chin & Chen (1987) *in* Boyd (1990) 96-h LC50 and 24-h LC50 for tiger shrimp postlarvae were only about 1.26 mg/L and 5.71 mg/L, respectively. This means that the concentration of ammonia-nitrogen in the culture media of this present research was relatively dangerous to tiger shrimp postlarvae. This might be the cause a direct or indirect shrimp mortality through the increase of TBV/TPC ratio (percentage ratio of total bacteria of vibrios and total common bacteria) in the culture water during PL-4 stage in the control tank (19.68%) and during PL-10 stage in the commercial probiotic treated tank

Table 1. Survival rate and average weight of tiger shrimp postlarvae reared in fiberglass tank with different probiotics

	Probiotics	Survival rate (%)	Postlarvae weight (mg/pcs)
A.	Liquid-formed RICA	61.5 ± 4.7[a]	1.7 ± 0.2[a]
B.	Powder-formed RICA	48.6 ± 6.8[a]	2.4 ± 0.5[a]
C.	Commercial powder probiotic	21.7 ± 9.9[b]	2.0 ± 0.5[a]
D.	Control (without probiotic)	51.2 ± 4.4[a]	1.3 ± 0.6[a]

Table 2. Ranges of ammonia-nitrogen concentrations in tiger shrimp culture tank water at different larvae stages treated with different probiotics

Probiotics	Zoea-1 (mg/L)	PL-1 (mg/L)	PL-4 (mg/L)	PL-10 (mg/L)
A. Liquid-formed RICA	0.076-0.217	2.332-2.560	4.520-5.083	1.595-2.435
B. Powder-formed RICA	0.038-0.167	2.477-2.660	4.171-4.690	1.644-2.115
C. Commercial powder probiotic	0.014-0.271	2.392-2.535	3.966-5.058	1.462-2.989
D. Control (without probiotic)	0.039-0.074	2.298-2.346	4.231-4.835	1.051-1.858

(37.52%). This finding was in line with the results reported by Atmomarsono & Nurbaya (2014) and Susianingsih *et al*. (2017) where the increase of TBV/TPC ratio in the culture water of more than 10% could be dangerous to the cultured shrimp.

Based on the individual weight size of the harvested postlarvae, postlarvae in treatment A (liquid-form RICA probiotic) were relatively more homogenous than that of the other treatments. However, the postlarvae in treatment B (powder-form RICA probiotic) were relatively heavier (2.4 ± 0.5) than that of the other treatments. The smallest, lightest, and varying in weight of harvested tiger shrimp postlarvae were found in the control (1.3 ± 0.6) (Table 1). These results showed that all kinds of probiotics were needed to maintain the culture water clean, so that the cultured tiger shrimp could grow well homogenously. Besides that, without any addition probiotics, the natural bacteria were not enough to demineralize some of organic matter and ammonia-nitrogen. As a result, the ratio of total bacteria of vibrios and total common bacteria (TBV/TPC) in the control tank water during PL-4 stage had increased over 10%.

In general, vibrio numbers in the cultured shrimp water in this experiment were not significantly high (1-6,860 cfu/mL). According to Defoirdt (2007) *Vibrio harveyi* could be dangerous when its population in the cultured shrimp water reached 10^4 cfu/mL. However, eventhough the vibrio numbers in this research were still lower than 10^4 cfu/mL, they might cause shrimp mortality when TBV/TPC ratio increased over 10% (Atmomarsono & Nurbaya, 2014; Susianingsih *et al*., 2017).

Table 3 shows that nitrite-nitrogen concentrations in the tiger shrimp culture tank water tended to increase following the culture period. Fortunately, the highest concentration of nitrite-nitrogen in this research (1.843 mg/L) was still considered as a safe level for tiger shrimp postlarvae. According to Chen & Chin (1988a) *in* Boyd (1990) the concentration of nitrite-nitrogen for tiger shrimp postlarvae should be less than 4.5 mg/L. However, since ammonia-nitrogen concentration in the culture tank water was relatively high, it could cause the cultured shrimps to be more susceptible to pathogenic *Vibrio harveyi* especially when TBV/TPC ratio increased over 10% such as in treatments C and D (Table 5).

In general, a high concentration of ammonia-nitrogen followed by relatively low concentration of nitrite-nitrogen indicated that in the culture water might be not enough nitrification bacteria. In treatments A and B, RICA-4 and RICA-5 probiotics (*Bacillus subtilis* BM12 and *Bacillus licheniformis* BM58 respectively) could function as nitrification bacteria. Unfortunately, RICA-4 and RICA-5 probiotics were not used anymore in this research after tiger shrimp reached postlarvae one (PL-1). Instead of using RICA-4 and RICA-5 probiotics alternately, RICA-1 probiotic was used everyday from PL-1 to postlarvae 12 stage following the procedure used for the commercial probiotic application in treatment C. It is suggested that for the future experiments, RICA-1, RICA-4, and RICA-5 probiotics should be used alternately to prevent the increase of total organic matter, ammonia-nitrogen, nitrite-nitrogen concentrations, and TBV/TPC ratio in the shrimp culture water.

Based on Table 4, the ranges of total organic matter concentrations in the tiger shrimp culture tank water were quite high (29.4-64.4 mg/L). According to Madeali *et al*. (2009) the concentration of total organic matter in the shrimp culture water should be less than 30 mg/L, otherwise, the total organic matter could trigger the growth of pathogenic microorganisms that might be dangerous to the cultured shrimp.

Since total organic matter concentrations in the shrimp culture water were relatively high in all tanks,

Table 3. Ranges of nitrite-nitrogen concentrations in tiger shrimp culture tank water at different larvae stages treated with different probiotics

Probiotics	Zoea-1 (mg/L)	PL-1 (mg/L)	PL-4 (mg/L)	PL-10 (mg/L)
A. Liquid-formed RICA	0.001-0.002	0.014-0.043	0.028-0.034	1.385-1.843
B. Powder-formed RICA	0.007-0.033	0.040-0.064	0.053-0.188	0.823-1.469
C. Commercial powder probiotic	0.007-0.024	0.023-0.031	0.033-0.042	0.938-1.672
D. Control (without probiotic)	0.007-0.007	0.042-0.054	0.032-0.200	0.360-1.686

Table 4. Ranges of total organic matter concentrations in tiger shrimp culture tank water at different larvae stages treated with different probiotics

Probiotics	Zoea-1 (mg/L)	PL-1 (mg/L)	PL-4 (mg/L)	PL-10 (mg/L)
A. Liquid-formed RICA	29.4-52.6	46.9-57.6	45.7-60.1	50.0-64.4
B. Powder-formed RICA	40.0-51.3	50.0-63.8	48.8-53.2	41.9-57.6
C. Commercial powder probiotic	40.0-54.4	50.0-61.3	37.5-63.8	43.2-53.8
D. Control (without probiotic)	46.9-50.7	47.6-54.4	42.5-52.6	45.0-48.2

any kind of probiotic which could demineralize total organic matter was vitally important. The three treatments in this experiment had this kind of probiotic. Unfortunately, only RICA-1 probiotic (*Brevibacillus laterosporus* BT951) was used in treatments A and B after reaching PL-1. *Bacillus subtilis* is one of the commonly used probiotic bacteria in the commercial probiotic products. This could explain the high level of the general total organic matter concentration and the high concentration of ammonia-nitrogen. These two water quality parameters might trigger the growth of pathogenic vibrios, in TBV/TPC ratio over 10% in the control (19.68%) and treatment C (37.52%) (Table 5). This level of TBV/TPC ratios was relatively dangerous to shrimp (Atmomarsono & Nurbaya, 2014; Susianingsih *et al*., 2017). Madeali *et al*. (2009) similarly reported that the pathogenicity of *Vibrio harveyi* was enhanced by the high concentration of total organic matter (more than 30 mg/L) in the cultured shrimp water.

During the experiment, other water quality parameters in the treatment tanks were also monitored daily. Ranges of water quality parameters were as follows 30.0-32.4 ppt for water salinity, 30.0°C-32.2°C for water temperature, 7.88-8.22 for water pH, and 4.45-5.34 mg/L for dissolved oxygen. These water quality parameters were within the standard for the cultured tiger shrimp based on the study of Atmomarsono *et al*. (2009).

Table 5. Ranges of percentage of total bacteria of vibrios and total plate count ratio (TBV/TPC) in tiger shrimp culture tank water at different larvae stages treated with different probiotics

Probiotics	Zoea-1 (mg/L)	PL-1 (mg/L)	PL-4 (mg/L)	PL-10 (mg/L)
A. Liquid-formed RICA	0.20-0.40	0.38-1.12	0.31-1.96	0.86-1.98
B. Powder-formed RICA	0.04-3.00	0.51-7.85	1.25-1.75	1.25-8.37
C. Commercial powder probiotic	0.20-0.67	0.92-8.03	2.09-5.61	2.26-**37.52**[*)]
D. Control (without probiotic)	0.03-0.33	0.68-3.10	2.36-**19.68**[*)]	1.93-2.84

*) Bolded values mean lethal level for the cultured shrimp

CONCLUSION

This current study concludes that in comparison with the control (without probiotic), the application of liquid-form or powder-form RICA probiotics could not increase ($P>0.05$) survival rate of tiger shrimp postlarvae. Compared with commercial probiotic, the application of liquid-form or powder-form RICA probiotics had produced a better survival rate ($P<0.05$) of tiger shrimp postlarvae. The survival rate of tiger shrimp postlarvae might be decreased by the high concentration of ammonia-nitrogen after PL-1 stage (2.30-5.08 mg/L) and the increase of TBV/TPC ratio after PL-4 stage (more than 10%). This study suggests that the three different species of probiotic bacteria should be used alternately started from zoea-1 until PL-12 at ten times concentration compared to the current research use to demineralize total organic matter, ammonia-nitrogen, and nitrite-nitrogen.

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